Charles University
Faculty of Science

Department of Biochemistry

Mgr. Květoslava Mlčochová

The preparation and characterisation of analogues of insulin and IGF-2 selective for both isoforms of insulin receptor and IGF-1 receptor

Příprava a charakterizace selektivních analogů insulin a IGF-2 pro obě isoformy insulinového receptoru a IGF-1 receptoru

Doctoral thesis

Scientific Supervisor: RNDr. Lenka Žáková, Ph.D.

Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences

Prague, 2019

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně pod vedením školitelky RNDr. Lenky Žákové, Ph.D. Tato práce popisuje mé vlastní výsledky s výjimkou výsledků získaných ve spolupráci s mými kolegy. Spolupráce i její rozsah jsou uvedeny v textu. Všechny použité zdroje jsou řádně citovány.

Tato práce, ani její podstatná část nebyly použity k získání jiného nebo stejného akademického titulu.

Praha, 29. září 2019

.....

Mgr. Květoslava Mlčochová

ACKNOWLEDGEMENT

First of all, I would like to express my thanks to my supervisor Dr. Lenka Žáková for her patient, guidance, and unlimited encouragement. This project could hardly be done without her target orientation and humanity.

My special thanks belong to all my colleagues from the research group of Dr. Jiráček as well as to Dr. Jiráček himself. They were my mentors and they were always ready to help me with practical or theoretical obstacles of my project. Therefore, I would like to thank all of them for being such a great team.

Last but not least I thank to my family for the encouragement, support and the patient. Particularly, I would like to thank to my husband Tomáš for his care, unlimited encouragement, and valuable advice during the whole my studies.

CONTENT

ABBREVIATIONS

Å	Ångström
Arg	Arginine
Akt	Protein kinase B
Ala	Alanine
Asn	Asparagine
ATP	Adenosine triphosphate
C_1 domain	C domain of IGF-1
C_2 domain	C domain of IGF-2
CNS	Central nervous system
CR domain	Cystein-rich domain
CryoEM	Cryogenic electron microscopy
CT	Carboxy-terminal tail
Cys	Cystein
D_1 domain	D domain of IGF-1
D_2 domain	D domain of IGF-2
Da	Dalton
DNA	Deoxyribonucleic acid
Erk	Extracellular regulated kinase
FnIII-1-3 domain	Fibronectin type III domains 1-3
Gln	Glutamate
Glu	Glutamic acid
GLUT4	Na^+ independent glucose transporters 4
Gly	Glycine
GPCR	G-protein coupled receptor
ID	Insert domain
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
IGF-2	Insulin-like growth factor 2
IGF-2R	Insulin-like growth factor 2 receptor
IGFBP 1-6	Insulin-like growth factor binding proteins
Ile	Isoleucine
IR	Insulin receptor
IRS	Insulin receptor substrates
JM	Juxtamembrane domain
K_d	Equilibrium dissociation constant
L1 domain	Leucine-rich repeat domain
L2domain	Leucine-rich repeat domain
Leu	Leucine
Lys	Lysine
MAP kinase	Mitogen-activated protein kinase
Met	Methionine
nM	nanomolar
NMR	Nuclear magnetic resonance
Phe	Phenyalanine

PI3K	Phosphoinositol-3 kinase
Pro	Proline
Ras	p21 protein
RTK	Receptor tyrosine-kinase
Ser	Serine
Shc	Src homology collagen
Thr	Threonine
TK	Tyrosine-kinase
TM	Transmembrane domain
Tyr	Tyrosine
Val	Valine
Zn	Zinc
αCT-peptide	αC-terminal peptide

ABSTRACT

Insulin and insulin-like growth factor 1 (IGF-1) and 2 (IGF-2) are related protein hormones with different but overlapping biological functions. All the hormones interact with a receptor within the insulin-IGF system (insulin receptor A and B, IGF-1 receptor), however with different affinity. The different interaction with individual receptors is just one of the main tools for regulation of the system that is essential for the proper functioning of the organism.

Although the residues directly interacting with receptors are mainly located in A and B domains, the C and D domains probably play a role in receptor specificity. Here, we firstly focused on the impact of D domains of IGF-1 and 2 (D_1 and D_2 domains) and C domain of IGF-2 (C_2 domain). To probe the impact of C and D domains, we prepared insulin analogues containing a part of or an entire domain following a pattern seen in IGFs. The receptor-binding affinities of these analogues and their receptor autophosphorylation potentials were characterised.

Our results revealed that the initial part of D_1 domain has a detrimental effect on IR affinity that is only slightly enhanced by the rest of the D_1 domain. D_2 domain has rather neutral effect on IR affinity. We further showed that the addition of amino acids derived from the C_2 domain to the C-terminus of the B-chain led to increased IR-B affinity and ability of its activation. This unexpected finding opens a new possibility of enhancement of IR-B specificity.

To prepare new IGF-2 analogues, we developed a novel and straightforward protocol of IGF-2 production. The first set of IGF-2 analogues contained unique IGF-1-like mutations in the B and C domains (i.e.Asn29, Gly30-Ser31, and Pro35-Gln36). All analogues exhibited significantly reduced affinity towards IR-A, particularly the analogues with a Pro-Gln insertion in the C domain where a displacement of the C-loop and more open C-loop conformation were confirmed by the NMR characterisation. The combination of Pro-Gln insertion and Ser29Asn mutation led to an almost 2-fold increase in IGF-1R affinity. Due to the decrease in IR-A affinity and concurrent increase in IGF-1R affinity, Asn29,Ser29(Pro-Gln)-IGF-2 showed an almost 10-fold higher IGF-1R/IR-A binding specificity compared to native IGF-2.

The second set of IGF-2 analogues were inspired by HisA4HisA8 insulin known for its disproportionate effects on IR binding and activation. We systematically modified IGF-2

positions 44, 45 and 48, which correspond to or are close to insulin sites A4 and A8. The IGF-1R and IR-A binding and autophosphorylation potencies of these analogues were characterised. Of the intended analogues, Gln45-IGF-2, Gln45, His48-IGF-2, and His48-IGF-2 were successfully prepared. They retained the main IGF-1R-related properties, but the His48 substitution showed a high affinity for IR-A and for IR-B, leading to the strongest IGF-2 binders yet reported. All analogues activated IR-A and IGF-1R without major discrepancies between their ability of receptor activation and binding affinities. Thus, the disproportion between receptor affinity and the ability of its activation is probably specific for insulin-IR interaction.

Recently, CryoEM studies revealed details of insulin interaction with IR-A through binding sites 1 and 2. But this picture does not fully match the results of mutagenesis studies, as several “supposedly site 2 residues” were not included in the interaction. Therefore, in the last part of this project, we focused on the “neglected” site 2 residues (50, 52, 53, 57). We modified these residues in two ways for His or for a similar amino acid. The study revealed that IGF-2 positions 50, 53 and 57 are relatively tolerant to modifications. Our results did not provide ambiguous evidence on the role of positions 50, 52, 53 and 57 of IGF-2 in site 2 interactions. However, computational metadynamics of corresponding mutations in insulin indicate that these modifications can affect the internal dynamics and inhibit its ability to adopt receptor-bound conformation, which is mainly important for binding to receptor site 1.

ABSTRAKT

Insulin a insulinu podobné růstové faktory 1 (IGF-1) a 2 (IGF-2) jsou příbuzné proteinové hormony s rozdílnými, avšak překrývajícími se biologickými funkcemi. Všechny tři hormony interagují, i když s různou afinitou, s receptory, patřícími do systému insulinu-IGF (insulinové receptory A a B (IR-A, B), IGF-1 receptor (IGF-1R)). Právě rozdílná schopnost interakce s jednotlivými receptory je jeden z hlavních nástrojů regulace tohoto systému, který je nezbytný pro správné fungování organismu.

Ačkoli aminokyseliny, které se přímo účastní interakce s receptory, se nacházejí zejména v doménách A a B, domény C a D mají patrně určitou úlohu ve vazebné specificitě. V rámci tohoto projektu jsme se nejprve zaměřili na vliv domén D z molekul IGF-1 a 2 (D_1 a D_2) a domény C z IGF-2 (C_2). Abychom mohli sledovat vliv domén C a D, připravili jsme insulinové analogy s celými nebo částmi jednotlivých domén tak, aby uspořádání odpovídalo přirozené pozici v molekule IGF. U takto připravených analogů byly studovány vazebné afinity a jejich schopnost aktivovat receptor.

Výsledky odhalily, že úvodní část domény D_1 má ničivý účinek na afinitu k IR. Tento účinek je pouze mírně zvýrazněn přítomností zbytku domény (D_1). Doména D_2 nemá téměř žádný účinek na afinitu k IR. Dále bylo zjištěno, že přidání aminokyselin odvozených z domény C_2 na C-konec řetězce B vede ke zvýšení afinity a schopnosti aktivovat IR-B. Toto nečekané zjištění otevírá nové možnosti zvýšení specificity vůči isoformě B IR.

Abychom byli schopni připravovat nové analogy IGF-2, vyvinuli jsme jednoduchou metodu přípravy IGF-2. První soubor analogů IGF-2 obsahoval unikátní mutace v rámci domén B a C, které byly odvozeny z IGF-1 (tj. Asn29, Gly30-Ser31 a Pro35-Gln36). Takto změněné analogy vykazovaly významně sníženou afinitu k IR-A, přičemž nejvýznamnější snížení bylo pozorováno u analogů s vloženou sekvencí Pro-Gln v doméně C. U těchto analogů byl pomocí NMR charakterizace potvrzen posun C-smyčky společně s jejím „otevřenějším“ prostorovým uspořádáním. Kombinace insertu Pro-Gln a mutace Ser29Asn vedla k téměř 2násobnému zvýšení afinity k IGF-1R. U analogu Asn29,Ser29(Pro-Gln)-IGF-2 byla rovněž díky snížené afinitě k IR-A a současně zvýšené afinitě k IGF-1R pozorována téměř 10násobně vyšší IGF-1R/IR-A vazebná specificita v porovnání s přirozeným IGF-2.

Druhý soubor analogů byl inspirován HisA4HisA8 insulinem, který je známý pro svou disproporci ve vazebné afinitě a schopnosti aktivace IR. Systematicky jsme upravovali pozice

44, 45 a 48 v molekule IGF2, které odpovídají pozicím A4 a A8 v molekule insulinu či jsou v jejich blízkém kontaktu. U těchto analogů byla měřena vazebná afinita k IR-A a IGF-1R a schopnost jejich autofosforylace. Z původně zamýšlených analogů se podařilo připravit pouze Gln45-IGF-2, Gln45, His48-IGF-2, a His48-IGF-2. Vytvořené analogy si zachovaly původní schopnost vázat a aktivovat IGF-1R, avšak substituce His48 vedla k významně vysokým afinitám k obou isoformám IR. IGF-2 analogy obsahující His48 váží IR s doposud nejvyšší známou afinitou. U žádného z analogů nebyl pozorován výrazný nesoulad mezi vazebnou afinitou k IR-A a IGF-1R a schopností jejich aktivace. Disproporce mezi schopností vázat a aktivovat receptor je patrně omezená na interakci insulin-IR.

Nedávná CryoEM strukturní analýza vazby insulinu na IR-A odhalila detaily interakce v oblastech vazebných míst 1 a 2. Publikované výsledky ale nejsou zcela v souladu s výsledky substitučních studií, jelikož interakce v oblasti vazebného místa 2 nezahrnuje všechny doposud předpokládané aminokyseliny. Z toho důvodu jsme se v poslední fázi našeho výzkumného projektu zaměřili na „opomenuté“ aminokyseliny spadající do vazebné oblasti 2 (50, 52, 53, 57). Aminokyselina na těchto pozicích jsme zaměňovali za His a za aminokyselinu s podobnými vlastnostmi. Na základě výsledků studie je patrné, že pozice 50, 53 a 57 jsou relativně tolerantní k zavedení změnám. Zjištěné výsledky nepodávají jednoznačnou evidenci o roli pozic 50, 52, 53 a 57 v interakci v oblasti vazebného místa 2. Avšak výsledky studie metadynamiky odpovídajících mutací v molekule insulinu naznačují, že uvedené substituce mohou ovlivňovat vnitřní dynamiku molekuly a inhibovat schopnost zaujmout vazebné uspořádání, což je důležité zejména pro interakce v oblasti vazebného místa 1.

1 INTRODUCTION

1.1 Insulin

Insulin, together with insulin-like growth factors 1 (IGF-1) and 2 (IGF-2) (1–3), relaxin (4) relaxin-related factors (5,6) and others (6–10) belong to an ancient insulin protein super-family. Members of this family play a crucial role at the level of cell mechanisms, such as survival, apoptosis, cell migration, proliferation, and differentiation, as well as at the level of the whole organism, such as body growth, metabolism, reproduction and life span. Insulin itself participates to a greater or lesser extent in the majority of these processes (11).

1.1.1 Biosynthesis and physiological function

Insulin is a peptide hormone produced by β-cells in the Islets of Langerhans. The insulin gene is located on the short arm of chromosome 11 (11p15.5). The transcription yields insulin in its pre-proform, with the primary structure consisting of 4 domains (a signal sequence, B, C, and A domains named in the N-terminal order; see Figure 1). The 22 amino acid-long signal sequence targets the molecule to the endoplasmic reticulum where insulin is further processed. While the signal sequence is removed immediately after translocation, the intra-molecular C-peptide contributes to the proper molecular folding and is cleaved during insulin maturation. The biosynthesis yields a two-chain molecule connected by disulphide bridges, the mature insulin (12,13).

At the final step of biosynthesis, insulin is stored in secretory granules in the β-cells (see Figure 2). Here, thanks to the high insulin concentration, the molecules form dimers and further hexamers. Hexamers are coordinated around Zn^{2+} ions which stabilise their arrangement. The coordination is mediated via imidazole in the HisB10 side chain. The precise *in vivo* conformation of insulin is not yet completely understood, even though numerous *in vitro* structures are available (reviewed in (14,15)).

The insulin monomer can reach two distinct conformations, differing mainly in the organisation of the N-terminus of the B-chain which are called T (tense) or R (relaxed) states. While in the T-conformation, the N-terminal residues (B1-6) are extended, in the R-conformation, they prolong the central helix, resulting in the B1-19 helix. One additional conformation was reported, known as the R^f state due to its similarity to the R-conformation. In the R^f conformation, the B-chain helix is shortened to a B3-19 segment, allowing B1-3

residues to "fray" from the α-helix. The T/R^f/R states differ from each other in their stability, ability to bind other substances (such as phenolic substances) and other aspects (16,17). The physiological relevance of the individual states for the storage of insulin and its activity is not yet completely understood (15).

Figure 1: *Insulin biosynthesis.* Steps of the biosynthesis are described in the right site. Location of the individual steps is indicated on the left. The figure is based on **(18)**.

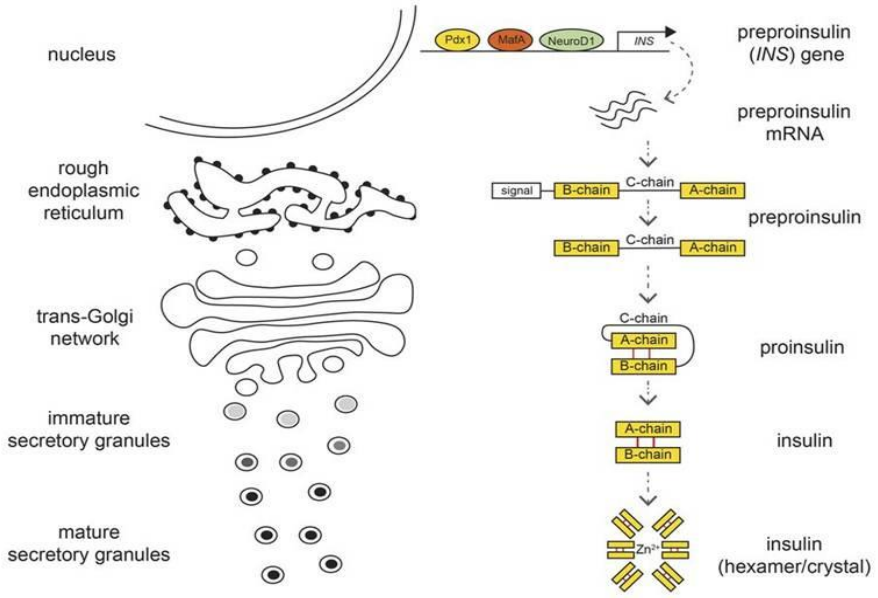
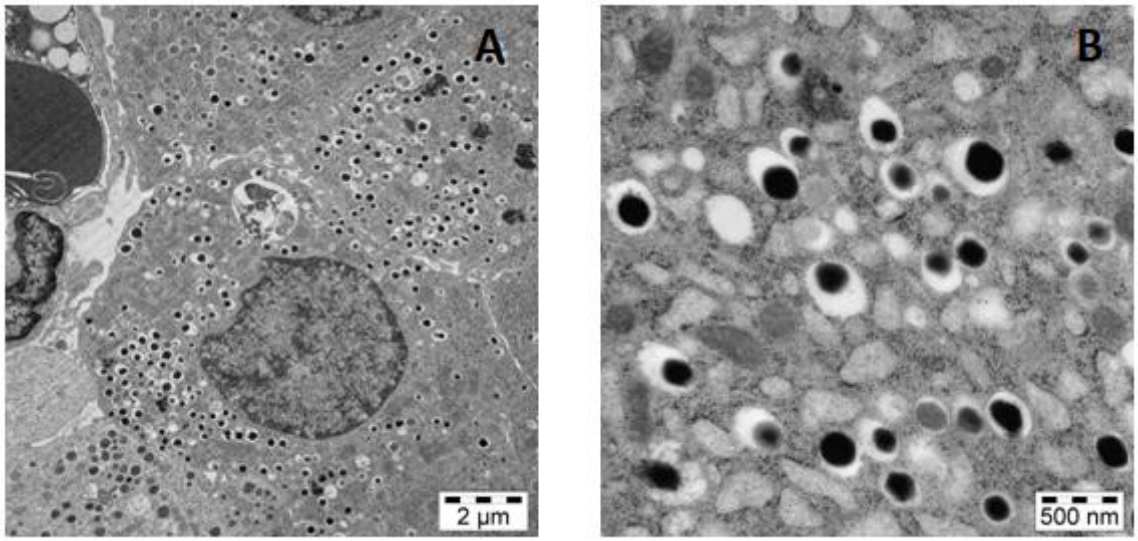
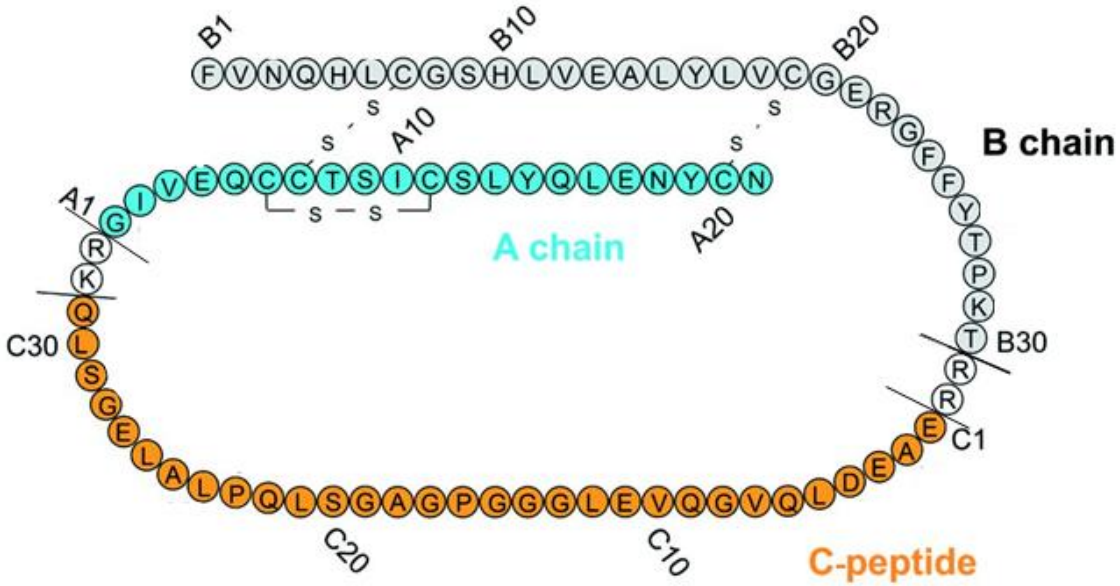


Figure 2: *Storage of insulin in secretory granules in the β-cells.* A: Human pancreatic β-cells as seen by electron microscopy (magnification × 10 000); B: A detail image of secretory granules (magnification × 40 000).



The situation *in vivo* is quite intricate, as the composition of the storage granules is not fully characterised. There are many other factors that can affect the insulin state. Apart from Zn^{2+} ions which were mentioned above, those with a recognised function are phenolic neurotransmitters, such as dopamine and serotonin participating in the regulation of insulin release (19,20). Another factor affecting both conformation and secretion could be arginine that accumulates upon the processing of proinsulin (see Figure 3).

Figure 3: *The structure of proinsulin with marked sites of enzymatic cleavage***.** Adapted from **(21)**



An increase in plasma, and consequently interstitial glucose levels, triggers pathways leading to insulin secretion. Secretory granules fuse with the plasmatic membrane and insulin enters the interstitial area. The change in the pH of the surrounding environment leads to deprotonation of carboxyl acid in the GluB13 side chain and the imidazole group of HisB10. A bond responsible for coordination of Zn^{2+} ions is weakened and the hexamers disintegrate. Due to progressive dilution of insulin concentration, insulin reaches its biologically active form, a monomer (12,22).

The predominant role of insulin is decreasing blood glucose level in the circulation; by doing so it contributes to the sustainability of glucose homeostasis and also enables further glucose utilisation. Insulin mediates its activity by enabling the transfer of glucose into insulin sensitive tissues (muscle, adipose) and concurrently by preventing the output of glucose from the liver. Glucose is also transported into the cells independently of insulin. Such mechanisms

ensure basal glucose uptake, nutrition of vital tissues (neuronal, placental) and contribute to system regulation. In insulin-sensitive tissue, insulin triggers a signalling cascade leading to translocation of Na^+ independent glucose transporters 4 (GLUT4) into the plasma membrane. In liver, insulin inhibits glycogen degradation. Apart from this, insulin stimulates biosynthesis of glycogen, proteins, fatty acids, and nucleic acids and contributes to the regulation of cell growth and differentiation (23,24).

1.1.2 Structure and invariant positions

Insulin is a heterodimer with cysteine linked A- and B-chains. The C-peptide/domain is cleaved out in many members of the insulin family. This is probably also the reason why the amino acid sequence of the C-peptide is not strictly conserved and varies among species (12). The structure of insulin was thoroughly characterised by X-ray crystallography and NMR spectroscopy (22,25–27). The A- and B-chains/domains are relatively invariant among proteins of the insulin family, but also among corresponding hormones in different species (orthologues) (28). One of the main common structural features is the position of Cys residues. The cysteine motif in the A-chain (CC-3X-C-8X-C) has been termed an insulin signature (7). In the B-chain, cysteine residues are separated by 11 amino acids consistently in insulin and IGFs. Disruption of these bridges leads to the loss of the original three-dimensional structure and severe impairment of the binding affinity to the targets' receptors (29). The proper position of cysteine residues is responsible for the proper formation of the characteristic pattern of the tertiary structure and contributes to molecular stability. Other dominant elements of the insulin structure are α-helixes. In the A-chain, an N-terminal α-helix (A2-A8) is followed by a non-canonical turn and another α-helix (A13-A19). In the B-chain, a central α-helix (B9-B19) is surrounded by two β-turns (B7-B10 and B20-B23). In hexamers, the N-terminus of the B-chain reaches two conformations, T or R (see above), and the C-terminal residues B24-B28 are in a β-sheet structure. The non-polar amino acids IleA2, ValA3, CysA11 LeuA16, LeuB11, LeuB15 are usually burned and build a hydrophobic core of the molecule (28).

Besides the cysteines, another 10 residues are strictly conserved across vertebrate evolution. These residues are GlyA1, IleA2, ValA3, TyrA19, LeuB6, GlyB8, LeuB11, ValB12, GlyB23, and PheB24 (28). The constant presence of these residues across evolution indicates their importance in the proper structure, function and maturation process.

Insulin forms dimers as a function of increasing concentration. It was shown that the amino acids involved in dimer formation are mostly presented in the B-chain; namely, GlyB8, SerB9, ValB12, TyrB16, GlyB23, PheB24, PheB25, TyrB26, ThrB27, ProB28, AsnA21. As already mentioned, dimers tend to form hexamers in the presence of Zn^{2+} ions. The amino acids contributing to hexamer formation are LeuA13, TyrA14, GluA17, PheB1, ValB2, GlnB4, GlnB13, AlaB14, LeuB17, ValB18, CysB19, GlyB20 and HisB10 that is responsible for Zn^{2+} ions' coordination (22,28,30).

Insulin residues involved in dimer and hexamer formation participate not only in the maturation process and storage of the mature molecule, but the majority of them also have a role in ligand-receptor interaction (see below).

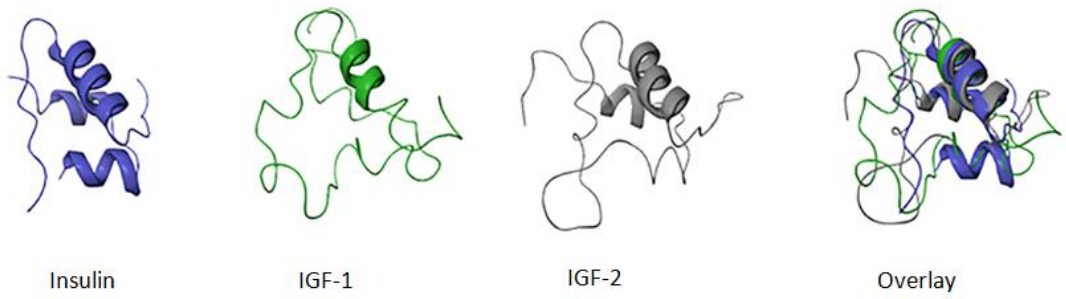
1.2 Insulin like growth factors (IGFs)

The existence of IGF was first assumed by Salomon and Daughaday in the late 1950s (31). They predicted that the growth hormone mediates its activity via growth-promoting peptides. Consequently, insulin-like activity together with growth-promoting effects were identified in vertebrate blood (32,33). Due to the sequence similarity with insulin, the peptides were named insulin-like growth factor 1 and 2 (IGF-1 and 2, Figure 4) (34). Some studies suggested that insulin and IGFs became distinct molecules only after vertebrates evolved and that they have one common ancestor (35). However, other studies pointed to evidence indicating the existence of individual precursors for both molecules (36).

Both IGFs participate in the regulation of cell proliferation, growth, migration, differentiation and survival. Proper regulation and function of both IGFs are necessary during the prenatal as well as postnatal life. IGF-1 and 2 share a high pattern of mutual structural similarity. However, each substance has its own biological role. This is particularly evident from the different pattern of expression and different affinities to respective receptors. Both factors display a wide range of developmental and tissue-specific production and are able to act in endocrine, paracrine as well as autocrine fashion. IGF-1 is preferentially expressed after birth, with peak production in juvenile life and with the liver being the predominant site of production. IGF-2 has a crucial role during embryonic and foetal development, when it is produced by a variety of somatic tissues (35). While IGF-1 is a known mediator of growth hormone activity, the role of IGF-2 in postnatal life is not as yet completely understood. This is particularly interesting when considering that the physiological concentration of IGF-2 is

about 60-70nM in adults (depending on the method used), approximately 3-times higher than that of IGF-1 (37,38). The levels of biologically active IGFs are tightly controlled by their expression, 6 binding proteins (IGFBP 1-6) and by the IGF-2 receptor in the case of IGF-2 (see below) (13,39).

Figure 4: *Structure of insulin, IGF-1, and IGF-2 and their overlay***.** Adapted from**(40)**



1.3 IGF-2

The human IGF-2 gene is located on the short arm of chromosome 11 (11p15.5), where it is in close proximity to the insulin gene (41). The gene contains 4 promoters and 10 exons (with only the last 3 being the coding ones). The transcription driven from individual promoters leads to a different 5'-untranslated leader exons. Transcription activity differs among organs and also during embryonic and foetal development. However, in the majority of cases, it is driven by promoters 2 to 4, with promoter 4 being predominantly active. Transcripts derived from promoter 1 were only found in liver and choroid plexus-leptomeninges in adults. IGF-2 transcription declines after birth. However, the transcription driven from promoter 3 and mainly promoter 4 continues to some extent in adulthood (35,42).

The IGF-2 gene is a subject of imprinting. The loss of imprinting was reported to be associated with pathological disorders during prenatal as well as postnatal life (43). During foetal development, only the paternal allele is transcribed and the maternal allele is silent. Interestingly, a reciprocal situation was found with the IGF-2R gene, where the maternal allele is exclusively transcribed. In adulthood, both alleles are transcribed in the liver and central nervous system (CNS). The expression is regulated on the level of specific tissue, promoter imprinting, as well as on the level of regulator genes (42,44,45).

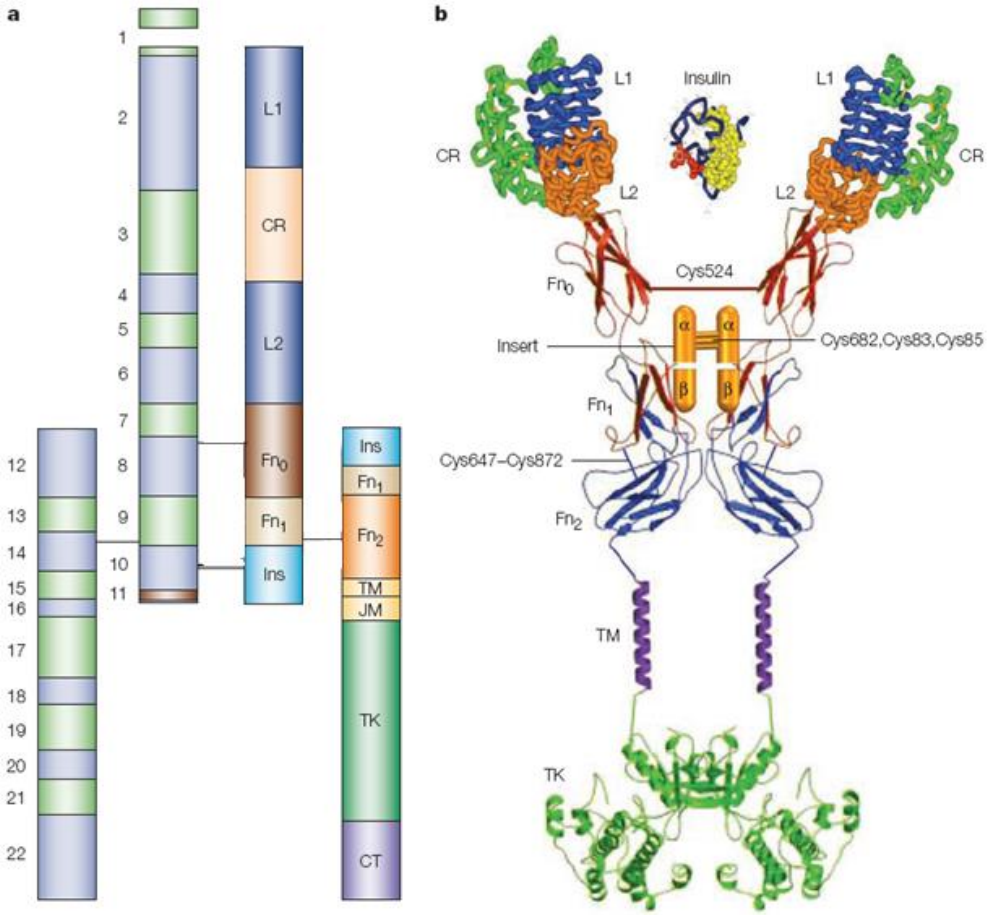
Transcription and subsequent translation of the IGF-2 gene result in several post-translation products with 180 or 236 amino acids. The former is a common case containing the biologically active IGF-2 in a preprohormone form; the latter contains additional N-terminal amino acids. During post-translation modification, a 24 amino acid-signal sequence is removed immediately after the product is translocated into the endoplasmic reticulum, which leads to the formation of pro-IGFs. Subsequent cleavage results in IGF-2(1-104) and IGF-2(1-87). Both IGF-2(1-104) and (1-87), called big-IGFs, were found in human plasma constituting 10 to 15 % of the total circulating IGF-2 levels (38,46–49). Contrary to the mature IGF-2, pro-IGF-2 and big-IGFs are O-glycosylated (48,50). Surprisingly, despite the higher molecular mass, big-IGFs and pro-IGF-2 possess affinity to IGF-1R, IGF-2R, and IGFBP-1, similar to those of mature IGF-2 and also display similar binding kinetics (51).

Mature IGF-2 contains 67 amino acids with the molecular weight of 7505 Da. The molecule consists of 4 domains B, C, A, and D (in the N-terminal order) and the tertiary structure is largely similar to that of insulin or IGF-1 (52,53). See Figure 4.

1.4 Receptors

There are three main receptors known to be the targets of insulin and IGFs' activity. These are insulin receptor (IR; in its two isoforms, A and B), insulin-like growth factor 1 receptor (IGF-1R) and insulin-like growth factor 2 receptor (IGF-2R). IR belongs to the family of receptor tyrosine-kinases (RTK) and is crucial for insulin function. It is a transmembrane homodimeric receptor, where each monomer consists of α (extracellular) and β (extracellular, transmembrane, and intracellular) subunits (see Figure 5). The intracellular domain (subunit β) possesses an intrinsic tyrosine-kinase (TK) activity. The two isoforms of IR differ by the presence (IR-B) or absence (IR-A) of a 12 amino-acid sequence at the C-terminal of the α-subunit. The presence or absence of this sequence is a consequence of an alternative splicing of exon 11 of the IR gene. IGF-1R also belongs to the RTK family and has a similar structure to IR. The structural similarity of the three receptors (IRA/B and IGF-1R) allows formation of hybrid receptors, where a monomer of individual receptors is included. IGF-2R is structurally unrelated to the receptors mentioned so far. It exclusively binds IGF-2. Neither IGF-1 nor insulin can interact with IGF-2R at clinically relevant concentrations (11,13).

Figure 5: *The structure of IR***.** a) Cartoon of the IR. The left half of the diagram indicates 22 exons of the insulin-receptor gene. The right half of the diagram shows the protein modules. L1 and L2, leucine-rich repeat domains, CR, cystein-rich domain; Fn0 (FnIII-1), Fn1 (FnIII-2), Fn2 (FnIII-3) fibronectin type III domains; Ins, insert domain; TM, transmembrane domain; JM, juxtamembrane domain; TK, tyrosine-kinase domain; CT, carboxy-terminal tail. b) The supra-domain organisation of the insulin receptor with marked disulphide bridges. The sequence corresponds to IR-B. Adapted from **(54)**.



1.4.1 Insulin-like growth factor 2 receptor (IGF-2R)

IGF-2R is a type I transmembrane glycoprotein receptor. It comprises a large extracellular subunit, a small transmembrane domain (23 amino acid residues) and a cytoplasmic domain (167 residues). The extracellular part consists of a signal sequence (40 amino acids) and 15 homologues' repeat domains containing between 124 and 192 amino acids (55). Truncation of the transmembrane region leads to the formation of a soluble form of the receptor (56).

IGF-2R is also known as a cation-independent monosa-6 phosphate receptor, as it binds manosa-6-phosphate with high affinity. Therefore, it can interact with lysosomal enzymes, other growth factors and cytokines. Individual ligands have their distinct binding sites (57,58). The binding site for IGF-2 is located in domain 11 of the extracellular region (59,60). The structure of the complex of IGF-2 with domain 11 was already solved (61). IGF-2R seems to be expressed ubiquitously with high expression during development, especially in sites where IGF-2 is also expressed. The soluble form of IGF-2R levels are high in infancy, during pre-pubertal life and fall in adolescent and adult life, with the exception of pregnancy when the IGF-2R levels are significantly elevated (62). Most of the newly synthesised transmembrane receptor is inside the cells around the *trans*-Golgi network and endosomal compartment, being redistributed to the cell surface following phosphorylation of Tyr26 (an activating signal) (55,63,64).

IGF-2R modulates the amount of circulating or tissue IGF-2 by binding the molecule and its subsequent degradation. In so doing, it decreases the biologically active form of IGF-2 and acts as a clearance receptor or a natural scavenger. Several studies emphasised the signalling through IGF-2R (64,65). A possible mechanism is interaction with the G-protein coupled receptor (GPCR). However, there are still many questions needing to be answered. Thus, the signalisation through IGF-2Rremains poorly understood (66).

1.4.2 IGF-2R specific analogues

Studies with IGF-2 analogues showed that substitution in particular positions could lead to analogues with favourable affinity to IGF-2R. One of these positions is Tyr27, where the introduction of Leu instead of the original Tyr (Leu27IGF-2) led to an analogue with a high affinity to IGF-2R and almost no affinity to IGF-1R. Leu27IGF-2 blocks IGF-2 removal and increases bioavailability of the wild-type circulating IGF-2 (67–69). By introducing a negative charge (Glu) at this position, the affinity to IGF-2R significantly decreased (13%) (67,70). Similarly, the substitution of neighbouring amino acid, Phe26, by Ser led to a 2-fold decrease in affinity to IGF-2R, but a 5- and 25-fold decrease in affinity to IGF-1R and IR, respectively (69).

A reported interesting position is Val43, where a substitution of Val for Met with S-methyl thio-ester in its side chain nearly did not affect the affinity to IGF-2R (72%), but destroyed the

affinity to IGF-1R and IR-A (below 1 %) (71). By introducing Leu at this position, the affinity to IGF-2R was also nearly unaffected and the affinities to IGF-1R and IR were destroyed (69).

Another important position for IGF-2R affinity is Phe19. The substitution of the original Phe by Ala led to no affinity to IGF-2R and decreased affinities to IGF-1R (40%) and IR-A (30%) as compared to the wild-type IGF-2. On the other hand, introduction of the amino acid with a non-polar aliphatic side chain, Leu, at position 19 led to an analogue with a 6-fold higher binding affinity for IGF-2R compared to the wild-type IGF-2. The affinities of Leu19IGF-2 to IGF-1R and IR-A were 70 and 80 %, respectively (71).

A sequence important for IGF-2R binding could be Phe48-Arg-Ser50. Substitution of this sequence by corresponding amino acids present in insulin (Thr-Ser-Ile) led to almost no affinity (<1%) to IGF-2R. The introducing of a positive charge (Arg) at positions 54 and 55 following the pattern in IGF-1 had a similarly devastating effect on IGF-2R affinity. Interestingly, the affinities to IR and IGF-1R were almost unaffected, or slightly increased, in all the aforementioned analogues (69).

Delaine et al. took advantage of studies published to date and performed a comprehensive mutagenesis study probing putative amino acids that are crucial for proper binding to IGF-2R. They prepared 14 novel analogues and defined a new binding surface which contributes to the binding interaction. The binding surface encompasses Thr16, Phe19, Asp52, and Leu53. These residues form a compact patch that is adjacent to residues Ala54 and Leu55 previously shown to be important for proper binding. Attention should particularly be drawn to Thr16 that represents a major difference between IGF-2 and IGF-1. Thus, the authors supposed it could be a major determinant of binding specificity for IGF-2R (72).

1.4.3 Insulin receptor (IR)

As mentioned earlier, insulin receptor (IR) is a predominant actor in mediating insulin activity. IR is a member of RTK which crosses the membrane once and its intracellular TK domain is inactive in the absence of the ligand. IR is a covalently bonded homodimer, regardless of the presence or absence of the ligand, which has a low basal “ligand-free” TK activity (73). The binding of a ligand in the extracellular region leads to activation of the TK domain and the triggering of the signalling pathway.

1.4.3.1 Biosynthesis and structure

A gene for the IR is located on chromosome 19 and contains 22 exons and 21 introns. IR occurs in two isoforms, A and B. The two isoforms differ in the presence (IR-B) or absence (IR-A) of a 12 amino-acid sequence that is a consequence of an alternative splicing of exon 11. Individual isoforms differ slightly in their affinity to insulin, but differ significantly in their affinity to IGF-2 when IR-A only is a high affinity receptor for IGF-2 (74). On the other hand, IGF-1 binds both isoforms of IR with low affinity. Interestingly, exon 11 has been found exclusively in mammals, which suggests functional diversification during evolution (13). The expression of the IR gene is strictly regulated on various levels during the biosynthesis (reviewed in (75)).

Each monomer subunit of the IR is synthesised as a single chain precursor that is further glycosylated, folded and forms dimers under guidance of chaperones (76). The precursor is subsequently transported to the Golgi apparatus where it is cleaved by furine protease to yield a mature receptor ($\alpha_2\beta_2$) (See Figure 5) (11).

The α-subunit (723 amino acids) and a part of the β-subunit (194 amino acids) form an extracellular region of the IR monomer (ectodomain). The β-subunit further flows through a membrane as a single transmembrane chain into the intracellular space, where it forms a cytoplasmic domain comprising 403 residues. The N-terminus of the α-subunit consists of 2 homologues leucine-rich repeat domains, L1 and L2, separated by a cysteine-rich (CR) region. The CR region contains 7 repeat domains, each comprising approximately 150 amino acids with 1 or 2 disulphide bonds (77,78). The L2 domain is followed by 3 fibronectin type III domains (FnIII-1, FnIII-2 and FnIII-3). The middle fibronectin domain (FnIII-2) is divided into 2 distinct regions by an insert domain (ID domain, 120 amino acids). ID connects the C-terminus of the α-subunit with the N-terminus of the β-subunit via a single disulphide bond, Cys647–Cys860 (IR-A sequence; see Figure 5). In the ID domain, IR-B contains an additional sequence of 12 amino acids inserted in an αC-terminal peptide (αCT-peptide) between positions 716 and 717 (that of IR-A).

Two monomers of the extracellular part of the IR are linked via disulphide bridges at Cys524 in FnIII-1 and Cys682, Cys683 and Cys685 in the ID (79). The ectodomain of each receptor monomer forms an inverted V-arrangement, where L1, CR and L2 domains form one part (leg) and 3 FnIII domains form the other part. Both monomers are oriented in an anti-parallel symmetry to the cell surface (see Figure 6) (13,77,80).

Inside the cell, the juxtamembrane region contributes to the docking of receptor substrates as well as to receptor internalisation (81,82). The intracellular part of the receptor further comprises a domain with TK activity followed by a C-tail. The domain with TK activity contains a catalytic loop that is autophosphorylated in response to insulin binding. Activation of this loop triggers the signalling pathways in the intracellular space.

1.4.3.2 IR isoforms A and B

IR is expressed not only in insulin-sensitive tissues but also in other tissues such as brain, kidney, pancreatic acini, erythrocytes and many others, where it probably mediates insulin or IGF-2 pleotropic functions. The highest human expression of IR is evident in the adipose tissue, with approximately 60 % of IR being isoform B (83,84). A high expression level of IR was further identified in liver, heart and lung. Lower IR expression was found in muscle, brain, spleen and placenta. The highest relative representation of isoform B is in the liver and muscles, where it reaches about 80 % of the total amount of IR. On the other hand, exclusive IR-A production was identified in lymphocytes (84).

Whereas IR-A is expressed ubiquitously, IR-B is expressed preferentially in tissues contributing to the target metabolic effects of insulin (insulin sensitive tissues) (13,85). IR-A has an essential role during embryonic development, when it contributes to growth-promoting effects by mediating IGF-2 activity. The role of IR-A in embryonic development was also confirmed in the study published by Louvi et al (86). They analysed dwarfing phenotypes resulting from targeted mutagenesis of genes encoding IGFs and their cognate receptors. The most devastating effect on growth (~30 % of normal weight) was observed in mice lacking both receptors (IGF-1R and IR), both IGFs or IGF-1R and IGF-2. The absence of only IR led to growth retardation of 10-15 %. Interestingly, the destructive growth retardation caused by the absence of IGF-1R (50 %) was fully prevented (93 % of normal weight) in animals concurrently lacking IGF-2R, indicating that the interaction of IGF-2 and IR was sufficient for growth development.

A tight control of IR expression, expression of individual isoforms as well as their ratio is crucial for the proper functioning of the organism. Deregulation of the system is associated with various pathological conditions (87–89). Several players involved in the regulation of IR-A/B expression were reported recently. These include, for example, hormones (90),

growth factors and insulin levels (91), glucose level (92) and splicing factors expressed in developmental and tissue-specific manner (93,94). Despite significant discoveries being made (reviewed in (13,75)), the exact mechanism leading to alternative splicing remains poorly understood.

Individual isoforms differ slightly in their affinity to insulin, with IR-A possessing the higher affinity (95–97). As previously mentioned, both isoforms markedly differ in their affinity to IGF-2, where IR-A only is a high-affinity receptor for IGF-2 (74). This difference is crucial for the whole insulin/IGF system. The binding affinities are shown in

Table 1. The other potentially important difference between both isoforms is the rate of internalisation and recycling time triggered upon ligand binding (see below) (95,98,99). Several studies also reported different signalling pathways upon insulin interaction with individual isoforms (A/B) (99,100).

Table 1: *Binding affinities of insulin, IGF-1 and IGF-2 to IR and IGF-1R.*

	IR-A		**IR-B**		**IGF-1R**	
	K_d (nM)	Relative affinity (%)	K_d (nM)	Relative affinity (%)	K_d (nM)	Relative affinity (%)
Insulin	0.25-0.55[a]	100%	0.67[a]	100%	290[b]	<0.1%
IGF-1	24[a]	1 %	220[a]	< 1%	0.24[b]	100%
IGF-2	2.9[a]	9 %	36[a]	2 %	2.3[a]	10%

[a] Reference (101)
[b]Reference (102)

1.4.4 Human insulin-like growth factor 1 receptor (IGF-1 R)

IGF-1R is a ubiquitously expressed homodimeric tyrosine kinase receptor that is essential for normal growth, differentiation and proliferation and other physiological conditions. Deregulation of this receptor is associated with cancer and other pathologies (103,104).

IGF-1R is an essential receptor for mediating IGF-1 activity. However, the receptor can also bind IGF-2 with an affinity similar to that of IR-A (see Table 1). As already mentioned, there is notable structural homology between IR and IGF-1R. Both receptors are disulphide-linked homodimers ($\alpha_2\beta_2$) with identical domain organisation and sequence homology reaching from 45 to 65 % in ligand binding region and from 60 to 85 % in the TK domain (13,78).

The complementary DNA for IGF-1R was first sequenced in 1986. leading to the determination of its primary structure (105). The human IGF-1R gene contains 21 exons, with

10 leading to α-chain and 11 to β-chain formation. There is no evidence of an exon that would be an equivalent to exon 11 in the IR gene. The receptor precursor contains 1367 amino acids, including an N-terminal signal peptide (30 amino acids). A furin protease cleavage site (Arg-Lys-Arg-Arg) is located at residues 708–711 and the cleavage results in the production of an α-chain (residues 1 – 707) and a β-chain (residues 712 – 1337). Analogues to IR, the α-chain together with 195 residues of the β-chain form the extracellular domain which contains several potential N-linked glycosylation sites (16 sites). The extracellular domain is followed by a single transmembrane chain (comprising residues 906 – 929) and a 408-residue intracellular domain with the TK activity (106).

To further probe the extracellular and especially the binding region, the IGF-1RΔβ construct was introduced as a model of the ectodomain (107). Structural analyses revealed that the quaternary structure of the IGF-1RΔβ ectodomain construct exhibits the same folded-over conformation as IR-Δβ. However, two differences were reported. Firstly, the site of the membrane entry (C-termini of domains FnIII-3 and FnIII-3') are closer in IGF-1RΔβ (67 Ǻ) than in IR-Δβ (115 Ǻ), making the overall shape of IGF-1R more compact. The cumulative differences in orientation between consecutive domains in the frame of one monomer result in the 17° deflection in the alignment of the L1 domain to the Fn-III-2 domain of the adjustment monomer (marked as FnIII-2') (108).

Secondly, the position of αCT'-peptide residues (698 to 704) is located C-terminally to the αCT'-helix (648 to 696). These residues pack against the surface of domain FnIII-2' in the case of IGF-1R. On the contrary, the axis of the IR αCT'-helix (694 to 710, His710 corresponds to His697 in IGF-1R) is probably directed away from the domain FnIII-2' in IR-Δβ (80,108,109).

1.5 Ligand- receptor interaction

1.5.1 Insulin-Insulin receptor interaction

Insulin-receptor interaction is a complex process showing negative cooperativity (110). The insulin molecule has two binding sites on its surface. The first is termed as a classic binding site and comprises predominantly hormone dimer-forming residues. The second consists predominantly of hormone hexamer-forming residues. Two binding sites are also present at each receptor monomer subunit in its extracellular part (see Figure 6). Binding site 1 on the

insulin receptor includes αCT-peptide from one α-receptor subunit and the central β-sheet of L1 domain from the other α-receptor subunit (L1') (111–113). Binding site 1 has a relatively high affinity for insulin (K_d of 6.4 nM). Binding site 2 is located in the FnIII-1 domain, and L2 domain contributes to the interaction. Site 2 has a markedly lower affinity to insulin, with a dissociation constant being about 400 nM (See Figure 6a) (114–116). To date, it has been assumed that binding site 2 is localised at the junction of FnIII-1 and FnIII-2 domains (80,116,117). However, recent data have indicated that the FnIII-2 domain may be too far to be involved in the interaction (114). Newly proposed site 2, however, corresponds to restricted interacting residues in insulin, which is in conflict with the results of mutagenesis studies (118,119). Thus, the precise location of site 2 remains open to discussion. Further discussion and potentially studies are needed to shed more light on this issue.

For successful binding and subsequent signal transduction, one insulin molecule has to interact with both types of binding sites and crosslink both monomers in a specific manner (see Figure 6 B and E). The classic binding site of insulin interacts with site 1 on the receptor. Binding site 2 of insulin interacts with site 2 on the insulin receptor (FnIII-1/2, L2 domains), located at the α-subunit opposite to that contributing to L1 interaction at site 1.

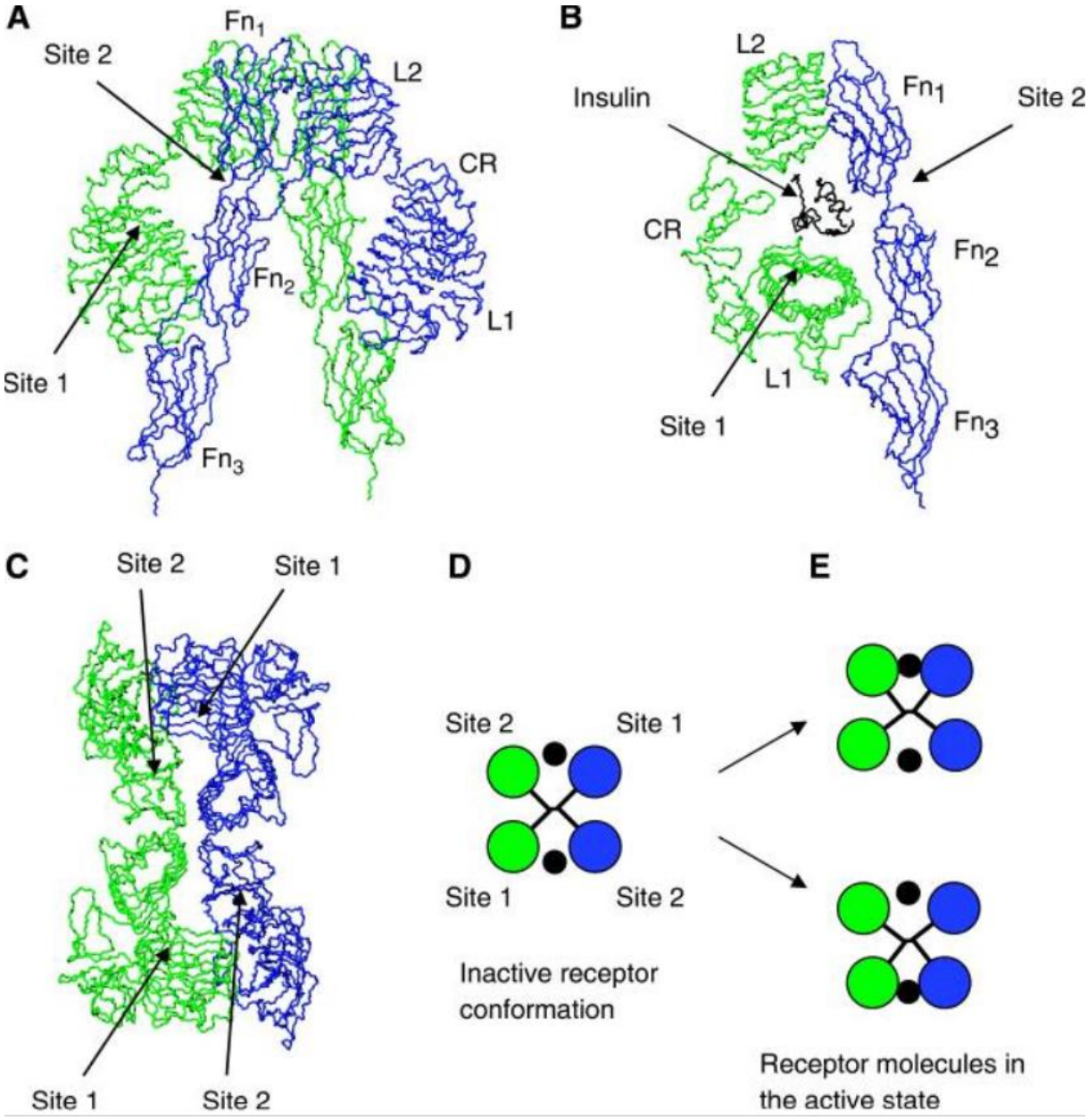
It is assumed that interaction with binding site 1 induces conformational changes in insulin and receptor molecules, leading to the high affinity interaction with K_d of 6-200 pM (120). Concurrently, the high affinity crosslink between site 1 and 2' (binding site of the receptor monomer counterpart) reduces the ability of the receptor to interact with another ligand molecule at alternative binding sites 1' and 2 (negative cooperativity).

Effective interaction in the extracellular domain concurrently triggers conformational changes, which result in activation of the intracellular TK domains. Initial steps in signal transduction are probably triggered by the shift of the αCT-peptide and subsequent conformational changes in the insert domains of both receptor monomers (114). According to the current state of knowledge, insulin probably causes both crucial steps in the interaction, the rearrangement of the L1-CR-L2 part and recruitment of the αCT-helix (114). However, further studies are needed to elucidate the precise mechanisms leading to signal propagation through the receptor (121).

Recently, several structural-analysis studies elucidating insulin-receptor interaction have been published (109,114,117). A crystallographic study using receptor constructs as models of the ectodomain studied the interaction at site 1 in detail (117). It was confirmed that insulin

interacts with the L1 domain and αCT-peptide. The interaction with the L1 domain is, however, limited to B-chain residues. The study further revealed that the C-terminal part of the insulin B-chain is probably deflected, as αCT-helix occupies the space of its expected location. Therefore, it is expected that the conformation of insulin should differ from that of a receptor-free state. This hypothesis was already mentioned previously (122) and confirmed recently in an cryo-EM analysis of the insulin-receptor complex (114). The cryo-EM analysis demonstrated that the C-terminal region of the B-chain actually takes a different position than in the receptor-free form in order to allow B-chain core residues to interact with the αCT-helix. However, from the data obtained, it was not possible to identify which receptor monomer the αCT-peptide originated from. As both receptor monomers were implicated in the interaction, the authors endorsed the trans-binding model hypothesis generally accepted so far.

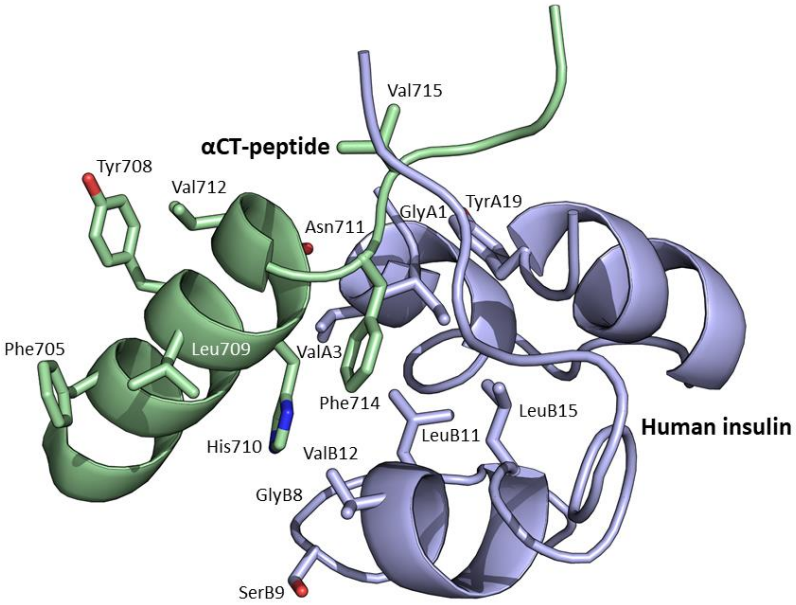
Figure 6: *Insulin receptor*. Green and blue colours represent individual monomers of IR. **A**: Crystal structure of the ectodomain (PDB code: 2DTG). **B**: Half of the IR dimer with an approximate position of insulin in the binding cavity. **C**: A view of the IR dimer as seen from the 'top'. **D**: Simplified inactive receptor conformation with insulin noted as black dots. **E**: Simplified active receptor conformation. Adapted from **(113)**



The crystallographic models revealed two positions in the αCT-peptide which are crucial for interaction at site 1. These are His710, which inserts into a pocket formed by ValA3, GlyB8, SerB9 and ValB12, and Phe714, which occupies the position surrounded by GlyA1, IleA2, TyrA19, LeuB11, ValB12 and LeuB15. A further important position could be Asn711, which is oriented to GlyA1, ValA3 and GluA4 of the insulin molecule. Hydrophobic residues at the surface of αCT-peptide (Phe705, Tyr708, Leu709, Val712 and Val713) interact with hydrophobic residues on the L1 domain (Leu36, Leu37, Leu62, Phe64, Phe88, Phe89, Val94

and Phe96) (117). Residues A8 and A21 generally included in the classic binding site probably do not have direct contact with receptor-binding site 1. However, the latest CryoEM analysis of the insulin –IR complex showed that ThrA8 is included in interactions at site 2 (114,123). The role of A21 in the binding process remains to be elucidated. A detailed picture of the aforementioned interactions of insulin with αCT-peptide residues is depicted in Figure 7.

Figure 7: *Detail of interaction of insulin with αCT-peptide* (based on PDB code: 6HN5).The complex represents interactions at site 1. αCT-peptide is shown in green and the insulin molecule is shown in light blue. The crucial amino acids are depicted with marked oxygen (red) and nitrogen (blue) molecules. L1 domain is not shown.



The interaction at site 2 is much less understood, compared to the interaction at site 1. The recent cryo-EM analysis revealed that the FnIII-1 domain (Pro495-Arg498 and Arg539-Asn541) interacts with residues in the N-terminal part of the insulin B-chain including HisB10. Further, there is an interaction between the αCT-peptide and the L2 and Fn-III-1 domains (114).

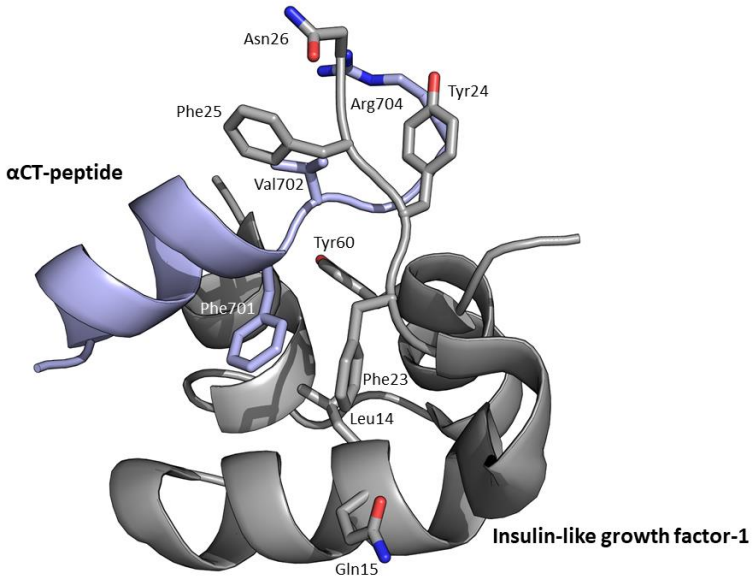
1.5.2 Interaction of IGF-1 with IGF-1R

Due to the high sequence homology of IR and IGF-1R, it is thought that the IGF-1R ectodomain has a similar conformation as IR with two binding sites in each receptor subunit. Both receptors are able to interact with all three ligands (insulin, IGF-1 and IGF-2), although

with variable affinity. Therefore, it could be assumed that IGF-1 binds to the primary binding site of the IGF-1R in a similar fashion to that of insulin interacting with IR. This assumption is further endorsed by mutagenesis studies, which revealed that binding sites on the IGF-1 surface correspond to those of insulin (118,124), although with additional interaction of the C domain of IGF-1 with the CR region of the IGF-1R which contributes to high affinity binding (125).

This hypothesis is in line with the results of the structural analysis of the IGF-1RΔβ–IGF-1 complex performed by Xu et al. Conformational changes seen upon ligand interaction with binding site 1 are similar to those seen upon interaction of insulin with IR binding site 1. The most crucial are the re-arrangement of the αCT-helix on the L1-β_2 surface and diversion of the C-terminal region of the B domain from the hormone core of IGF-1. The latter allows the B-chain residues to be engaged in interaction with αCT'-helix (108). Details of interactions with αCT-peptide are shown in Figure 8.

Figure 8: *Interaction of αCT-peptide with IGF-1*(based on PDB code: 4XSS). The picture represents details of interaction between IGF-1 and αCT-peptide from IGF-1R. αCT-peptide is shown in light blue. IGF-1 is shown in grey. Crucial amino acids are depicted with marked oxygen (red) and nitrogen (blue) molecules.

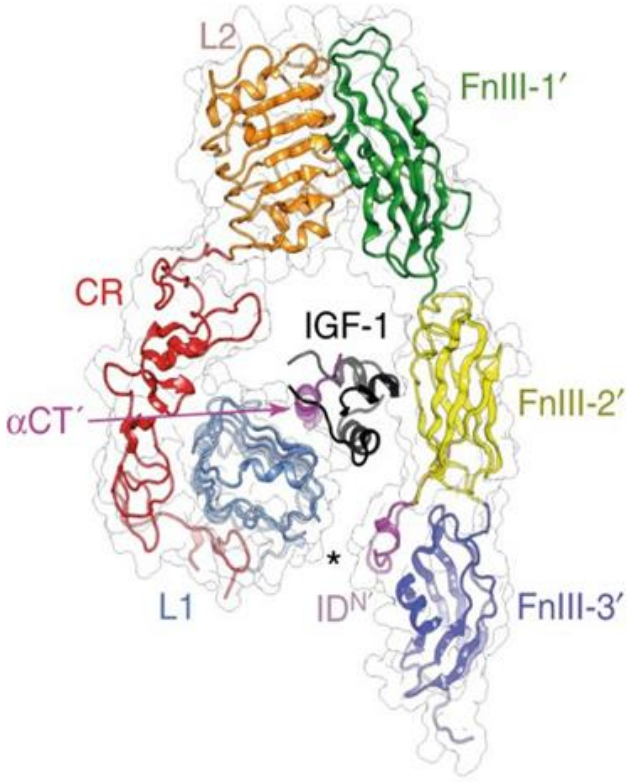


Analysis of the receptor-ligand construct further revealed that the IGF-1-L1-CR complex separates away from the FnIII-2' domain, which is effected by the hinge motion at the junction between the CR and L2 domains. The bound IGF-1 also largely interacts with the FnIII-2'domain (see Figure 9). The interface includes residues Ile583, Ser788, Asn789 and Phe790 of the receptor and Asp53, Leu54 and Arg55 of IGF-1. However, the effective

interface is sparse, thus only Leu54 is thought to contribute to site 2 interactions (108,118,126).

Only distinct pieces of information are available about the interaction of ligands with site 2 on IGF-1R as well as its precise location. Despite the high similarity between IR and IGF-1R, differences in site 2 interactions are probably more pronounced than those seen in interactions at site 1 (121).

Figure 9: *IGF-I between the site 1 components L1 and αCT′ and FnIII-2′.* The separation (asterisked) of the L1-CR module away from FnIII-2′ and $ID^{N'}$ is noted by asterisk.Adapted from **(108)**



1.5.3 Interaction of IGF-2

IGF-2 binds IR as well as IGF-1R with high affinity, but lower than those of maternal ligands (i.e. insulin and IGF-1). To date, there is no published structure of IGF-2 in complexes with IR or IGF-1R. Information regarding the receptor affinity is mainly based on mutagenesis studies (127,128). However, the high sequence and structural homology of IGF-1 and 2 led to the assumption that the mechanism of binding to and consequently activation of the IGF-1R is similar to those of IGF-1. Interaction with the IR is also proposed to be roughly the same as

that of insulin. However, there must be differences in binding of IGF-2 to IR-A in comparison with insulin, because different signalling pathways were reported upon the binding of IGF-2 to IR-A. While stimulation with IGF-2 led to growth-promoting effects in murine fibroblasts over-expressing human IR-A, glucose uptake was activated upon stimulation with insulin (74).

1.6 Signal transduction

Effective interaction with the receptor results in signal transmission from the extracellular to intracellular space. Very little is known about the signal transduction through the receptor for both IR and IGF-1R. To date, it has been demonstrated that interaction in the binding region causes a series of conformational changes in the receptor molecule. These changes lead to facilitation of ATP binding, β-subunit phosphorylation and subsequent recruitment of intracellular proteins or substrates responsible for signal propagation (13).

In the ligand-free state, TK domains are maintained at a distance preventing interaction with each other. Ligand binding enables TK domains to become in proximity, allowing mutual trans-phosphorylation of TK activation loops. During receptor activation, Tyr residues are phosphorylated in all three domains of the intracellular part, in the juxtamembrane region (Tyr at positions 953, 960, and 972), in the TK domain (1146, 1150, and 1151), and in the C-tail (1316 and 1322) where Tyr1146, 1150 and Tyr1151 are the most important for signal generation and its transmission. The positions correspond to human IR-A nomenclature (13,129–131).

The activated receptor (mainly the TK domain) forwards the signal to the receptor substrates and messengers (adapter molecules) that are responsible for its intensification and recruitment of the crucial signal molecules. Insulin substrates (IRS) and Shc represent the most important molecules transmitting the signal from the receptor. They are responsible for activating two major signalling pathways, PI3K/Akt and Ras/Erk (see Figure 10) (132).

Activation of phosphoinositol-3 kinase (PI3K) and consequently Akt kinase results in insulin-dependent translocation of GLUT4 glucose transporters in muscle and adipose tissue as a predominant metabolic effect. The activated Akt cascade, however, participates in various other biological processes with metabolic as well as mitogenic outcomes. Akt activity leads to

direct regulation of target enzymes or to regulation of gene expression at a level of transcription as well as translation (133,134).

The other major signalling pathway is mediated through the activation of Ras and subsequently Erk (extracellular regulated kinase), leading solely to mitogenic outcomes. Erk is a promiscuous kinase which could phosphorylate a huge variety of different substrates (more than 100). This signalling pathway results in the modulation of proliferation and differentiation through regulation of gene transcription (135,136).

It is generally assumed that superiority of one of the abovementioned cascades is partly conferred by features intrinsic to the ligand (insulin or IGF) and partly to the receptor (IR-A/B or IGF-1R). However, it should not be ignored that the signalling pathways are also determined by the (un)availability of the substrates and downstream targets in individual cells. Therefore, different biological outcomes can be seen after interaction of the same ligand with the same receptor in various tissues (130). Notwithstanding, the IGF/insulin system initiates different signalling pathways leading to different outcomes when these three hormones interact with the two receptors even in the same cell (74,137).

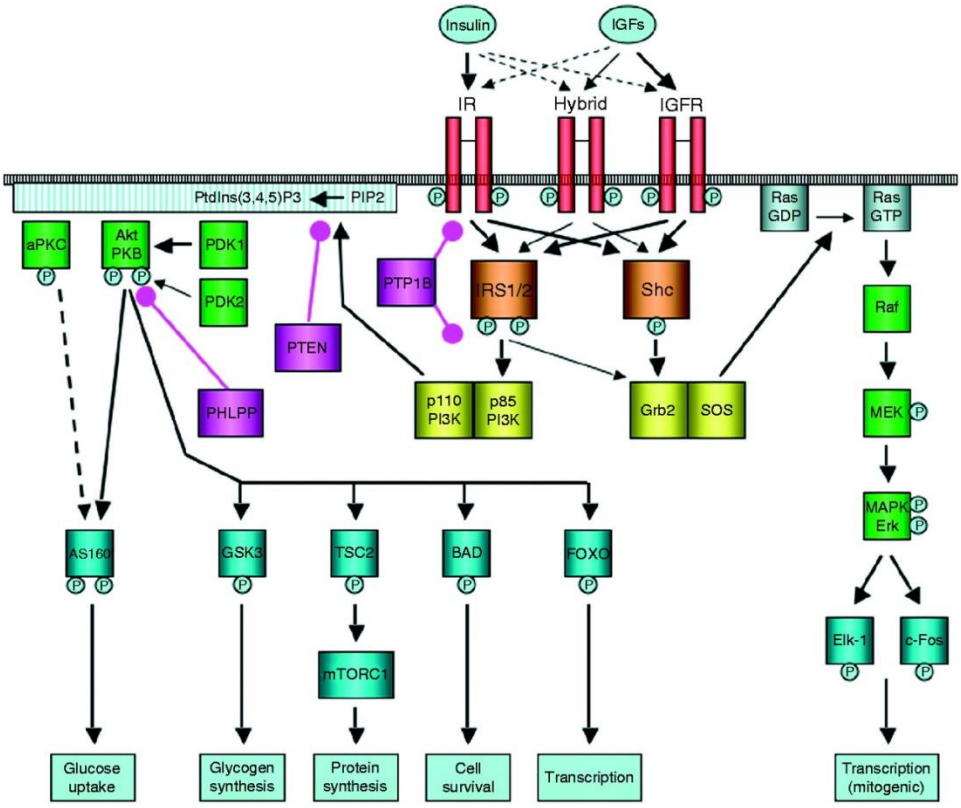
The TK domains of IR and IGF-1R are largely similar, reaching 84 % of sequence similarity. Tyr residues, which are crucial for auto-phosphorylation, are strictly conserved, as well as residues (motifs) directly interacting with protein substrates (mainly IRS and Shc) (138). However, IGF-1R and IR can differ in the efficacy of substrate recruitment. This was also confirmed by the recent study showing that the juxtamembrane region represents one of the crucial aspects for mitogenic/metabolic signalling cascade preferences (139). In addition, differences in the C-terminal (CT) region, where sequence homology only reaches 48 %, can influence the interaction with signalling proteins and thereby modulate the triggered cascades (130). Indeed, different signalling was reported through IR and IGF-1R intracellular domains when attached to the same extracellular part (139,140).

Regarding the interaction with the same receptor, the target biological outcomes are regulated by the different affinity of individual hormones. However, different affinity itself cannot explain the different signalling triggered upon the binding of individual hormones (74,141,142). Previous data indicated that signalling outcomes can be modulated by the kinetics of the binding interaction (129). Several insulin analogues with hyper-mitogenic activity showed slow dissociation kinetics and persistent receptor occupancy (143), which is in accordance with the hypothesis that the high affinity and persistent occupancy of the

receptor could prefer mitogenic signalisation. This is also consistent with the higher affinity of insulin to the more “mitogenic” isoform A of the IR. However, slower dissociation kinetics were observed in interaction with the “metabolic” isoform B (13,95). Moreover, IGF-2 binds to IR-A with meaningfully lower affinity compared to insulin, but the signalling leads to prolonged Erk activation, whereas Akt activation is prolonged after insulin binding (144). Therefore, binding affinity, persistent occupancy, and dissociation kinetics contribute to different biological outcomes, but are probably not the main reason for it. Considering that both IR isoforms have an identical intracellular part, the different signalling is caused by the minor difference in the binding region given by the presence or absence of 12 extra amino acids.

Internalisation is another feature which could distinguish individual receptors and contribute to the superiority of one of the triggered signalling pathways (98). The speed of internalisation could affect the effectiveness of phosphorylation of Shc and consequently activation of the Erk cascade. While the substrates leading to metabolic effects are probably fully activated from the receptor on the cell surface, the mitogenic pathway continues during or possibly even requires internalisation in both IR and IGF-1R (129). Several studies indeed confirmed that the internalisation is required for the signalling pathway initiated by Shc-activation but not IRS phosphorylation (reviewed in (145)). IR-A undergoes faster internalisation compared to IR-B (98). Interestingly, Morcavallo et al. reported that interaction of insulin with IR-A promotes receptor internalisation contrary to IGF-2 which only modestly influences the receptor internalisation. The authors hypothesised that the lower affinity of IGF-2 promotes lower phosphorylation and recruitment of downstream effectors, but concurrently protects IR-A from negative feedback regulation mediated by sustained mitogenic stimuli (145,146). A schema of signalling pathways triggered upon hormone receptor interaction is shown in Figure 10.

Figure 10: *Signalling pathways*. The principal components of the PI3K/Akt and Ras/MAP kinase pathways are indicated: receptors (red), tyrosine-phosphorylated substrates (orange), adaptors and transducers (yellow and grey), serine/threonine kinases (green), serine/threonine phosphorylated substrates and downstream components (blue) and negative regulators (purple). Adapted from **(132)**. PKC- kinase C, Akt– kinase B, PDK 1/ 2- 3-phosphatidylinositol dependent kinase -1/ 2, PHLPP- PH domain and leucine rich repeat protein phosphatases, PTEN- phosphatase and tensin homology protein, PTP– protein tyrosine phosphatase, IRS- insulin receptor substrate, Shc- Src homology collagen, PI3K - phosphatidylinositol 3-kinase, Grb2- growth factor receptor binding protein 2, Sos- Son of sevenless, MEK - MAPK and Erk kinase, MAPK- mitogen activated kinase, Erk - extracellular signal-regulated kinase, AS160– Akt substrate of 160 kDa, GSK3 - glycogen synthase kinase-3, TSC2- tuberous sclerosis complex protein 2, mTORC1- mammalian target of rapamycin complex 1, BAD – Bcl-xL/Bcl-2 associated death promoter, FOXO- Forkhead box O, Elk 1- ETS Like-1 protein, Fos- oncogene originally identified as causative agent in Finkel-Biskis-Jinkins (FBJ) murine osteogenic sarcoma virus, PIP2-phosphatidylinositol-4,5-bisphosphate, PIP3- phosphatidylinositol-3,4,5-triphosphate, Ras – p21 protein, GDP–guanosine 5'-bisphosphate GTP- guanosine triphosphate



Considering all these data, binding mechanism is a complex event where the binding affinity, binding kinetics as well as ability to induce internalisation overall contribute to modulation of signalling cascades and consequently biological outcomes. Further studies are necessary to provide additional insights to contribute to the determination of crucial steps in signalling modulation.

1.6.1 Hybrid receptors

All three tyrosinekinase receptors i.e. IR-A/B, IGF-1R are co-expressed in the majority of tissue in various ratios. Due to structural homology, they can form hybrid receptors. Hybrid (insulin) receptors may comprise either different isoforms of IR (A/B) or one IR monomer (A or B) and one IGF-1R monomer.

The existence of a hybrid (insulin) receptor was already postulated by Kasuga in 1983 (147) and confirmed by Soos and Siddle in 1989 (148). Blanquart et al. showed that IR-A/IR-B hybrids are randomly formed in cells (149). Formation of hetero-receptors probably occurs with a similar effectiveness as that of homo-receptors (13). Therefore, hybrid (insulin) receptors may be more abundant than homo-receptors in some tissues.

Hybrid insulin receptor recruits the effector molecules with the same affinity as the IR-A homodimer, while maintaining the high affinity to insulin as well as IGF-2 (149). On the contrary, IGF-1R/IR hybrid receptors (HR) bind insulin with a much lower affinity than IR, while maintaining the high affinity for IGFs (96,150). These receptors probably arise stoichiometrically from IR and IGF-1R (83). However, recent data have indicated possible regulatory mechanisms (reviewed in Belfiore et al 2017 (75)).

The physiological role of HRs is still unknown. All three native ligands (insulin, IGF-1 and IGF-2) are able to stimulate phosphorylation of HR, with insulin being much less effective than IGFs. Binding of IGF-1 to either hybrid receptor (HR-A or HR-B) leads to the production of substrates specific to IGF-1 signalisation. Such substrates were also detected upon insulin stimulation of HR-A (but not upon stimulation of HR-B), which indicates a shift towards IGF-1R signalisation (151). These results are nonetheless in conflict with those published by Benyoucef et al (96). They repeatedly demonstrated that HRs are responsive to IGFs and unresponsive to insulin, regardless of the IR isoform involved. According to the authors, both hybrid isoforms (HR-A, HR-B) bind insulin with an almost similar affinity that is meaningfully lower than those of IGFs. This assumption is consistent with results of another study, where the binding properties of HRs were found to be similar to those of native IGF-1R. Thus, signalling of insulin through HRs probably has no physiological relevance (152).

1.7 Targeting the binding affinity of IGF-2 to individual receptors

1.7.1 IGF-2 analogues

Sequence homology among insulin, IGF-1 and 2 enables cross-reactivity with the target receptors. There are several amino acids which are conserved in insulin and IGFs or in IGFs only. These positions are thought to have an important impact on the structural stability or binding affinity of these hormones (see Figure 13). Nonetheless, despite this homology, each molecule binds individual receptors of the insulin/IGF family and binding proteins with different affinity and possibly uses a slightly different way when interacting with the same receptor. This subsequently leads to a diverse biological activity (74,137,141,142). Identification of the structural determinants responsible for receptor specificity is a matter of current interest, as receptor-specific analogues offer an important therapeutic application. IGF-2 is the least studied protein among the three hormones despite its high abundance (37,38). Only several targeted mutagenesis studies have been published to date (126–128).

1.7.1.1 Naturally occurring IGF-2 analogues

Rather interesting examples represent two naturally occurring IGF-2 analogues with substitution either at position 29, where original Ser is replaced by tetrapeptide Arg-Leu-Pro-Gly (Arg29-Leu-Pro-Gly29-IGF-2), or at position 33, where Ser is replaced by tripeptide Cys-Gly-Asp (Cys33-Gly-Asp33-IGF-2). Both analogues (Arg29-Leu-Pro-Gly29-IGF-2 and Cys33-Gly-Asp33-IGF-2) were identified circulating in blood. These analogues maintain a relatively high affinity to IGF-1R (85 and 39 % for Arg29-Leu-Pro-Gly29-IGF-2 and Cys33-Gly-Asp33-IGF-2, respectively) and to IGF-2R (110 and 71 % forArg29-Leu-Pro-Gly29-IGF-2 and Cys33-Gly-Asp33-IGF-2, respectively), compared to native IGF-2. The affinities allow us to get the impression that relatively significant interventions in the primary structure could be tolerated by IGF-1R and IGF-2R at these positions. The ability to incorporate thymidine correlates with IGF-1R affinity (Cys33-Gly-Asp33-IGF-2, 45 %) or is slightly lower (Arg29-Leu-Pro-Gly29-IGF-2, 77 %). These analogues are believed to be formed due to different splicing or potentially due to the presence of different alleles (46,153,154).

Table 2 : *The affinity of naturally occurring analogues to IGF-1R and IGF-2R*

	IGF-1R [%]	**IGF-2R [%]**	**Reference**
IGF-2	100	100	
Arg29-Leu-Pro-Gly29-IGF-2	85	110	(153)
Cys33-Gly-Asp33-IGF-2	39	71	(153)

1.7.1.2 N- and C-terminal analogues

Several mutagenesis studies examined the importance of both N- and C-terminal amino acids. It was shown that removal of one or two N-terminal amino acids did not significantly reduce or even slightly increased the affinity to IGF-2R and IGF-1R (29). Conflicting results were reported for analogues lacking six N-terminal amino acids. Lüthi et al demonstrated that the removal of the six amino acids resulted in a decrease in the affinity to IGF-2R (25 % of IGF-2) while the affinity to IGF-1R remained almost unaffected (96 %) (153). Similar affinity for IGF-1R (79 %) was reported by Hashimoto et al (155). Slightly different results were obtained by Forbes et al. (2002) when des(1-6)IGF-2 had approximately 40 % of the affinity of IGF-2 when using recombinant human IGF-1R (rhIGF-1R) and BIO-core technology (68). The observed differences could have been caused by the different methods used, as slightly different affinities were previously reported with soluble and membrane receptors. Nonetheless, in all cases, the des(1-6)-IGF-2 still maintained relatively high affinity to IGF-1R. Removal of the next one or the next two amino acids (resulting in des(1-7) and des (1-8)-IGF-2) led to a rapid decrease in the affinity to IGF-1R. Hashimoto et al. focused on position 8. The substitution of Leu8 by Gly alone resulted in a similar effect as the removal of the initial 8 amino acids (binding affinity less than 1 %) (155). This indicates that Leu8 has an important role in retaining the affinity to IR and IGF-1R. Conflicting results have been reported for affinity to IGF-2R. See Table 3.

Removal of the first (Ala1) and the last two C-terminal amino acids (Ser66, Glu67) resulted in an almost 2-fold reduction of the affinity to IGF-1R (40 %) compared to IGF-2 (29).

In conclusion, it was demonstrated that removal of one or two N-terminal residues does not severely impact the binding affinity. Moreover, Oh et al. demonstrated that addition of one (Trp) or three (Met-His-Trp) amino acids at the N-terminus of IGF-2 even increases the affinity to IGF-1R, leading to 140 % or 160 % of IGF-2 affinity, respectively. The affinity to IR is not affected (10 0%, Trp-IGF-2) or only slightly (70 %, Met-His-Trp-IGF-2) (29).

Table 3: *Relative binding affinities of IGF-2 C- and N-terminal analogues*

	IGF-1R [%]	IGF-2R [%]	IR * [%]	Reference
IGF-2	100	100	100	
DesAla1-IGF-2	80	140	80	(29)
Des(1-2)-IGF-2	80	110	60	(29)
Des(1-5)-IGF-2	55	350[b]	50	(155)
Des(1-6)-IGF-2	79	700[b]	41	(155)
Des(1-6)-IGF-2	96	25		(153)
Des(1-6)-IGF-2	41			(68)
Des(1-6)-IGF-2	56	42		(156)
Des(1-7)-IGF-2	3.3	1200[b]	11	(155)
Des(1-8)-IGF-2	15	23		(153)
Des(1-8)-IGF-2	0.1	0.5[b]	0.1	(155)
Gly8-IGF-2	0.3	0.9[b]	0.4	(155)
Des(Ala1,Ser66,Glu67)-IGF-2	40	120	100	(29)
Trp-IGF-2	140	90	100	(29)
Met-His-Trp-IGF-2	160	110	70	(29)

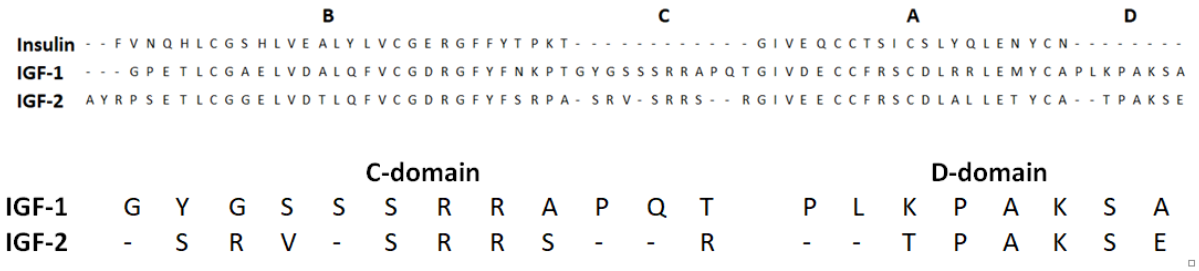
* measured on membranes from fresh human or rat placentas, blood and blood vessels
[b] Rat IGF-2/CI-P receptor

1.7.2 The importance of C and D domains of IGFs

Several positions were identified in the A- and B-chains of insulin, where substitution led to almost IGF-like activity to IR-A. Inversely, IGF-2 containing 6 amino acids (His7, Ala16, Tyr18, Thr48, Ile50, Asn58) from insulin showed a 3-fold higher affinity to IR-A as compared to native IGF-2 (128). C and D domains, however, still represent one of the major determinants suspected significantly contributing to receptor specificity and consequently the diverse biological activity of individual hormones. Mature insulin lacks C and D domains. The C-peptide of proinsulin is cleaved off during maturation and does not share any sequential similarity with the C domains of IGFs. Considering the poor affinity of proinsulin to IR, the C-peptide plays a role mainly during maturation and folding, and contributes to other physiological functions (157). D domains are unique to IGFs only.

As already mentioned, IGF-1 possesses a high affinity to IGF-1R and almost no affinity to both isoforms of IR, while IGF-2 is able to interact with IGF-1R and IR-A with high affinity (86,141,158). C and D domains of IGF-1 (C_1 and D_1 domains) are longer, compared to their counterparts in IGF-2 (C_2 and D_2 domains), but share several conserved positions (see Figure 11). The swapping of C and D domains between IGF-1 and 2 did not lead to any global structural changes (159).

Figure 11: *The comparison of sequence of individual domains in insulin and IGFs*. The C and D domains of IGF-1 and 2 are depicted in detail.



The C_1 domain in IGF-1 is crucial for binding and activation of IGF-1R, as demonstrated by the substitution of C_1 with a four-glycine bridge, which led to 30-fold loss of affinity to IGF-1R. The binding to IR, IGF-2R and binding proteins were unaffected, indicating that the loss of affinity was not a consequence of the loss of the original tertiary structure, but rather due to the importance of the C_1 domain in binding to IGF-1R and its activation (160). Attachment of the C_1 domain to the C-terminus of the insulin B-chain also increased the affinity to IGF-1R. The two-chain hybrid of insulin and IGF-1 reached from 11 % (161) to 20 % (162) of the IGF-1 affinity. Not surprisingly, the addition of the C_1 domain to the B-chain resulted in a decrease in the affinity to soluble IR (2-fold) (161), as well as the membrane anchored receptor (162). However, when the C_1 domain connected B and A domains, following a pattern seen in IGFs and proinsulin, the affinity to IR-A was retained (113 %), together with high affinity to IGF-1R (19-28 % of IGF-1) (163).

The necessity of the presence of the C_1 domain for proper binding to and activation of IGF-1R was further confirmed in a study with IGF-1/IGF-2 hybrid analogues. While the presence of the D_1 domain in IGF-1 had only little impact on the binding affinity and activation of IGF-1R (159,160), the substitution of C_1 with C_2 domains decreased the affinity of the IGF-1 analogue to IGF-1R and its activation close to those of IGF-2. Surprisingly, both domains (C_1 and D_1) equally contributed to the increasing affinity of IGF-2 analogues to IGF-1R (2.9- or 2.6-fold higher affinity for C and D domains, respectively, as compared to native IGF-2) (159).

The C_2 and D_2 domains proved to be important for binding to IR-A, as they increased the binding affinity of the IGF-1 analogue 1.9-fold (C_2 domain) and 1.5-fold (D_2 domain). The contribution of both domains was additive and resulted in an affinity close to that of IGF-2. The IGF-2 hybrid containing C_1 and D_1 had only a slightly higher IR-A affinity than IGF-1. The effect on activation of IR-A followed the pattern of binding abilities but was more

pronounced. C and D domains also regulated affinity to IR-B in a similar way. However, the affinity to isoform B was lower than that to isoform A (159).

Henderson et al (2015) provided a more detailed insight into the C_2 domain. They preserved the amino acid motif conserved in both IGFs (Ser36/35, Arg37/36 and Arg38/37 numbering corresponds to IGF-1/2) and substituted C- or N-terminal flanks of the C_2 domain with amino acids from the C_1 domain or shortened C_1 domain to be similar in size to C_2, while retaining its original charge distribution (see

Figure **12**). The presence of either N- or C-terminus of the C_1 domain had only little impact on IR-A affinity, but the affinities to IGF-1R were close to those of IGF-1, with the C-tail being slightly more effective. Interestingly, insertion of part of the C_1-domain only (C- or N-tail; C_1N and C_1C) led to a higher affinity to IGF-1R than observed in IGF-2C_1. Shortening of the C_1 domain (8 amino acids; C_1S) resulted in a decrease in the IR-A affinity below that seen in IGF-2C_1, as well as meaningful reduction of IGF-1R affinity (164). The decrease of IGF-1R affinity as compared to IGF-2C_1 (6.5-fold) was accounted for by the absence of Tyr31,which has been shown to reduce IGF-1 binding to IGF-1R 6-fold (165). The affinities are summarised in Table 4.

Figure 12: *C domains of IGF-2 analogues.* Based on (164).

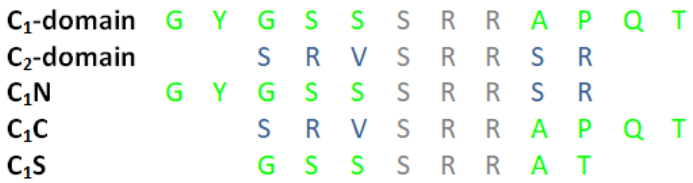


Table 4: *Relative affinities of IGF-2 analogues to IR-A and IGF-1R*

	IR-A IC_{50} relative to IGF-2(%)	IGF-1R IC_{50} relative to IGF-2(%)
IGF-2	100	100
IGF-2C_1N	79[a]	158[a]
IGF-2C_1C	87[a]	190[a]
IGF-2C_1S	43[a]	21
IGF-2C_1	49	136[a]
IGF-1	23	211
Insulin	492	1

[a] not significant when compared to IGF-2

The presence of the D_1 domain in the insulin molecule decreased the binding potency to IR to one-third (measured on rat adipocytes) and the ability to stimulate glucose oxidation to 20 % of the activity of native insulin. On the other hand, the ability to incorporate thymidine into

the DNA of human fibroblast was 2.8-fold higher than that of insulin (166). On the contrary, the D_2 domain itself was not sufficient to increase the mitogenic potency of insulin (167).

The contribution of both domains needs to be further investigated. Nevertheless, on the basis of the observed results, the C and D domains of both IGFs play a crucial role in receptor specificity. Regarding IGF-1R, the role of the C_1 domain exceeds the role of the D_1 domain. The C_2 domain promotes the affinity to IR-A compared to the C_1 domain (168). The presence of the D_1 domain has a detrimental effect on the affinity to IR-A and leads to an increase in the mitogenic potential, while the D_2 domain fails to have any mitogenic effect (167).

1.7.3 Insulin/IGFs substitution leading to disproportionate binding and activation ability

Modulation of biological activity of the insulin/IGF-system could be aimed either at binding-specific analogues or by the determination of the aspects leading to modification of signal intensity when the binding potency is not affected or only slightly affected. Binding studies as well as signalling studies provide us with valuable pieces of information about hormone/receptor interaction and overall modulation of the system.

Due to the long-term research in this field, there are standardised assays of binding potency providing accurate and reliable results. On the other hand, the interpretation of signalling assays could be quite intricate. There are many factors which should be taken into consideration. The main shortcoming is that commonly used cell models over-express the target receptors, thus, the intensity of signalling could also be affected by the cell equipment. Nonetheless, despite these limitations, signalling studies are invaluable for further research. Specific antagonists of IR-A and mainly IGF-1R could serve as a useful tool in the treatment of a wide spectrum of diseases, as several types of cancer with poor prognoses are accompanied by IR-A and IGF-1R over-expression (89).

Schaffer et al. (169) prepared a series of peptides targeting site 1 or site 2 of the insulin receptor. They showed that covalent linkage of these fragments can lead to agonists or antagonists of the IR, depending on the linkage pattern. Consequently, Whittaker and colleagues (170) followed up their results and focused on insulin analogues with disproportionately impaired TK activation and binding potency. The insulin analogue with His substitution at positions A4 and A8 (HisA4HisA8-insulin) showed a native IR binding

affinity, but poor ability of IR activation. In human insulin, Glu and Thr are at positions A4 and A8, respectively. Glu at position A4 contributes to the classic binding site (binding site 1) (22,171). This position has not been strictly conserved during development. However, only mild mutations keeping the negative charge (Asp) or similar size (Gln) can be seen in the insulin of other vertebrates (28). By the introduction of positively-charged His at this position, binding affinity, together with the ability to stimulate Akt activation and IR autophosphorylation, significantly decreased (170). Whittaker and colleagues proposed that interaction of GluA4 with Asp707 in the αCT-segment of the insulin receptor probably contributes to the conformational changes leading to receptor activation.

Position A8 is less conserved. A wide pattern of amino acids is presented in vertebrate insulin. Introduction of His with the positive charge and bulkier side chain led to higher affinity to soluble human IR, also with higher efficacy of Akt activation and IR autophosphorylation (170). ThrA8 was considered as participating in the binding interaction on the basis of mutagenesis studies. The latest CryoEM analysis of the insulin –IR complex showed that ThrA8 is included in interactions at site 2 (114,117,119,123).

GluA4 in the insulin molecule has its equivalent in both IGFs, Asp45 (IGF-1) and Glu44 (IGF-2). The presence of Asp or Glu is in the frame of variability observed in the insulin of vertebrates (28) and further highlights the significance of the position within the insulin-IGF system.

To date, no analogue of IGF-1 or IGF-2 has been reported that acts as an antagonist of IGF-1R. The interesting properties of HisA4HisA8-insulin open up a further opportunity for analysing positions in IGF-1 and 2, which could lead to the modulation of antagonistic or agonistic properties.

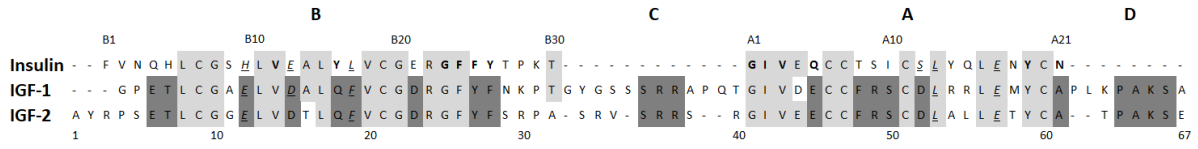
1.7.4 Interaction of IGF-2 with binding site 2 of the receptor (IGF-1R /IR)

To date, there is no structure of the IGF-2/receptor (IR or IGF-1R) complex. It is proposed that IGF-2 uses similar mechanisms as insulin or IGF-1 when binding to cognate receptors. However, there are probably differences in the contribution of individual residues (126,128).

While the importance of site 1 is evident, it is still not clear how site 2 is important in the IGF-IGF-1R/IR interaction. This question is endorsed by the fact that IGFs bind a hybrid receptor with high affinity comparable to that of IGF-1R (96).

Alvino and colleagues (126) took advantage of sequence and structural similarity between insulin and IGFs and proposed IGF-2 site 1 and site 2 residues, inspired by known receptor-binding surface of the insulin molecule. The site 1 surface includes Val14, Gln18, Gly41, Ile42, Glu45, Phe48, Tyr59, Ala61, Gly25, Phe28, and residues Val43, Phe26, Tyr27 that were previously shown to be important for IR and IGF-1R and also IGF-2R binding (69). Site 2 residues supposedly consist of Glu12, Asp15, Phe19, Asp52, Leu53, and Glu57 (see Figure 13). The authors focused on the less probed area of site 2 residues. They prepared 6 analogues with Ala substitution of original residues and characterised their binding affinity to receptors (IR-A and IGF-1R) and the ability of receptor activation. All analogues showed a significant decrease (more than 2-fold) in binding affinity to IR-A as well as to IGF-1R, except Ala substitution of Asp52 which led to a slight increase in affinity to both receptors (approx. 130 % of the native affinity of IGF-2 for both receptors). Only a mild effect was observed in Ala53-IGF-2 for the affinity to IR-A (90 %) and in Ala19-IGF-2 for the affinity to IGF-1R (112 %). However, it should be mentioned that there was a marked difference in affinity to soluble and membrane-anchored receptors for both analogues, when the affinity to the soluble form was significantly decreased (more than 2-fold) in both cases. Substitution of Asp15 unexpectedly led to a marked decrease (more than 2-fold) in affinity to the mini IGF-1R construct that contains binding site 1 only. Furthermore, Asp15 is adjacent to Val14, Gln18 and hydrophobic residues that form site 1 (Val14, Phe28, Val43, Phe26 and Tyr27). Thus, it is proposed that Asp15 contributes to interaction at site 1 rather than at site 2. Unfortunately, the low affinity of IGF-2 to insulin the mini-receptor construct precludes evaluation of the interaction with site 1 only in IR.

Figure 13: *The amino acid sequence alignment of IGF-2, IGF-1, and insulin*. Insulin residues important for IR binding and defined as site 1 residues (ValB12, TyrB16, GlyB23, PheB24, PheB25, TyrB26, GlyA1, IleA2, ValA3, GlnA5, TyrA19, AsnA21) are shown in bold type and those defined as site 2 residues (HisB10, GluB13, LeuB17, SerA12, LeuA13, GluA17) are underlined and in italics. IGF-1 and IGF-2 site 2 residues are underlined and in italics. Conserved residues are boxed in light grey, residues conserved between IGF-2 and IGF-1 are boxed in dark grey. Based on **(127)**



The role of Glu12 seems to be particularly interesting. In an follow-up study (127), Alvino and colleagues demonstrated that Glu12 is crucial for high affinity binding to IGF-1R, as well as IR-A. The corresponding position in the insulin molecule is occupied by HisB10 which has an irreplaceable role in Zn^{2+} coordination and overall insulin activity. Substitution of HisB10 to negatively-charged Asp or Glu led to "super-active insulin" with markedly enhanced mitogenic activity (172–174). Glutamic acid is in the same position in IGF-1 as well, (Figure 13), which highlights the importance of the negative charge at this position for high mitogenic activity. Introduction of mild substitution into IGF-2, keeping negative charge (Asp) or similar size (Gln), led to a significant decrease in affinity to IR-A as well as IGF-1R (20 - 40 % of the affinity of native IGF-2). Surprisingly, Asp was better tolerated than Gln at IR-A at this position (40 and 24 %, respectively). Positively-charged substitutions (Lys, Arg, His) had a detrimental effect on the IGF-1R affinity (less than 12 % of the native affinity of IGF-2) and were better tolerated on IR-A (32-35 %), with the exception of Arg (9 % of the native IGF-2 affinity) (127).

Negative charge is also important for maintaining the ability of receptor activation. Asp12IGF-2 was only slightly less potent than native IGF-2 in receptors' phosphorylation (80 % of the maximum response). Other investigated amino acids (Gln, His, Lys, Arg, Ala) were significantly less able to activate both receptors (IR-A and IGF-1R). The ability of receptor activation overall mirrored the binding affinity However, the impact of several substitutions was more apparent on the ability of receptor activation than on the binding affinity. These include Ala, Lys and Arg substitutions that showed a greater decrease in ability to activate both IR-A and IGF-1R than expected on the basis of binding affinities.

Furthermore, despite the similar affinity of Lys12IGF-2 and His12IGF-2 to IR-A, Lys12IGF-2 was significantly weaker in activation of the receptor. Interestingly, Lys12 substitution also had a lower impact on IR-A affinity than on IGF-1R binding (32 versus 7 % of IGF-2 affinities, respectively), but the effect on IR-A activation was even more detrimental.

Thus far, there are only a few analogues probing the affinity to binding site 2 or exploring the binding surface of site 2 on the IGF-2 molecule. Further analogues could shed some light on the importance of site 2 and consequently expand our knowledge about IGF-2 and its physiological role.

2 RESEARCH AIMS

- Completion of the set of insulin analogues with added amino acids derived from the C_2 domain and evaluation of the impact of C_2, D_1, and D_2 domains of IGF on the affinity to IR and IGF-1R and the ability of their activation
- Simplification of the novel method of IGF-2 production leading to a completely native IGF-2 molecule
- Completion of the set of IGF-2 analogues containing IGF-1-like motifs derived from B and C domains of IGF-1;evaluation of the impact of these mutations on IR and IGF-1R potency
- Preparation of IGF-2 analogues with substitution in positions 44, 45 and 48 and their characterisation with a focus on antagonistic effects
- Evaluation of the role of positions 50, 52, 53, and 57 in interactions at site 2 on IR and IGF-1R

3 PUBLICATIONS

3.1 List of publications

1. **Křížková K.**, Chrudinová M., Povalová A., Selicharová I., Collinsová M., Vaněk V., Brzozowski A.M., Jiráček J., and Žáková L. 2016. "*Insulin-Insulin-like Growth Factors Hybrids as Molecular Probes of Hormone:Receptor Binding Specificity*." Biochemistry 55 (21): 2903–13. https://doi.org/10.1021/acs.biochem.6b00140

2. Hexnerová R., **Křížková K.**, Fábry M., Sieglová I., Kedrová K., Collinsová M., Ullrichová P., Srb P., Williams C., Crump M.P., Tošner Z., Jiráček J., Veverka V., and Žáková L. 2016. "*Probing Receptor Specificity by Sampling the Conformational Space of the Insulin-like Growth Factor II C-Domain.*" Journal of Biological Chemistry 291 (40): 21234–45. https://doi.org/10.1074/jbc.M116.741041

3. Macháčková K., Chrudinová M., Radosavljević J., Potalitsyn P., **Křížková K.**, Fábry M., Selicharová I., Collinsová M., Brzozowski A.M., Žáková L. and Jiráček J. 2018. *"Converting Insulin-like Growth Factors 1 and 2 into High-Affinity Ligands for Insulin Receptor Isoform A by the Introduction of an Evolutionarily Divergent Mutation."* Biochemistry 57 (16): 2373–82. https://doi.org/10.1021/acs.biochem.7b01260.

4. Macháčková K., **Mlčochová K.**, Potalitsyn, P., Hanková K., Socha O., Buděšínský M., Muždalo A., Lepšík M., Černeková M., Radosavljević J., Fábry M., Mitrová M., Chrudinová M., Lin L., Yurenko J., Hobza P., Selicharová I., Žáková L. and Jiráček J. 2019. *"Mutations at hypothetical binding site 2 in insulin and insulin-like growth factors 1 and 2 result in receptor- and hormone-specific responses."* Journal of Biological Chemistry 294, in press

Publication not related to the topic

Palivec V., Cristina M. V., Kozak M., Ganderton T.R., **Křížková K.**, Turkenburg J.P., Halušková P., Žáková L., Jiráček J., Jungwirth and Brzowski A.M.2017. "*Computational and Structural Evidence for Neurotransmitter-Mediated Modulation of the Oligomeric States of Human Insulin in Storage Granules."* Journal of Biological Chemistry, March, jbc.M117.775924. https://doi.org/10.1074/jbc.M117.775924.

3.2 Insulin-Insulin-like Growth Factors Hybrids as Molecular Probes of Hormone: Receptor Binding Specificity.

3.2.1 Background

Although insulin and both IGFs have a similar side chain pattern of binding surfaces, each hormone probably uses a slightly different binding mechanism when interacting with IR and IGF-1R, consequently leading to the triggering of different signalling cascades.

There is currently a relatively good insight into the roles of A and B-chains/domains of insulin and IGF-1 in the binding interaction. The impact of C and D domains of IGFs is much less understood. It is, however, envisaged that they play some specific role in the different binding affinities and abilities of receptor activation. Studies of the functional importance of the IGF-1 C domain (C_1 domain) indicated that it plays an important role in IGF-1:IGF-1R interaction. However, it seems to have a detrimental effect on IGF-1:IR binding. It was also proposed that the C_1 domain interacts with the CR domain on the IGF-1R, but the crystal structure of the complex of IGF-1 with IR L1-CR domains mediated by the IGF-1R αCT-segment did not clarify contacts of C and D domains with its receptor, due to the invisibility of the flexible C domain in the complex (108). The role of the C domain of IGF-2 (C_2 domain) has been even less studied. It is shorter compared to its counterpart in IGF-1 and contact with the CR domain is not anticipated. The importance of C domains for hormone receptor interaction is highlighted by the fact that $C_1 \rightarrow C_2$ swap in IGF-1 doubled the affinity to IR-A and IR-B, but diminished the affinity to IGF-1R to 25 %. D_1 and D_2 domains also play some roles in the activation of IR and IGF-1R, but they are probably less important than C domains.

Here we aimed to elucidate in detail the roles of D_1, D_2 and C_2 domains in receptor specificity. We used the human insulin molecule as a template, as it has a high affinity to both isoforms of IR, hence is sensitive to these receptors. To address these issues, we prepared (Table 5) 1) insulin analogues with the extended C-terminus of the A-chain by amino acids from D_1 and D_2 domains (Analogues 1-3); 2) insulin analogues with the C-terminus of the B-chain extended by amino acids mimicking the C_2 domain (Analogues 4-7). The structure of the complex of IGF-1 with the IGF-1R αCT/IR L1-CR construct showed that the conformation of IGF-1 B domain residues 21-26 is almost identical to the structure of equivalent insulin B22-B27 residues on the same receptor. This indicates that at least the first residues of the C_1 domain probably follow the direction of the end of the B domain.

In the prepared analogues, the binding affinities and the ability to stimulate autophosphorylation of the receptor were evaluated.

3.2.2 Summary

Addition of D_1 and D_2 domain amino acids had a negative impact on binding potency to IR. As we expected, the D_1 domain had a more negative impact on binding to IR than the D_2 domain, where the initial sequence (Pro-Leu-Lys) of D_1 domain showed the main detrimental effect which was only slightly pronounced by the presence of the entire domain. The results further revealed that the initial part of the D_1 sequence contributes to or possibly is a crucial factor in different affinity of IGF-1 to IR-A and IR-B.

Binding studies with analogues containing a part mimicking the C_2 domain provided surprising results. In IR-A, analogues with only one or two additional amino acids were less active than analogues containing three or four extra residues. In the case of IR-B, the effect was even more significant. The analogue containing the longest part of the C_2 domain (Analogue 7, see Table 5) had the highest binding potency (150 %).

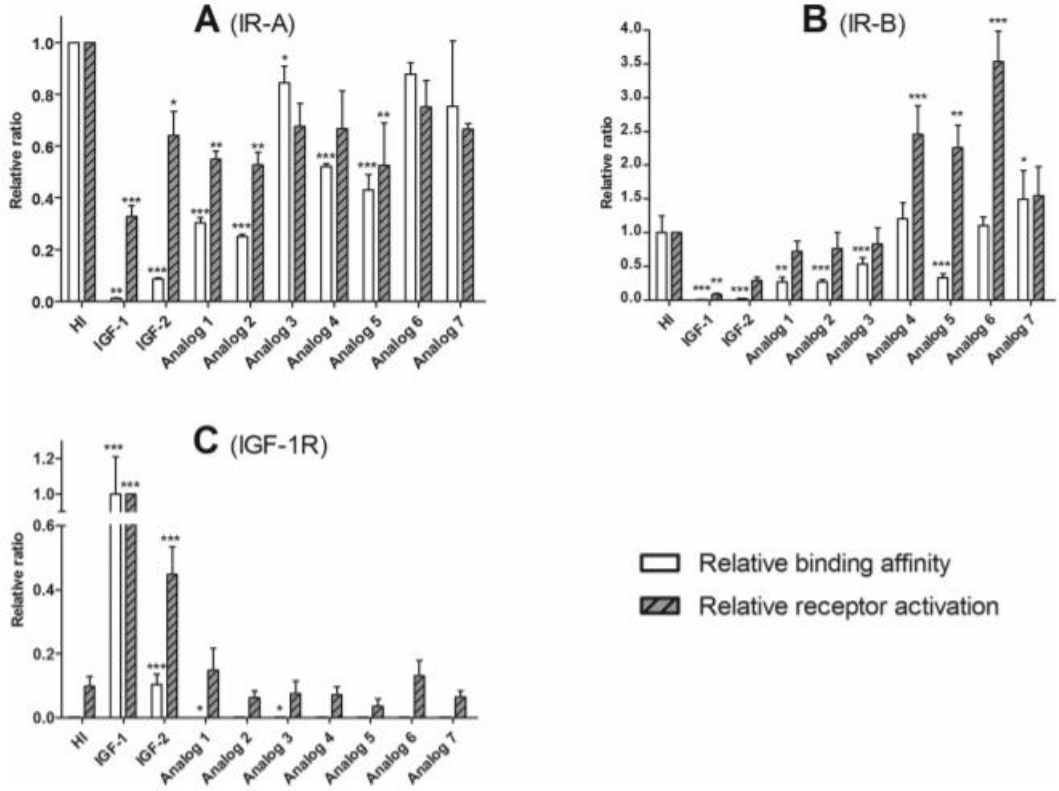
Addition of the D_1, D_2 or C_2 domain did not significantly alter the binding affinity for IGF-1R. The exceptions were Analogue 1 with a 3-times lower affinity, and Analogue 3 which bound IGF-1R 2-times more strongly than insulin.

Activation of the receptor was measured in murine fibroblast over-expressing IR-A, IR-B or IGF-1R. Autophosphorylation of Tyr1158 (IR; IR-B numbering) or Tyr1131 (IGF-1R) was detected. The results are shown in Figure 14. In IR-A and IGF-1R, the autophosphorylation abilities followed their binding trends. Analogues with D domains stimulated IR-B comparably to insulin. Interestingly, analogues with parts of the C_2 domain showed significantly higher autophosphorylation (2- to 3-times higher than human insulin). However, the analogue with enhanced binding potency (150 %; analogue 7) stimulated IR-B similarly to insulin.

Table 5: **Insulin analogues**

	Structure
Analogue 1	AlaA21Pro-Leu-LysA24-insulin
Analogue 2	AlaA21Pro-Leu-Lys-Pro-Ala-Lys-Ser-AlaA24-insulin
Analogue 3	AlaA21Trp-Pro-Ala-Lys-Ser-GluA24-insulin
Analogue 4	SerB31-insulin
Analogue 5	SerB31-LysB32-insulin
Analogue 6	SerB31-Lys-ValB33-insulin
Analogue 7	SerB31-Lys-Val-SerB34-insulin

Figure 14: Comparison of relative binding affinities (white bars) for IR-A (A), IR-B (B), and IGF-1R (C) and relative abilities to activate these receptors (grey bars) of human insulin (HI), IGF-1, IGF-2, and insulin analogues containing sequences derived from the D domain of IGF-1 (1 and 2) or IGF-2 (3) or from the C domain of IGF-2 (4−7). The experimental values are related to the binding potency and biological activity of HI (for IRA and IR-B) or IGF-1 (for IGF-1R). Asterisks indicate that binding of the ligand or autophosphorylation of a particular receptor induced by the ligand differs significantly from that of insulin (*p < 0.05; **p < 0.01; ***p < 0.001).



3.2.3 My contribution

I prepared insulin analogues containing amino acids mimicking the C_2 domain. Three of them (Analogues 4, 6, 7) were also included in my diploma thesis. The analogues containing D_1, D_2 domains or their parts were the main subject of the diploma thesis of my colleague Anna

Povalová. I measured the binding potencies to all three types of receptors. I contributed to the writing of the manuscript.

Křížková K., Chrudinová M., Povalová A., Selicharová I., Collinsová M., Vaněk V., Brzozowski A.M., Jiráček J., and Žáková L. 2016. "*Insulin-Insulin-like Growth Factors Hybrids as Molecular Probes of Hormone: Receptor Binding Specificity*." Biochemistry 55 (21): 2903–13.

The article *in extenso* can be found in Appendix I.

3.3 Probing Receptor Specificity by Sampling the Conformational Space of the Insulin-like Growth Factor II C-domain

3.3.1 Background

The mechanism of insulin and IGFs binding to the receptors was originally proposed on the mutagenesis studies only. However, several reports based on the crystal structure on the insulin:IR complex and the first bound structure of IGF-1 through complexation with the IR/IGF-1R hybrid construct have recently revealed the binding mode of the hormones at receptor site 1. Details of the precise arrangement of the C domain of bound IGF-1 are still unknown. Nonetheless, it has been proposed that the C domain is rearranged compare to the receptor-free structure to prevent unfavourable steric clashes mainly with the αCT-peptide of the receptor. C domains also represent regions with major differences between IGFs, in amino acid composition as well as length.

To gain a greater insight into the structural basis of IGF-2 binding specificity to IR-A and IGF-1R, we generated a series of mutants containing amino acid substitution within the B and C domains. These were designed to make IGF-2 more IGF-1-like. To do this, a new efficient and cost-effective protocol for the recombinant production of IGF-2 had to be developed to gain sufficient quantities for biological and NMR characterisation.

3.3.2 Summary

We developed the efficient production of correctly folded IGF-2, which served as a platform for the production of new IGF-2 analogues. The method provided high yields of IGF-2 analogues (0.8-1.8 mg/l of culture) with only a single additional Gly residue at the N-terminus. The correct folding was confirmed by HPLC retention time, ^{1}H-$^{15}NHSQC$, CD spectra, and binding affinities to cognate receptors (IR-A, IGF-1R).

In total, 6 analogues were designed. The modifications were as follows: 1) a point mutation at position Ser29(Asn29-IGF-2); 2) an insertion of dipeptide Gly-Ser after Arg34 (Arg34(Gly-Ser)-IGF-2); 3) an insertion of dipeptide Pro-Gln after Ser39 (Ser39(Pro-Gln)-IGF-2); 4) a combination of both insertions; 5) a combination of the Asn29 mutation with the Pro-Gln insertion; 6) a combination of the Asn29 mutation with both insertions.

As expected, all modifications led to significantly impaired IR-A binding. The Asn29-IGF-2 B domain mutant gave a 2-fold reduction in IR-A affinity and the insertions in the C domain exhibited even stronger negative effects. All analogues bearing the Pro-Gln motif showed low affinity to IR-A (1.1 to 1.8% of insulin affinity). Further combinations did not appear to have any additive effect.

Interestingly, an insertion of IGF-1-like features generally led to a moderate decrease of binding potency to IGF-1R. One exception was a combination of Asn29 mutation and a Pro-Gln insertion, which resulted in an almost 2-fold increase in binding potency (18.8 % versus 10.9 % of native IGF-2). A similar effect was not observed in the analogue containing a combination with Asn29 mutation and both insertions (see Table 6).

Table 6: *The receptor binding affinities of hormones and IGF-2 analogues reported in this work*

Analogue	Relative binding affinity for human IR-A [%]	Relative binding affinity for human IGF-1R [%]
Insulin	100±5;100±8	0.08±0.01[a]
IGF-1	1±0.3[b]	100±21[a]; 100±4
Commercial IGF-2	8.2±0.4[b]	10.8±3.3[b]
IGF-2	7.9±0.7	10.9±5.0
Asn29-IGF-2	4.2±0.4	5.3±1.3
Arg34(Gly-Ser)-IGF-2	2.8±1.1	5.8±1.3
Ser29(Pro-Gly)-IGF-2	1.1±0.1	4.8±1.1
Arg34(Gly-Ser),Ser29(Pro-Gly)-IGF-2	1.8±0.4	4.2±1.6
Asn29,Ser29(Pro-Gly)-IGF-2	1.4±0.3	18.8±5.1
Asn29,Arg34(Gly-Ser) Ser29,(Pro-Gly) -IGF-2	1.2±0.3	7.8±2.7

[a]from Vikova et al (102)
[b]from Krizkova et al (101)

We selected two IGF-2 analogues with the most pronounced impact on receptor binding, Ser39(Pro-Gln)-IGF-2 (lowest IR-A and IGF-1R binding) and Asn29,Ser39(Pro-Gln)-IGF-2 (decreased in the IR-A and enhanced IGF-1R affinity) for NMR characterisation. Results revealed that both analogues preserved their overall structural organisation. The C domain insertion led to a significant change in its conformational space, with the main differences residing between residues 29 and 42. Insertion of Pro-Gln after Ser39 led to increased conformational freedom within the C-loop that generated a rearrangement stabilised by several new packing interactions. The newly formed hydrophobic contacts led to the formation of a better-defined C-loop that bends around the bulky side chains of Tyr27 and Tyr61 of both C domain modified analogues. In comparison with native IGF-2, the extended C domain is spatially constrained and bent toward the triad of aromatic residues at the C-terminus of the B domain (Phe26, Tyr27, and Phe28). In native IGF-2, Ser29 has no

significant contacts to the neighbouring residues. The Pro-Gln extension led to repositioning of it in close proximity to Tyr27. The hydroxyl proton from the Ser29 side chain may be involved in hydrogen bonds with the backbone carboxyl group either from Pro31 or Arg42. The modification of Ser29 to Asn29 led to loss of this hydrogen bond. The Asn29 side chain is pointing out of the C-loop and the Asn29 substitution probably leads to further stabilisation of contacts between the C domain and aromatic triad (Phe26, Tyr27, and Phe28), which consequently leads to the stabilisation of the interaction between Tyr27 and Arg30/Pro31.

3.3.3 My contribution

I developed a new method for recombinant production of IGF-2 in collaboration with my colleague, Rozálie Hexnerová. The new method of production of IGF-2 was included in my as well as her diploma thesis. I participated in the design of new analogues. In all analogues, I participated in the determination of binding affinities to IR and IGF-1R. In collaboration with Rozalie Hexnerová, I prepared all analogues including those with isotope labelling (^{13}C, ^{15}N). Analogues Ser29(Asn29-IGF-2), Arg34(Gly-Ser)-IGF-2, Ser39(Pro-Gln)-IGF and Arg34(Gly-Ser), Ser39(Pro-Gln)-IGF2 were also included in Rozalie's diploma thesis. I contributed to the writing of the manuscript.

Hexnerová R., Křížková K., Fábry M., Sieglová I., Kedrová K., Collinsová M., Ullrichová P., Srb P., Williams C., Crump M.P., Tošner Z., Jiráček J., Veverka V., and Žáková L. 2016. "*Probing Receptor Specificity by Sampling the Conformational Space of the Insulin-like Growth Factor II C-Domain*." Journal of Biological Chemistry 291 (40): 21234–45. https://doi.org/10.1074/jbc.M116.741041

The article *in extenso* can be found in Appendix II.

3.4 Converting Insulin-like Growth Factors 1 and 2 into High-Affinity Ligands for Insulin Receptor Isoform A by the Introduction of an Evolutionarily Divergent Mutation

3.4.1 Background

In recent years, the role of the IGF/insulin system in cancer development and growth has been widely studied. Efforts have been focused on the development of anti-IGF-1R targeted therapies, mostly tyrosine kinase inhibitors and anti-receptor antibodies. However, the results of clinical trials have not been satisfactory, either because of toxicity or lack of the specificity. Due to unmet expectations, a new therapy concerning antagonists of IGF-1R could represent a promising strategy for IGF-1R-related malignancies.

The binding of insulin and IGFs to the receptors (IGF-1R, IR) triggers two major signalling pathways. The former leads to activation of PI3K and subsequently Akt kinase, which predominantly results in metabolic responses. The second involves the activation of the Ras/Raf/MAPK/Erk cascade, mediating proliferative effects through gene transcription regulation. Insulin acts via both isoforms of IR and mediates mainly metabolic responses. IGFs promote their mitogenic activity mainly via IGF-1R. However, similar mitogenic stimulation also results from binding of IGF-2 to IR-A. The complexity of the system is further amplified by the presence of hybrid receptors that can be effectively activated by IGFs but not by insulin.

Recently, it was reported that a combination of GluA4His and ThrA8His mutations results in an insulin analogue with native IR-A binding affinity, but poor ability to stimulate IR autophosphorylation and downstream Akt activation (170). Corresponding positions to A4 in insulin are Asp45 and Glu44 in IGF-1 and 2, respectively. A neighbouring position in insulin is neutral GlnA5, contrary to Glu (44/45, 45/46) which is present in both IGFs.

Here, we focused on IGF analogues with mutations at positions corresponding to or closely related to A4 and A5. Some of the mutations were also combined with the Phe49His in IGF-1 and the Phe48His in IGF-2, as it has been shown that the insulin corresponding mutation ThrA8His significantly increased IR-A binding potency.

3.4.2 Summary

IGF-1 and IGF-2 analogues were produced by recombinant expression in *E. Coli*. The method of production of IGF-2 was further modified to yield a native IGF-2 without additional Gly at the N-terminus (position -1).

The first series of analogues was “insulin-inspired” mutations at positions 45 and 46 (Asn, His, Ala) in IGF-1 and at position 44 and 45 (Gln, His, Ala) in IGF-2. While the production of IGF-1 analogues was successful in all designed analogues, only Gln45-IGF-2 was made in a yield that allowed its characterisation.

Gln45-IGF-2 had reduced affinity to IGF-1R compared to the native IGF-2, but activated the receptor in a similar manner. Interestingly, the affinity to IR-A was significantly increased compared to the native IGF-2 (20 % of the affinity of human insulin). However, despite the changes in affinities to IR-A, the ability of receptor activation remained unaffected.

The second series of analogues was inspired by ThrA8His substitution in insulin, which showed enhanced potency for IR-A with antagonistic properties. IGF-2 has Phe at an equivalent position (48).

Neither single His48 substitution nor the combination with Gln45 had any effect on the affinity to IGF-1R or the ability of its activation. The affinity to IR-A was significantly enhanced in both new analogues (5- to 7-fold as compared to native IGF-2). Both analogues were able to interact with IR-A at nanomolar concentration, which makes them half-equipotent to insulin with respect to IR-A (Table 7). Moreover, the activation abilities were also high, with potency similar to that of human insulin. The higher affinity of Gln45His48-IGF-2 for IR was also reflected in the affinity to isoform B (IR-B), which was approximately 8-times higher when compared to the native IGF-2.

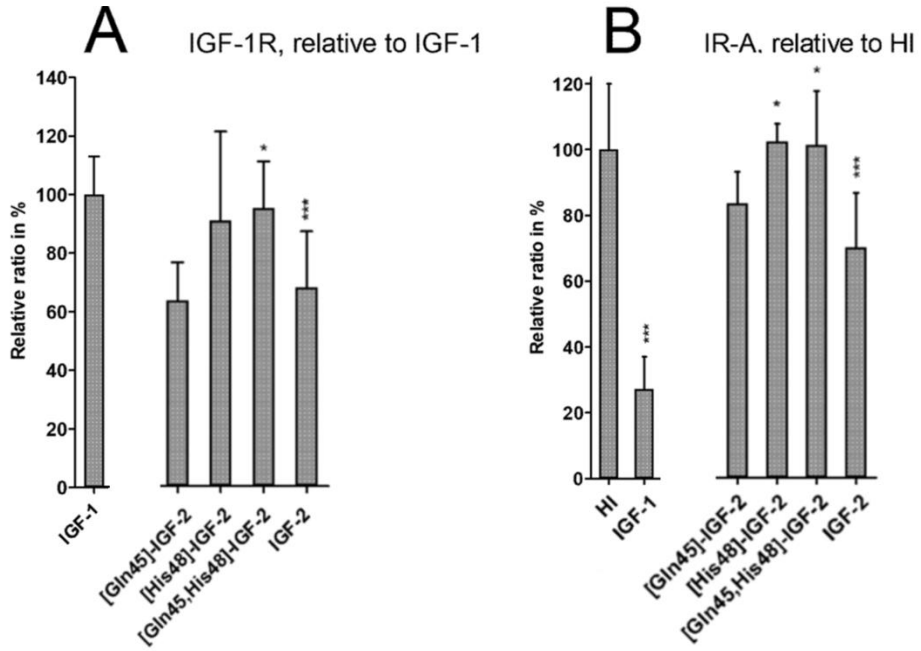
The binding affinities of all analogues were proportionate to their abilities to activate IR or IGF-1R without any important discrepancies.

Table 7: *IR-A and IGF-1R binding affinities of native hormones and analogues*

	relative binding affinity for human IGF-1R (%) relative to IGF-1	relative binding affinity for human IR-A (%) relative to HI
Human IGF-1	100±37a 100±12b	
Human insulin		100±20c 100±28d 100±43e
Human IGF-2	10.9±5.7*	8.6±0.6c
First series of IGF-2 analogues		
Gln45-IGF-2	5.5±0.6*a	20±3.8
Second series of IGF-2 analogues		
His48-IGF-2	18.2±4.7a	59.3±14.3
Gln45,His48-IGF-2	18.0±1.0a	46.2±8.5e

*p < 0.05
aRelative to the human IGF-1 Kd value of 0.16 ± 0.06 (n= 3).
bRelative to the human IGF-1 Kd value of 0.25 ± 0.03 (n = 4)
c Relative to the human insulin Kd value of 0.25 ± 0.05 (n = 5)
dRelative to the human insulin Kd value of 0.32 ± 0.09 (n = 4)
eRelative to the human insulin Kd value of 0.30 ± 0.13 (n = 5)

Figure 15: *Relative abilities to activate (A) IGF-1R and (B) IR-A of human insulin (HI), human IGF-1, human IGF-2, and IGF-1 and IGF-2 analogues.* Mean ± SD values were calculated from four independent experiments (n = 4). In panel A, the experimental values are related to the biological activity of IGF-1. In panel B, the experimental values are the biological activity of HI. Asterisks indicate that induction of autophosphorylation of a particular receptor induced by a ligand differs significantly (*p < 0.05; **p < 0.01; ***p < 0.001) from the effect of IGF-2. In panel A, the significance of the effect of native IGF-2 (asterisks) is related to human IGF-1, and in panel B to human insulin. In panel B, the significance of the effect of native IGF-1 (asterisks) is related to human insulin.



3.4.3 My contribution

I participated in the design of the new analogues and worked on the preparation of "insulin inspired" IGF-2 analogues, including all unsuccessful ones. I participated in the determination of binding affinities to IR and IGF-1R of all analogues. This publication also includes the results of IGF-1 and its analogues, which are not discussed here as the results of IGF-1 analogues are not covered by the topic of this thesis. The results of IGF-1 analogues were included in the doctoral thesis of my colleague, Kateřina Macháčková.

Macháčková K., Chrudinová M., Radosavljević J., Potalitsyn P., **Křížková K.**, Fábry M., Selicharová I., Collinsová M., Brzozowski A.M., Žáková L. and Jiráček J. 2018. *"Converting Insulin-like Growth Factors 1 and 2 into High-Affinity Ligands for Insulin Receptor Isoform A by the Introduction of an Evolutionarily Divergent Mutation."* Biochemistry 57 (16): 2373–82. https://doi.org/10.1021/acs.biochem.7b01260.

The article *in extenso* can be found in Appendix III.

3.5 Mutations at hypothetical binding Site 2 in insulin and insulin-like growth factors 1 and 2elicit receptor- and hormone-specific responses

3.5.1 Background

It is generally accepted that insulin interacts with the receptor by an interplay of two binding sites: the primary binding site 1, which binds receptors with a high affinity (~6 nM), and the secondary binding site 2, which binds receptors with a lower affinity (~400 nM). Binding of insulin sites 1 and 2 to respective sites 1 and 2 on the receptor creates a high-affinity complex (~0.2 nM).

Crystallographic studies provided a structural insight into interactions of site 1 of insulin or IGF-1 with receptor site 1 formed by L1 and αCT-peptide of IR-A or IGF-1R. Interactions of insulin and IGF-1 with site 1 of the cognate receptors are similar. The results of structural studies confirmed the available evidence from previous mutagenesis studies. To date, no structural information is available for a complex of IGF-2 with either IR or IGF-1R, but it is assumed that at least interactions at site 1are similar to those of insulin and IGF-1.

Characterisation of contacts at site 2 is intricate, probably due to the highly dynamic character of the interactions. Recently, two studies provided insight into the site 2 interactions of insulin with IR-A (114,123). The studies restricted site 2 to ThrA8, CysA7, GlnB4–GlyB8, HisB10, and GluB13 residues in the insulin molecule and to the FnIII-1 domain on the receptor molecule. These structural results, however, do not fully match the results of mutagenesis studies with insulin that also assumed involvement of amino acids IleA10, SerA12, LeuA13 and GluA17.

Therefore, we focused on these “neglected” insulin residues IleA10, SerA12, LeuA13, and GluA17 and prepared a series of mutants to study their interactions with the receptors. In parallel, similar mutations were prepared in homologous positions of IGF-1 and IGF-2 (positions Ser51/50, Asp53/52, Leu54/53 and Glu58/57).

3.5.2 Summary

Insulin and both IGFs were modified at positions A10, A12, A13, and A17 in insulin and at equivalent positions in IGFs. Based on the mutagenesis studies, they were considered as parts of hormone sites 2 (72,126).

We intended to mutate each position in two ways. We either introduced a homologous exchange; i.e. Leu→Val, Ser→Thr, Glu→Asp and Asp→Glu or exchanged the wild-type amino acid for His. IGF-2 analogues were prepared in *E. coli* cells as described previously (158,175). Neither of the intended analogues with substitution in position 52 of IGF-2 was successfully prepared. It seems that this position is important for folding of the molecule. This hypothesis is also supported by the fact that only ThrA12-insulin was prepared. In addition, His57-IGF-2 was not successfully prepared, despite many attempts. However, on the contrary, Asp57-IGF-2 was prepared in a sufficient quantity.

All IGF-2 analogues tolerated the substitutions at positions 50 and 53 relatively well. Asp57-IGF-2 bound the IR (A/B) similarly or slightly better than the native molecule. The affinity to IGF-1R was even stronger (see Table 8). Interestingly, homologous Glu-to-Asp mutations at position 58 in IGF-1 and position 57 in IGF-2 resulted in different biological effects. Glu-to-Asp change at position 58 of IGF-1 had reducing effects on the analogue's binding affinities and especially for IGF-1R. Therefore, the NMR structure of ^{15}N- and ^{13}C-labelled Asp58-IGF-1 was determined to investigate the interesting effects of Glu-to-Asp substitution at position 58 of IGF-1.

Table 8: *Simplified overview of relative receptor-binding affinities of IGF-2 analogs;* the relative binding affinities are shown in % of the native hormone, which has 100 % binding affinity for the specific receptor

Position in the native hormone	Analog	Result of production	Binding affinity (in % of the native hormone) for		
			IR-A	IR-B	IGF-1R
Ser50-IGF-2	Thr50-IGF-2	+	78	153	264
	His50-IGF-2	+	100	68	136
Asp52-IGF-2	Glu52-IGF-2	-	nd	nd	nd
	His52-IGF-2	-	nd	nd	nd
Leu53-IGF-2	Val53-IGF-2	+	72	37	145
	His53-IGF-2	+	51	84	182
Glu57-IGF-2	Asp57-IGF-2	+	106	166	200
	His57-IGF-2	-	nd	nd	nd

We also employed molecular dynamics (MD) analysis for insulin analogues modified at positions A10, A12, A13 and A17 to analyse the effect of mutations on the dynamics of the insulin molecule, especially the crucial detachment of the B-chain C-terminus. MD indeed revealed that the dynamics of the low-affinity His-mutants are qualitatively different in that they are less likely to assume conformation with the detached B-chain C-terminus compatible with IR-binding. Wild-type insulin is likely to assume a wide range of open conformations, with the global minimum exceeding the defined threshold for the wide-open state. In comparison, HisA10-insulin and HisA13-insulin mutants are partially collapsed, especially in the B-chain α-helix. The HisA17-insulin is stabilised in a closed conformation, with both N- and C-termini of the B-chain remaining close to the insulin core. Interestingly, the native-like affinity ThrA12-insulin shows a free energy profile similar to native insulin. The results of computational metadynamics demonstrated that mutations can affect the internal dynamics of insulin and inhibit its ability to adopt receptor-bound conformation, which is important for binding to receptor site 1.

Overall, considering all the data, we show that insulin's positions A10, A13 and A17 are important for the biological activity of the hormone. Positions 51/50 and 54/53 of IGF-1/IGF-2 probably do not play any important role in receptor binding. We propose that position 58 in IGF-1 may be involved in direct interaction with site 1 of the IGR-1R.

3.5.3 My contribution

The publication also includes the results of the IGF-1 and insulin analogues which are not discussed here in detail. IGF-1 analogues were also the subject of the doctoral thesis of my colleague, Kateřina Macháčková, who is a co-author of this paper.

I participated in the design of IGF-2 analogues and contributed to the simplification of IGFs' production. I prepared all the IGF-2 analogues with the support of my colleague, Pavlo Potalitsyn. I contributed to the measurement of the binding affinities of all analogues to the three receptors. I participated in the writing of the manuscript.

Macháčková K., Mlčochová K., Potalitsyn, P., Hanková K., Socha O., Buděšínský M., Muždalo A., Lepšík M., Černeková M., Radosavljević J., Fábry M., Mitrová M., Chrudinová M., Lin L., Yurenko J., Hobza P., Selicharová I., Žáková L. and Jiráček J. (2019). *"Mutations*

at hypothetical binding site 2 in insulin and insulin-like growth factors 1 and 2 result in receptor- and hormone-specific responses." Journal of Biological Chemistry 294, *in press*

The article *in extenso* can be found in Appendix IV.

4 DISCUSSION

The high similarity among insulin and IGFs enables cross-reactivity of these hormones with the receptors of insulin-IGFs' system (IR-A/B and IGF-1R) and also led to the presumption that they use a similar binding mechanism. Indeed, several mutagenesis studies (118,124,126–128) revealed that insulin and both IGFs have a similar side chain pattern of the binding surface. However, detailed studies often using more advanced methods (114,117,123,123) indicate that, despite the significant homology, each hormone probably uses a slightly different binding mechanism when interacting with IR and IGF-1R. This consequently leads to different signalling cascades in response to the interaction of the hormone with its cognate receptor (74).

Although the amino acids included in the binding surface of all three hormones are probably mainly in the A and B domains/chains (118,119), C and D domains represent an important difference between insulin and IGFs, as well as between IGF-1 and 2, which probably contributes to the modulation of binding affinity or the ability of receptor activation. The C_1 domain significantly contributes to the high affinity binding to IGF-1R (108) and negatively impacts the affinity to IR-A (161,162). However, when C_1 domain connected A and B domains of insulin following the pattern seen in IGFs, the high affinity to IR was retained (163). The role of the C_2 domain has been less studied. The different IGF-1 and IGF-2 affinities to IR-A indicate that the C_2 domain has a less negative or even a slightly positive impact on IR-A affinity, and negatively affects IGF-1R affinity. The role of D domains has not been sufficiently probed. Although the absence of the D_1 domain itself did not negatively impact the affinity of IGF-1 to IGF-1R (159,160), C_1 and D_1 domains almost equally contributed to the increase in the affinity of the IGF-2C_1D_1 hybrid to IGF-1R. Similarly, C_2 and D_2 domains almost equally contributed to the increase in the affinity to IR-A of the IGF-1C_2D_2 hybrid. As there are many uncertainties in the role of C and D domains in binding to and activation of the receptors, we decided to focus on their roles, attempting to shed more light on this field.

In the first part, we prepared hybrid insulin analogues with extension derived from D_1, D_2 and C_2 domains. Insulin is a high affinity maternal ligand for both IR; therefore, it represents a sensitive probe for evaluation of the role of individual domains or its parts, ignoring the difference in A and B domains between IGFs and insulin.

The prolongation of the C-terminus of the insulin A-chain by amino acids from the D_1 domain had a negative effect on the binding affinities to IR-A and IR-B (25-30 % of the affinity of insulin), which is in accordance with previous studies (160,163,167). Our data revealed that the initial part of D_1 (Pro-Leu-Lys) had a pronounced negative effect, that was only slightly increased by the presence of the entire D_1 domain. The initial part of D_1 domain is also probably important factor for different IR-A versus IR-B affinity. On the contrary, the D_2 domain had only a marginally negative effect on IR-A binding (85 %), and decreased the affinity to IR-B to one-half (53 %). Here it should also be mentioned that all analogues containing the D domains included an AsnA21Ala substitution. This substitution itself reduced the affinity of insulin to IR-A to 65 % (129,176). Based on the results, we assume that the negative effect of the D_1 domain on IR affinity is caused by its initial specific sequence (Pro-Leu-Lys), which is unique to IGF-1 and probably determines a specific conformation. The D_2 domain has a rather neutral role in binding to IR-A and at least a slightly negative role in interaction with IR-B. The different binding affinities were not translated into the different ability of receptor activation. All analogues containing D domains or its part had a similar ability to activate IR-A, which was lower than that of insulin and IGF-2. Surprisingly, all analogues also retained the ability to activate IR-B on the same level as insulin did and better than IGF-2. Nevertheless, it seems that the first amino acids from the D_1 domain are one of the most important determinants responsible for low affinity of IGF-1 to both isoforms of IR.

Furthermore, we expected that the addition of the D_2 and especially D_1 domain would increase the binding affinity toward IGF-1R in accordance with previously published results (159). Despite our expectations, the increase in binding affinity was observed only in the analogue containing D_2 domain, where a more than 2-fold increase was observed. In analogues with a part or the entire D_1 domain, the binding affinities were similar to (Analogue 2, 107 %) or even decreased (Analogue 1, 33 %) below the value of native insulin. These results agreed with the hypothesis suggesting that the D_1 domain itself does not play a critical role in IGF-1R binding (159) and D_2 domain does not have a deleterious effect on IGF-1R binding (167). However, this may also indicate that the insulin molecule is not sufficiently sensitive or a suitable probe for IGF-1R. Our results underline that the role of D domains is evidently different in the context of IR and IGF-1R.

The other set of analogues contained analogues which extended the C-terminus of the B-chain with amino acids derived from the C_2 domain. We originally proposed preparing analogues

containing parts or the entire C_2 domain, as we did in the case of analogues with the D domains. However, the low solubility of peptides did not allow us to do so. Therefore, we prepared analogues with one to four initial C_2 domain-derived amino acids. The original Arg was substituted with Lys, enabling better protection of the side chain during the synthesis. This substitution was previously used in studies with the C_1 domain (162). As the amino acids were derived from the C_2 domain, we expected that their addition to the insulin molecule would increase the mitogenic properties of analogues. However, only a moderate and non-significant increase in the binding affinities to IGF-1R (104 – 150 %) and a slight decrease in the IR-A affinity (43-88%) were noted, with the ability of receptor activation reflecting the binding potencies.

The affinities to IR-B were much more surprising. Despite the presence of C_2-derived amino acids, the affinity and ability of IR-B activation were equipotent or even increased (Figure 14), with only the exception of Analogue 5 (SerB31LysB32-insulin) (33 % of the binding affinity of insulin), where the negative effect could be caused by a positively-charged C-terminal Lys. However, Analogue 5 retained a high ability of receptor activation (see Figure14). Although we were not successful in the preparation of analogues containing the entire or parts of the C_2 domain, our results provide information about the impact of the initial part of the C_2 domain to IR-B binding and its activation. The high affinity to IR-B revealed the important fact that the C-terminus of the B-chain could be a target for increasing potency to IR-B and consequently for receptor selectivity. These results are supported by papers demonstrating the increase in IR-B-specificity through modifications in the C-terminus of the B-chain (102,146,177).

To provide a more detailed insight into the structural elements responsible for different binding specificities of IGF-1 and 2, we prepared six IGF-2 analogues with unique IGF-1-like mutations in the B and C domains (i.e.Asn26,Gly30-Ser31, and Pro35-Gln36). The recent structure of bound IGF-1 to the IGF-1R/IR complex revealed that the structural rearrangement of the C_1 domain is probably necessary to prevent unfavourable steric clashes. Therefore, we probed the role of two parts of the C_1 domain. The introduced insertions were chosen to counterbalance the different lengths of the C_1 and C_2 domains (Gly-Ser; Pro-Gln). Apart from that, Pro-Gln insertion also represents a marked structural aspect of the C_1 domain. Ser29Asn substitution was chosen as a novel IGF-1 derived mutation. Corresponding residue in IGF-1 (Asn26) is at the C-terminus of the B domain, which undergoes structural changes upon binding to IGF-1R or IR. Although not specific contact was reported between Asn26 and IR

L1 domain or IGF-IR αCT-peptide in the complex with hybrid IGF-1R/IR receptor, Asn26 was captured to the binding site 1 of IGF-1 (117).

In order to prepare the analogues, we established and further simplified a novel protocol of IGF-2 production. Our protocol is user-friendly and cost-effective. The total chemical synthesis of IGF-2 is extremely difficult due to the lengths and amino acids' sequence of IGF-2. For this reason, we used the recombinant IGF-2 expression in *Escherichia coli* as a fusion with an N-terminal and cleavable His6-tagged GB1 protein (immunoglobulin binding domain B1 of streptococcal Protein-G). The method takes advantage of the on-column refolding step initiated by the one-step transition from denaturing and reducing conditions to non-denaturing and non–reducing conditions. Subsequent cleavage of the fusion protein and His-tag yields a completely native IGF-2 molecule, without any additional amino acids. The correct structure was confirmed by HPLC retention time, ^{1}H-$^{15}NHSQC$, CD spectra, and binding affinities to cognate receptors (IR-A, IGF-1R).

By introducing the IGF-1-like mutations, we aimed to shift the binding properties of the analogues towards those of IGF-1 and concurrently to explore the impact of individual aspects on the binding specificity. The negative role of the C_1 domain in the binding to IR-A was mentioned above. However, it is not clear if the negative effect is caused by the difference in length of the C_1 and C_2 domains or by the particular amino acids. It was already shown that the longer C_1 domain can cause structural restrictions during the binding to IR-A, which is supported by evidence that IGF-1 analogues with a shorter C domain exhibit enhanced binding affinity to IR-A (160). Indeed, all six IGF-2 analogues showed reduced IR-A affinity compared to native IGF-2. In addition, four of these showed as low an affinity as IGF-1. The most significant reduction (7- to 8-fold as compared to native IGF-2) was observed in Ser39(Pro-Gln)-IGF-2. Interestingly, this reduction was greater than the effect of swapping the entire C_2 for C_1 domain (3.7-fold) (159). The low IR-A affinity of single insertion (Pro-Gln), which was not further reduced in a combined insertion (Gly-Ser, Pro-Gln), indicates that, despite the undeniable role of the length of C_1 versus C_2 domains, the specific amino acid sequence is at least as important as the length. In this context, attention should be paid to the role of Pro36 (IGF-1 sequence, reflected in Pro-Gln insertion) which introduces a specific structural aspect to the C_1 domain.

Our assumption was confirmed in NMR studies which revealed that the Pro-Gln insertion leads to a displacement of the C-loop and more open C-loop conformation compared to the

native IGF-2. A comparable decrease in IR-A binding affinities of Ser39(Pro-Gln)-IGF-2 and Asn29,Ser39(Pro-Gln)-IGF-2 also indicates that it is caused mainly by their similarly altered C-loop structures rather than Ser29Asn replacement, which was relatively well tolerated by IR-A.

Regarding IGF-1R affinity, the combination of the Pro-Gln insertion with Ser29Asn substitution in Asn29,Ser29(Pro-Gln)-IGF-2 led to an analogue exhibiting an almost 2-fold increased binding affinity. Surprisingly, neither Asn29 mutation nor Pro-Gln insertion itself increased the binding affinity to IGF-1R compared to the native IGF-2. Their combination with the other insertion Gly-Ser led to an affinity lower than that of native IGF-2. Moreover, all our selected changes were insufficient to recover the IGF1-like binding affinity toward IGF-1R. Only Asn29,Ser29(Pro-Gln)-IGF-2, together with its markedly lowered affinity for IR-A and increased binding affinity toward IGF-1R, resulted in an almost 10-fold enhanced IGF-1R/IR-A binding specificity in comparison with IGF-2. Besides the aforementioned substitutions, there are other important determinants (e.g. IGF-1 Tyr31) (165,178,179) which, on their own or in combination, are crucial for the high affinity interaction toward IGF-1R. Taking together, we suppose that IGF-2 specificity toward IGF-1R is determined by the amino acid composition of the C domain together with other important determinants, rather than by the length of the C domain.

Analogues with high receptor specificity, but also those exhibiting a disproportion between receptor affinity and the ability of its activation, have medical potential as IGF-2 over-production has been associated with poor prognosis in cancer or other diseases (180). Whittaker and colleagues prepared an insulin analogue, HisA4,ThrA8-insulin, that had an almost native affinity, but poor ability of receptor activation. Because there are not any IGFs' antagonists reported thus far, we focused on the corresponding and neighbouring positions in IGF-2, positions 44 and 45 (Glu44, Glu45). Three types of mutations were planned 1) substitution of the residues at positions 44 and 45 for Ala; 2) their substitution for His; 3) substitution of the acids by corresponding amides (e.g. Glu→Gln). These mutations were also planned to combine with the Phe48His substitution. However, the low solubility during the folding step precluded preparation of the majority of analogues. Only three of the originally planned IGF-2 analogues were successfully prepared. These were Gln45-IGF-2, Gln45,His48-IGF-2, and His48-IGF-2.

The introduced mutations did not dramatically alter the binding affinities to IGF-1R. Gln45 substitution decreased the binding potency to one-half of the native IGF-2 and the His48 substitution increased the binding potency almost 2-times (18.8% of the IGF-1 affinity). Interestingly, the combination of both mutations exhibited the same binding potency as a single His48 substitution did. Despite the difference seen in the binding potencies, all analogues activated IGF-1R similarly to that which native IGF-2 did. Interestingly, different trends were observed for the His48 substitution in IGF-1 and 2. While in IGF-2 this mutation led to an increase in binding affinity to IGF-1R, the corresponding substitution (His49) had a rather negative effect on the binding potency to IGF-1R in IGF-1 (68 %). This indicates the different nature of interactions of IGF-1 and IGF-2 with IGF-1R, at least at this position.

The changes in affinities to IR-A were much more fascinating. All analogues are better IR-A binders than the native molecule. The most pronounced change was seen in the analogue with single His48 substitution (59 % of the insulin affinity). Although Gln45 substitution also led to the increase in the IR-A affinity in comparison with IGF-2 (20 % of the insulin affinity), the combination of both substitutions did not lead to a synergistic effect. Nonetheless, both His48-IGF-2 and His48,Gln45-IGF-2, to our knowledge, are the strongest IR-A binders reported to date. The remarkably high affinity of His48,Gln45-IGF-2 was also seen for IR-B, where the analogue reached almost 12 % of the binding affinity of insulin (8-fold stronger than the native IGF-2). Although we expected the increase in IR binding affinity by the introduction of His48 as the corresponding substitution in insulin (HisA8) to increase the affinity to IR (181–184), the scope of the impact is surprising.

In insulin, modification of HisA8 enhances the binding affinity more than 3 times and is present in fish and birds. It was proposed that this high-affinity mutation in birds and fish was selected by evolution to rapidly activate metabolic mobilisation to escape from predators. ThrA8 in human insulin was proposed to be included in the receptor binding interaction on the basis on mutagenesis studies (119,170). The structural analysis of binding site 1 did not, however, reveal a direct contact with the receptor, and the role of A8 was unclear. The latest CryoEM analyses of the insulin –IR complex showed that ThrA8 is included in interactions at site 2 (114,123). Our data demonstrate the greater impact of the substitution on the interaction with IR than IGF-1R. This may indicate a different binding surface at site 2 interactions in both receptors, but also a diverse role of site 2 interaction for IR and IGF-1R. While in IR the role of site 2 is undeniable, it is not completely elucidated in the case of IGF-1R (96,152). The

other possibility is also that His at this position enhances some contacts with elements of site 1 interactions which are more favourable for IR rather than IGF-1R.

We did not meet the original goal to design analogues with disproportionate character between binding affinity and ability of receptor activation. Thus, the observed significant disproportionate binding and activation of insulin analogues mutated at A4 and A8 (170) are probably specific to and limited to insulin-IR interactions. Nonetheless, our data confirmed the important role of position A8 of insulin in IR affinity and provided the evidence that position 48 in IGF-2 has an important role in IR affinity as well.

Interactions at site 2 are much less understood compared to those at site 1. Recently, new and innovative cryoEM structural analyses were published. However, their results do not completely match the results of mutagenesis studies and the knowledge about site 2 interactions accepted to date. Therefore, in the last part of our project, we focused on site 2 interactions. The aforementioned latest data have provided evidence that site 2 in insulin is structurally restricted to ThrA8, CysA7, GlnB4–GlyB8, HisB10, and GluB13 residues (114,123). Mutagenesis studies and biochemical data also included amino acids IleA10, SerA12, LeuA13 and GluA17 in site 2 (114,119,123). Therefore, we concentrated on these residues in insulin and the corresponding residues in IGFs (50, 52, 53, and 57 for IGF-2). Each residue investigated was mutated in two ways. Firstly, all mutated positions were substituted for His with its relatively large side chain containing the imidazole group. Secondly, each of the modified residues was mutated with a similar amino acid, maintaining a similar size and charge characteristics.

Despite many attempts, either we were not able to prepare analogues Glu52-IGF-2, His52-IGF-2 and His57-IGF-2 or the quantities were considerably lower compared to others and yields did not allow any biological characterisations. This indicates that residues at positions 52 and 57 in IGF-2 may play a specific role in folding. Our results are relatively surprising, because others were more successful in several modifications of positions 52 and 57 in IGF-2 (Ala, Asp or Lys52, and Ala57) (72,126). The reasons for these different production yields could be the different production strategies, e.g. yeast vs *E. coli*, different fusion protein partners, etc.

Although we were not able to prepare analogues with substituted position 52, the mutation of the corresponding position in insulin together with available data do not indicate any crucial role of this position in binding to receptors (IR, IGF-1R) or their activation. Positions 50 and

53 in IGF-2 were relatively tolerant to modifications (Table 8). The introduced substitution led to a 1.4- to 2.6-fold increase in the affinity to IGF-1R while the affinity to IR-A remained unchanged or slightly decreased (50-100 % of the native binding affinity). On the contrary, mutations in insulin at A10 (corresponding to position 50 in IGF-2) led to severe impairment of binding affinity to all three receptors. The mutation of A13 impaired the affinity to IR but not to IGF-1R. The different response to substitution at position 53 and especially A13 in the insulin molecule further indicates that site 2 is different on IR and IGF-1R which has already been previously proposed (78,121,172,175).

Mutation in position 57 provided quite interesting results. While mutations in corresponding positions in insulin (A17) and IGF-1 (58) led to a decrease in binding affinity to IR-A and IGF-1R, the affinities of Asp57-IGF-2 remained unaffected or markedly increased, especially toward IR-B and IGF-1R. A different response of a similar mutation (Glu to Asp) in both IGFs deserves attention. This supports the hypothesis that both molecules (IGF-1 and 2) use slightly different binding mechanisms. In the recently reported crystal structure of the IGF-1-IGF-1R complex (PDB 5U8Q), Glu58 can create close contact (2.7-3.0 Å, probably a salt bridge) with Arg704 of the αCT-peptide. Thus, the decrease in binding affinity may be caused by the absence of this salt bridge in Asp58-IGF-1. No such stabilisation was seen in insulin-IR-A complexes, PDB 4OGA (117) or PDB 6HN5 (123), or in the complex of IGF-1 with IR L1 domain and IGF-1R αCT-peptide (PDB 4XSS) (185). To date, there is no structural data showing a complex of IGF-2 with either receptor. If our result with the structure of Asp58-IGF-1 indicated involvement of amino acid at position 58 in site 1 of IGF-1R, the positive (IGF-1R) or rather neutral (IR) effect of Glu→Asp substitution in IGF-2 does not indicate an irreplaceable role of Glu57 in binding to either receptor.

Our data did not provide unambiguous evidence of the role of A10/A12/A13/A17 of insulin or corresponding residues in IGFs in site 2 interactions. Therefore, we decided to provide a more detailed insight into the impact of A10/A12/A13/A17 mutations on overall insulin structural integrity and consequently the interaction with site 1. We initiated a series of computational experiments with insulin mutants and with native insulin. The insulin receptor complex is characterised by a partial detachment of the C-terminus of the B-chain (B25-B30) which is crucial for potent binding (121,185,186). Our data suggest that introduction of the His mutation (A10/A13/A17) probably negatively affects the ability to adopt the so-called open/active conformation that is necessary for interaction with site 1. Therefore, these residues previously proposed as being involved in site 2 interactions could rather impact the

structural integrity and consequently interaction at site 1. These results are in good agreement with the hypothesis of Weis and colleagues, who predicted that A12, A13, A17 and B17 residues can play a role during the initial docking of insulin to the receptor (123). This partially explains the significant and different impact of mutation at these positions which are hormone receptor-specific.

5 SUMMARY

- In the first part of the project, we systematically investigated the role of the C and D domains of IGF. Our data demonstrated that the role of D domains is evidently different in the context of IR and IGF-1R. The addition of the D_1 domain to insulin had a negative effect on binding affinities to IR (25-30 % of the insulin affinity), where the initial sequence (Pro-Leu-Lys) showed the main detrimental effect which was only slightly pronounced by the presence of the entire D_1 domain. Further, the initial part of the D_1 domain is probably an important factor for different IR-A versus IR-B affinity. The D_2-domain had a neutral role in binding to IR-A and at least a slightly negative role in the interaction with IR-B. Despite the differences seen in receptor affinities, all analogues showed a similar ability of receptor activation, which was lower than those of insulin and IGF-2. Regarding IGF-1R, the addition of the D_2 domain led to a more than 2-fold increased IGF-1R binding affinity. On the other hand, analogues with a part or entire D_1 domain had IGF-1R affinity similar to or lower than that of native insulin. Analogues with attached one to four initial C_2 domain-derived amino acids (Ser-Lys-Val-Ser) exhibited only a moderate increase in the binding affinities to IGF-1R (104-150 % of the insulin affinity) and a slight decrease in the IR-A affinity (43-88 %), with the ability of receptor activation reflecting the binding potencies. Surprisingly, their affinity to and ability of IR-B activation were equipotent to or even increased compared to native insulin with only the exception of Ser31Lys32-insulin.
- In the second part of the project, we focused on IGF-2 analogues. To be able to prepare new IGF-2 analogues, we established and subsequently simplified a novel protocol of IGF-2 production. Our protocol takes advantage of the spontaneous folding on a Ni^{2+}column. It is user-friendly and cost-effective.
- The first set of IGF-2 analogues contained unique IGF-1-like mutations in the B and C domains (i.e.Asn26, Gly30-Ser31, and Pro35-Gln36). The introduced mutations showed

reduced IR-A affinity compared to native IGF-2, especially analogues containing Pro-Gln insertion where a displacement of the C-loop and more open C-loop conformation was determined by the NMR characterisation. The low IR-A affinity of single insertion (Pro-Gln) was not further pronounced in a combined insertion (Gly-Ser, Pro-Gln). This indicates that, despite the undeniable role of the length of C_1 versus C_2 domains, the specific amino acid sequence is at least as important as the length in IR-A affinity.

Neither of our selected changes was sufficient to recover IGF-1-like binding affinity to IGF-1R. However, the combination of the Pro-Gln insertion with Ser29Asn substitution led to an almost 2-fold increased binding affinity. Similarly to IR, the IGF-2 specificity toward IGF-1R is probably determined by the amino acid composition of the C domain together with other important determinants rather than by its length.

The markedly lowered affinity for IR-A together with increased binding affinity toward IGF-1R in Asn29,Ser29(Pro-Gln)-IGF-2 led to a 10-fold enhanced IGF-1R/IR-A binding specificity in comparison with IGF-2.

- The second set of IGF-2 analogues was inspired by the HisA4, HisA8-insulin, exhibiting a disproportion between receptor affinity and ability of its activation. In positions corresponding or close to insulin A4 (Glu44, Glu45), the original amino acid was substituted by Ala, His or corresponding amide. The mutation was then combined with Phe48His substitution. Of the intended analogues, Gln45-IGF-2, Gln45, His48-IGF-2, and His48-IGF-2 were successfully prepared. The binding potency to IGF-1R was not dramatically altered and the ability of receptor activation remained unaffected. However, all analogues were better IR-A binders than IGF-2, especially those with His48 substitution. Both His48-IGF-2 and His48, Gln45-IGF-2 are the strongest IR-A binders reported so far. The remarkably high affinity of His48, Gln45-IGF-2 was also seen for IR-B (12 % of the insulin affinity). Thus, position 48 in IGF-2 has an important role in IR affinity, similarly to position A8 in insulin. The ability of receptor activation reflected binding potencies. The original intention was not met, as the antagonistic effect of HisA4, HisA8 substitutions is probably limited to insulin–IR interaction.
- In the last part of our project, we concentrated on residues in IGF-2 (50, 52, 53, and 57) which were not included in site 2 interactions on the basis of the latest structural analyses. However, they were considered as participating in site 2 interactions on the basis of mutagenesis studies. Each residue investigated was substituted in two ways: with His and with a similar amino acid. We were not able to prepare analogues with substituted position 52. Positions 50, 53, and 57 in IGF-2 were relatively tolerant to

modifications. Our results did not provide unambiguous evidence on the role of positions 50, 52, 53 and 57 of IGF-2 in site 2 interactions. The different response of IGF-1, IGF-2 and insulin to modification at corresponding positions to 53 and 57 endorses the hypothesis that each hormone uses a slightly different way in interaction with the receptor (IR, IGF-1R).

6 REFERENCES

1. Dull TJ, Gray A, Hayflick JS, Ullrich A. Insulin-like growth factor II precursor gene organization in relation to insulin gene family. Nature. 1984 Sep 30;310(5980):777–81.

2. Hua Q, Mayer JP, Jia W, Zhang J, Weiss MA. The Folding Nucleus of the Insulin Superfamily a flexible peptide model foreshadows the native state. J Biol Chem. 2006 Sep 22;281(38):28131–42.

3. Murray-Rust J, McLeod AN, Blundell TL, Wood SP. Structure and evolution of insulins: implications for receptor binding. BioEssays News Rev Mol Cell Dev Biol. 1992 May;14(5):325–31.

4. Bathgate RAD, Samuel CS, Burazin TCD, Layfield S, Claasz AA, Reytomas IGT, et al. Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. J Biol Chem. 2002 Jan 11;277(2):1148–57.

5. Burkhardt E, Adham IM, Hobohm U, Murphy D, Sander C, Engel W. A human cDNA coding for the Leydig insulin-like peptide (Ley I-L). Hum Genet. 1994 Jul;94(1):91–4.

6. Conklin D, Lofton-Day CE, Haldeman BA, Ching A, Whitmore TE, Lok S, et al. Identification of INSL5, a new member of the insulin superfamily. Genomics. 1999 Aug 15;60(1):50–6.

7. Lu C, Lam HN, Menon RK. New members of the insulin family: regulators of metabolism, growth and now ... reproduction. Pediatr Res. 2005 May;57(5 Pt 2):70R-73R.

8. Wu Q, Brown MR. Signaling and function of insulin-like peptides in insects. Annu Rev Entomol. 2006;51:1–24.

9. Shabanpoor F, Separovic F, Wade JD. The human insulin superfamily of polypeptide hormones. Vitam Horm. 2009;80:1–31.

10. Iwami M. Bombyxin: An Insect Brain Peptide that Belongs to the Insulin Family. Zoolog Sci. 2000 Nov 1;17(8):1035–44.

11. De Meyts P, Sajid W, Palsgaard J, Theede AM, Gauguin L, Aladdin H, et al. Insulin and IGF-I receptor structure and binding mechanism. Mech Insul Action Med Intell Unit. 2007;1–32.

12. Weiss M, Steiner DF, Philipson LH. Insulin Biosynthesis, Secretion, Structure, and Structure-Activity Relationships. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000. Available from: http://www.ncbi.nlm.nih.gov/books/NBK279029/

13. Belfiore A, Frasca F, Pandini G, Sciacca L, Vigneri R. Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocr Rev. 2009 Oct;30(6):586–623.

14. Ward CW, Lawrence MC. Landmarks in Insulin Research. Front Endocrinol. 2011 Nov 22;2.

15. Weiss MA. The structure and function of insulin: decoding the TR transition. Vitam Horm. 2009;80:33–49.

16. Derewenda U, Derewenda Z, Dodson EJ, Dodson GG, Reynolds CD, Smith GD, et al. Phenol stabilizes more helix in a new symmetrical zinc insulin hexamer. Nature. 1989 Apr 13;338(6216):594–6.

17. Jacoby E, Krüger P, Karatas Y, Wollmer A. Distinction of structural reorganisation and ligand binding in the T<==>R transition of insulin on the basis of allosteric models. Biol Chem Hoppe Seyler. 1993 Sep;374(9):877–85.

18. Tokarz VL, MacDonald PE, Klip A. The cell biology of systemic insulin function. J Cell Biol. 2018 Jul 2;217(7):2273–89.

19. Lundquist I, Ekholm R, Ericson LE. Monoamines in the pancreatic islets of the mouse. 5-hydroxytryptamine as an intracellular modifier of insulin secretion, and the hypoglycaemic action of monoamine oxidase inhibitors. Diabetologia. 1971 Dec;7(6):414–22.

20. Ustione A, Piston DW, Harris PE. Minireview: Dopaminergic regulation of insulin secretion from the pancreatic islet. Mol Endocrinol Baltim Md. 2013 Aug;27(8):1198–207.

21. Culina S, Brezar V, Mallone R. Mechanisms in endocrinology: Insulin and type 1 diabetes: immune connections. Eur J Endocrinol. 2013 Feb 1;168(2):R19–31.

22. Baker EN, Blundell TL, Cutfield JF, Cutfield SM, Dodson EJ, Dodson GG, et al. The structure of 2Zn pig insulin crystals at 1.5 A resolution. Philos Trans R Soc Lond B Biol Sci. 1988 Jul 6;319(1195):369–456.

23. De Meyts P. Insulin and its receptor: structure, function and evolution. BioEssays News Rev Mol Cell Dev Biol. 2004 Dec;26(12):1351–62.

24. Khan AH, Pessin JE. Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways. Diabetologia. 2002 Nov;45(11):1475–83.

25. Lisgarten DR, Palmer RA, Lobley CMC, Naylor CE, Chowdhry BZ, Al-Kurdi ZI, et al. Ultra-high resolution X-ray structures of two forms of human recombinant insulin at 100 K. Chem Cent J. 2017 Aug 1;11:73.

26. Jacoby E, Hua QX, Stern AS, Frank BH, Weiss MA. Structure and dynamics of a protein assembly. 1H-NMR studies of the 36 kDa R6 insulin hexamer. J Mol Biol. 1996 Apr 26;258(1):136–57.

27. Olsen HB, Ludvigsen S, Kaarsholm NC. Solution structure of an engineered insulin monomer at neutral pH. Biochemistry. 1996 Jul 9;35(27):8836–45.

28. Conlon JM. Evolution of the insulin molecule: insights into structure-activity and phylogenetic relationships. Peptides. 2001 Jul;22(7):1183–93.

29. Oh Y, Beukers MW, Pham HM, Smanik PA, Smith MC, Rosenfeld RG. Altered affinity of insulin-like growth factor II (IGF-II) for receptors and IGF-binding proteins, resulting from limited modifications of the IGF-II molecule. Biochem J. 1991 Aug 15;278 (Pt 1):249–54.

30. Blundell T, Dodson G, Hodgkin D, Mercola D. Insulin: The Structure in the Crystal and its Reflection in Chemistry and Biology by. In: Anfinsen CB, Edsall JT, Richards FM, editors. Advances in Protein Chemistry 26, Academic Press; 1972. p. 279–402.

31. Salmon WD, Daughaday WH. A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro. J Lab Clin Med. 1957 Jun;49(6):825–36.

32. Froesch ER, Bürgi H, Ramseier EB, Bally P, Labhart A. Antibody-supressible and nonsupressible insulin-like activities in human serum and their physiologic significance. An insulin assay with adipose tissue of increased precision and specificity*. J Clin Invest. 1963 Nov;42(11):1816–34.

33. Rinderknecht E, Humbel RE. Polypeptides with nonsuppressible insulin-like and cell-growth promoting activities in human serum: isolation, chemical characterization, and some biological properties of forms I and II. Proc Natl Acad Sci U S A. 1976 Jul;73(7):2365–9.

34. Rinderknecht E, Humbel RE. Amino-terminal sequences of two polypeptides from human serum with nonsuppressible insulin-like and cell-growth-promoting activities: evidence for structural homology with insulin B chain. Proc Natl Acad Sci U S A. 1976 Dec;73(12):4379–81.

35. Engström W, Shokrai A, Otte K, Granérus M, Gessbo A, Bierke P, et al. Transcriptional regulation and biological significance of the insulin like growth factor II gene. Cell Prolif. 1998 Dec;31(5–6):173–89.

36. McRory JE, Sherwood NM. Ancient divergence of insulin and insulin-like growth factor. DNA Cell Biol. 1997 Aug;16(8):939–49.

37. Renehan AG, Jones J, O'Dwyer ST, Shalet SM. Determination of IGF-I, IGF-II, IGFBP-2, and IGFBP-3 levels in serum and plasma: comparisons using the Bland-Altman method. Growth Horm IGF Res Off J Growth Horm Res Soc Int IGF Res Soc. 2003 Dec;13(6):341–6.

38. Khosla S, Hassoun AA, Baker BK, Liu F, Zein NN, Whyte MP, et al. Insulin-like growth factor system abnormalities in hepatitis C-associated osteosclerosis. Potential insights into increasing bone mass in adults. J Clin Invest. 1998 May 15;101(10):2165–73.

39. De Meyts P, Sajid W, Palsgaard J, Theede A-M, Gauguin L, Aladdin H, et al. Insulin and IGF-I Receptor Structure and Binding Mechanism [Internet]. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013. Available from: https://www.ncbi.nlm.nih.gov/books/NBK6192/

40. Andersen M, Nørgaard-Pedersen D, Brandt J, Pettersson I, Slaaby R. IGF1 and IGF2 specificities to the two insulin receptor isoforms are determined by insulin receptor amino acid 718. PloS One. 2017 Jun 1;12(6):e0178885.

41. Brissenden JE, Ullrich A, Francke U. Human chromosomal mapping of genes for insulin-like growth factors I and II and epidermal growth factor. Nature. 1984 Sep 30;310(5980):781–4.

42. Bergman D, Halje M, Nordin M, Engström W. Insulin-Like Growth Factor 2 in Development and Disease: A Mini-Review. Gerontology. 2013;59(3):240–9.

43. Chao W, D'Amore PA. IGF2: Epigenetic regulation and role in development and disease. Cytokine Growth Factor Rev. 2008 Apr;19(2):111–20.

44. Reik W, Constancia M, Dean W, Davies K, Bowden L, Murrell A, et al. Igf2 imprinting in development and disease. Int J Dev Biol. 2000;44(1):145–50.

45. DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. Cell. 1991 Feb 22;64(4):849–59.

46. Zumstein PP, Luthi C, Humbel RE. Amino Acid Sequence of a Variant Pro-Form of Insulin-Like Growth Factor II. Proc Natl Acad Sci U S A. 1985;82(10):3169–72.

47. Khosla S, Ballard FJ, Conover CA. Use of site-specific antibodies to characterize the circulating form of big insulin-like growth factor II in patients with hepatitis C-associated osteosclerosis. J Clin Endocrinol Metab. 2002 Aug;87(8):3867–70.

48. Daughaday WH, Trivedi B. Measurement of derivatives of proinsulin-like growth factor-II in serum by a radioimmunoassay directed against the E-domain in normal subjects and patients with nonislet cell tumor hypoglycemia. J Clin Endocrinol Metab. 1992 Jul;75(1):110–5.

49. Qiu Q, Jiang J-Y, Bell M, Tsang BK, Gruslin A. Activation of Endoproteolytic Processing of Insulin-Like Growth Factor-II in Fetal, Early Postnatal, and Pregnant Rats and Persistence of Circulating Levels in Postnatal Life. Endocrinology. 2007 Oct 1;148(10):4803–11.

50. Hudgins WR, Hampton B, Burgess WH, Perdue JF. The identification of O-glycosylated precursors of insulin-like growth factor II. J Biol Chem. 1992 Apr 25;267(12):8153–60.

51. Valenzano KJ, Heath-Monnig E, Tollefsen SE, Lake M, Lobel P. Biophysical and biological properties of naturally occurring high molecular weight insulin-like growth factor II variants. J Biol Chem. 1997 Feb 21;272(8):4804–13.

52. Terasawa H, Kohda D, Hatanaka H, Nagata K, Higashihashi N, Fujiwara H, et al. Solution structure of human insulin-like growth factor II; recognition sites for receptors and binding proteins. EMBO J. 1994 Dec 1;13(23):5590–7.

53. Torres AM, Forbes BE, Wallace JC, Francis GL, Norton RS. Solution structure of human insulin-like growth factor II. Relationship to receptor and binding protein interactions. J Mol Biol. 1995 Apr 28;248(2):385–401.

54. De Meyts P, Whittaker J. Structural biology of insulin and IGF1 receptors: implications for drug design. Nat Rev Drug Discov. 2002 Oct;1(10):769–83.

55. Hassan AB. Keys to the hidden treasures of the mannose 6-phosphate/insulin-like growth factor 2 receptor. Am J Pathol. 2003 Jan;162(1):3–6.

56. MacDonald RG, Tepper MA, Clairmont KB, Perregaux SB, Czech MP. Serum form of the rat insulin-like growth factor II/mannose 6-phosphate receptor is truncated in the carboxyl-terminal domain. J Biol Chem. 1989 Feb 25;264(6):3256–61.

57. Olson LJ, Yammani RD, Dahms NM, Kim J-JP. Structure of uPAR, plasminogen, and sugar-binding sites of the 300 kDa mannose 6-phosphate receptor. EMBO J. 2004 May 19;23(10):2019–28.

58. Reddy ST, Chai W, Childs RA, Page JD, Feizi T, Dahms NM. Identification of a Low Affinity Mannose 6-Phosphate-binding Site in Domain 5 of the Cation-independent Mannose 6-Phosphate Receptor. J Biol Chem. 2004 Oct 9;279(37):38658–67.

59. Schmidt B, Kiecke-Siemsen C, Waheed A, Braulke T, Figura K von. Localization of the Insulin-like Growth Factor II Binding Site to Amino Acids 1508–1566 in Repeat 11 of the Mannose 6-Phosphate/Insulin-like Growth Factor II Receptor. J Biol Chem. 1995 Jun 23;270(25):14975–82.

60. Brown J, Delaine C, Zaccheo OJ, Siebold C, Gilbert RJ, van Boxel G, et al. Structure and functional analysis of the IGF-II/IGF2R interaction. EMBO J. 2008 Jan 9;27(1):265–76.

61. Williams C, Rezgui D, Prince SN, Zaccheo OJ, Foulstone EJ, Forbes BE, et al. Structural insights into the interaction of insulin-like growth factor 2 with IGF2R domain 11. Struct Lond Engl 1993. 2007 Sep;15(9):1065–78.

62. Costello M, Baxter RC, Scott CD. Regulation of Soluble Insulin-Like Growth Factor II/Mannose 6-Phosphate Receptor in Human Serum: Measurement by Enzyme-Linked Immunosorbent Assay. J Clin Endocrinol Metab. 1999 Feb 1;84(2):611–7.

63. Liu Q, Grubb JH, Huang SS, Sly WS, Huang JS. The Mannose 6-Phosphate/Insulin-like Growth Factor-II Receptor Is a Substrate of Type V Transforming Growth Factor-β Receptor. J Biol Chem. 1999 Sep 7;274(28):20002–10.

64. El-Shewy HM, Luttrell LM. Chapter 24 Insulin-Like Growth Factor-2/Mannose-6 Phosphate Receptors. In: Vitamins & Hormones [Internet]. Academic Press; 2009 [cited 2018 Oct 17]. p. 667–97. (Insulin and IGFs; vol. 80). Available from: http://www.sciencedirect.com/science/article/pii/S0083672908006249

65. Harris LK, Westwood M. Biology and significance of signalling pathways activated by IGF-II. Growth Factors Chur Switz. 2012 Feb;30(1):1–12.

66. El-Shewy HM, Lee M-H, Obeid LM, Jaffa AA, Luttrell LM. The insulin-like growth factor type 1 and insulin-like growth factor type 2/mannose-6-phosphate receptors independently regulate ERK1/2 activity in HEK293 cells. J Biol Chem. 2007 Sep 7;282(36):26150–7.

67. Roth BV, Bürgisser DM, Lüthi C, Humbel RE. Mutants of human insulin-like growth factor II: expression and characterization of analogs with a substitution of TYR27 and/or a deletion of residues 62-67. Biochem Biophys Res Commun. 1991 Dec 16;181(2):907–14.

68. Forbes BE, Hartfield PJ, McNeil KA, Surinya KH, Milner SJ, Cosgrove LJ, et al. Characteristics of binding of insulin-like growth factor (IGF)-I and IGF-II analogues to the type 1 IGF receptor determined by BIAcore analysis. Eur J Biochem. 2002 Feb;269(3):961–8.

69. Sakano K, Enjoh T, Numata F, Fujiwara H, Marumoto Y, Higashihashi N, et al. The design, expression, and characterization of human insulin-like growth factor II (IGF-II) mutants specific for either the IGF-II/cation-independent mannose 6-phosphate receptor or IGF-I receptor. J Biol Chem. 1991 Nov 5;266(31):20626–35.

70. Bürgisser DM, Roth BV, Giger R, Lüthi C, Weigl S, Zarn J, et al. Mutants of human insulin-like growth factor II with altered affinities for the type 1 and type 2 insulin-like growth factor receptor. J Biol Chem. 1991 Jan 15;266(2):1029–33.

71. Ziegler AN, Chidambaram S, Forbes BE, Wood TL, Levison SW. Insulin-like growth factor-II (IGF-II) and IGF-II analogs with enhanced insulin receptor-a binding affinity promote neural stem cell expansion. J Biol Chem. 2014 Feb 21;289(8):4626–33.

72. Delaine C, Alvino CL, McNeil KA, Mulhern TD, Gauguin L, De Meyts P, et al. A novel binding site for the human insulin-like growth factor-II (IGF-II)/mannose 6-phosphate receptor on IGF-II. J Biol Chem. 2007 Jun 29;282(26):18886–94.

73. De Meyts P. The Insulin Receptor and Its Signal Transduction Network [Updated 2016 Apr 27]. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000 [cited 2018 Oct 19]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK378978/

74. Frasca F, Pandini G, Scalia P, Sciacca L, Mineo R, Costantino A, et al. Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol Cell Biol. 1999 May;19(5):3278–88.

75. Belfiore A, Malaguarnera R, Vella V, Lawrence MC, Sciacca L, Frasca F, et al. Insulin Receptor Isoforms in Physiology and Disease: An Updated View. Endocr Rev. 2017 01;38(5):379–431.

76. Bass J, Chiu G, Argon Y, Steiner DF. Folding of insulin receptor monomers is facilitated by the molecular chaperones calnexin and calreticulin and impaired by rapid dimerization. J Cell Biol. 1998 May 4;141(3):637–46.

77. De Meyts P. The insulin receptor: a prototype for dimeric, allosteric membrane receptors? Trends Biochem Sci. 2008 Aug;33(8):376–84.

78. Lawrence MC, McKern NM, Ward CW. Insulin receptor structure and its implications for the IGF-1 receptor. Curr Opin Struct Biol. 2007 Dec 1;17(6):699–705.

79. Sparrow LG, McKern NM, Gorman JJ, Strike PM, Robinson CP, Bentley JD, et al. The Disulfide Bonds in the C-terminal Domains of the Human Insulin Receptor Ectodomain. J Biol Chem. 1997 Nov 21;272(47):29460–7.

80. McKern NM, Lawrence MC, Streltsov VA, Lou M-Z, Adams TE, Lovrecz GO, et al. Structure of the insulin receptor ectodomain reveals a folded-over conformation. Nature. 2006 Sep 14;443(7108):218–21.

81. Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol. 2006 Feb;7(2):85–96.

82. Backer JM, Shoelson SE, Weiss MA, Hua QX, Cheatham RB, Haring E, et al. The insulin receptor juxtamembrane region contains two independent tyrosine/beta-turn internalization signals. J Cell Biol. 1992 Aug;118(4):831–9.

83. Bailyes EM, Navé BT, Soos MA, Orr SR, Hayward AC, Siddle K. Insulin receptor/IGF-I receptor hybrids are widely distributed in mammalian tissues: quantification of individual receptor species by selective immunoprecipitation and immunoblotting. Biochem J. 1997 Oct 1;327 (Pt 1):209–15.

84. Benecke H, Flier JS, Moller DE. Alternatively spliced variants of the insulin receptor protein. Expression in normal and diabetic human tissues. J Clin Invest. 1992 Jun;89(6):2066–70.

85. Ramalingam L, Oh E, Thurmond DC. Novel roles for insulin receptor (IR) in adipocytes and skeletal muscle cells via new and unexpected substrates. Cell Mol Life Sci CMLS. 2013 Aug;70(16):2815–34.

86. Louvi A, Accili D, Efstratiadis A. Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. Dev Biol. 1997 Sep 1;189(1):33–48.

87. Belfiore A, Malaguarnera R. Insulin receptor and cancer. Endocr Relat Cancer. 2011 Aug;18(4):R125-147.

88. Wang C-F, Zhang G, Zhao L-J, Qi W-J, Li X-P, Wang J-L, et al. Overexpression of the insulin receptor isoform A promotes endometrial carcinoma cell growth. PloS One. 2013;8(8):e69001.

89. Denley A, Wallace JC, Cosgrove LJ, Forbes BE. The Insulin Receptor Isoform Exon 11- (IR-A) in Cancer and Other Diseases: A Review. Horm Metab Res. 2003 Nov;35(11/12):778–85.

90. García-Arencibia M, Molero S, Dávila N, Carranza MC, Calle C. 17beta-Estradiol transcriptionally represses human insulin receptor gene expression causing cellular insulin resistance. Leuk Res. 2005 Jan;29(1):79–87.

91. Kaminska D, Hämäläinen M, Cederberg H, Käkelä P, Venesmaa S, Miettinen P, et al. Adipose tissue INSR splicing in humans associates with fasting insulin level and is regulated by weight loss. Diabetologia. 2014 Feb;57(2):347–51.

92. Malakar P, Chartarifsky L, Hija A, Leibowitz G, Glaser B, Dor Y, et al. Insulin receptor alternative splicing is regulated by insulin signaling and modulates beta cell survival. Sci Rep. 2016 Aug 16;6:31222.

93. Sen S, Talukdar I, Liu Y, Tam J, Reddy S, Webster NJG. Muscleblind-like 1 (Mbnl1) Promotes Insulin Receptor Exon 11 Inclusion via Binding to a Downstream Evolutionarily Conserved Intronic Enhancer. J Biol Chem. 2010 Aug 13;285(33):25426–37.

94. Talukdar I, Sen S, Urbano R, Thompson J, Yates JR, Webster NJG. hnRNP A1 and hnRNP F modulate the alternative splicing of exon 11 of the insulin receptor gene. PloS One. 2011;6(11):e27869.

95. Yamaguchi Y, Flier JS, Yokota A, Benecke H, Backer JM, Moller DE. Functional properties of two naturally occurring isoforms of the human insulin receptor in Chinese hamster ovary cells. Endocrinology. 1991 Oct;129(4):2058–66.

96. Benyoucef S, Surinya KH, Hadaschik D, Siddle K. Characterization of insulin/IGF hybrid receptors: contributions of the insulin receptor L2 and Fn1 domains and the alternatively spliced exon 11 sequence to ligand binding and receptor activation. Biochem J. 2007 May 1;403(3):603–13.

97. Mosthaf L, Grako K, Dull TJ, Coussens L, Ullrich A, McClain DA. Functionally distinct insulin receptors generated by tissue-specific alternative splicing. EMBO J. 1990 Aug;9(8):2409–13.

98. Vogt B, Carrascosa JM, Ermel B, Ullrich A, Häring HU. The two isotypes of the human insulin receptor (HIR-A and HIR-B) follow different internalization kinetics. Biochem Biophys Res Commun. 1991 Jun 28;177(3):1013–8.

99. Leibiger B, Leibiger IB, Moede T, Kemper S, Kulkarni RN, Kahn CR, et al. Selective insulin signaling through A and B insulin receptors regulates transcription of insulin and glucokinase genes in pancreatic beta cells. Mol Cell. 2001 Mar;7(3):559–70.

100. Nevado C, Valverde AM, Benito M. Role of insulin receptor in the regulation of glucose uptake in neonatal hepatocytes. Endocrinology. 2006 Aug;147(8):3709–18.

101. Křížková K, Chrudinová M, Povalová A, Selicharová I, Collinsová M, Vaněk V, et al. Insulin-Insulin-like Growth Factors Hybrids as Molecular Probes of Hormone:Receptor Binding Specificity. Biochemistry. 2016 May 31;55(21):2903–13.

102. Viková J, Collinsová M, Kletvíková E, Buděšínský M, Kaplan V, Žáková L, et al. Rational steering of insulin binding specificity by intra-chain chemical crosslinking. Sci Rep. 2016 Jan 21;6:19431.

103. Pollak MN, Schernhammer ES, Hankinson SE. Insulin-like growth factors and neoplasia. Nat Rev Cancer. 2004 Jul;4(7):505–18.

104. Pollak M. The insulin receptor/insulin-like growth factor receptor family as a therapeutic target in oncology. Clin Cancer Res Off J Am Assoc Cancer Res. 2012 Jan 1;18(1):40–50.

105. Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, et al. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J. 1986 Oct;5(10):2503–12.

106. Adams TE, Epa VC, Garrett TP, Ward CW. Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci CMLS. 2000 Jul;57(7):1050–93.

107. Whitten AE, Smith BJ, Menting JG, Margetts MB, McKern NM, Lovrecz GO, et al. Solution structure of ectodomains of the insulin receptor family: the ectodomain of the type 1 insulin-like growth factor receptor displays asymmetry of ligand binding accompanied by limited conformational change. J Mol Biol. 2009 Dec 18;394(5):878–92.

108. Xu Y, Kong GK-W, Menting JG, Margetts MB, Delaine CA, Jenkin LM, et al. How ligand binds to the type 1 insulin-like growth factor receptor. Nat Commun. 2018 Feb 26;9(1):821.

109. Croll TI, Smith BJ, Margetts MB, Whittaker J, Weiss MA, Ward CW, et al. Higher-Resolution Structure of the Human Insulin Receptor Ectodomain: Multi-Modal Inclusion of the Insert Domain. Struct Lond Engl 1993. 2016 Mar 1;24(3):469–76.

110. De Meyts P, Roth J, Neville DM, Gavin JR, Lesniak MA. Insulin interactions with its receptors: experimental evidence for negative cooperativity. Biochem Biophys Res Commun. 1973 Nov 1;55(1):154–61.

111. Ward CW, Lawrence MC. Ligand-induced activation of the insulin receptor: a multi-step process involving structural changes in both the ligand and the receptor. BioEssays News Rev Mol Cell Dev Biol. 2009 Apr;31(4):422–34.

112. Ward CW, Lawrence MC. Similar but different: ligand-induced activation of the insulin and epidermal growth factor receptor families. Curr Opin Struct Biol. 2012 Jun;22(3):360–6.

113. Kiselyov VV, Versteyhe S, Gauguin L, De Meyts P. Harmonic oscillator model of the insulin and IGF1 receptors' allosteric binding and activation. Mol Syst Biol. 2009 Feb 17;5:243.

114. Scapin G, Dandey VP, Zhang Z, Prosise W, Hruza A, Kelly T, et al. Structure of the insulin receptor-insulin complex by single-particle cryo-EM analysis. Nature. 2018 05;556(7699):122–5.

115. Hao C, Whittaker L, Whittaker J. Characterization of a second ligand binding site of the insulin receptor. Biochem Biophys Res Commun. 2006 Aug 18;347(1):334–9.

116. Whittaker L, Hao C, Fu W, Whittaker J. High-affinity insulin binding: insulin interacts with two receptor ligand binding sites. Biochemistry. 2008 Dec 2;47(48):12900–9.

117. Menting JG, Whittaker J, Margetts MB, Whittaker LJ, Kong GK-W, Smith BJ, et al. How insulin engages its primary binding site on the insulin receptor. Nature. 2013 Jan 10;493(7431):241–5.

118. Gauguin L, Delaine C, Alvino CL, McNeil KA, Wallace JC, Forbes BE, et al. Alanine scanning of a putative receptor binding surface of insulin-like growth factor-I. J Biol Chem. 2008 Jul 25;283(30):20821–9.

119. De Meyts P. Insulin/receptor binding: the last piece of the puzzle? What recent progress on the structure of the insulin/receptor complex tells us (or not) about negative cooperativity and activation. BioEssays News Rev Mol Cell Dev Biol. 2015 Apr;37(4):389–97.

120. Tatulian SA. Structural Dynamics of Insulin Receptor and Transmembrane Signaling. Biochemistry. 2015 Sep 15;54(36):5523–32.

121. Ward CW, Menting JG, Lawrence MC. The insulin receptor changes conformation in unforeseen ways on ligand binding: sharpening the picture of insulin receptor activation. BioEssays News Rev Mol Cell Dev Biol. 2013 Nov;35(11):945–54, doi/10.1002/bies.201370111.

122. Hua QX, Shoelson SE, Kochoyan M, Weiss MA. Receptor binding redefined by a structural switch in a mutant human insulin. Nature. 1991 Nov 21;354(6350):238–41.

123. Weis F, Menting JG, Margetts MB, Chan SJ, Xu Y, Tennagels N, et al. The signalling conformation of the insulin receptor ectodomain. Nat Commun. 2018 Oct 24;9(1):1–10.

124. Houde D, Demarest SJ. Fine details of IGF-1R activation, inhibition, and asymmetry determined by associated hydrogen /deuterium-exchange and peptide mass mapping. Struct Lond Engl 1993. 2011 Jun 8;19(6):890–900.

125. Whittaker J, Groth AV, Mynarcik DC, Pluzek L, Gadsbøll VL, Whittaker LJ. Alanine scanning mutagenesis of a type 1 insulin-like growth factor receptor ligand binding site. J Biol Chem. 2001 Nov 23;276(47):43980–6.

126. Alvino CL, McNeil KA, Ong SC, Delaine C, Booker GW, Wallace JC, et al. A novel approach to identify two distinct receptor binding surfaces of insulin-like growth factor II. J Biol Chem. 2009 Mar 20;284(12):7656–64.

127. Alvino CL, Ong SC, McNeil KA, Delaine C, Booker GW, Wallace JC, et al. Understanding the mechanism of insulin and insulin-like growth factor (IGF) receptor activation by IGF-II. PloS One. 2011;6(11):e27488.

128. Gauguin L, Klaproth B, Sajid W, Andersen AS, McNeil KA, Forbes BE, et al. Structural basis for the lower affinity of the insulin-like growth factors for the insulin receptor. J Biol Chem. 2008 Feb 1;283(5):2604–13.

129. Jensen M, De Meyts P. Molecular mechanisms of differential intracellular signaling from the insulin receptor. Vitam Horm. 2009;80:51–75.

130. Siddle K. Molecular Basis of Signaling Specificity of Insulin and IGF Receptors: Neglected Corners and Recent Advances. Front Endocrinol. 2012 Feb 28;3.

131. Hubbard SR, Wei L, Ellis L, Hendrickson WA. Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature. 1994 Dec 22;372(6508):746–54.

132. Siddle K. Signalling by insulin and IGF receptors: supporting acts and new players. J Mol Endocrinol. 2011 Aug;47(1):R1-10.

133. Whiteman EL, Cho H, Birnbaum MJ. Role of Akt/protein kinase B in metabolism. Trends Endocrinol Metab TEM. 2002 Dec;13(10):444–51.

134. Proud CG. Regulation of protein synthesis by insulin. Biochem Soc Trans. 2006 Apr;34(Pt 2):213–6.

135. Avruch J. MAP kinase pathways: the first twenty years. Biochim Biophys Acta. 2007 Aug;1773(8):1150–60.

136. Ramos JW. The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. Int J Biochem Cell Biol. 2008;40(12):2707–19.

137. Versteyhe S, Klaproth B, Borup R, Palsgaard J, Jensen M, Gray SG, et al. IGF-I, IGF-II, and insulin stimulate different gene expression responses through binding to the IGF-I receptor. Front Endocrinol. 2013 Aug 9;4.

138. Favelyukis S, Till JH, Hubbard SR, Miller WT. Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. Nat Struct Biol. 2001 Dec;8(12):1058–63.

139. Cai W, Sakaguchi M, Kleinridders A, Gonzalez-Del Pino G, Dreyfuss JM, O'Neill BT, et al. Domain-dependent effects of insulin and IGF-1 receptors on signalling and gene expression. Nat Commun. 2017 27;8:14892.

140. Mulligan C, Rochford J, Denyer G, Stephens R, Yeo G, Freeman T, et al. Microarray analysis of insulin and insulin-like growth factor-1 (IGF-1) receptor signaling reveals the selective up-regulation of the mitogen heparin-binding EGF-like growth factor by IGF-1. J Biol Chem. 2002 Nov 8;277(45):42480–7.

141. Morrione A, Valentinis B, Xu S, Yumet G, Louvi A, Efstratiadis A, et al. Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. Proc Natl Acad Sci U S A. 1997 Apr 15;94(8):3777–82.

142. Baudry A, Lamothe B, Bucchini D, Jami J, Montarras D, Pinset C, et al. IGF-1 receptor as an alternative receptor for metabolic signaling in insulin receptor-deficient muscle cells. FEBS Lett. 2001 Jan 19;488(3):174–8.

143. Hansen BF, Danielsen GM, Drejer K, Sørensen AR, Wiberg FC, Klein HH, et al. Sustained signalling from the insulin receptor after stimulation with insulin analogues exhibiting increased mitogenic potency. Biochem J. 1996 Apr 1;315 (Pt 1):271–9.

144. Sacco A, Morcavallo A, Pandini G, Vigneri R, Belfiore A. Differential signaling activation by insulin and insulin-like growth factors I and II upon binding to insulin receptor isoform A. Endocrinology. 2009 Aug;150(8):3594–602.

145. Morcavallo A, Stefanello M, Iozzo RV, Belfiore A, Morrione A. Ligand-mediated endocytosis and trafficking of the insulin-like growth factor receptor I and insulin receptor modulate receptor function. Front Endocrinol. 2014 Dec 17;5.

146. Morcavallo A, Genua M, Palummo A, Kletvikova E, Jiracek J, Brzozowski AM, et al. Insulin and insulin-like growth factor II differentially regulate endocytic sorting and stability of insulin receptor isoform A. J Biol Chem. 2012 Mar 30;287(14):11422–36.

147. Kasuga M, Fujita-Yamaguchi Y, Blithe DL, White MF, Kahn CR. Characterization of the insulin receptor kinase purified from human placental membranes. J Biol Chem. 1983 Sep 25;258(18):10973–80.

148. Soos MA, Siddle K. Immunological relationships between receptors for insulin and insulin-like growth factor I. Evidence for structural heterogeneity of insulin-like growth factor I receptors involving hybrids with insulin receptors. Biochem J. 1989 Oct 15;263(2):553–63.

149. Blanquart C, Achi J, Issad T. Characterization of IRA/IRB hybrid insulin receptors using bioluminescence resonance energy transfer. Biochem Pharmacol. 2008 Oct 1;76(7):873–83.

150. Soos MA, Field CE, Siddle K. Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity. Biochem J. 1993 Mar 1;290(Pt 2):419–26.

151. Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R, Belfiore A. Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. J Biol Chem. 2002 Oct 18;277(42):39684–95.

152. Slaaby R, Schäffer L, Lautrup-Larsen I, Andersen AS, Shaw AC, Mathiasen IS, et al. Hybrid receptors formed by insulin receptor (IR) and insulin-like growth factor I receptor (IGF-IR) have low insulin and high IGF-1 affinity irrespective of the IR splice variant. J Biol Chem. 2006 Sep 8;281(36):25869–74.

153. Lüthi C, Roth BV, Humbel RE. Mutants of human insulin-like growth factor II (IGF II). Expression and characterization of truncated IGF II and of two naturally occurring variants. Eur J Biochem. 1992 Apr 15;205(2):483–90.

154. Hampton B, Burgess WH, Marshak DR, Cullen KJ, Perdue JF. Purification and characterization of an insulin-like growth factor II variant from human plasma. J Biol Chem. 1989 Nov 15;264(32):19155–60.

155. Hashimoto R, Fujiwara H, Higashihashi N, Enjoh-Kimura T, Terasawa H, Fujita-Yamaguchi Y, et al. N-terminal deletion mutants of insulin-like growth factor-II (IGF-II) show Thr7 and Leu8 important for binding to insulin and IGF-I receptors and Leu8 critical for all IGF-II functions. J Biol Chem. 1995 Jul 28;270(30):18013–8.

156. Francis GL, Aplin SE, Milner SJ, McNeil KA, Ballard FJ, Wallace JC. Insulin-like growth factor (IGF)-II binding to IGF-binding proteins and IGF receptors is modified by deletion of the N-terminal hexapeptide or substitution of arginine for glutamate-6 in IGF-II. Biochem J. 1993 Aug 1;293(Pt 3):713–9.

157. Steiner DF. The proinsulin C-peptide--a multirole model. Exp Diabesity Res. 2004 Mar;5(1):7–14.

158. Hexnerová R, Křížková K, Fábry M, Sieglová I, Kedrová K, Collinsová M, et al. Probing Receptor Specificity by Sampling the Conformational Space of the Insulin-like Growth Factor II C-domain. J Biol Chem. 2016 Aug 10;jbc.M116.741041.

159. Denley A, Bonython ER, Booker GW, Cosgrove LJ, Forbes BE, Ward CW, et al. Structural determinants for high-affinity binding of insulin-like growth factor II to insulin receptor (IR)-A, the exon 11 minus isoform of the IR. Mol Endocrinol Baltim Md. 2004 Oct;18(10):2502–12.

160. Bayne ML, Applebaum J, Underwood D, Chicchi GG, Green BG, Hayes NS, et al. The C region of human insulin-like growth factor (IGF) I is required for high affinity binding to the type 1 IGF receptor. J Biol Chem. 1989 May 7;264(19):11004–8.

161. Schäffer L, Kjeldsen T, Andersen AS, Wiberg FC, Larsen UD, Cara JF, et al. Interactions of a hybrid insulin/insulin-like growth factor-I analog with chimeric insulin/type I insulin-like growth factor receptors. J Biol Chem. 1993 Feb 15;268(5):3044–7.

162. Cara JF, Mirmira RG, Nakagawa SH, Tager HS. An insulin-like growth factor I/insulin hybrid exhibiting high potency for interaction with the type I insulin-like growth factor and insulin receptors of placental plasma membranes. J Biol Chem. 1990 Oct 15;265(29):17820–5.

163. Kristensen C, Andersen AS, Hach M, Wiberg FC, Schäffer L, Kjeldsen T. A single-chain insulin-like growth factor I/insulin hybrid binds with high affinity to the insulin receptor. Biochem J. 1995 Feb 1;305 (Pt 3):981–6.

164. Henderson ST, Brierley GV, Surinya KH, Priebe IK, Catcheside DEA, Wallace JC, et al. Delineation of the IGF-II C domain elements involved in binding and activation of the IR-A, IR-B and IGF-IR. Growth Horm IGF Res Off J Growth Horm Res Soc Int IGF Res Soc. 2015 Feb;25(1):20–7.

165. Bayne ML, Applebaum J, Chicchi GG, Miller RE, Cascieri MA. The roles of tyrosines 24, 31, and 60 in the high affinity binding of insulin-like growth factor-I to the type 1 insulin-like growth factor receptor. J Biol Chem. 1990 Sep 15;265(26):15648–52.

166. King GL, Kahn CR, Samuels B, Danho W, Bullesbach EE, Gattner HG. Synthesis and characterization of molecular hybrids of insulin and insulin-like growth factor I. The role of the A-chain extension peptide. J Biol Chem. 1982 Sep 25;257(18):10869–73.

167. De Vroede MA, Rechler MM, Nissley SP, Ogawa H, Joshi S, Burke GT, et al. Mitogenic activity and receptor reactivity of hybrid molecules containing portions of the insulin-like growth factor I (IGF-I), IGF-II, and insulin molecules. Diabetes. 1986 Mar;35(3):355–61.

168. Denley A, Cosgrove LJ, Booker GW, Wallace JC, Forbes BE. Molecular interactions of the IGF system. Cytokine Growth Factor Rev. 2005 Aug 1;16(4):421–39.

169. Schäffer L, Brissette RE, Spetzler JC, Pillutla RC, Østergaard S, Lennick M, et al. Assembly of high-affinity insulin receptor agonists and antagonists from peptide building blocks. Proc Natl Acad Sci U S A. 2003 Apr 15;100(8):4435–9.

170. Whittaker J, Whittaker LJ, Roberts CT, Phillips NB, Ismail-Beigi F, Lawrence MC, et al. α-Helical element at the hormone-binding surface of the insulin receptor functions as a signaling element to activate its tyrosine kinase. Proc Natl Acad Sci U S A. 2012 Jul 10;109(28):11166–71.

171. Pullen RA, Lindsay DG, Wood SP, Tickle IJ, Blundell TL, Wollmer A, et al. Receptor-binding region of insulin. Nature. 1976 Feb;259(5542):369–73.

172. Mynarcik DC, Williams PF, Schaffer L, Yu GQ, Whittaker J. Identification of common ligand binding determinants of the insulin and insulin-like growth factor 1 receptors. Insights into mechanisms of ligand binding. J Biol Chem. 1997 Jul 25;272(30):18650–5.

173. Schwartz GP, Burke GT, Katsoyannis PG. A superactive insulin: [B10-aspartic acid]insulin(human). Proc Natl Acad Sci U S A. 1987 Sep;84(18):6408–11.

174. Glendorf T, Sørensen AR, Nishimura E, Pettersson I, Kjeldsen T. Importance of the solvent-exposed residues of the insulin B chain alpha-helix for receptor binding. Biochemistry. 2008 Apr 22;47(16):4743–51.

175. Macháčková K, Chrudinová M, Radosavljević J, Potalitsyn P, Křížková K, Fábry M, et al. Converting Insulin-like Growth Factors 1 and 2 into High-Affinity Ligands for Insulin Receptor Isoform A by the Introduction of an Evolutionarily Divergent Mutation. Biochemistry. 2018 Apr 24;57(16):2373–82.

176. Kristensen C, Kjeldsen T, Wiberg FC, Schäffer L, Hach M, Havelund S, et al. Alanine Scanning Mutagenesis of Insulin. J Biol Chem. 1997 May 16;272(20):12978–83.

177. Žáková L, Kletvíková E, Lepšík M, Collinsová M, Watson CJ, Turkenburg JP, et al. Human insulin analogues modified at the B26 site reveal a hormone conformation that is undetected in the receptor complex. Acta Crystallogr D Biol Crystallogr. 2014 Sep 27;70(Pt 10):2765–74.

178. Maly P, Lüthi C. The binding sites of insulin-like growth factor I (IGF I) to type I IGF receptor and to a monoclonal antibody. Mapping by chemical modification of tyrosine residues. J Biol Chem. 1988 May 25;263(15):7068–72.

179. Keyhanfar M, Booker GW, Whittaker J, Wallace JC, Forbes BE. Precise mapping of an IGF-I-binding site on the IGF-1R. Biochem J. 2007 Jan 1;401(Pt 1):269–77.

180. Dynkevich Y, Rother KI, Whitford I, Qureshi S, Galiveeti S, Szulc AL, et al. Tumors, IGF-2, and Hypoglycemia: Insights From the Clinic, the Laboratory, and the Historical Archive. Endocr Rev. 2013 Dec 1;34(6):798–826.

181. Simon J, Freychet P, Rosselin G, DeMeyts P. Enhanced binding affinity of chicken insulin in rat liver membranes and human lymphocytes: relationship tothe kinetic properties of the hormone- receptor interaction. Endocrinology. 1977 Jan;100(1):115–21.

182. Weiss MA, Hua Q-X, Jia W, Nakagawa SH, Chu Y-C, Hu S-Q, et al. Activities of Monomeric Insulin Analogs at Position A8 Are Uncorrelated with Their Thermodynamic Stabilities. J Biol Chem. 2001 Oct 26;276(43):40018–24.

183. Weiss MA, Wan Z, Zhao M, Chu Y-C, Nakagawa SH, Burke GT, et al. Non-standard insulin design: structure-activity relationships at the periphery of the insulin receptor. J Mol Biol. 2002 Jan 11;315(2):103–11.

184. Wan Z, Xu B, Huang K, Chu Y-C, Li B, Nakagawa SH, et al. Enhancing the activity of insulin at the receptor interface: crystal structure and photo-cross-linking of A8 analogues. Biochemistry. 2004 Dec 28;43(51):16119–33.

185. Menting JG, Lawrence CF, Kong GK-W, Margetts MB, Ward CW, Lawrence MC. Structural Congruency of Ligand Binding to the Insulin and Insulin/Type 1 Insulin-like Growth Factor Hybrid Receptors. Struct Lond Engl 1993. 2015 Jul 7;23(7):1271–82.

186. Jirácek J, Záková L, Antolíková E, Watson CJ, Turkenburg JP, Dodson GG, et al. Implications for the active form of human insulin based on the structural convergence of highly active hormone analogues. Proc Natl Acad Sci U S A. 2010 Feb 2;107(5):1966–70.

7 APPENDIX

Appendix I

BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

Insulin−Insulin-like Growth Factors Hybrids as Molecular Probes of Hormone:Receptor Binding Specificity

Květoslava Křížková,[†,‡] Martina Chrudinová,[†,‡] Anna Povalová,[†,‡] Irena Selicharová,[†] Michaela Collinsová,[†] Václav Vaněk,[†] Andrzej M. Brzozowski,[§] Jiří Jiráček,[†] and Lenka Žáková*[,†]

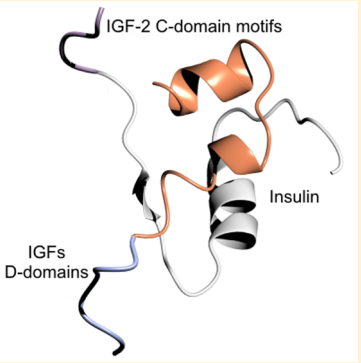
[†]Institute of Organic Chemistry and Biochemistry, Academy of Science of the Czech Republic v.v.i., Flemingovo nám. 2, 166 10 Praha 6, Czech Republic

[‡]Charles University in Prague, Faculty of Science, Department of Biochemistry, Hlavova 8, 128 43 Praha 2, Czech Republic

[§]York Structural Biology Laboratory, Department of Chemistry, University of York, Heslington, York YO10 5DD, United Kingdom

Supporting Information

ABSTRACT: Insulin, insulin-like growth factors 1 and 2 (IGF-1 and -2, respectively), and their receptors (IR and IGF-1R) are the key elements of a complex hormonal system that is essential for the development and functioning of humans. The C and D domains of IGFs (absent in insulin) likely play important roles in the differential binding of IGF-1 and -2 to IGF-1R and to the isoforms of IR (IR-A and IR-B) and specific activation of these receptors. Here, we attempted to probe the impact of IGF-1 and IGF-2 D domains (D_I and D_{II}, respectively) and the IGF-2 C domain (C_{II}) on the receptor specificity of these hormones. For this, we made two types of insulin hybrid analogues: (i) with the C-terminus of the insulin A chain extended by the amino acids from the D_I and D_{II} domains and (ii) with the C-terminus of the insulin B chain extended by some amino acids derived from the C_{II} domain. The receptor binding affinities of these analogues and their receptor autophosphorylation potentials were characterized. Our results indicate that the D_I domain has a more negative impact than the D_{II} domain does on binding to IR, and that the D_I domain Pro-Leu-Lys residues are important factors for a different IR-A versus IR-B binding affinity of IGF-1. We also showed that the additions of amino acids that partially "mimic" the C_{II} domain, to the C-terminus of the insulin B chain, change the binding and autophosphorylation specificity of insulin in favor of the "metabolic" IR-B isoform. This opens new venues for rational enhancement of insulin IR-B specificity by modifications beyond the C-terminus of its B chain.



Insulin, insulin-like growth factors 1 and 2 (IGF-1 and -2, respectively), and their cognate cell surface receptors, together with IGF-binding proteins (IGFBP-1−6), form a complex hormonal/signaling system that is essential for the development and functioning of humans. Its deregulation leads to increased cancer risk, diabetes mellitus type 1 and 2, and other disorders, such as obesity and metabolic syndrome.[1,2]

Insulin is a small (51 amino acids) protein hormone that is the main regulator of glucose homeostasis. The mature insulin is the post-translational product of a single-chain (pre)-proinsulin, where the C peptide connecting A and B chains is proteolytically, and specifically, cleaved off. This results in a two-chain (A1−A21 and B1−B30) hormone, with two interchain disulfide bridges (A7−B7 and A20−B19) and one intrachain disulfide bridge (A6−A11) (Figure 1). The overall tertiary structure, chain organization, and arrangement of the disulfide bridges are conserved in members of the insulin-like family such as IGFs, relaxins, bombyxins, insulin-like peptides, etc.[3]

In contrast to insulin, IGF-1 and IGF-2 are single-chain hormones (70 and 67 amino acids, respectively) involved mainly in cell growth and protection against apoptosis.[4,5] Both IGFs share a high degree of sequence homology, which is also extended to insulin (see Figure 1). They consist of four domains, B, C, A, and D, with their B and A domains corresponding to the B and A chains of insulin. C segments of IGFs that span the B and A domains are structural analogues of insulin C peptide, but without sequence similarity. The unique D domains of IGFs [without equivalents in (pro)insulin] extend the C-terminus of the A domains (Figure 1).

Insulin and IGFs elicit their biological effects by binding to the insulin receptor isoforms (IR-A and IR-B), and the insulin-like growth factor receptor 1 (IGF-1R), while circulating IGF-binding proteins modulate bioavailability of both IGFs.[6] Moreover, IGF-2 binds specifically to the distinct insulin-like growth factor receptor 2 (IGF-2R), which is a cation-independent mannose 6-phosphate receptor that is likely responsible for the clearance of IGF-2 from the cell surface and for the preclusion of IGF-2:IGF-1R/IR interactions.[4] The IR and IGF-1R receptors are transmembrane glycoproteins consisting of two α and two β subunits, which are connected by

Received: February 16, 2016
Revised: May 12, 2016
Published: May 12, 2016

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903−2913

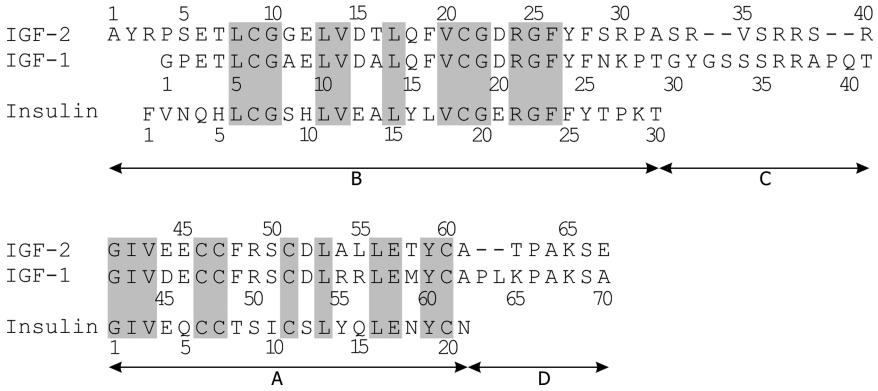


Figure 1. Alignment of primary sequences of human IGF-1, IGF-2, and insulin. The gray background highlights evolutionarily conserved residues. Arrows indicate the residues associated with the A–D domains of IGFs and the A and B chains of insulin.

disulfide bridges to create $(\alpha\beta)_2$ dimers. The extracellular α parts of the IR contain hormone-binding regions, and the membrane-anchored parts β contain intracellular tyrosine kinase domains.[7−9] The functional heterogeneity of IR arises from an alternative splicing of exon 11 located at the C-terminus of the α subunit, which results in two isoforms, IR-B and IR-A, with different C-terminal αCT peptides (IR-B and IR-A, ±12 amino acids). The IR isoforms are expressed in a tissue-specific manner in humans. IR-B is the main IR form for insulin glycemic response-sensitive tissues (liver, muscles, and adipose tissue), while IR-A is a dominant IR isoform in the brain.[10−12] Insulin and IGF-1 bind preferentially to their cognate receptors (IR-A/IR-B and IGF-1R, respectively) at subnanomolar concentrations. However, both hormones can also cross-bind to their receptors (but with significantly lower affinities), with the exception of IGF-2 that binds with relatively high affinity to both IR-A and IGF-1R.[13−15]

Simultaneous engagement of two, distinct hormone-binding sites, so-called site 1 and site 2, on insulin and IGF receptors is required for the high-affinity binding complex.[16] The recent crystallographic studies showed that site 1 on both IR and IGF-1R receptors is similar, involving some L1 domain surface and the αCT peptide, which interact with the respective binding sites 1 on insulin and IGFs (in A and B chains/domains)[17,18] or IGF-1.[19] However, the nature of IR/IGF-1R-binding site 2 is still understood only on the basis of mutagenesis studies.[20−23]

Although there is now relatively good insight into the roles of A and B chains/domains of insulin and IGF-1 in the hormone:IR/IGF-1R site 1 interactions, the functional impact of the C and D domains of IGFs is much less understood. It is envisaged, however, that they play some role in a differential binding of IGF-1 and -2 to IR-A, IR-B, and IGF-1R, and activation.[24,25] The studies of the functional importance of the IGF-1 C domain (C_I domain), investigated in the context of different constructs of insulin,[26,27] IGF-1,[25,28] and IGF-2,[24] revealed that it may play an important role in the IGF-1:IGF-1R interaction, and in eliciting biological activity of this hormone. However, it is detrimental to IGF-1:IR binding. It was also proposed that the C_I domain may interact with the CR domain of the IGF-1R,[29,30] but the recent crystal structure of the complex of IGF-1 with IR L1-CR domains, mediated by the IGF-1R αCT segment[19] (Figure 2), did not yet clarify contacts of the C and D domains with the receptor. In contrast to IGF-1, the interaction of the shorter C domain of IGF-2 (C_{II}) with the CR domain is not anticipated.[31,32] Although the role of the C_{II} domain has been much less studied, it has been shown that

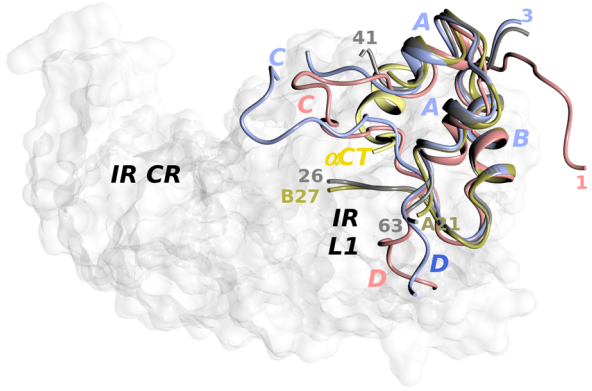


Figure 2. Structures of insulin, IGF-1, and IGF-2 on the insulin receptor L1-CR domains. All individual hormone structures have been superimposed on the crystal structure of the IR L1-CR/IGF-1/IGF-1R αCT-peptide complex (PDB entry 4xss), with the IR as the white surface, IGF-1 in the 4xss complex colored dark gray, and the αCT peptide colored bright yellow. The insulin molecule (gold/dark yellow) was derived from the homologous IR L1-CR/insulin/1R αCT-peptide structure (PDB entry 4oga). Free, noncomplexed IGF-1 [blue, PDB entry 1gzr (to show C and D domains, not defined in the 4xss structure)], IGF-2 [pink, PDB entry 1igl (NMR model 1)], and insulin were superimposed on the 8–18 Cα atoms of the B domain α helix in the IGF-1 4xss complex. There is a 36–38 gap observed in the free IGF-1 (1gzr) structure. A–D denote domains in these hormones (color coding as in the individual molecules). Numbers assist terminal residues seen in the individual structures. The conformations of the C and D domains on the IR are putative, i.e., unchanged from their noncomplexed structure, hence their clash with the IGF-1R αCT peptide.

the $C_I \rightarrow C_{II}$ swap in IGF-1 doubled the binding affinities of this IGF-1 analogue for IR-A and IR-B but diminished its binding affinity for IGF-1R to ∼25%.[24]

The role of D_I and D_{II} domains has been studied by several groups,[24,33,34] which showed that both D_I and D_{II} domains play some roles in the activation of IR and IGF-1R, but they are possibly less important in this process than the C domains.

Although the receptor-binding surfaces of insulin and both IGFs have a similar side chain pattern,[7,21,23,35] it is assumed that each molecule uses a slightly different IR-A, IR-B, or IGF-1R binding mechanism,[32] triggering subsequently specific signaling cascades.[14,36,37] Moreover, the differential binding of insulin and IGFs to so-called hybrid receptors (receptor heterodimers formed by IR-A/B and IR-A/IGF-1R $\alpha\beta$ subunits) brings even more complexity to the IGF/insulin system.[38,39]

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903−2913

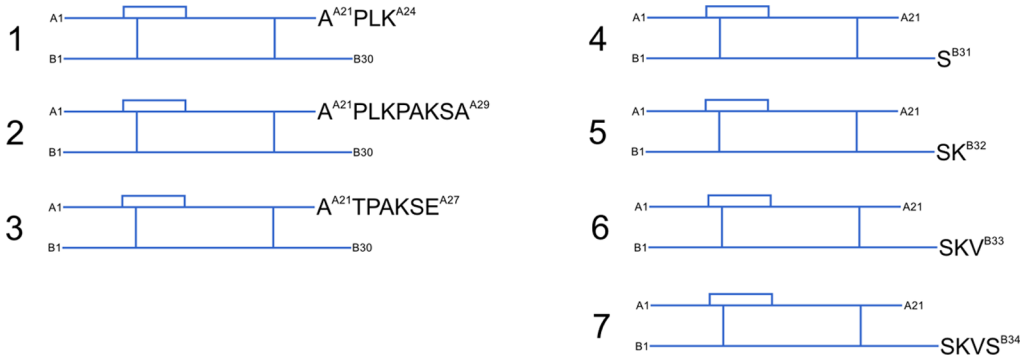


Figure 3. Schematic representation of structures of insulin analogues **1**−**7** prepared in this study. Insulin A and B chains and disulfide bridges are shown as blue lines. The additional residues derived from the D_I, D_{II}, or C_{II} domains are drawn in single-letter codes, with insulin numbering.

Here, we aimed to elucidate further the roles of the D_I, D_{II}, and C_{II} domains in the specific functionality of these hormones: their binding to IR-A, IR-B, and IGF-1R and their impact on the autophosphorylation of these receptors. The human insulin molecule was selected here as a template, as it has a high affinity for both IR isoforms; hence, it is much more sensitive probe against these receptors. To address these issues, we made (i) insulin analogues with the C-terminus of the A chain extended by amino acids from the D domains of IGF-1 and IGF-2 and (ii) insulin analogues with the C-terminus of the B chain extended by amino acids mimicking the C_{II} domain (Figure 3). Despite a single-chain organization of IGF-1, the structure of the IGF-1/IGF-1 α-CT/IR L1-CR complex[19] showed that the conformation of IGF-1 B domain residues 21−26 is almost identical to the structure of equivalent insulin B22−B27 residues on the same receptor (Figure 2). This suggests that at least the first residues of the C_I domain (invisible in this complex) can follow the direction of the end part of the B domain. As the IGF-2 and IGF-1 IR binding modes should be similar, the insulin molecule can be considered as a useful structural scaffold for the study of the role of C_{II} residues in binding of hormones to receptors. Receptor binding affinities of all analogues and their abilities to stimulate autophosphorylation of the receptors were characterized, to correlate the impact of the hormones' modifications on their receptor specificity.

EXPERIMENTAL PROCEDURES

Synthesis of Analogues 1−3. *Synthesis of Insulin Chains.* The individual modified A chains and wild-type B chain were prepared by total chemical solid-phase synthesis, and SH groups of cysteines were converted to *S*-sulfonates as previously described.[40,41] Briefly, Wang resins preloaded with Fmoc amino acids (Novabiochem-Merck) were used to synthesize the human insulin B chain and modified insulin A chains (A^{A21}-T-P-A-K-S-E^{A27}-, A^{A21}-P-L-K-P-A-K-S-A^{A29}-, and A^{A21}-P-L-K^{A24}- A chains) on an automatic solid-phase synthesizer (ABI 433A, Applied Biosystems, Foster City, CA). The used coupling reagents were HBTU/HOBt in DMF. The Cys, His, Gln, and Asn side chains were protected with Trt. The side chains of Tyr, Asp, Glu, Ser, and Thr were protected with tBu, and the lysine side amino group was protected with Boc. Peptides were cleaved from the resin with a TFA/H_2O/thioanisole/EDT/phenol/TIS mixture (92/2.2/2.2/1/2.2/0.4) and precipitated from cold Et_2O. Crude chains (100 μmol) were dissolved and stirred in 25 mL of sulfitolysis buffer [100 mM Tris, 250 mM Na_2SO_3, 80 mM $Na_2S_4O_6$, and 7 M guanidine hydrochloride (pH 8.6)] for 3 h at room temperature (RT). The chains were desalted on a Sephadex G-10 column (4 cm × 85 cm) in 50 mM NH_4HCO_3 and purified using reverse-phase high-performance liquid chromatography (RP-HPLC) (Nucleosil C18 column, 250 mm × 21 mm, 5 μm).

Recombination of Insulin Chains. The method for the formation of disulfide bonds in insulin analogues has been described previously in detail.[40,41] Briefly, *S*-sulfonate derivatives of the insulin A chain (30 mg) and B chain (15 mg) were dissolved in 2 and 1 mL of degassed 0.1 M Gly/NaOH buffer (pH 10.5), respectively. The exact molar concentration of each chain was determined by UV spectroscopy at 280 nm using molar extinction coefficients of 3480 and 3230 M^{-1} cm^{-1} for the A and B chains, respectively. Dithiothreitol (aliquots from Pierce, catalog no. 20291) was added rapidly to the mixture of both chains to give an SH/SSO_3 molar ratio of 1.1/1. The mixture was stirred in a capped vessel for 30−45 min at RT. After the reduction, aerated 0.1 M Gly/NaOH (pH 10.5) buffer was added to a final 2/3 ratio of degassed and aerated buffers. The resulting solution was stirred for an additional 48 h at 4 °C in an open vessel to permit air oxidation.[42] Glacial acetic acid (4 mL) was added to the mixture to terminate the reaction. The resulting mixture was applied to a low-pressure column (Sephadex G-50 in 1 M acetic acid, 2 cm × 75 cm). The fractions containing analogues were purified using RP-HPLC (Nucleosil C18 column, 250 mm × 8 mm, 5 μm). The molecular weight of products was confirmed by a HR mass spectroscopy instrument (LTQ, Orbitrap XL, Thermo Fisher Scientific, Waltham, MA). The purity of the analogues was analyzed by RP-HPLC (Nucleosil C18 column, 250 mm × 4.6 mm, 5 μm) and was >95%.

Synthesis of Analogues 4−7. *Synthesis of Peptide Precursors.* The G-F-F-Y-T-P-K(Pac)-T-S and G-F-F-Y-T-P-K(Pac)T-S-K-V-S peptides were synthesized manually by a stepwise coupling of the corresponding Fmoc-protected amino acid on a 2-chlorotrityl resin using HBTU/DIPEA in 1-methyl-2-pyrrolidinone (NMP). The completeness of the reaction was controlled by a Kaiser test and quantified by measuring the absorbance of the piperidine−dibenzofulvene complex after Fmoc group deprotection. Synthesized peptides were cleaved from the resin with a DCM/AcOH/trifluoroethanol mixture (6/2/2) for 2 h at RT. The residues were evaporated to dryness and treated with a DCM/TFA/TIS/H_2O mixture (44/50/3/3) for 2 h at RT. The mixture was evaporated *in vacuo* and treated with diethyl ether. The solid residue after the diethyl ether

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903−2913

extraction was dissolved in 40% acetonitrile in water with 0.1% TFA and purified using RP-HPLC.

G-F-F-Y-T-P-K(Pac)T-S-K(Pac)V and G-F-F-Y-T-P-K(Pac)-T-S-K(Pac) peptides were synthesized using an automatic solid-phase synthesizer (ABI 433A, Applied Biosystems) by a similar method described above but using 2-chlorotrityl resin.

Enzymatic Semisynthesis. Analogues **4**−**7** were prepared by the enzymatic semisynthesis starting from *des*(B23−B30)-octapeptide-insulin (DOI) and respective peptides. Analogue **4** was prepared according to the previously described protocol.[43,44] Semisyntheses of analogues **5**−**7** were performed according to the slightly different protocol published by Nakagawa and Tager[45] because of the lower solubility of their precursor peptides. Briefly, a peptide (30 mM) and DOI (7.7 mM) were dissolved in an *N*,*N*-dimethylacetamide (DMA)/1,4-butanediol/0.2 M Tris-HCl mixture (pH 8.0) (7/7/6), supplemented with 10 mM $Ca(Ac)_2$ and 1 mM EDTA in a total volume of 400 μL. The reaction was initiated by the addition of 2 mg of TPCK-treated trypsin. The pH of the solution was adjusted to 6.9−7.0 by *N*-methylmorpholine. The reaction mixture was incubated at 37 °C and monitored by RP-HPLC (Nucleosil 120-5 C-18 column, 250 mm × 4.6 mm). After 24−48 h, the reaction was stopped by the addition of acetone and the product was isolated by RP-HPLC (Nucleosil C18 column, 250 mm × 8 mm). The molecular weight was confirmed by HR mass spectroscopy (LTQ, Orbitrap XL, Thermo Fisher Scientific).

Enzymatic Deprotection of Precursors of Insulin Analogues. The enzymatic deprotection of Pac-protected analogues was performed according to the previously described protocol.[43,46] The respective precursor of an insulin analogue (**4**, **5**, **6**, or **7**, ∼1 mg of each) with a phenylacetyl protecting group (Pac) on N^{ε} groups of lysine(s) was dissolved in 1 mL of 50 mM potassium phosphate buffer (pH 7.5). Protease inhibitor cocktail (5 μL, Sigma-Aldrich, catalog no. P8465) was added. The reaction was initiated by the addition of soluble penicillin amidohydrolase (PA) and monitored via RP-HPLC (Nucleosil C18 column, 250 mm × 4 mm). The reaction proceeded at 37 °C. After 16 h, an extra amount of PA was added. After completion of the deprotection (∼28 h), an analogue was purified by RP-HPLC as described above. The molecular weight was confirmed by HR mass spectroscopy (LTQ, Orbitrap XL, Thermo Fisher Scientific). The purity (>96%) of analogues **4**−**7** was verified by RP-HPLC.

Cell Cultures. IM-9 cells (ATCC) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in humidified air with 5% CO_2 at 37 °C.

Mouse embryonic fibroblasts used for binding and signaling were derived from animals with targeted disruption of the IGF-1 receptor gene[47] and stably transfected with expression vectors containing either A (R^-/IR-A) or B (R^-/IR-B) isoforms of human insulin receptor or human IGF-1 receptor (R^{+39}).[14,48] The cell lines were kindly provided by A. Belfiore (University of Magna Graecia, Catanzaro, Italy) and R. Baserga (Thomas Jefferson University, Philadelphia, PA). Cells were grown in DMEM medium with 5 mM glucose (Biosera) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.3 μg/mL puromycin, 100 units/mL penicillin, and 100 μg/mL streptomycin in humidified air with 5% CO_2 at 37 °C.

Receptor Binding Studies. *Human IM-9 Lymphocytes (human IR-A isoform).* Receptor binding studies with the insulin receptor in membranes of human IM-9 lymphocytes (containing only the human IR-A isoform) were performed and K_d values determined according to the procedure described recently in detail by Morcavallo et al.[13] Binding data were analyzed using the Excel algorithms specifically developed for the IM-9 cell system in the laboratory of P. De Meyts (A. V. Groth and R. M. Shymko, Hagedorn Research Institute, Gentofte, Denmark, a kind gift of P. De Meyts) using a method of nonlinear regression and a one-site fitting program and taking into account potential depletion of free ligand. Each binding curve was determined in duplicate, and the final dissociation constant (K_d) of an analogue was calculated from at least three ($n \geq 3$) binding curves (K_d values) determined independently. The dissociation constant of human ^{125}I-labeled insulin (PerkinElmer) was set to 0.3 nM.

Mouse Embryonic Fibroblasts (human IR-B isoform). Receptor binding studies with the insulin receptor in membranes of mouse embryonic fibroblasts derived from IGF-1 receptor knockout mice that solely expressed the human IR-B isoform were performed as described in detail previously.[40,49] Binding data were analyzed, and the dissociation constant (K_d) was determined with GraphPad Prism 5 using a method of nonlinear regression and a one-site fitting program and taking into account potential depletion of free ligand. Each binding curve was determined in duplicate, and the final dissociation constant (K_d) of each analogue was calculated from at least three ($n \geq 3$) binding curves (K_d values) determined independently. The dissociation constant of human ^{125}I-labeled insulin (PerkinElmer) was set to 0.3 nM.

Mouse Embryonic Fibroblasts (human IGF-1R). Receptor binding studies with the IGF-1 receptor in membranes of mouse embryonic fibroblasts derived from IGF-1R knockout mice and transfected with human IGF-1R were performed as described previously.[40] Binding data were analyzed and the dissociation constants determined by the same method that was used for IR-B. The dissociation constant of human [^{125}I]IGF-1 (PerkinElmer) was set to 0.2 nM. Here we should note that the use of bovine serum albumin in the binding buffer (e.g., Sigma-Aldrich A6003) void of "IGF-binding-like" proteins, which interfere with the binding assay, is essential.[50]

The significance of the changes in binding affinities of the analogues, related to the insulin binding for all types of receptors, was calculated using a two-tailed *t* test.

Stimulation of Cells. Cells (cell lines R^{+39}, R^-/IR-A, and R^-/IR-B) were seeded in 24-well plates (4×10^4 cells per well) in 300 μL of DMEM and incubated for 24 h. Cells were afterward starved for 4 h in serum-free medium. A ligand (insulin, IGF-1, IGF-2, or an analogue) at final concentrations of 10^{-8} M was added to the medium in each well for 10 min. The reaction was terminated by removal of the medium, and the mixture was washed with ice-cold 0.9% NaCl and snap frozen until the next manipulation. The series of ligands were tested four times using different batches of cells.

Immunoblotting. Cells were lysed in 50 μL of lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS (w/v), 10% glycerol (v/v), 0.01% Bromphenol Blue (w/v), 0.1 M DTT (w/v), 50 mM NaF, 1 mM Na_3VO_4, and 0.5% protease inhibitor coctail (Sigma-Aldrich) by sonication. Proteins were routinely analyzed using immunoblotting and horseradish peroxidase-labeled secondary antibodies (Sigma-Aldrich). Cell extracts (10 μL containing 10 ± 0.8 μg of proteins) were separated on 10% SDS−polyacrylamide gels and electroblotted to a PVDF membrane. The membranes were probed with the following antibodies: anti-phospho-IGF-1R β subunit (Tyr1131)/IRβ

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903−2913

Table 1. Receptor Binding Affinities of Human Insulin, IGF-1, IGF-2, and the Insulin Analogues Reported in This Work

analogue	K_d ± SE (nM) (n) for human IR-A in IM-9 lymphocytes	relative binding affinity[a] for human IR-A (%)	K_d ± SE (nM) (n) for human IR-B in mouse fibroblasts	relative binding affinity for human IR-B (%)	K_d ± SE (nM) (n) for human IGF-1R in mouse fibroblasts	relative binding affinity for human IGF-1R (%)	
human insulin[b]	0.55 ± 0.04 (7)[1] 0.36 ± 0.06 (5)[2] 0.43 ± 0.00 (5)[3] 0.25 ± 0.02 (5)[4]	100	0.67 ± 0.17 (4)	100 ± 25	292 ± 31 (3)[c]	100 ± 11	0.08 ± 0.01
$A^{A21}PLK^{A24}$-insulin (**1**)	1.19 ± 0.08*** (3)[2]	30.3 ± 2.0	2.51 ± 0.63** (4)	26.7 ± 6.7	877 ± 378* (3)	33.3 ± 14.4	0.03 ± 0.01
$A^{A21}PLKPAKSA^{A29}$-insulin (**2**)	1.44 ± 0.06*** (3)[2]	25.0 ± 1.0	2.51 ± 0.36*** (4)	26.7 ± 3.8	274 ± 29 (3)	107 ± 11	0.09 ± 0.01
$A^{A21}TPAKSE^{A27}$-insulin (**3**)	0.51 ± 0.04* (3)[3]	84.3 ± 6.6	1.26 ± 0.23*** (4)	53.2 ± 9.7	124 ± 22* (3)	235 ± 42	0.19 ± 0.03
S^{B31}-insulin (**4**)	0.48 ± 0.01*** (3)[4]	52.1 ± 1.1	0.56 ± 0.11 (3)	120 ± 24	280 ± 41 (3)	104 ± 15	0.09 ± 0.01
$S^{B31}K^{B32}$-insulin (**5**)	1.28 ± 0.18*** (3)[1]	43.0 ± 6.0	2.03 ± 0.36*** (3)	33.0 ± 5.9	257 ± 16 (2)[d]	114 ± 7	0.09 ± 0.01
$S^{B31}KV^{B33}$-insulin (**6**)	0.41 ± 0.02 (3)[2]	87.8 ± 4.3	0.61 ± 0.07 (3)	110 ± 13	195 ± 33 (3)	150 ± 25	0.12 ± 0.02
$S^{B31}KVS^{B34}$-insulin (**7**)	0.57 ± 0.19 (3)[3]	75.4 ± 25.1	0.45 ± 0.13* (4)	149 ± 43	234 ± 99 (2)[d]	125 ± 53	0.10 ± 0.03
human IGF-1	23.8 ± 6.6*** (3)[4]	1.1 ± 0.3	224 ± 16*** (4)	0.30 ± 0.02	0.24 ± 0.05*** (5)[c]	1217 ± 254	100 ± 21
human IGF-2	2.92 ± 0.14*** (3)[4]	8.6 ± 0.4	35.5 ± 5.6*** (4)	1.9 ± 0.3	2.32 ± 0.72*** (3)	126 ± 39	10.3 ± 3.2

[a]The relative receptor binding affinity (potency) is defined as (K_d of human insulin or IGF/K_d of analogue) × 100. [b]The K_d of human insulin for IR-A was determined in four independent measurements (1−4). The individual values of K_d of insulin analogues are relative to one of these K_d values of human insulin (e.g., 1 to 1, etc.). [c]From ref 61. [d]The K_d value represents the mean of two independent measurements ± range. Asterisks indicate that binding of the ligand to a particular receptor differs significantly from that of insulin (*p < 0.05; **p < 0.01; ***p < 0.001).

(Tyr1146), (Cell Signaling Technology), anti-human IR β subunit (Invitrogen), and anti-IGF-1R β subunit (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were developed using the SuperSignal West Femto maximum sensitivity substrate (Pierce) and analyzed using the ChemiDoc MP Imaging System (Bio-Rad). The signal density generated by each ligand in a particular experiment was expressed as the contribution of phosphorylation relative to the respective human insulin (R^-/IR-A and R^-/IR-B) IGF-1 (R^{+39}) signal in the same experiment. Mean ± standard error of the mean (SEM) values were calculated from four independent experiments. The significance of the changes in stimulation of autophosphorylation related to the stimulation by insulin was calculated using one-way analysis of variance.

RESULTS

Design of the Analogues. The first three insulin analogues [**1**−**3** (Figure 3)] have the C-terminus of the A chain extended by amino acids from the N-terminal parts of the IGF's D domains. In analogue **1**, the D_I domain is represented only by its first three P-L-K amino acids, which are an "insert" (or addition) to the D_{II} domain (Figure 1); hence, they can be considered as the D_I domain "unique" feature. Here, we were interested in their "isolated" (i.e., purely D_I-like "signature") effect on binding of these hormones. Analogues **2** and **3** contain the entire D domains of IGF-1 and IGF-2, respectively. Additionally, insulin's A chain C-terminal Asn^{A21} has been substituted in analogues **1**−**3** with alanine that is present in both IGFs at that site; hence, the A21 site could be considered here as part of the D domains.

The studies of the effect of the C_{II} domain on insulin functionality were originally planned on hormone analogues containing the full (or large fragments of) C_{II} domain, which would be enzymatically introduced into *des*(B23−B30)-octapeptide-insulin (DOI), as the extensions of insulin C-terminal octapeptide B22−B30.[43] An alternative recombination of the already extended B chain with the A chain was also tried as described previously.[40,41] However, all these attempts were unsuccessful (see below); hence, for synthetic reasons, only the first four amino acids of the C_{II} domain (with arginines also replaced with lysines) were added after B30. This work resulted in analogues **4**−**7** (Figure 3).

Synthesis of the Analogues. Analogues **1**−**3** were prepared by the total chemical synthesis and chain recombination (folding) of insulin A and B chain *S*-sulfonates.[40,41] Addition of amino acids from the D domains of IGFs to the C-terminus of insulin A chain resulted in reduced yields of chain recombination (2−5%), in comparison with the average recombination efficiencies of native insulin (~8−12%).[40] Analogue **3** (with the D_{II} domain) was obtained with a yield (5.4%) better than those of analogues **1** and **2** containing D_I domain motifs (~2%). Furthermore, all trials for synthesizing the $A^{A21}PL^{A23}$-insulin analogue, which was designed to probe the effect of P-L substitution alone (like P-L-K), have never yielded a sufficient amount of the material for its biological characterization.

In the first attempt toward the total chemical synthesis of an insulin analogue with the whole C_{II} domain, the insulin B chain extended by the C_{II} S-R-V-S-R-R-S-R amino acids of IGF-2 was successfully made. However, its recombination with the insulin A chain *S*-sulfonate form[40,41] failed because of the insolubility of this B chain derivative in the recombination buffer. Therefore, a fully protected (*t*-Bu, Boc) G-F-F-Y-T-P-K-T-S-K-V-S-K-K-S-K peptide containing C-terminal insulin octapeptide and amino acids of the C_{II} domain was prepared. Here, arginine residues in the original C_{II} were substituted with lysines to allow both easier side chain protection and enzymatic coupling with DOI. The N^{ε}-Boc-Lys protection was necessary to shield this sequence against trypsin proteolysis during analogue semisynthesis. Although a similar approach was already used in the preparation of insulin with the C_I domain,[27] the semisynthetic attachment of this peptide to DOI failed

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903−2913

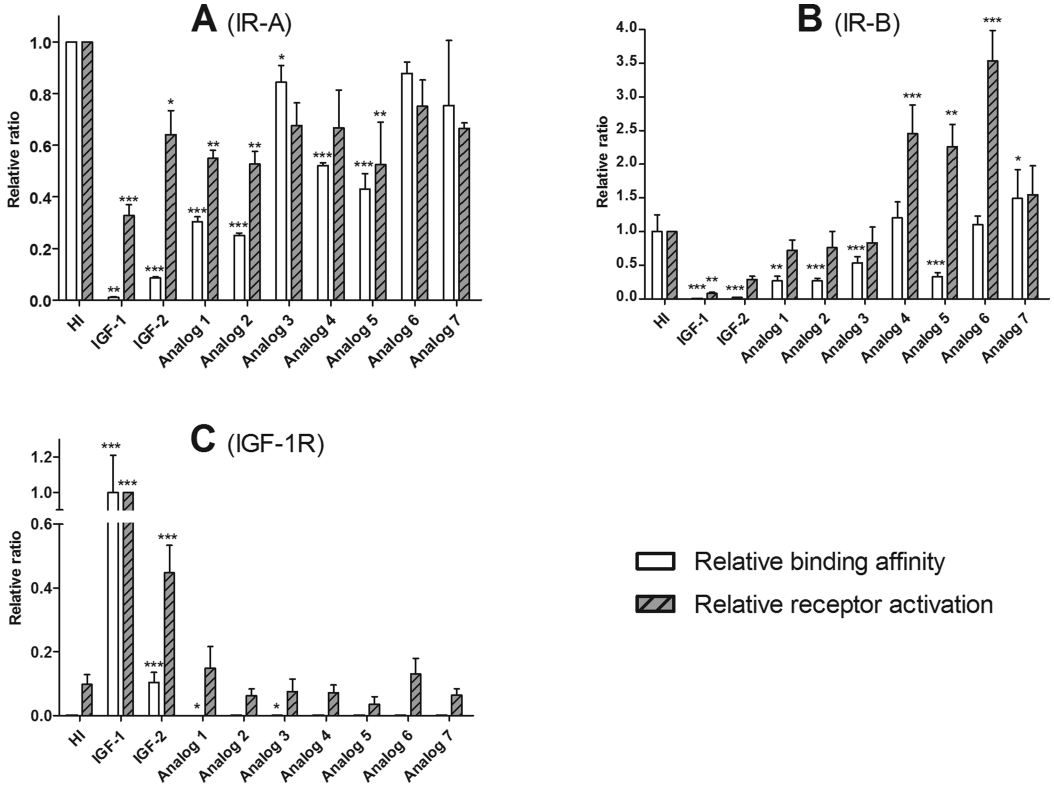


Figure 4. Comparison of relative binding affinities (white bars) for IR-A (A), IR-B (B), and IGF-1R (C) and relative abilities to activate these receptors (gray bars) of human insulin (HI), IGF-1, IGF-2, and insulin analogues containing sequences derived from the D domain of IGF-1 (1 and 2) or IGF-2 (3) or from the C domain of IGF-2 (4−7). The experimental values are related to binding potency and biological activity of HI (for IR-A and IR-B) or IGF-1 (for IGF-1R). Asterisks indicate that binding of the ligand or autophosphorylation of a particular receptor induced by the ligand differs significantly from that of insulin (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). See also Table 1 and Experimental Procedures.

again because of its insolubility. Hence, we employed a more limited and pragmatic approach, in which the C-terminus of insulin B chain octapeptide was systematically expanded by the subsequent residues from the C_{II} domain. This strategy resulted in four C_{II} derivatives of insulin B23−30 octapeptide, with addition of S, S-K, S-K-V, or S-K-V-S amino acids, and all lysine free N^{ε}-groups were phenylacetyl-protected. In contrast to the whole C_{II} domain-modified B23−30 octapeptide, these peptides were successfully attached enzymatically to the DOI. However, the yields of the individual semisyntheses of analogues **4**−**7** were only within the range of ∼1.5−5%. Analogue **7** was obtained with the lowest yield, and the efficiencies of semisyntheses for **4**−**7** were directly proportional to the length of the respective peptide. Furthermore, the final removal of the phenylacetyl protection required use of the cocktail protease inhibitors to protect these analogues against proteolytic contaminants present in the penicillin amidohydrolase solution.

Receptor Binding Studies. The binding affinities of all analogues, human insulin, and human IGF-1 and IGF-2 for human IR-A, IR-B, and IGF-1R are listed in Table 1.

IR-A Binding Affinities. All insulin analogues **1**−**7** have either similar, or lower, affinity for IR-A in comparison with that of human insulin. Hybrid molecules **1**−**3** containing motifs from the D domains of IGF-1 and IGF-2 have different impacts on IR-A binding. Whereas analogue **3** (whole D_{II} domain) has a binding affinity comparable to that of HI (84%), the binding potencies of both analogues **1** (with the APLK fragment of the D_I domain) and **2** (with the whole D_I domain) decreased to less (or approximately) than one-third of HI binding potency.

The addition of amino acids mimicking the C_{II} domain to human insulin (analogues **4**−**7**) has lowered their level of IR-A binding to 43−88%. Interestingly, analogues with only one (S^{B31}, **4**) or two ($S^{B31}K^{B32}$, **5**) additional amino acids are less active than analogues with three (**6**) or four (**7**) extra residues from the C_{II} domain (Table 1, Figure 4A, and Figure S1).

IR-B Binding Affinities. The additions of the D domain amino acids of IGF-1 and -2 to HI have a similar negative effect on the binding of analogues to both IR isoforms, but the negative impact of the addition of the whole D_{II} domain (analogue **3**) is more significant on IR-B (53%) than on IR-A (85%) affinity.

The effects of additions of C_{II} domain amino acids to HI on its binding to IR-B were rather surprising. Except analogue **5**, with only 33% HI binding affinity, the other C_{II} domain-derived analogues have affinities of IR-B similar to (**4** and **6**), or slightly higher than (**7**), that of HI. Interestingly, the most potent analogue, **7**, contains the longest, four extra amino acids, modification from the C_{II} domain (Table 1, Figure 4B, and Figure S2).

IGF-1R Binding Affinities. All analogues exhibited very low binding affinity for IGF-1R compared to the affinity of IGF-1 for this receptor. In general, the analogues have binding potencies similar to that of human insulin. The exceptions are analogue **1**, the IGF-1R binding affinity of which is 3 times lower, and analogue **3**, which binds IGF-1R 2 times stronger than HI (Table 1, Figure 4C, and Figure S3).

Autophosphorylation of IR and IGF-1R. Activation of IR-A, IR-B, and IGF-1R was measured in R^-/IR-A, R^-/IR-B, and R^{+39} cells, respectively, and by the detection of the

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903−2913

autophosphorylation of IR Tyr1158 (IR-B numbering) or the analogous IGF-1R Tyr1131. Cells were stimulated either by the natural ligand (human IGF-1, IGF-2, or insulin) or by an insulin analogue for 10 min, at a ligand concentration of 10 nM each. The results are shown in Figure 4 in correlation with binding affinities of analogues. Representative immunoblots are shown in Figure S4.

Generally, IR-A autophosphorylation abilities of the analogues (Figure 4A) followed their IR-A binding trends. All analogues exhibited a slightly reduced capability to activate IR-A compared to that of human insulin; they were within IGF-2 activation range and were enhanced in relation to IGF-1.

The IR-B stimulation abilities of analogues **1**−**3** (with the D_I and D_{II} domains) (Figure 4B) were comparable with that of human insulin. However, "C_{II} domain analogues" **4**−**6** showed significantly higher (2−3 times higher than that of HI) levels of autophosphorylation of the IR-B. Interestingly, analogue **7**, with enhanced (150%) binding potency for IR-B, stimulated IR-B like HI. Therefore, the IR-B receptor autophosphorylation abilities of insulin analogues modified by fragments of the C_{II} domain are not really correlated with their binding potencies for this receptor: analogues **4** and **6**, strong activators of IR-B, are equipotent with HI in IR-B binding, while analogue **5** (also a good stimulant of IR-B) has only 33% of the IR-B binding potency of HI.

The addition of sequences derived from D_I, D_{II}, and C_{II} domains to human insulin did not significantly influence the ability of these ligands to stimulate the autophosphorylation of IGF-1R. All analogues **1**−**7** stimulated this receptor with an efficiency comparable to that of human insulin (Figure 4C).

DISCUSSION

The C and D domains of IGF-1 and IGF-2 represent the major structural differences between these growth factors and insulin (Figure 1). However, it was proposed that the A and B domains of IGFs are the main determinants of their specific binding and activation of IGF-1R.[16,20] This has been recently supported by a similar mode of binding of insulin and IGF-1 to IR/IGF-1R constructs observed in the respective crystal structures.[17−19] Therefore, we can envisage that the roles of the C and D domains are limited to more subtle modulation of binding of IGF-1 and IGF-2 to IR-A, IR-B, and IGF-1R, and subsequent tuning of their activation.[24,51]

Initial studies of insulin:D domain hybrids were reported in the 1980s.[33,34,52] Although they provided valuable data, insight into binding affinities of these analogues for individual "isolated" IR isoforms and IGF-1R was not gained at that time. Moreover, the understanding of the role of the IGF-2 C_{II} domain in the receptor selectivity of this hormone is still limited.

These structural and functional ambiguities prompted us to investigate whether insulin analogues, which carry D_I, D_{II}, and C_{II} domains, could be molecular probes for testing the functionalities of these structural segments.

The sensitivity of interactions of insulin with IR-A and IR-B and the feasibility of the chemical synthesis of insulin-based analogues (in comparison to IGF-based scaffolds) were the decisive factors in selection of this hormone as a working template in this study.

The addition of amino acids from the D_I domain (analogues **1** and **2**) had a visible negative effect on insulin IR-A and IR-B binding affinities [25−30% of that of HI (Table 1)]. This confirmed the previously proposed unfavorable interference of the D_I domain with binding to IR[34] and agrees with the observation that deletion of the D_I domain in IGF-1 doubles its IR binding affinity.[25,26] Moreover, the $D_{II} \rightarrow D_I$ swap in IGF-2 also decreased its IR-A affinity to 37%.[24] Here, we have shown that addition of the first three amino acids of the D_I domain (P-L-K, analogue **1**) to insulin A21 site is sufficient to lower its level of IR-A binding. Unfortunately, we have not succeeded in synthesizing the A^{A21}-P-L^{A23} analogue with the short, initial D_I sequence. This analogue would help in understanding better the functional significance of these particular two amino acids of the D_I domain, as they represent a unique IGF-1 D domain insert (in comparison with the shorter D_{II} domain) (Figure 1). Nevertheless, it seems that not the length of the D_I domain but its particular P-L-K sequence is the determining factor for the different IR-A and IR-B binding abilities of IGF-1 and IGF-2. This underlines further the presence and role of the usually structurally significant proline residue at position 62 in the D_I domain, which may have a specific effect on the conformation of the D domain. It would amplify the interference of the D_I domain with the hormone's binding site at IR, as its receptor-bound conformation is likely different from the D_{II}-specific fold.

In contrast to that of the D_I domain, the addition of the D_{II} domain to the insulin molecule (analogue **3**) has a marginal effect (85%) on its IR-A binding and a relatively small negative effect (53%) on its affinity for IR-B (Table 1). Although the peculiar role of $N^{A21} \rightarrow A^{A21}$ substitution in analogues **1**−**3** cannot be ignored (this single mutation reduces the level of insulin IR-A binding to ∼65%[53,54]), we assume that the IR binding trends of these analogues are meaningful, as all of them contain the Ala^{A21} mutation. Therefore, the different binding affinities of analogue **3** for IR isoforms, compared to that of HI, can result from the presence of the whole additional D_{II} domain, rather than from the impact of the Ala^{A21} mutation. The relatively more important decrease in the IR-B binding affinity of analogue **3** compared to its effect on IR-A could also indicate that (i) the D_{II} domain is, at least partly, responsible for a lower affinity of IGF-2 for IR-B and (ii) the D_{II} domain does not play a major role in the interaction with IR-A. Because only the 12 additional amino acids at the C-terminus of the α subunit (encoded by exon 11) are the difference between IR-A and IR-B, some specific, unfavorable interaction of the D_{II} domain with the αCT segment of IR-B could be behind the mechanism of the "D_{II}-mediated" lower affinity for IR-B. This could also mean that the D_{II} domain does not interact, or does so in an only marginal fashion, with IR-A.

It may be expected that the addition of D_I and D_{II} domains to insulin will, somehow, increase the IGF-1R affinity of these analogues. However, this effect was only partially noted in the D_{II} domain-containing analogue **3**. Its binding affinity for IGF-1R is >2 times higher than that of HI (Table 1 and Figure 4). The nondeleterious effect of the D_{II} domain for IGF-1R was observed also by Denley et al.,[24] who found that the IGF-1 analogue with the added D_{II} domain had a similar binding affinity for native IGF-1. It was rather surprising that the binding affinity for IGF-1R was not increased in the analogues containing the whole D_I domain (or its fragment, analogue **2** or **1**, respectively), and that their binding affinities were similar to or significantly lower than the affinity of HI [analogue **2**, 107%; analogue **1**, 33% (Table 1)]. Denley at al[24] observed that addition of the D_I domain to IGF-2 enhanced 3-fold the IGF-1R binding affinity of the hybrid in comparison with that of native IGF-2. Hence, it is possible that the role of the D_I

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903−2913

domain in binding to IGF-1R can differ in the context of insulin and IGF molecules.

All three (**1**−**3**) D_I and D_{II} analogues show a similar ability to activate IR-A (Figure 4A and Figure S4), despite their relatively different IR-A binding affinities [25−84% (Table 1)]; these IR-A stimulation properties are also lower than those of HI and IGF-2. Moreover, the autophosphorylation of IR-A by analogues **1**−**3** does not simply, and fully, correlate with their binding affinities, and the insertion of D_I and D_{II} domains into insulin has a less negative impact on IR-A autophosphorylation than on binding. The activations of IR-B by analogues **1**−**3** are also similar; however, in comparison with the autophosphorylation of IR-A, their effect is less pronounced here, and these analogues are also able to activate IR-B better than IGF-2 (Figure 4B and Figure S4). This suggests that the core of the hormone(s) (insulin or IGF) plays a more important role in these processes than the D domains. Similar effects can be seen in the autophosphorylation of IGF-1R, where analogues **1**−**3** stimulate the receptor at the HI level, and much more weakly than both IGFs (Figure 4C and Figure S4).

The second part of our work here concerned whether the incorporation of the elements of the C_{II} domain into insulins template (analogues **4**−**7**) would allow us to monitor their impact on hormone:receptor specificity. It must be stressed that the reports concerning the significance of the C_I and C_{II} domains (or particular amino acids) are infrequent, and studies of the C_{II} domain are especially limited.[24−28,55,56] This results likely from the methodological barrier, i.e., difficult synthesis of this particular amino acid sequence. For example, the high content of arginine in the C_{II} domain decreases significantly the solubility of these peptides. Hence, this was also the main reason behind our unsuccessful synthesis of the insulin analogue with the entire C_{II} domain. Furthermore, the Arg residues interfere also with the trypsin-catalyzed semisynthesis of the analogue, as the Arg-involving peptide bonds are digested even under the tryptic-digest unfavorable conditions (e.g., pH 7 and organic solvent). Therefore, we had to include Arg → Lys substitutions in the C_{II} domain, which is similar to the approach of the previous study concerning the C_I domain of IGF-1.[27] Here, we also tried to use the Boc protection of Lys to prepare an analogue containing the entire C_{II}-like domain (G^{B23}FFY-TPKTSKVSKKSK^{B38}) by trypsin-catalyzed semisynthesis. Unfortunately, the 16-amino acid peptide precursor composed of the B23−B30 C-terminal segment of insulin that was followed by the eight amino acids of the C_{II} domain was insoluble in solvents necessary for enzymatic semisynthesis. Therefore, we focused here on the systematic enzymatic semisynthesis of insulin analogues with the C-terminus of B chain extended by one, two, three, and four residues from the C_{II} domain; they also contained Arg → Lys substitutions, with lysine side chains temporarily N^{ε}-protected by the Pac groups. This approach was more successful, and the Pac Lys protection was removed enzymatically with penicillin amidohydrolase after the semisynthesis.[43,46] However, some proteolytic side reactions were also experienced here, probably because of a prolonged Pac cleavage time that was required for the removal of these multiple protective groups. Hence, the use of a cocktail of protease inhibitors was needed because of the poor yield of these reactions (in the range of 1.5−5%), as well.

Despite these synthetic difficulties, we made four (**4**−**7**) new hybrid analogues of human insulin containing one to four C_{II} domain residues (with Arg → Lys substitutions); they were subsequently characterized with all three types of receptors. We expected that the addition of fragments of the C_{II} domain to insulin will enhance its binding to the "mitogenic" IR-A and IGF-1R receptors, weakening simultaneously its interaction with the "metabolic" IR-B isoform. Although only moderate, and nonsignificant, increases in the binding affinities of **4**−**7** for IGF-1R were observed (Table 1) [paralleled by similar small IGF-1R autophosphorylation effects (Figure 4C)], the binding affinities and autophosphorylation ability of these analogues for IR-A and IR-B were unexpected. Analogues **4**−**7** have moderately decreased binding affinities [from 43 to 88% (Table 1)] for IR-A compared to HI, paralleled by their similar ability to activate this receptor (Figure 4A). More surprising were the equipotent, or slightly increased, IR-B binding affinities of analogues **4**, **6**, and **7**, with only analogue **5** being a significantly weaker IR-B binder [33% (Table 1 and Figure 4B)]. Its lower binding affinity for IR-B [33%, and to some extent also for IR-A, as well (43%)] could be caused by a negative effect of the C-terminal LysB32. This effect is similar to the impact of the C-terminal arginine residues in insulin glargine, which has two extra arginines at the C-terminus of the B chain, and its IR-B binding affinity is also lower than that of HI.[57−59] Some C domain-related reports suggest that the C_I domain (but not the C_{II} domain) should be considered as an important factor in hormone−IGF-1R binding, but with negligible effect on their IR binding.[25] In contrast, the C_{II} domain was supposed to be critical for signaling through IR-A.[51] However, a slightly positive effect of the C_{II} domain on IR-B binding was observed for the IGF-1 analogue with this whole IGF-2 segment, as well.[24]

Interestingly, the IR-B autophosphorylation abilities of analogues **4**−**6** do not fully follow their IR-B binding affinities as they are significantly enhanced. Here, only analogue **7** has its IR-B autophosphorylation ability proportional to its IR-B binding affinity (Figure 4B). The preferential activation of IR-B by analogues **4**−**6** is interesting, as it was already indicated that a relatively moderate IR isoform specificity of insulin analogues may have a significant impact on their biological effects.[12] Hence, IR-B-specific analogues could indeed present important applications in assuring a more physiological profile of clinical insulins *in vivo*, with an enhanced hepatic mode of action.[60]

It should be stressed that the extra amino acids added to the C-terminus of the B chain in analogues **4**−**7** might not represent the true C_{II} mimics, as their selection was significantly driven here by the semisynthetic yields. Our modifications should be thus rather considered as new (but IGF-derived), artificial structural motifs that add new properties to the insulin molecule; this is different from the "natural", biological impact of the C_{II} domain. Nevertheless, they showed that the use of even significantly constrained/modified sequences within the C_{II} domain could result in protein probes that provide valuable insight into the hormone's functionality. Moreover, they could open a new path into the design and creation of novel and important analogues with enhanced IR-B specificity, needed for clinical applications.

The preferential binding/autophosphorylation of IR-B by analogues **4**−**7** evokes also questions about a direct interaction of their B chain C-terminal "extra" residues (B31−B34) with α-CT peptide of IR-B. The position of 12 amino acids of IR-B exon 11 in the receptor structure is still unknown. However, it can be assumed that the conformation of insulin B21−B27 residues (visible in the insulin:IR-A complex) on the L1 domain may be similar upon binding to both IR isoforms. If this is indeed the case, then a direct interaction between the C-

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903−2913

terminus of the insulin B chain and exon 11-encoded additional IR-B residues cannot be excluded. Therefore, our results concerning analogues **4**–**7**, together with the recently published evidence of the increased hormone's IR-B sensitivity obtained through modification of the C-terminal part of the insulin B chain,[12,49,61] underline and indicate the importance of this region for achieving IR-B-specific hormone analogues. This opens new venues for a rational manipulation of the insulin B26-onward part of the hormone, which was usually though to be unimportant for its functionality. We show here that a careful extension of the B chain beyond the B30 site can introduce new and exciting properties into the insulin molecule.

In summary, our insulin-based hybrid hormonal probes with elements of IGF-1 and IGF-2 presented here suggest that (i) the D_I domain plays a more negative role in binding to IR than the D_{II} domain does, (ii) D_I domain P-L-K residues are a determining factor for a different IR-A and IR-B binding affinity of IGF-1 and IGF-2, and (iii) the addition of amino acids "mimicking" the C_{II} domain to the C-terminus of the insulin B chain may result in an unexpected, specifically deepened, autophosphorylation of "metabolic" IR-B. Our research evidence underlines also the sophistication and complexity of the insulin/IGF/IR/IGF-1R signaling system, in which hormone:receptor binding and receptor activation strengths are frequently not fully correlated and are, likely, modulated further by the half-life of these complexes and their endocytotic fate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b00140.

Supplementary Figures S1–S4 (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: zakova@uochb.cas.cz.

Funding

This work was supported by the Czech Science Foundation (Grant 15-19018S, to L.Ž.), the Medical Research Council (Grant MR/K000179/1, A.M.B.), the Charles University Grant Agency (Grant 638613, to K.K.), and the Research Project of the Academy of Sciences of the Czech Republic (RVO:61388963).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

C_I, IGF-1 C domain; C_{II}, IGF-2 C domain; D_I, IGF-1 D domain; D_{II}, IGF-2 D domain; DIPEA, *N*,*N*-diisopropylethylamine; EDT, 1,2-ethanedithiol; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HI, human insulin; IR, insulin receptor; HOBt, 1-hydroxybenzotriazole; IGF, insulin-like growth factor; IGF-1R, insulin-like growth factor 1 receptor; Pac, phenylacetyl; PDB, Protein Data Bank; TIS, triisopropylsilane.

■ REFERENCES

(1) Vigneri, P., Frasca, F., Sciacca, L., Pandini, G., and Vigneri, R. (2009) Diabetes and cancer. *Endocr.-Relat. Cancer 16*, 1103–1123.

(2) Cohen, D. H., and LeRoith, D. (2012) Obesity, type 2 diabetes, and cancer: the insulin and IGF connection. *Endocr.-Relat. Cancer 19*, F27–F45.

(3) Murray-Rust, J., McLeod, A. N., Blundell, T. L., and Wood, S. P. (1992) Structure and evolution of insulins: implications for receptor binding. *BioEssays 14*, 325–331.

(4) Gallagher, E. J., and LeRoith, D. (2011) Minireview: IGF, Insulin, and Cancer. *Endocrinology 152*, 2546–2551.

(5) LeRoith, D. (2006) Insulin-like growth factors. *Seminars in Medicine of the Beth Israel Deaconess Medical Center 336*, 633–640.

(6) Denley, A., Cosgrove, L. J., Booker, G. W., Wallace, J. C., and Forbes, B. E. (2005) Molecular interactions of the IGF system. *Cytokine Growth Factor Rev. 16*, 421–439.

(7) Adams, T. E., Epa, V. C., Garrett, T. P., and Ward, C. W. (2000) Structure and function of the type 1 insulin-like growth factor receptor. *Cell. Mol. Life Sci. 57*, 1050–1093.

(8) De Meyts, P. (2004) Insulin and its receptor: structure, function and evolution. *BioEssays 26*, 1351–1362.

(9) De Meyts, P., and Whittaker, J. (2002) Structural biology of insulin and IGF1 receptors: implications for drug design. *Nat. Rev. Drug Discovery 1*, 769–783.

(10) Moller, D. E., Yokota, A., Caro, J. F., and Flier, J. S. (1989) Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man. *Mol. Endocrinol. 3*, 1263–1269.

(11) Seino, S., and Bell, G. I. (1989) Alternative splicing of human insulin receptor messenger RNA. *Biochem. Biophys. Res. Commun. 159*, 312–316.

(12) Vienberg, S. G., Bouman, S. D., Sorensen, H., Stidsen, C. E., Kjeldsen, T., Glendorf, T., Sorensen, A. R., Olsen, G. S., Andersen, B., and Nishimura, E. (2011) Receptor-isoform-selective insulin analogues give tissue-preferential effects. *Biochem. J. 440*, 301–308.

(13) Morcavallo, A., Genua, M., Palummo, A., Kletvikova, E., Jiracek, J., Brzozowski, A. M., Iozzo, R. V., Belfiore, A., and Morrione, A. (2012) Insulin and Insulin-like Growth Factor II Differentially Regulate Endocytic Sorting and Stability of Insulin Receptor Isoform A. *J. Biol. Chem. 287*, 11422–11436.

(14) Frasca, F., Pandini, G., Scalia, P., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I. D., Belfiore, A., and Vigneri, R. (1999) Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol. Cell. Biol. 19*, 3278–3288.

(15) Ziegler, A. N., Chidambaram, S., Forbes, B. E., Wood, T. L., and Levison, S. W. (2014) Insulin-like Growth Factor-II (IGF- II) and IGF- II. Analogs with Enhanced Insulin Receptor- a Binding Affinity Promote Neural Stem Cell Expansion. *J. Biol. Chem. 289*, 4626–4633.

(16) De Meyts, P. (2015) Insulin/receptor binding: The last piece of the puzzle? *BioEssays 37*, 389–397.

(17) Menting, J. G., Whittaker, J., Margetts, M. B., Whittaker, L. J., Kong, G. K. W., Smith, B. J., Watson, C. J., Zakova, L., Kletvikova, E., Jiracek, J., Chan, S. J., Steiner, D. F., Dodson, G. G., Brzozowski, A. M., Weiss, M. A., Ward, C. W., and Lawrence, M. C. (2013) How insulin engages its primary binding site on the insulin receptor. *Nature 493*, 241–245.

(18) Menting, J. G., Yang, Y. W., Chan, S. J., Phillips, N. B., Smith, B. J., Whittaker, J., Wickramasinghe, N. P., Whittaker, L. J., Pandyarajan, V., Wan, Z. L., Yadav, S. P., Carroll, J. M., Strokes, N., Roberts, C. T., Ismail-Beigi, F., Milewski, W., Steiner, D. F., Chauhan, V. S., Ward, C. W., Weiss, M. A., and Lawrence, M. C. (2014) Protective hinge in insulin opens to enable its receptor engagement. *Proc. Natl. Acad. Sci. U. S. A. 111*, E3395–E3404.

(19) Menting, J. G., Lawrence, C. F., Kong, G. K., Margetts, M. B., Ward, C. W., and Lawrence, M. C. (2015) Structural Congruency of Ligand Binding to the Insulin and Insulin/Type 1 Insulin-like Growth Factor Hybrid Receptors. *Structure 23*, 1271–1282.

(20) Gauguin, L., Delaine, C., Alvino, C. L., McNeil, K. A., Wallace, J. C., Forbes, B. E., and De Meyts, P. (2008) Alanine scanning of a putative receptor binding surface of insulin-like growth factor-I. *J. Biol. Chem. 283*, 20821–20829.

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903–2913

(21) Gauguin, L., Klaproth, B., Sajid, W., Andersen, A. S., McNeil, K. A., Forbes, B. E., and De Meyts, P. (2008) Structural basis for the lower affinity of the insulin-like growth factors for the insulin receptor. *J. Biol. Chem. 283*, 2604−2613.
(22) Renteria, M. E., Gandhi, N. S., Vinuesa, P., Helmerhorst, E., and Mancera, R. L. (2008) A Comparative Structural Bioinformatics Analysis of the Insulin Receptor Family Ectodomain Based on Phylogenetic Information. *PLoS One 3* (11), e3667.
(23) Alvino, C. L., McNeil, K. A., Ong, S. C., Delaine, C., Booker, G. W., Wallace, J. C., Whittaker, J., and Forbes, B. E. (2009) A Novel Approach to Identify Two Distinct Receptor Binding Surfaces of Insulin-like Growth Factor II. *J. Biol. Chem. 284*, 7656−7664.
(24) Denley, A., Bonython, E. R., Booker, G. W., Cosgrove, L. J., Forbes, B. E., Ward, C. W., and Wallace, J. C. (2004) Structural determinants for high-affinity binding of insulin-like growth factor II to insulin receptor (IR)-A, the exon 11 minus isoform of the IR. *Mol. Endocrinol. 18*, 2502−2512.
(25) Bayne, M. L., Applebaum, J., Underwood, D., Chicchi, G. G., Green, B. G., Hayes, N. S., and Cascieri, M. A. (1989) The C region of human insulin-like growth factor (IGF) I is required for high affinity binding to the type 1 IGF receptor. *J. Biol. Chem. 264*, 11004−11008.
(26) Kristensen, C., Andersen, A. S., Hach, M., Wiberg, F. C., Schaffer, L., and Kjeldsen, T. (1995) A Single-Chain Insulin-Like Growth-Factor-I Insulin Hybrid Binds with High-Affinity to the Insulin-Receptor. *Biochem. J. 305*, 981−986.
(27) Cara, J. F., Mirmira, R. G., Nakagawa, S. H., and Tager, H. S. (1990) An Insulin-Like Growth Factor-I Insulin Hybrid Exhibiting High Potency for Interaction with the Type-I Insulin-Like Growth-Factor and Insulin-Receptors of Placental Plasma-Membranes. *J. Biol. Chem. 265*, 17820−17825.
(28) Gill, R., Wallach, B., Verma, C., Urso, B., DeWolf, E., Grotzinger, J., MurrayRust, J., Pitts, J., Wollmer, A., DeMeyts, P., and Wood, S. (1996) Engineering the C-region of human insulin-like growth factor-1: Implications for receptor binding. *Protein Eng., Des. Sel. 9*, 1011−1019.
(29) Keyhanfar, M., Booker, G. W., Whittaker, J., Wallace, J. C., and Forbes, B. E. (2007) Precise mapping of an IGF-I-binding site on the IGF-1R. *Biochem. J. 401*, 269−277.
(30) Whittaker, J., Groth, A. V., Mynarcik, D. C., Pluzek, L., Gadsboll, V. L., and Whittaker, L. J. (2001) Alanine scanning mutagenesis of a type 1 insulin-like growth factor receptor ligand binding site. *J. Biol. Chem. 276*, 43980−43986.
(31) Henderson, S. T., Brierley, G. V. B., Surinya, K. H., Priebe, I. K., Catcheside, D. E. A., Wallace, J. C., Forbes, B. E., and Cosgrove, L. J. (2015) Delineation of the IGF-II C domain elements involved in binding and activation of the IR-A, IR-B and IGF-IR. *Growth Horm. IGF Res. 25*, 20−27.
(32) Sorensen, H., Whittaker, L., Hinrichsen, J., Groth, A., and Whittaker, J. (2004) Mapping of the insulin-like growth factor II binding site of the type I insulin-like growth factor receptor by alanine scanning mutagenesis. *FEBS Lett. 565*, 19−22.
(33) King, G. L., Kahn, C. R., Samuels, B., Danho, W., Bullesbach, E. E., and Gattner, H. G. (1982) Synthesis and Characterization of Molecular Hybrids of Insulin and Insulin-Like Growth Factor-I - the Role of the A-Chain Extension Peptide. *J. Biol. Chem. 257*, 869−873.
(34) Devroede, M. A., Rechler, M. M., Nissley, S. P., Ogawa, H., Joshi, S., Burke, G. T., and Katsoyannis, P. G. (1986) Mitogenic Activity and Receptor Reactivity of Hybrid Molecules Containing Portions of the Insulin-Like Growth Factor-I (Igf-I), Igf-II, and Insulin Molecules. *Diabetes 35*, 355−361.
(35) Sakano, K. I., Enjoh, T., Numata, F., Fujiwara, H., Marumoto, Y., Higashihashi, N., Sato, Y., Perdue, J. F., and Fujitayamaguchi, Y. (1991) The Design, Expression, and Characterization of Human Insulin-Like Growth Factor-II (Igf-II) Mutants Specific for Either the IGF-II Cation-Independent Mannose 6-Phosphate Receptor Or IGF-I Receptor. *J. Biol. Chem. 266*, 20626−20635.
(36) Morrione, A., Valentinis, B., Xu, S. Q., Yumet, G., Louvi, A., Efstratiadis, A., and Baserga, R. (1997) Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. *Proc. Natl. Acad. Sci. U. S. A. 94*, 3777−3782.
(37) Versteyhe, S., Klaproth, B., Borup, R., Palsgaard, J., Jensen, M., Gray, S. G., and De Meyts, P. (2013) IGF-I, IGF-II, and Insulin Stimulate Different Gene Expression Responses through Binding to the IGF-I Receptor. *Front. Endocrinol. 4*, 98.
(38) Slaaby, R. (2015) Specific insulin/IGF1 hybrid receptor activation assay reveals IGF1 as a more potent ligand than insulin. *Sci. Rep. 5*, 7911.
(39) Belfiore, A., Frasca, F., Pandini, G., Sciacca, L., and Vigneri, R. (2009) Insulin Receptor Isoforms and Insulin Receptor/Insulin-Like Growth Factor Receptor Hybrids in Physiology and Disease. *Endocr. Rev. 30*, 586−623.
(40) Kosinova, L., Veverka, V., Novotna, P., Collinsova, M., Urbanova, M., Moody, N. R., Turkenburg, J. P., Jiracek, J., Brzozowski, A. M., and Zakova, L. (2014) Insight into the Structural and Biological Relevance of the T/R Transition of the N-Terminus of the B-Chain in Human Insulin. *Biochemistry 53*, 3392−3402.
(41) Krizkova, K., Veverka, V., Maletinska, L., Hexnerova, R., Brzozowski, A. M., Jiracek, J., and Zakova, L. (2014) Structural and Functional Study of the GlnB22-Insulin Mutant Responsible for Maturity-Onset Diabetes of the Young. *PLoS One 9* (11), e112883.
(42) Chance, R. E., Hoffmann, J. A., Kroeff, E. P., Johnson, M. G., Schirmer, E. W., and Bromer, W. W. (1981) The production of human insulin using recombinant DNA technology and a new chain combination procedure. In *Proceedings of the 7th American Peptide Symposium* (Rich, D. H., and Gross, E., Eds.) pp 721−728.
(43) Zakova, L., Zyka, D., Jezek, J., Hanclova, I., Sanda, M., Brzozowski, A. M., and Jiracek, J. (2007) The use of Fmoc-Lys(Pac)-OH and penicillin G acylase in the preparation of novel semisynthetic insulin analogs. *J. Pept. Sci. 13*, 334−341.
(44) Zakova, L., Kazdova, L., Hanclova, I., Protivinska, E., Sanda, M., Budesinsky, M., and Jiracek, J. (2008) Insulin analogues with modifications at position B26. Divergence of binding affinity and biological activity. *Biochemistry 47*, 5858−5868.
(45) Nakagawa, S. H., and Tager, H. S. (1992) Importance of aliphatic side-chain structure at positions 2 and 3 of the insulin A chain in insulin-receptor interactions. *Biochemistry 31*, 3204−3214.
(46) Antolikova, E., Zakova, L., Turkenburg, J. P., Watson, C. J., Hanclova, I., Sanda, M., Cooper, A., Kraus, T., Brzozowski, A. M., and Jiracek, J. (2011) Non-equivalent Role of Inter- and Intramolecular Hydrogen Bonds in the Insulin Dimer Interface. *J. Biol. Chem. 286*, 36968−36977.
(47) Sell, C., Dumenil, G., Deveaud, C., Miura, M., Coppola, D., Deangelis, T., Rubin, R., Efstratiadis, A., and Baserga, R. (1994) Effect of a Null Mutation of the Insulin-Like Growth-Factor-I Receptor Gene on Growth and Transformation of Mouse Embryo Fibroblasts. *Mol. Cell. Biol. 14*, 3604−3612.
(48) Miura, M., Surmacz, E., Burgaud, J. L., and Baserga, R. (1995) Different Effects on Mitogenesis and Transformation of a Mutation at Tyrosine-1251 of the Insulin-Like Growth-Factor-I Receptor. *J. Biol. Chem. 270*, 22639−22644.
(49) Zakova, L., Kletvikova, E., Lepsik, M., Collinsova, M., Watson, C. J., Turkenburg, J. P., Jiracek, J., and Brzozowski, A. M. (2014) Human insulin analogues modified at the B26 site reveal a hormone conformation that is undetected in the receptor complex. *Acta Crystallogr., Sect. D: Biol. Crystallogr. 70*, 2765−2774.
(50) Slaaby, R., Andersen, A. S., and Brandt, J. (2008) IGF-I binding to the IGF-I receptor is affected by contaminants in commercial BSA: The contaminants are proteins with IGF-I binding properties. *Growth Horm. IGF Res. 18*, 267−274.
(51) Denley, A., Brierley, G. V., Carroll, J. M., Lindenberg, A., Booker, G. W., Cosgrove, L. J., Wallace, J. C., Forbes, B. E., and Roberts, C. T. (2006) Differential activation of insulin receptor isoforms by insulin-like growth factors is determined by the C domain. *Endocrinology 147*, 1029−1036.
(52) Devroede, M. A., Rechler, M. M., Nissley, S. P., Joshi, S., Burke, G. T., and Katsoyannis, P. G. (1985) Hybrid Molecules Containing the B-Domain of Insulin-Like Growth Factor-I Are Recognized by Carrier

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903−2913

Proteins of the Growth-Factor. *Proc. Natl. Acad. Sci. U. S. A. 82*, 3010−3014.
(53) Kristensen, C., Kjeldsen, T., Wiberg, F. C., Schaffer, L., Hach, M., Havelund, S., Bass, J., Steiner, D. F., and Andersen, A. S. (1997) Alanine scanning mutagenesis of insulin. *J. Biol. Chem. 272*, 12978−12983.
(54) Jensen, A. M. (2000) Analysis of structure-function relationships of the insulin molecule by alanine-scanning mutagenesis. Master's Thesis, University of Copenhagen, Copenhagen.
(55) Forbes, B. E., Hartfield, P. J., McNeil, K. A., Surinya, K. H., Milner, S. J., Cosgrove, L. J., and Wallace, J. C. (2002) Characteristics of binding of insulin-like growth factor (IGF)-I and IGF-II analogues to the type 1 IGF receptor determined by BIAcore analysis - Correlation of binding affinity with ability to prevent apoptosis. *Eur. J. Biochem. 269*, 961−968.
(56) Wang, P., Cai, R. R., Feng, Y. M., and Zhang, Y. S. (2000) Studies on insulin/IGF-1 hybrid and IGF-1 growth-promoting functional region. *IUBMB Life 49*, 321−325.
(57) Werner, U., Korn, M., Schmidt, R., Wendrich, T. M., and Tennagels, N. (2014) Metabolic effect and receptor signalling profile of a non-metabolisable insulin glargine analogue. *Arch. Physiol. Biochem. 120*, 158−165.
(58) Le Roith, D. (2007) Insulin glargine and receptor-mediated signalling: Clinical implications in treating type 2 diabetes. *Diabetes/Metab. Res. Rev. 23*, 593−599.
(59) Kurtzhals, P., Schaffer, L., Sorensen, A., Kristensen, C., Jonassen, I., Schmid, C., and Trub, T. (2000) Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes 49*, 999−1005.
(60) Herring, R., Jones, R. H., and Russell-Jones, D. L. (2014) Hepatoselectivity and the evolution of insulin. *Diabetes, Obes. Metab. 16*, 1−8.
(61) Vikova, J., Collinsova, M., Kletvikova, E., Budesinsky, M., Kaplan, V., Zakova, L., Veverka, V., Hexnerova, R., Avino, R. J., Strakova, J., Selicharova, I., Vanek, V., Wright, D. W., Watson, C. J., Turkenburg, J. P., Brzozowski, A. M., and Jiracek, J. (2016) Rational steering of insulin binding specificity by intra-chain chemical crosslinking. *Sci. Rep. 6*, 19431.

DOI: 10.1021/acs.biochem.6b00140
Biochemistry 2016, 55, 2903−2913

Appendix II

⌘ *Author's Choice*

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 40, pp. 21234–21245, September 30, 2016

Probing Receptor Specificity by Sampling the Conformational Space of the Insulin-like Growth Factor II C-domain*[S]

Received for publication, June 2, 2016, and in revised form, July 29, 2016 Published, JBC Papers in Press, August 10, 2016, DOI 10.1074/jbc.M116.741041

Rozálie Hexnerová[‡§1], Květoslava Křížková[‡§1], Milan Fábry[‡¶], Irena Sieglová[‡], Kateřina Kedrová[‡§], Michaela Collinsová[‡], Pavlína Ullrichová[‖], Pavel Srb[‡], Christopher Williams[**], Matthew P. Crump[**], Zdeněk Tošner[§], Jiří Jiráček[‡], Václav Veverka[‡2], and Lenka Žáková[‡3]

*From the [‡]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i., Flemingovo nám 2, 166 10 Prague 6, Czech Republic, [§]Faculty of Science, Charles University in Prague, Albertov 6, Prague 128 43, Czech Republic, [‖]Department of Analytical Chemistry, University of Chemistry and Technology, Technická 5, 166 28 Prague 6, Czech Republic, [¶]Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic, and **Department of Organic and Biological Chemistry, School of Chemistry, Cantock's Close, University of Bristol, Bristol BS8 1TS, United Kingdom*

Insulin and insulin-like growth factors I and II are closely related protein hormones. Their distinct evolution has resulted in different yet overlapping biological functions with insulin becoming a key regulator of metabolism, whereas insulin-like growth factors (IGF)-I/II are major growth factors. Insulin and IGFs cross-bind with different affinities to closely related insulin receptor isoforms A and B (IR-A and IR-B) and insulin-like growth factor type I receptor (IGF-1R). Identification of structural determinants in IGFs and insulin that trigger their specific signaling pathways is of increasing importance in designing receptor-specific analogs with potential therapeutic applications. Here, we developed a straightforward protocol for production of recombinant IGF-II and prepared six IGF-II analogs with IGF-I-like mutations. All modified molecules exhibit significantly reduced affinity toward IR-A, particularly the analogs with a Pro-Gln insertion in the C-domain. Moreover, one of the analogs has enhanced binding affinity for IGF-1R due to a synergistic effect of the Pro-Gln insertion and S29N point mutation. Consequently, this analog has almost a 10-fold higher IGF-1R/IR-A binding specificity in comparison with native IGF-II. The established IGF-II purification protocol allowed for cost-effective isotope labeling required for a detailed NMR structural characterization of IGF-II analogs that revealed a link between the altered binding behavior of selected analogs and conformational rearrangement of their C-domains.

The insulin-insulin-like growth factor (IGF)[4] axis is a complex signaling pathway mediated by a group of three sequentially and structurally homologous peptide hormones, their membrane receptors, and several circulating IGF-binding proteins. Insulin and IGF-I and -II are all capable of higher or lower affinity binding toward the transmembrane tyrosine kinase receptors insulin receptor isoform A (IR-A), insulin receptor isoform B (IR-B), and insulin-like growth factor type I receptor (IGF-1R) (1, 2). All three receptors also share a high degree of homology, which is manifested by overlapping biological responses upon ligand binding (3–5). Binding of insulin and IGFs to the receptors triggers two major signaling pathways via autophosphorylation of tyrosines within their intracellular tyrosine kinase domains. The first, usually referred to as a phosphoinositide 3-kinase (PI3K)/Akt pathway, is key for the metabolic effects of ligand binding such as a decrease in plasma glucose levels (6). The second signaling pathway, referred to as Ras/ERK, involves activation of the Ras/Raf/MAPK/ERK1/2 cascade, which mediates proliferative effects through gene transcription regulation (7). Whereas insulin signals mainly via both IR isoforms (8), IGF-I and IGF-II promote the mitogenic signaling through IGF-1R (9, 10), and similar mitogenic stimulation results from IGF-II binding to IR-A (11).

Both IGFs are essential for embryonic development and are present in serum at nanomolar concentrations in adults (12) with IGF-II levels being 3-fold higher than IGF-I levels (13). Whereas the role of IGF-II in tumor development is well doc-

* This work was supported by Czech Science Foundation Grant 15-19018S, Medical Research Council Grant MR/K000179/1, Ministry of Education of the Czech Republic Programs "NAVRAT" LK11205 and "NPU I" LO1304, Charles University Grant Agency Grant 227020, Specific University Research (Ministry of Education of the Czech Republic Grant 20/2013, A1_FCHI_2014_003), and Research Project of the Academy of Sciences of the Czech Republic RVO:61388963. The authors declare that they have no conflicts of interest with the contents of this article.

[S] This article contains supplemental Figs. S1–S8 and Table S1.

The atomic coordinates and structure factors (codes 5L3L, 5L3M, and 5L3N) have been deposited in the Protein Data Bank (http://wwpdb.org/).

The assigned chemical shifts have been deposited into the BioMagResBank under accession numbers 34000, 34001, and 34002.

[1] Joint first authors.

[2] To whom correspondence may be addressed. Tel.: 420-220-183-135; E-mail: vaclav.veverka@uochb.cas.cz.

[3] To whom correspondence may be addressed: Inst. of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i., Flemingovo nám 2, 166 10 Prague 6, Czech Republic. Tel.: 420-220-183-441; E-mail: zakova@uochb.cas.cz.

[4] The abbreviations used are: IGF, insulin-like growth factor; IR, insulin receptor; IR-A, insulin receptor isoform A; IR-B, insulin receptor isoform B; IGF-1R, insulin-like growth factor type I receptor; IGF-2R, insulin-like growth factor type II receptor; L1, leucine-rich repeat region; α-CT, C-terminal helix; GB1, immunoglobulin binding domain B1 of streptococcal Protein-G; TEV, tobacco etch virus; RP-HPLC, reversed phase HPLC; HSQC, heteronuclear single quantum coherence; D11, Domain 11; HMQC, heteronuclear multiple quantum coherence.

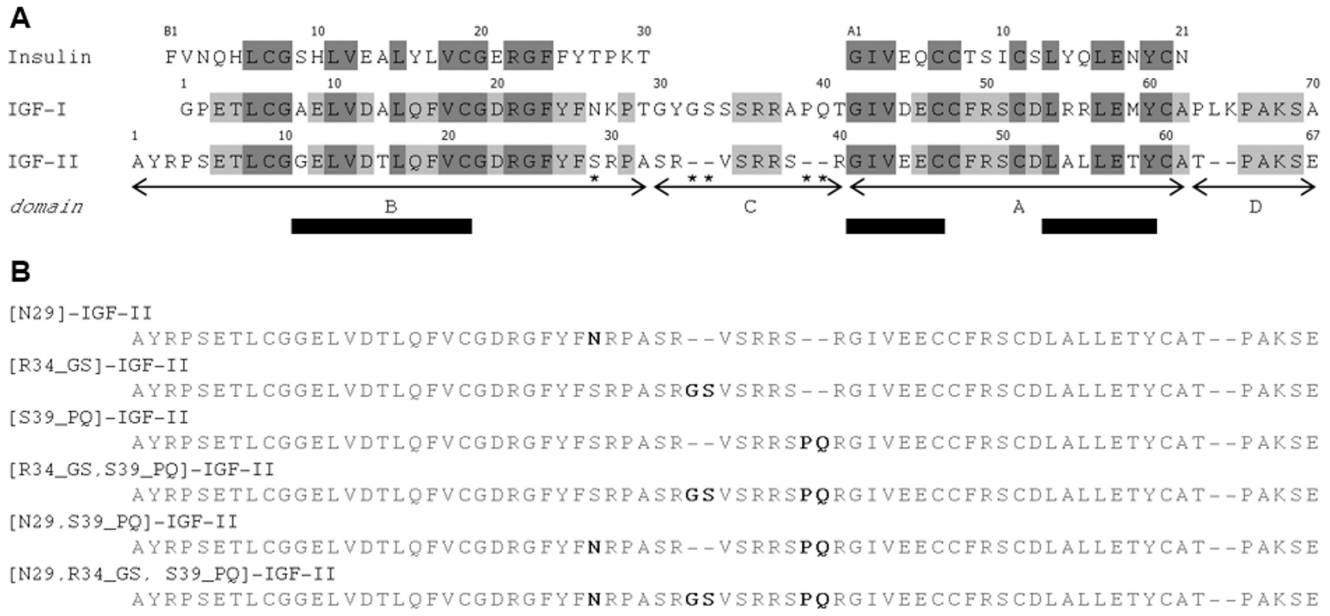


FIGURE 1. *A*, the amino acid sequence alignment of insulin, IGF-I, and IGF-II. It illustrates their high primary structure homology with the conserved residues highlighted in *dark gray* and the residues conserved between IGF-I and IGF-II in *light gray*. The organization of IGF-I and IGF-II into B-, C-, A-, and D-domains is shown below the sequences; domains A and B correspond to insulin A and B chains. The positions of conserved α-helices are shown as *bars* above the sequences. IGF-II residues mutated in this study are labeled with an *asterisk*. *B*, the amino acid sequence of the six prepared IGF-II analogs with highlighted mutations.

umented (14), its physiological role remains unclear. It is known that IGF-II is important for fetal development and placental function (15, 16), and several animal studies indicate an important role for IGF-II in memory enhancement (17–19). The availability of IGF ligands for signaling is modulated by a family of high affinity IGF-binding proteins 1–6 (20, 21) and insulin-like growth factor type II receptor (IGF-2R) (22). The equilibrium of individual components and the appropriate function of the entire insulin-IGF system are essential for biological responses such as regulation of basal metabolism, cellular growth, proliferation, survival, and migration (23).

IGF-I and IGF-II are single chain peptides composed of 70 and 67 amino acids, respectively. Mature IGFs consist of four domains: B, C, A, and D in order from the N terminus. IGF-I and -II share over 60% sequence identity, mostly in the B- and A-domains that correspond to the B and A chains in insulin (Fig. 1). The 3D structure of IGF-I was obtained by both NMR and x-ray (24–34), whereas the structure of IGF-II has been determined only by NMR (35, 36). Together with insulin, these hormones share the insulin-like conformation consisting of three highly conserved α-helices (Fig. 1) further stabilized by three characteristic disulfide bonds (28, 36, 37).

IR-A, IR-B, and IGF-1R are homodimeric, and each monomer consists of an extracellular subunit (α) and transmembrane subunit (β) that are linked via four disulfide bonds into a functional β-α-α-β homodimer (38–40). The alternative splicing of IR exon 11 generates a 12-amino acid sequence in the C terminus of the α-subunit or IR-B that is absent in IR-A (41–43). Each monomer contains two insulin/IGF binding sites termed the primary (1) and second (2) site on one monomer and 1′ and 2′ on the partner. The primary binding site is formed from a leucine-rich repeat region (L1) and C-terminal helix (α-CT) region that combine with the second site of the partner monomer (2′) to form the complete binding pocket. The two sites (1-2′) bind a single molecule of insulin/IGF, triggering structural rearrangements and negative cooperativity for binding at the 1′-2 site (44–46). The mechanisms of insulin or IGF binding to their cognate receptors were originally proposed on the basis of extensive mutagenesis studies only (47, 48). More recently, however, several reports based on the crystal structures of the insulin·IR complexes (49, 50), "activated" insulin analogs (51–53), and the first bound structure of IGF-I through complexation with a IR/IGF-1R hybrid construct (54) have revealed the binding mode of the hormones at the receptor site 1 represented by the L1 subunit and α-CT segment. However, details of the precise arrangement of the C-domain of bound IGF-I are currently unknown, but structural rearrangement of this region in conjunction with the α-CT region has been proposed to be necessary to prevent unfavorable steric clashes. Moreover, the C-domain is a region with major differences between IGFs, both in the amino acid composition and length (Fig. 1), probably being a key determinant of receptor binding specificity.

Both insulin and IGF-I have been extensively studied through the preparation and functional analysis of numerous analogs (for extensive reviews, see Refs. 46, 48, and 55), whereas the structure-function of IGF-II is less developed (15, 56–62). To gain greater insight into the structural basis of IGF-II binding specificity to IR-A and IGF-1R, we generated a series of mutants containing amino acid substitutions within the B- and C-domains of IGF-II. These were designed to make IGF-II more IGF-I-like (Fig. 1) and were tested through binding affinities to their cognate receptors. This was enabled by the development of a new, efficient, and cost-effective protocol for recombinant production of IGF-II analogs in sufficient quantities for structural characterization by NMR. Our data revealed that the newly prepared IGF-II analogs display conserved or slightly increased IGF-1R affinities

but markedly reduced IR-A affinities, which correlates with the specific conformational changes in the structurally elusive C-domain of IGF-II.

Results

Recombinant Production of IGF-II—A prerequisite for this study was the efficient production of correctly folded IGF-II, which would serve as a reference molecule as well as a platform for the design and production of new IGF-II analogs. This was achieved by recombinant IGF-II expression in *Escherichia coli* as a fusion with an N-terminal and cleavable His_6-tagged GB1 protein (immunoglobulin binding domain B1 of streptococcal Protein-G) (63, 64). This technique provided high yields (0.8–1.8 mg liter^{-1} of culture) of IGF-II analogs with only a single additional glycine residue cloning artifact at the N terminus. The fusion protein was successfully expressed in *E. coli* and purified using immobilized metal ion affinity chromatography (supplemental Fig. S1). Two major peaks were observed; the first eluted at lower concentrations of imidazole (110–160 mM; fractions 1–2 in supplemental Fig. S1) and consisted of folded and misfolded monomeric IGF-II with slightly different migration of two bands observable in non-reducing SDS-PAGE (supplemental Fig. S1). The second peak eluted at higher concentrations of imidazole and consisted of multimeric forms (310–480 mM; fractions 4–5 in supplemental Fig. S1). Both monomeric and multimeric fusion proteins were subsequently cleaved using TEV protease under redox conditions of oxidized and reduced glutathione. Interestingly, the moderate reducing environment triggered disulfide bond reshuffling that resulted in liberation of monomeric IGF-II from multimeric aggregates. Following cleavage, IGF-II was separated from the His_6-tagged GB1 and TEV by immobilized metal ion affinity chromatography. RP-HPLC of this crude IGF-II product consisted of one major peak and two to four minor peaks (supplemental Fig. S1). The retention time of the major protein peak was nearly identical to that observed for native human IGF-II, and the correct molecular weight of the recombinantly produced purified IGF-II protein with formed disulfide bonds was confirmed by high resolution mass spectrometry. Both forms, monomeric and multimeric, yielded the desired product of correct mass and were combined after the correct protein fold was confirmed by 1D ^{1}H NMR (supplemental Fig. S2) and ^{1}H-^{15}N HSQC that was highly similar to the previously published data (65).

In total, six IGF-II analogs were designed to determine the effects of IGF-I motif incorporation into IGF-II. The modifications were as follows: (i) a point mutation at position Ser^{29} for Asn ([N29]IGF-II), (ii) an insertion of Gly-Ser after Arg^{34} ([R34_GS]IGF-II), (iii) an insertion of Pro-Gln after Ser^{39} ([S39_PQ]IGF-II), (iv) a combination of both insertions ([R34_GS,S39_PQ]IGF-II), (v) a combination of S29N mutation with Pro-Gln insertion ([N29,S39_PQ]IGF-II), and (vi) a combination of S29N mutation with both insertions ([N29,R34_GS,S39_PQ]IGF-II). All analogs gave comparable RP-HPLC elution profiles (data not shown) with that of IGF-II (supplemental Fig. S1) with one major product and several minor peaks. The characterization of minor by-products was prevented by their relatively low yields.

The structural integrity of the six analogs was confirmed using ^{1}H NMR and far-UV circular dichroism as illustrated in supplemental Figs. S2 and S3. The CD spectra obtained for prepared analogs are similar to the broadly α-helical secondary structure profile obtained for non-modified IGF-II. The presence of the expected tertiary structure was further confirmed by 1D ^{1}H (supplemental Fig. S2) NMR spectra, and each analog compared well with the native IGF-II profile.

Receptor Binding—The binding affinities of the IGF-II analogs toward human IR-A and IGF-1R together with binding affinities of selected analogs to IR-B are summarized in Table 1 and Fig. 2. The corresponding binding curves are shown in supplemental Figs. S4–S6.

IR-A Binding Affinities—All modifications led to a significantly impaired IR-A binding, ranging from 4.2 to 1.1% of the insulin affinity when compared with IGF-II (7.9%). The [N29]IGF-II B-domain mutant gave a 2-fold reduction in IR-A affinity, whereas the analogs with C-domain insertions exhibited stronger negative effects. [R34_GS]IGF-II showed an almost 3-fold reduction in binding (2.8%), whereas [S39_PQ]IGF-II showed an 8-fold reduction. All of the analogs bearing the Pro-Gln motif were significantly less active (1.1–1.8%), and further combinations did not appear to have any additive effect.

IGF-1R Binding Affinities—An insertion of IGF-I-like features, S29N, Gly-Ser, Pro-Gln alone, or a combination of Gly-Ser and Pro-Gln, within the IGF-II molecule led rather unexpectedly to a moderate decrease of binding potency toward IGF-1R (Table 1 and Fig. 2). However, the Pro-Gln insertion combined with the S29N mutation resulted in an increase in binding potency to that of 18.8% to IGF-I in comparison with IGF-II (10.9%). In contrast, this effect was negated when the S29N mutation was combined with both insertions.

IR-B Binding Affinities—Both reference molecules, commercial IGF-II and our recombinant IGF-II, show similar binding potency for IR-B compared with IGF-I (1.9 and 1.5% of human insulin, respectively; ~40 nM; Table 1). The IR-B binding affinity of [N29]IGF-II dropped to almost one-third of the potency obtained for IGF-II (0.6%; 108 nM).

Structural Characterization of IGF-II Analogs by NMR Spectroscopy—We selected two IGF-II analogs with the most pronounced impact on receptor binding [S39_PQ]IGF-II (with lowest IR-A and IGF-1R binding) and [N29,S39_PQ]IGF-II (with decreased IR-A and enhanced IGF-1R binding) (Table 1 and Fig. 2) for NMR structural characterization to understand the molecular basis of Pro-Gln and S29N modifications.

Undesirable dynamic and aggregation behavior of IGF-II severely affects the quality of NMR spectra of this protein and would prevent the accurate structural determination required for a detailed comparison between these analogs. Previously, it has been shown that upon binding to an engineered high affinity Domain 11 (D11) of the IGF-2R the spectral properties of IGF-II improve dramatically (65). The fact that the IGF-II modifications reported here are distributed on the opposing face to the D11 binding site allowed this system to be utilized for structural studies of the B- and C-domains. As expected, the binding of either ^{15}N- or ^{13}C/^{15}N-labeled IGF-II analogs to unlabeled D11 led to a significant line narrowing of the NMR signals as illustrated in supplemental Fig. S7 despite the more than a

TABLE 1
The receptor binding affinities of hormones and IGF-II analogs reported in this work
The values of K_d and relative binding affinities of human insulin, IGF-I, IGF-II, and IGF-II analogs were determined for human IR-A in membranes of human IM-9 lymphocytes and for human IR-B and human IGF-1R in membranes of mouse fibroblasts. Relative receptor binding affinity is defined as (K_d of human insulin or IGF/K_d of analog) × 100. ND is not determined.

Analog	K_d ± S.E. (nM) (*n*) for human IR-A in IM-9 lymphocytes	Relative binding affinity for human IR-A	K_d ± S.E. (nM) (*n*) for human IGF-1R in mouse fibroblasts	Relative binding affinity for human IGF-1R	K_d ± S.E. (nM) (*n*) for human IR-B in mouse fibroblasts	Relative binding affinity for human IR-B
		%		%		%
Commercial human insulin	0.43 ± 0.02 (5)	100 ± 5	292 ± 31 (3)[a]	0.08 ± 0.01	0.67 ± 0.17 (5)[b]	100 ± 18
	0.24 ± 0.02 (5)	100 ± 8			0.67 ± 0.08 (4)[a]	100 ± 12
Commercial human IGF-I	23.8 ± 6.6 (3)[b]	1.0 ± 0.3[c]	0.24 ± 0.05 (5)[a]	100 ± 21	224 ± 16 (4)[b]	0.3 ± 0.0[d]
			0.25 ± 0.01 (4)	100 ± 4		
Commercial human IGF-II	2.92 ± 0.14 (3)[b]	8.2 ± 0.4[e]	2.32 ± 0.72 (3)[b]	10.8 ± 3.3[e]	35.5 ± 5.6 (4)[b]	1.9 ± 0.3[d]
IGF-II	3.03 ± 0.27 (3)	7.9 ± 0.7[e]	2.29 ± 1.04 (4)	10.9 ± 5.0[e]	43.7 ± 5.3 (3)	1.5 ± 0.2[d]
[N29]IGF-II	10.3 ± 1.1 (3)	4.2 ± 0.4[f]	4.57 ± 1.09 (3)	5.3 ± 1.3[g]	108 ± 16 (3)	0.6 ± 0.1[h]
[R34_GS]IGF-II	15.4 ± 6.0 (3)	2.8 ± 1.1[f]	4.13 ± 0.90 (3)	5.8 ± 1.3[g]	ND	ND
[S39_PQ]IGF-II	38.0 ± 2.9 (3)	1.1 ± 0.1[f]	5.00 ± 1.10 (3)	4.8 ± 1.1[g]	ND	ND
[R34_GS,S39_PQ]IGF-II	23.4 ± 4.8 (3)	1.8 ± 0.4[f]	5.68 ± 2.13 (3)	4.2 ± 1.6[g]	ND	ND
[N29,S39_PQ]IGF-II	16.8 ± 3.8 (3)	1.4 ± 0.3[e]	1.33 ± 0.36 (3)	18.8 ± 5.1[e]	ND	ND
[N29,R34_GS,S39_PQ]IGF-II	19.9 ± 5.5 (2)[i]	1.2 ± 0.3[e]	3.19 ± 1.08 (3)	7.8 ± 2.7[e]	ND	ND

[a] From Vikova *et al.* (87).
[b] From Krizkova *et al.* (2).
[c] Relative to human insulin K_d value of 0.24 ± 0.02 (n = 5).
[d] Relative to human insulin K_d value of 0.67 ± 0.12 (n = 5).
[e] Relative to human IGF-I K_d value of 0.25 ± 0.01 (n = 4).
[f] Relative to human insulin K_d value of 0.43 ± 0.02 (n = 5).
[g] Relative to human IGF-I K_d value of 0.24 ± 0.05 (n = 5).
[h] Relative to human insulin K_d value of 0.67 ± 0.08 (n = 4).
[i] This K_d value represents mean of two independent measurements ±range.

2-fold increase in the total molecular mass of the system. First, we determined the structure of the D11-bound unmodified IGF-II that was utilized in the structural analysis of IGF-II analogs. As expected, it is highly similar to the previously published structure (65) with some regions being more resolved, especially around the sites modified in the analogs, reflecting the substantially higher number of experimental restraints (1039 *versus* 764 unambiguous NOE restraints (supplemental Table S1 and Ref. 65)). Next, we verified that binding to D11 did not significantly affect the IGF-II C-domain and C-terminal portion of the B-domain by comparison of assigned 2D ^{1}H-^{15}N HSQC spectra of free and D11-bound [S39_PQ]IGF-II (supplemental Fig. S8). Although significant chemical shift perturbations were observed over the A-domain and the first 75% of the B-domain, the regions containing the mutations showed very small or negligible chemical shift perturbations.

Both analogs, [S39_PQ]IGF-II and [N29,S39_PQ]IGF-II, preserved their overall structural organization with the three highly conserved α-helices further stabilized by three disulfide bonds. As expected, the D11 binding interface on the IGF-II analogs was not perturbed by the modifications, and structural changes were restricted to the modification sites (Fig. 3). In both analogs, the C-domain insertion led to a significant change in the conformational space sampled by this region of the protein compared with unmodified IGF-II with the main differences residing between residues 29 and 42. Detailed analysis (Fig. 4) revealed that the insertion of Pro-Gln after Ser^{39} led to increased conformational freedom within the C-loops of both analogs that generated a rearrangement stabilized by several new packing interactions in the remote part of the C-domain. In the native IGF-II sequence, Tyr^{27} points away from the C-loop and forms hydrophobic contacts with Ala^{61}, whereas the C-loop is unrestrained by additional contacts to the other parts of IGF-II (Fig. 4*A*). By contrast, the aromatic ring of Tyr^{27} forms contacts to the methyl group of Ala^{32} in [S39_PQ]IGF-II (Fig. 4*B*) and Arg^{30} and Pro^{31} in [N29,S39_PQ]IGF-II (Fig. 4*C*). Arg^{30} is no longer unrestrained in these analogs and interacts with the aromatic ring of Tyr^{61} (Tyr^{59} in unmodified IGF-II) via a cation-π interaction. These new hydrophobic contacts lead to the formation of a better defined C-loop that bends around the bulky side chains of Tyr^{27} and Tyr^{61} of both C-domain-modified analogs (Fig. 4, *B* and *C*). In comparison with unmodified IGF-II, the extended C-domain in both analogs is spatially constrained and bent toward the triad of aromatic residues at the C terminus of the B-domain (Phe^{26}, Tyr^{27}, and Phe^{28}). Ser^{29} in IGF-II (Fig. 4*A*) is located at the hinge of the semiflexible loop with no significant contacts to neighboring residues. The Pro-Gln extension in [S39_PQ]IGF-II led to the repositioning of Ser^{29} in close proximity to Tyr^{27}, although there are no observed NOE contacts between Ser^{29} protons and Tyr^{27} or surrounding residues. However, the hydroxyl proton from its side chain may be involved in hydrogen bonds, *e.g.* with the backbone carboxyl groups either from Pro^{31} (<2.8 Å in half of the structures), which is closer in the extended loop, or from Arg^{42} (<2.8 Å in a quarter of the structures) at the opposite side of the loop (Figs. 4*B* and 5). The modification of Ser^{29} to Asn^{29} in [N29,S39_PQ]IGF-II led to a loss of this hydrogen bond and a subtle conformational rearrangement of the C-loop backbone. In addition, the Asn^{29} side chain is pointing out of the C-loop

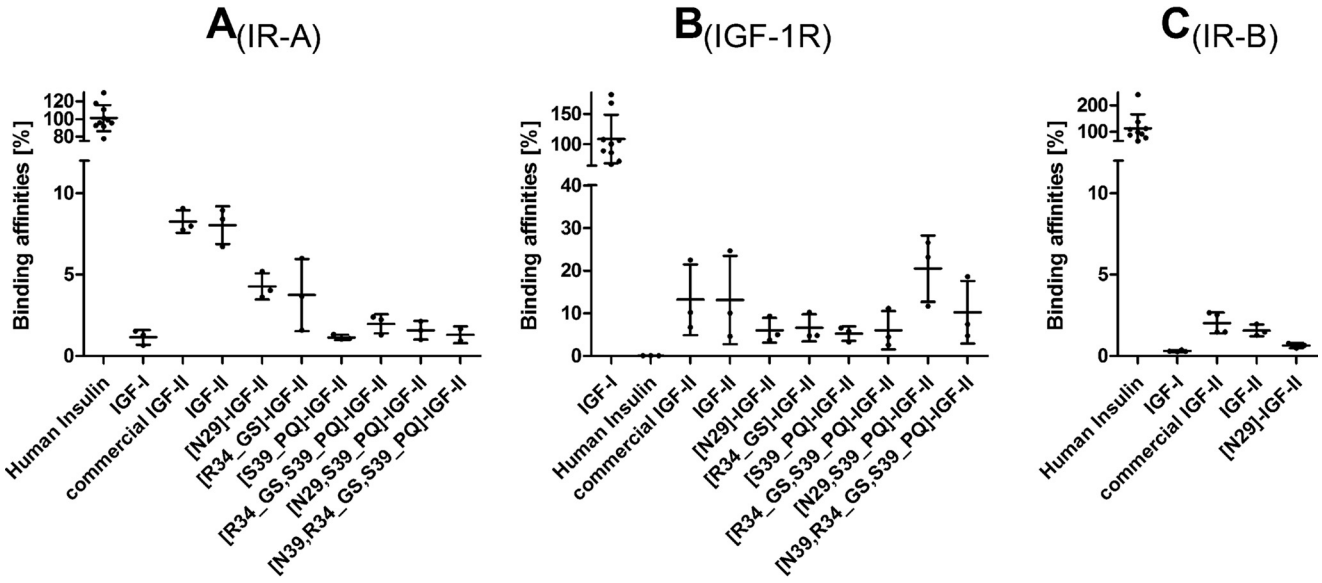


FIGURE 2. **Summary of receptor binding affinities.** Shown is a bar plot representation of relative binding affinities (from Table 1) of native hormones and IGF-II analogs prepared in this work for human IR-A (*A*), IGF-1R (*B*), and IR-B (*C*). *Error bars* represent S.D.

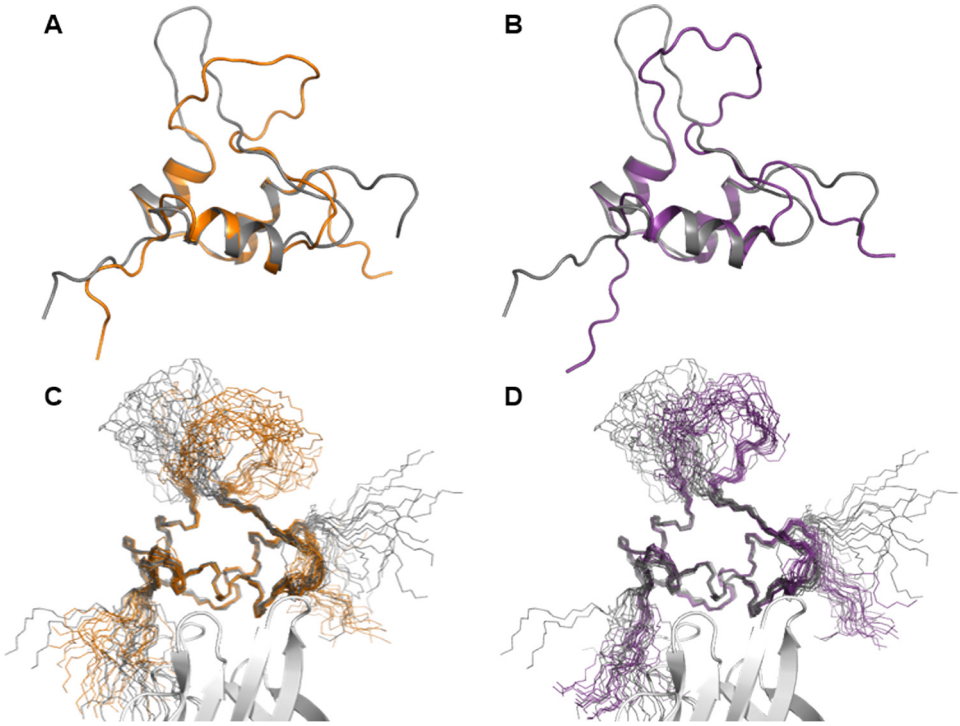


FIGURE 3. **Solution structures of [S39_PQ]IGF-II (*orange*) and [N29,S39_PQ]IGF-II (purple) compared with non-modified IGF-II (gray).** *A* and *B* show representative structures of the Domain 11-bound IGF-II analogs, and *C* and *D* show sets of 20 converged structures bound to D11 (*white*). The insertion of Pro-Gln in the C-domain after position 39 led to a significant structural rearrangement of the semiflexible loop.

and is fully solvent-exposed with NOE contacts between the NH_2 group from the Asn^{29} side chain and $H^{\beta 2}$ from Phe^{28}, perhaps further stabilizing the cluster of contacts between the C-domain and aromatic triad that in turn might stabilize the additional interactions seen between Tyr^{27} and Arg^{30}/Pro^{31} (Fig. 4*C*) that were not observed for the [S39_PQ]IGF-II analog.

Discussion

IGF-II is capable of binding to both IR-A and IGF-1R with single digit nanomolar affinity ($K_d \sim 3$ nM; Table 1) and to IR-B with lower affinity (~40 nM; Table 1). Although the binding affinities of the "parent" ligands, insulin and IGF-I, toward their cognate receptors are in the subnanomolar range (Table 1), IGF-II can still effectively signal through both IR-A and IGF-1R receptors or their hybrid forms *in vivo* (66, 67), which may trigger unfavorable biological responses. The knowledge about structural elements within these hormones responsible for differential binding specificity to each receptor could open a new path to the development of receptor-selective IGF and insulin analogs with potential medical applications. The analogs pre-

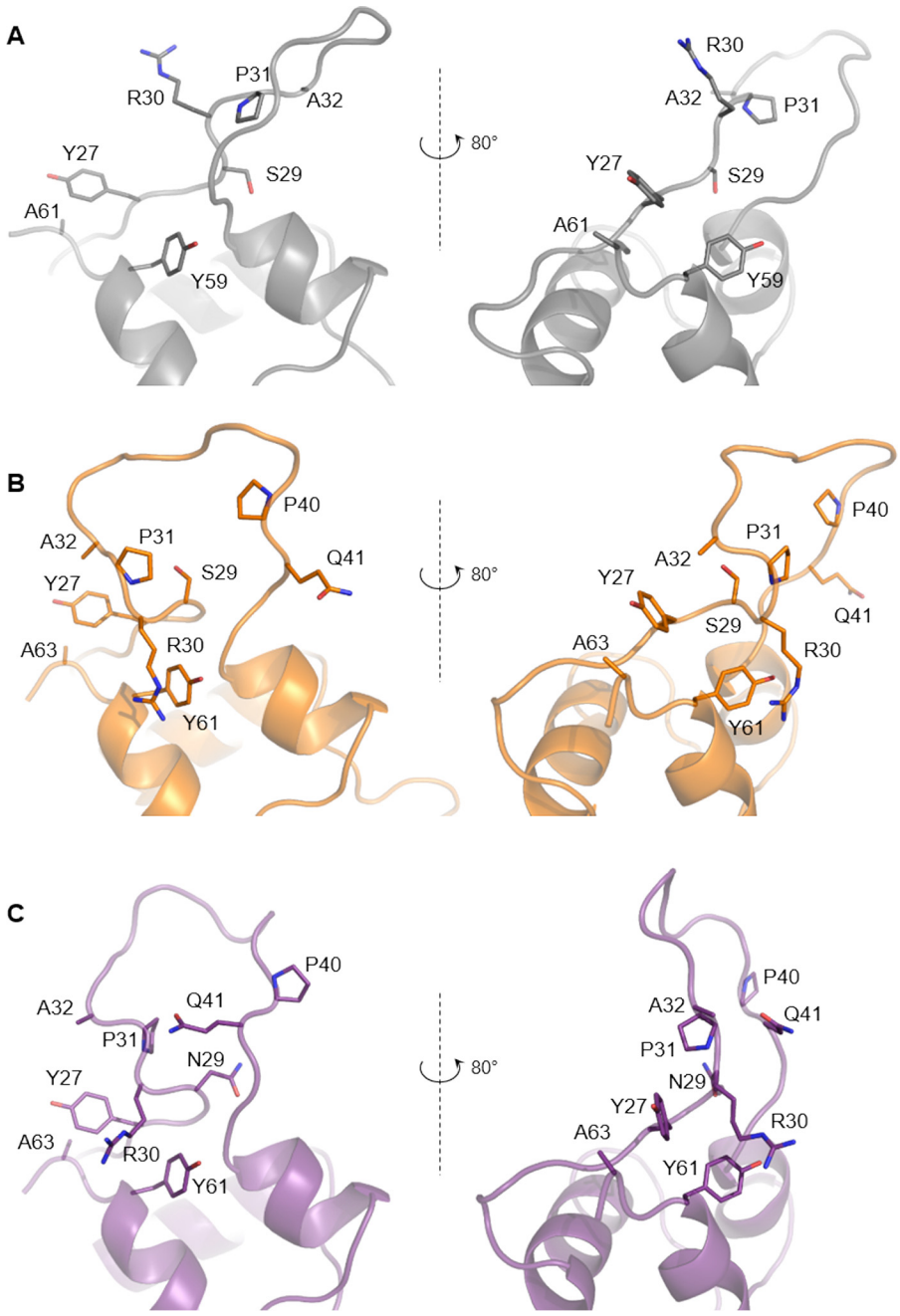


FIGURE 4. **Structural impact of the IGF-II modifications.** Non-modified IGF-II (*A*; *gray*) is compared with [S39_PQ]IGF-II (*B*; *orange*) and [N29,S39_PQ]IGF-II (*C*; *purple*), revealing different spatial orientation of highlighted residues. In particular, the rearrangement of the C-domain is driven by repositioning of Ala^{32} toward Tyr^{27} and Arg^{30} toward Tyr^{61} (Tyr^{59} in non-modified IGF-II) supported by additional contacts within this area.

pared and structurally characterized in this work were designed to investigate the effects of introducing unique IGF-I motifs (*i.e.* Asn^{26}, Gly^{30}-Ser^{31}, and Pro^{35}-Gln^{36}; Fig. 1) to IGF-II on receptor binding behavior. We hypothesized that such modifications may negatively affect the hormone's binding potency toward IR-A while enhancing the binding affinity for IGF-1R. Moreover, there are no reported analogs with the mutation of Ser^{29} in IGF-II, and there are only a few studies regarding alterations in the C-domain (57, 59).

The development of an efficient protocol for IGF-II production was a key step in being able to reliably prepare the IGF-II analogs. The total chemical synthesis of IGF-II is extremely difficult and time-consuming due to the length and unfavorable composition of the IGF-II sequence (68). The most frequently used recombinant approach, analogous to the production of IGF-I (69, 70), is based on preparation of a fusion comprising porcine growth hormone N-terminal residues 1–11 (plus N-terminal Met), a subtilisin-specific cleavage sequence (Val-Asn-Phe-Ala-His-Tyr ↓), and human IGF-II (71). However, specifically mutated subtilisin (H64A) used for the procedure is no longer commercially available. We therefore chose an alternative approach that includes an "on-column" refolding step of denatured IGF-II in a fusion with His_6-tagged GB1 protein (63, 64). Subsequent cleavage of the fusion protein in a redox envi-

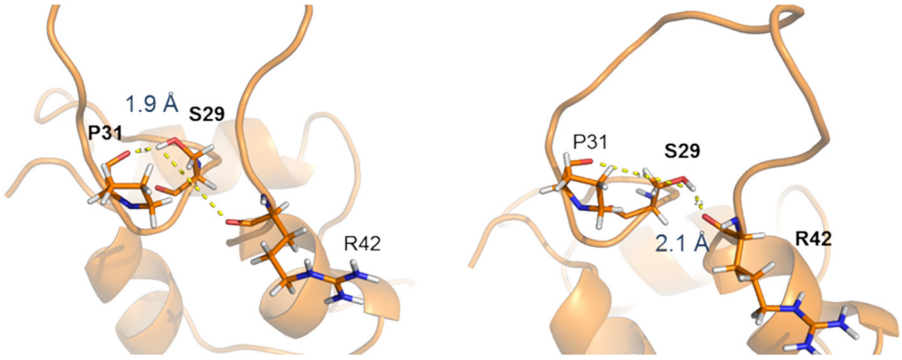


FIGURE 5. **The formation of stabilizing hydrogen bond in [S39_PQ]IGF-II.** The hydroxyl proton from the Ser[29] side chain is stabilizing the C-loop via a hydrogen bond to the backbone carboxyl groups either from Pro[31] or Arg[42].

ronment and RP-HPLC purification yields IGF-II with only a single additional glycine residue at the N terminus. This improves on the recently reported recombinant method that leaves three surplus N-terminal amino acids (glycine, alanine, and methionine) (65, 72) and therefore reduces uncertainty in interpreting structure and function of this protein in biological assays.

The binding affinities of recombinantly produced IGF-II toward IR-A, IR-B, and IGF-1R correlate with the values obtained for commercial IGF-II (Table 1 and Fig. 2). These comparable binding characteristics confirmed the correct disulfide pairing as misfolded IGFs do not bind to IGF-1R or IR-A with a measurable affinity (27, 73, 74). This method therefore leads to a rapid and cost-effective preparation of authentic IGF-II, providing us and others with an essential tool for studying IGF-II-related structure and function.

Our initial goal to reduce IR-A affinity of IGF-II was successful as all six IGF-II analogs showed reduced IR-A binding (Table 1) with four of these showing low affinity similar to IGF-I. The most significant was [S39_PQ]IGF-II with an ~7–8-fold reduction in affinity compared with IGF-II. Interestingly, this reduction was greater than the effect of swapping the entire IGF-II C-domain for IGF-I C-domain (3.7-fold) (57). Our data and data of others (57, 59) suggest that there are two main factors affecting the binding potency of IGF-II to IR-A. First, a longer C-domain may introduce structural restrictions during binding to the insulin receptor. This is in agreement with the finding that IGF-I analogs with a shorter C-domain exhibit enhanced binding affinity to IR-A (75). The second factor relates to specific C-domain amino acids (*e.g.* Pro[39] in IGF-I), which may affect the structure of the C-domain main chain and therefore binding to IR-A.

Although we only tested a single analog for binding to IR-B, the 2-fold reduction in binding observed for [N29]IGF-II suggests a similar sensitivity to changes in the C-domain (Table 1). Hence, we have not further pursued testing IR-B affinities of analogs and we focused on binding to IGF-1R.

The combination of the Pro-Gln insertion with S29N mutation in [N29,S39_PQ]IGF-II (Table 1 and Fig. 2) led to an analog exhibiting higher binding affinity to IGF-1R compared with native IGF-II. Our data suggest that the IGF-II specificity toward IGF-1R is determined by the amino acid composition of the C-domain rather than its length as demonstrated by the relatively lower binding affinity of the [R34_GS,S39_PQ]IGF-II analog. The selected mutations do not completely recover IGF-I-like binding to IGF-1R and cannot counterbalance the absence of other important IGF-I determinants (*e.g.* IGF-I Tyr[31] (76–78)). Nonetheless, the almost doubling in IGF-1R binding affinity of [N29,S39_PQ]IGF-II analog together with its markedly lowered affinity for IR-A resulted in almost 10-fold enhanced IGF-1R/IR-A binding specificity in comparison with IGF-II.

The comparison of D11-bound structures of IGF-II, [S39_PQ]IGF-II and [N29,S39_PQ]IGF-II, revealed that both analogs differ from IGF-II in the orientation and structuring of their C-loops (Figs. 3 and 4). The significant and similar displacement of the C-loops in both [S39_PQ]IGF-II and [N29,S39_PQ]IGF-II together with their more open C-loop conformations can be attributed to the effect of their PQ inserts. Moreover, the C-loop loops back to generate a turn stabilized by contacts between Tyr[27] and Ala[32] and a hydrogen bond between Ser[29] and Pro[31] or Arg[42] in [S39_PQ]IGF-II (Figs. 4*B* and 5). The absence of this hydrogen bond due to the S29N mutation in [N29,S39_PQ]IGF-II might be compensated for by Pro[31] packing against Tyr[27] (Fig. 4*C*). A comparable decrease in IR-A binding affinities of [S39_PQ]IGF-II and [N29,S39_PQ]IGF-II in comparison with IGF-II indicates it is caused mainly by their similarly altered C-loop structures rather than S29N mutation, which is well tolerated by IR-A.

In the crystal structure of human IGF-I (Protein Data Bank code 1GZR) (29), the Asn[26] side chain is solvent-exposed at the interface of the B- and C-domains with the Asn[26] presenting a potential polar hot spot. An equivalent Asn[29] in [N29,S39_PQ]IGF-II is in a similar position but is less exposed due to a partial overlap by the rearranged C-loop (Fig. 6*A*). Asn[26] is at the C terminus of the IGF-I B-domain, which is structurally altered upon binding to IGF-1R or IR (54) (Fig. 6, IGF-I receptor-bound structures in *cyan*). Analogous structural events are observed upon insulin binding to IR (50, 53), and it can be expected that receptor-driven activation of IGF-II is similar. In the Menting *et al.* (54) structure (Protein Data Bank code 4XSS), Asn[26] is the last IGF-I B-domain residue resolved in the complex with the hybrid IGF-IR/IR where it has been captured in the binding site formed from the IGF-IR α-CT and IR L1 domains (Fig. 6*B*). However, the structure of the complex did not reveal any specific contacts between IGF-I Asn[26] and IR L1 domain or IGF-IR α-CT. However, it cannot be ruled out that Ser[29] within the IGF-II molecule (or Asn[29] in

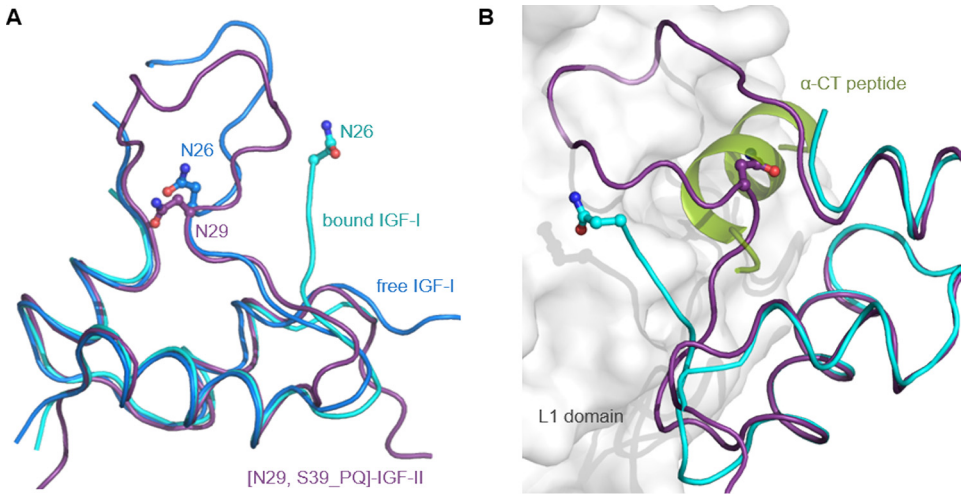


FIGURE 6. **A superposition of free or hybrid IR/IGF-1R fragment-bound forms of IGF-I with [N29,S39_PQ]IGF-II.** *A*, an overlay of the backbone of free human IGF-I (Protein Data Bank code 1GZR; in *blue*) with [N29,S39_PQ]IGF-II (in *purple*) and IGF-I from a complex with the L1 domain from IR and IGF-1R α-CT peptide (Protein Data Bank code 4XSS; in *cyan*). The positions of Asn^{26} in IGF-I and Asn^{29} in IGF-II side chains are highlighted. *B*, the crystal structure (Protein Data Bank code 4XSS) of IGF-I (in *cyan*) in a complex with IR L1 domain (in *white*) and IGF-1R α-CT peptide (in *green*) overlaid with [N29,S39_PQ]IGF-II in *purple*.

[N29,S39_PQ]IGF-II) may be involved in direct contacts to IGF-1R, and this hypothesis could be supported by a positive effect of S29N mutation on IGF-1R binding affinity of [N29,S39_PQ]IGF-II. Hence, Ser^{29} may represent an important site for engineering of the IGF-1R binding specificity in IGF-II analogs.

Concluding Remarks

We have developed a straightforward protocol for the production of recombinant IGF-II with an additional glycine at the N terminus. We prepared six IGF-II analogs with IGF-I-like mutations. All these mutants have markedly reduced affinity for IR-A, especially those analogs with Pro-Gln insertions in the C-domain. Moreover, one of the analogs, [N29,S39_PQ]IGF-II, shows the enhanced binding affinity for IGF-1R in comparison with IGF-II due to the synergistic effect of Pro-Gln insertion and S29N point mutation. Consequently, this analog has almost 10-fold enhanced IGF-1R/IR-A binding selectivity in comparison with IGF-II. Structural characterization of selected analogs revealed that the conformational rearrangement of the C-loop induced by insertion of two residues from IGF-I is manifested in the reduced affinity for IR-A. A combination of the effect of this insertion with an additional IGF-I like substitution, S29N, driving the additional subtle rearrangement of the C-loop forms a structural basis for the increased binding affinity of [N29,S39_PQ]IGF-II for IGF-1R. To our knowledge, the research reported here is a unique example of the determination of 3D structures of IGF-II analogs with modifications that have an impact on receptor binding affinities. Identification of structural determinants in IGFs and insulin that are responsible for specific binding to their cognate receptors is important for designing new, more specific hormone analogs with potential therapeutic applications.

Experimental Procedures

Recombinant Expression of IGF-II Analogs

The human IGF-II sequence was cloned into a modified pRSFDuet-1 expression vector fused with an N-terminal His_6 tag, GB1 protein, and TEV protease cleavage site (Glu-Asn-Leu-Tyr-Phe-Gln ↓ Gly). An additional N-terminal Gly (−1) was incorporated to facilitate TEV cleavage. Mutation S29N, Gly-Ser insertion following Arg^{34}, Pro-Gln insertion following Ser^{39}, and a combination of both insertions were obtained by site-directed mutagenesis (QuikChange kit, Agilent Technologies) performed with appropriate mutagenic primers of the IGF-II sequence subcloned into the pBluescript vector. After sequence verification, the mutant fragments were reintroduced into the full-length IGF-II in the expression vector. Constructs were transformed into *E. coli* BL21(λDE3) and cultivated using LB medium or minimal medium containing [^{15}N]ammonium sulfate and D-[^{13}C]glucose. The bacterial culture was grown at 37 °C to an optical density (550 nm) of ~1, induced with 1 mM isopropyl β-D-1-thiogalactopyranoside, and further cultured for 4–5 h. Cells were harvested by centrifugation for 20 min at 4,000 × *g*, and cell pellets were stored at −20 °C prior to further processing.

Isolation of Inclusion Bodies

Cells pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, 50 μM PMSF) using 10 ml of buffer/1 g of biomass and homogenized by three passes through an Avestin EmulsiFlex-C3® apparatus at 4 °C and homogenization pressure of 1,200 megapascals. Inclusion bodies from the cell lysate were obtained by centrifugation at 20,000 × *g* at 4 °C for 20 min and further washed as a suspension in a wash buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA) with 0.1% (v/v) Triton X-100, sonicated in an ice bath, and centrifuged (20,000 × *g*, 4 °C, 20 min). The wash procedure was repeated in the absence of 0.1% (v/v) Triton X-100, and wet paste consisting of inclusion bodies was stored at −20 °C.

Purification of IGF-II and Analogs

The inclusion bodies were resuspended in a minimum volume (2 ml/g of wet paste) of 50 mM Tris-HCl, pH 8.0 buffer with 300 mM NaCl and sufficient β-mercaptoethanol to yield a final

concentration of 0.02% (v/v) after the following dilution step. The suspension was gently diluted into 50 mM Tris-HCl, pH 8.0 buffer with 300 mM NaCl and 8 M urea to a final concentration of ~1 g (wet weight of inclusion bodies)/50 ml and incubated for 2–3 h at room temperature with moderate stirring. The solution of the denatured fusion protein was then loaded onto an equilibrated HisTrap HP (5 ml) column connected to an ÄKTA FPLC® system (GE Healthcare), and after washing with 50 mM Tris-HCl, pH 8.0 buffer with 300 mM NaCl, the retained protein was eluted using a 0–500 mM imidazole gradient in 50 mM Tris-HCl, pH 8.0 buffer with 300 mM NaCl within 10 column volumes. The presence of the fusion protein in collected fractions was verified by SDS-PAGE and anti-His_6 Western blotting, and the pooled fractions were dialyzed at 6 °C against 50 mM Tris-HCl, pH 8.0, 300 mM NaCl. The fusion partner was subsequently cleaved by an overnight TEV digestion in the presence of reduced and oxidized glutathione (1.5 mM GSH and 0.15 mM GSSG) at room temperature. Cleaved IGF-II was separated from the fusion protein by a gravity flow nickel chelating chromatography (HIS-Select Nickel Affinity Gel, Sigma-Aldrich) and further desalted on a Chromabond C_4 column (Macherey-Nagel) using 80% CH_3CN (v/v) with 0.1% TFA (v/v) for elution. The collected protein fraction was lyophilized; resuspended in 7% (v/v) acetic acid, 27% (v/v) CH_3CN, 0.03% TFA (v/v); and purified on a semipreparative RP-HPLC column (Vydac 214TP510-C4, 250 × 10 mm) using a CH_3CN/H_2O gradient supplemented with 0.1% TFA (v/v). The separated fractions were lyophilized, the purity of products was analyzed by analytical RP-HPLC, and the identity of the products was verified by high resolution electrospray ionization mass spectrometry (LTQ Orbitrap XL, Thermo Fisher Scientific, Waltham, MA).

NMR Spectroscopy

All NMR data for free IGF-II and analogs were acquired at 25 °C using 600- and 850-MHz Bruker Avance II spectrometers, both of which were equipped with $^1H/^{13}C/^{15}N$ cryoprobes. To confirm the correct fold of IGF-II analogs, 1D ^1H spectra (unlabeled samples) and 2D ^1H-^{15}N HSQC spectra were acquired. The NMR spectra were collected using 350-μl samples of protein (75–380 μM) dissolved in 50 mM d_4-acetic acid (pH 3.0), 5% D_2O (v/v), 0.01% (w/v) NaN_3. Data for IGF-II and analogs bound to a high affinity Domain 11 variant of IGF-2R (D11) (65, 72) were acquired from 350-μl samples of 200–400 μM IGF-II·D11 complex in acetate buffer (20 mM d_4-acetic acid, pH 4.2, 5% D_2O (v/v), 0.01% (w/v) NaN_3) at 35 °C.

To determine the structure of either free or bound IGF-IIs, a series of double and triple resonance spectra (79, 80) were recorded on $^{13}C/^{15}N$ uniformly labeled IGF-II or analogs to determine essentially complete sequence-specific resonance backbone and side chain assignments. Constraints for ^1H-^1H distances were derived from 3D ^{15}N-^1H NOESY-HSQC and ^{13}C-^1H NOESY-HMQC, which were acquired using an NOE mixing time of 100 ms.

The family of converged structures was initially calculated using Cyana 2.1 (81). The combined automated NOE assignment and structure determination protocol was used to automatically assign the NOE cross-peaks identified in NOESY spectra and to produce preliminary structures. In addition, backbone torsion angle constraints, generated from assigned chemical shifts using the program TALOS+ (82), were included in the calculations. Subsequently, five cycles of simulated annealing combined with redundant dihedral angle constraints were used to produce sets of converged structures with no significant restraint violations (distance and van der Waals violations <0.2 Å and dihedral angle constraint violation <5°), which were further refined in explicit solvent using YASARA software with the YASARA force field (83). The structures with the lowest total energy were selected. Analysis of the family of structures obtained was carried out using the Protein Structure Validation Software suite (Northeast Structural Genomics consortium) and MOLMOL (84). The statistics for the resulting structures are summarized in supplemental Table S1.

Circular Dichroism

CD spectra were measured in a quartz cuvette with an optical path length of 0.5 mm (Starna Cells) using a J-815 spectropolarimeter (Jasco, Japan) at room temperature. The far- and near-UV CD spectra were used to identify changes in protein secondary and tertiary structures. The spectral regions were 200–300 nm. The final spectra were obtained as an average of five accumulations. The spectra were corrected for the baseline by subtracting the spectra of the corresponding polypeptide-free solution. Analogs or IGF-II was dissolved and measured in 5% aqueous acetic acid (0.33 mg/ml; 45 μM).

Receptor Binding Studies

Commercial human insulin and IGF-II were provided by Sigma-Aldrich, and human IGF-I was provided by Tercica.

Human IM-9 Lymphocytes (Human IR-A Isoform)

Receptor binding studies with the insulin receptor in membranes of human IM-9 lymphocytes (containing only human IR-A isoform) were carried out, and K_d values were determined according to the procedure described recently (85). Binding data were analyzed by Excel algorithms especially developed for the IM-9 cell system in the laboratory of Prof. Pierre De Meyts (developed by A. V. Groth and R. M. Shymko, Hagedorn Research Institute, Denmark; a kind gift of P. De Meyts) using a method of non-linear regression and a one-site fitting program and taking into account potential depletion of free ligand. Each binding curve was determined in duplicate, and the final dissociation constant (K_d) of an analog was calculated from at least three ($n \geq 3$) independently determined binding curves. The dissociation constant of human ^{125}I-insulin was set to 0.3 nM.

Mouse Embryonic Fibroblasts

Human IR-B Isoform—Receptor binding studies with the insulin receptor in membranes of mouse embryonic fibroblasts derived from IGF-I receptor knock-out mice that solely expressed the human IR-B isoform were performed as described in detail previously (86, 87). Binding data were analyzed, and the dissociation constant (K_d) was determined with GraphPad Prism 5 software using a method of non-linear regression and a one-site fitting program and taking into account potential depletion of free ligand. K_d values of analogs were determined and calculated by the same procedure as for IR-A.

Human IGF-1R—Receptor binding studies with the IGF-I receptor in membranes of mouse embryonic fibroblasts derived from IGF-1R knock-out mice and transfected with human IGF-1R were performed as described previously (86, 87). Binding data were analyzed, and the dissociation constants were determined and calculated by the same method as for IR-B. The dissociation constant of human ^{125}I-IGF-I was set to 0.2 nM. Mouse embryonic fibroblasts expressing human IR-B or IGF-1R were a kind gift from Prof. Antonino Belfiore (University of Magna Grecia, Catanzaro, Italy) and Prof. Renato Baserga (Thomas Jefferson University, Philadelphia, PA). Here we should note that the use of bovine serum albumin (*e.g.* Sigma-Aldrich A6003) void of "IGF-binding-like" proteins, which interfere with these binding assays, is essential for the preparation of the binding buffer (88).

Author Contributions—R. H. and K. Křížková contributed equally to the paper. R. H., K. Křížková, K. Kedrová, and I. S. carried out protein expression and purification. R. H., P. S., Z. T., and V. V. carried out NMR experiments and structure refinement. K. Křížková, K. Kedrová, M. C., and L. Ž. tested the analogs. M. F. and C. W. carried out DNA cloning. P. U. measured CD spectra. J. J. and L. Ž. conceived the study, designed experiments, and analyzed data. R. H., K. Křížková, M. P. C., J. J., V. V., and L.Ž. wrote the paper. All authors discussed the results and commented on the manuscript.

Acknowledgment—We thank Prof. Marie Urbanová from the University of Chemistry and Technology in Prague for assistance with measuring CD spectra of analogs.

References

1. Pandini, G., Frasca, F., Mineo, R., Sciacca, L., Vigneri, R., and Belfiore, A. (2002) Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *J. Biol. Chem.* **277,** 39684–39695
2. Křížková, K., Chrudinová, M., Povalová, A., Selicharová, I., Collinsová, M., Vaněk, V., Brzozowski, A. M., Jiráček, J., and Žaková, L. (2016) Insulin-insulin-like growth factors hybrids as molecular probes of hormone:receptor binding specificity. *Biochemistry* **55,** 2903–2913
3. Lee, J., and Pilch, P. F. (1994) The insulin receptor: structure, function, and signaling. *Am. J. Physiol. Cell Physiol.* **266,** C319–C334
4. Boucher, J., Tseng, Y. H., and Kahn, C. R. (2010) Insulin and insulin-like growth factor-1 receptors act as ligand-specific amplitude modulators of a common pathway regulating gene transcription. *J. Biol. Chem.* **285,** 17235–17245
5. Siddle, K. (2012) Molecular basis of signaling specificity of insulin and IGF receptors: neglected corners and recent advances. *Front. Endocrinol.* **3,** 34
6. Hers, I., Vincent, E. E., and Tavaré, J. M. (2011) Akt signalling in health and disease. *Cell. Signal.* **23,** 1515–1527
7. Siddle, K. (2011) Signalling by insulin and IGF receptors: supporting acts and new players. *J. Mol. Endocrinol.* **47,** R1–R10
8. Bedinger, D. H., and Adams, S. H. (2015) Metabolic, anabolic, and mitogenic insulin responses: a tissue-specific perspective for insulin receptor activators. *Mol. Cell. Endocrinol.* **415,** 143–156
9. Esposito, D. L., Blakesley, V. A., Koval, A. P., Scrimgeour, A. G., and LeRoith, D. (1997) Tyrosine residues in the C-terminal domain of the insulin-like growth factor-I receptor mediate mitogenic and tumorigenic signals. *Endocrinology* **138,** 2979–2988
10. O'Connor, R., Kauffmann-Zeh, A., Liu, Y., Lehar, S., Evan, G. I., Baserga, R., and Blättler, W. A. (1997) Identification of domains of the insulin-like growth factor I receptor that are required for protection from apoptosis. *Mol. Cell. Biol.* **17,** 427–435
11. Sacco, A., Morcavallo, A., Pandini, G., Vigneri, R., and Belfiore, A. (2009) Differential signaling activation by insulin and insulin-like growth factors I and II upon binding to insulin receptor isoform A. *Endocrinology* **150,** 3594–3602
12. Le Roith, D. (1997) Seminars in medicine of the Beth Israel Deaconess Medical Center. Insulin-like growth factors. *N. Engl. J. Med.* **336,** 633–640
13. LeRoith, D., and Roberts, C. T. (2003) The insulin-like growth factor system and cancer. *Cancer Lett.* **195,** 127–137
14. Dynkevich, Y., Rother, K. I., Whitford, I., Qureshi, S., Galiveeti, S., Szulc, A. L., Danoff, A., Breen, T. L., Kaviani, N., Shanik, M. H., Leroith, D., Vigneri, R., Koch, C. A., and Roth, J. (2013) Tumors, IGF-2, and hypoglycemia: insights from the clinic, the laboratory, and the historical archive. *Endocr. Rev.* **34,** 798–826
15. Alvino, C. L., Ong, S. C., McNeil, K. A., Delaine, C., Booker, G. W., Wallace, J. C., and Forbes, B. E. (2011) Understanding the mechanism of insulin and insulin-like growth factor (IGF) receptor activation by IGF-II. *PLoS One* **6,** e27488
16. Gallagher, E. J., and LeRoith, D. (2011) Minireview: IGF, insulin, and cancer. *Endocrinology* **152,** 2546–2551
17. Alberini, C. M., and Chen, D. Y. (2012) Memory enhancement: consolidation, reconsolidation and insulin-like growth factor 2. *Trends Neurosci.* **35,** 274–283
18. Chen, D. Y., Stern, S. A., Garcia-Osta, A., Saunier-Rebori, B., Pollonini, G., Bambah-Mukku, D., Blitzer, R. D., and Alberini, C. M. (2011) A critical role for IGF-II in memory consolidation and enhancement. *Nature* **469,** 491–497
19. Pascual-Lucas, M., Viana da Silva, S., Di Scala, M., Garcia-Barroso, C., González-Aseguinolaza, G., Mulle, C., Alberini, C. M., Cuadrado-Tejedor, M., and Garcia-Osta, A. (2014) Insulin-like growth factor 2 reverses memory and synaptic deficits in APP transgenic mice. *EMBO Mol. Med.* **6,** 1246–1262
20. Clemmons, D. R. (1998) Role of insulin-like growth factor binding proteins in controlling IGF actions. *Mol. Cell. Endocrinol.* **140,** 19–24
21. Firth, S. M., and Baxter, R. C. (2002) Cellular actions of the insulin-like growth factor binding proteins. *Endocr. Rev.* **23,** 824–854
22. Kornfeld, S. (1992) Structure and function of the mannose 6-phosphate insulin-like growth factor-II receptors. *Annu. Rev. Biochem.* **61,** 307–330
23. Belfiore, A., and Malaguarnera, R. (2011) Insulin receptor and cancer. *Endocr.-Relat. Cancer* **18,** R125–R147
24. Schaffer, M. L., Deshayes, K., Nakamura, G., Sidhu, S., and Skelton, N. J. (2003) Complex with a phage display-derived peptide provides insight into the function of insulin-like growth factor I. *Biochemistry* **42,** 9324–9334
25. Laajoki, L. G., Francis, G. L., Wallace, J. C., Carver, J. A., and Keniry, M. A. (2000) Solution structure and backbone dynamics of long-[Arg^3]insulin-like growth factor-I. *J. Biol. Chem.* **275,** 10009–10015
26. De Wolf, E., Gill, R., Geddes, S., Pitts, J., Wollmer, A., and Grötzinger, J. (1996) Solution structure of a mini IGF-1. *Protein Sci.* **5,** 2193–2202
27. Sato, A., Nishimura, S., Ohkubo, T., Kyogoku, Y., Koyama, S., Kobayashi, M., Yasuda, T., and Kobayashi, Y. (1993) 3-Dimensional structure of human insulin-like growth factor-I (IGF-I) determined by ^{1}H-NMR and distance geometry. *Int. J. Pept. Protein Res.* **41,** 433–440
28. Cooke, R. M., Harvey, T. S., and Campbell, I. D. (1991) Solution structure of human insulin-like growth factor 1: a nuclear magnetic resonance and restrained molecular dynamics study. *Biochemistry* **30,** 5484–5491
29. Brzozowski, A. M., Dodson, E. J., Dodson, G. G., Murshudov, G. N., Verma, C., Turkenburg, J. P., de Bree, F. M., and Dauter, Z. (2002) Structural origins of the functional divergence of human insulin-like growth factor-I and insulin. *Biochemistry* **41,** 9389–9397
30. Siwanowicz, I., Popowicz, G. M., Wisniewska, M., Huber, R., Kuenkele, K. P., Lang, K., Engh, R. A., and Holak, T. A. (2005) Structural basis for the regulation of insulin-like growth factors by IGF binding proteins. *Structure* **13,** 155–167
31. Vajdos, F. F., Ultsch, M., Schaffer, M. L., Deshayes, K. D., Liu, J., Skelton, N. J., and de Vos, A. M. (2001) Crystal structure of human insulin-like growth factor-1: detergent binding inhibits binding protein interactions. *Biochemistry* **40,** 11022–11029
32. Yun, C. H., Tang, Y. H., Feng, Y. M., An, X. M., Chang, W. R., and Liang, D. C. (2005) 1.42 Å crystal structure of mini-IGF-1(2): an analysis of the

disulfide isomerization property and receptor binding property of IGF-1 based on the three-dimensional structure. *Biochem. Biophys. Res. Commun.* **326,** 52–59
33. Sitar, T., Popowicz, G. M., Siwanowicz, I., Huber, R., and Holak, T. A. (2006) Structural basis for the inhibition of insulin-like growth factors by insulin-like growth factor-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 13028–13033
34. Zesławski, W., Beisel, H. G., Kamionka, M., Kalus, W., Engh, R. A., Huber, R., Lang, K., and Holak, T. A. (2001) The interaction of insulin-like growth factor-I with the N-terminal domain of IGFBP-5. *EMBO J.* **20,** 3638–3644
35. Terasawa, H., Kohda, D., Hatanaka, H., Nagata, K., Higashihashi, N., Fujiwara, H., Sakano, K., and Inagaki, F. (1994) Solution structure of human insulin-like growth-factor-II; recognition sites for receptors and binding-proteins. *EMBO J.* **13,** 5590–5597
36. Torres, A. M., Forbes, B. E., Aplin, S. E., Wallace, J. C., Francis, G. L., and Norton, R. S. (1995) Solution structure of human insulin-like growth-factor-II. Relationship to receptor and binding-protein interactions. *J. Mol. Biol.* **248,** 385–401
37. Gursky, O., Li, Y., Badger, J., and Caspar, D. L. (1992) Monovalent cation binding to cubic insulin crystals. *Biophys. J.* **61,** 604–611
38. McKern, N. M., Lawrence, M. C., Streltsov, V. A., Lou, M. Z., Adams, T. E., Lovrecz, G. O., Elleman, T. C., Richards, K. M., Bentley, J. D., Pilling, P. A., Hoyne, P. A., Cartledge, K. A., Pham, T. M., Lewis, J. L., Sankovich, S. E., *et al.* (2006) Structure of the insulin receptor ectodomain reveals a folded-over conformation. *Nature* **443,** 218–221
39. Lawrence, M. C., McKern, N. M., and Ward, C. W. (2007) Insulin receptor structure and its implications for the IGF-1 receptor. *Curr. Opin. Struct. Biol.* **17,** 699–705
40. Ward, C. W., Menting, J. G., and Lawrence, M. C. (2013) The insulin receptor changes conformation in unforeseen ways on ligand binding: sharpening the picture of insulin receptor activation. *BioEssays* **35,** 945–954
41. Yamaguchi, Y., Flier, J. S., Benecke, H., Ransil, B. J., and Moller, D. E. (1993) Ligand-binding properties of the two isoforms of the human insulin receptor. *Endocrinology* **132,** 1132–1138
42. Seino, S., Seino, M., Nishi, S., and Bell, G. I. (1989) Structure of the human insulin receptor gene and characterization of its promoter. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 114–118
43. Mosthaf, L., Grako, K., Dull, T. J., Coussens, L., Ullrich, A., and McClain, D. A. (1990) Functionally distinct insulin-receptors generated by tissue-specific alternative splicing. *EMBO J.* **9,** 2409–2413
44. Schaefer, E. M., Siddle, K., and Ellis, L. (1990) Deletion analysis of the human insulin receptor ectodomain reveals independently folded soluble subdomains and insulin binding by a monomeric α-subunit. *J. Biol. Chem.* **265,** 13248–13253
45. Brandt, J., Andersen, A. S., and Kristensen, C. (2001) Dimeric fragment of the insulin receptor α-subunit binds insulin with full holoreceptor affinity. *J. Biol. Chem.* **276,** 12378–12384
46. De and Meyts, P. (2015) Insulin/receptor binding: the last piece of the puzzle? *BioEssays* **37,** 389–397
47. Kristensen, C., Kjeldsen, T., Wiberg, F. C., Schäffer, L., Hach, M., Havelund, S., Bass, J., Steiner, D. F., and Andersen, A. S. (1997) Alanine scanning mutagenesis of insulin. *J. Biol. Chem.* **272,** 12978–12983
48. Denley, A., Cosgrove, L. J., Booker, G. W., Wallace, J. C., and Forbes, B. E. (2005) Molecular interactions of the IGF system. *Cytokine Growth Factor Rev.* **16,** 421–439
49. Menting, J. G., Whittaker, J., Margetts, M. B., Whittaker, L. J., Kong, G. K., Smith, B. J., Watson, C. J., Záková, L., Kletvíková, E., Jiráček, J., Chan, S. J., Steiner, D. F., Dodson, G. G., Brzozowski, A. M., Weiss, M. A., *et al.* (2013) How insulin engages its primary binding site on the insulin receptor. *Nature* **493,** 241–245
50. Menting, J. G., Yang, Y., Chan, S. J., Phillips, N. B., Smith, B. J., Whittaker, J., Wickramasinghe, N. P., Whittaker, L. J., Pandyarajan, V., Wan, Z. L., Yadav, S. P., Carroll, J. M., Strokes, N., Roberts, C. T., Jr., Ismail-Beigi, F., *et al.* (2014) Protective hinge in insulin opens to enable its receptor engagement. *Proc. Natl. Acad. Sci. U.S.A.* **111,** E3395–E3404
51. Jirácek, J., Záková, L., Antolíková, E., Watson, C. J., Turkenburg, J. P., Dodson, G. G., and Brzozowski, A. M. (2010) Implications for the active form of human insulin based on the structural convergence of highly active hormone analogues. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 1966–1970
52. Žaková, L., Kletvíková, E., Veverka, V., Lepsík, M., Watson, C. J., Turkenburg, J. P., Jirácek, J., and Brzozowski, A. M. (2013) Structural integrity of the B24 site in human insulin is important for hormone functionality. *J. Biol. Chem.* **288,** 10230–10240
53. Záková, L., Kletvíková, E., Lepšík, M., Collinsová, M., Watson, C. J., Turkenburg, J. P., Jiráček, J., and Brzozowski, A. M. (2014) Human insulin analogues modified at the B26 site reveal a hormone conformation that is undetected in the receptor complex. *Acta Crystallogr. D Biol. Crystallogr.* **70**, 2765–2774
54. Menting, J. G., Lawrence, C. F., Kong, G. K., Margetts, M. B., Ward, C. W., and Lawrence, M. C. (2015) Structural congruency of ligand binding to the insulin and insulin/type 1 insulin-like growth factor hybrid receptors. *Structure* **23,** 1271–1282
55. Mayer, J. P., Zhang, F., and DiMarchi, R. D. (2007) Insulin structure and function. *Biopolymers* **88,** 687–713
56. Hashimoto, R., Fujiwara, H., Higashihashi, N., Enjoh-Kimura, T., Terasawa, H., Fujita-Yamaguchi, Y., Inagaki, F., Perdue, J. F., and Sakano, K. (1995) N-terminal deletion mutants of insulin-like growth factor-II (IGF-II) show Thr^{7} and Leu^{8} important for binding to insulin and IGF-I receptors and Leu^{8} critical for All IGF-II functions. *J. Biol. Chem.* **270,** 18013–18018
57. Denley, A., Bonython, E. R., Booker, G. W., Cosgrove, L. J., Forbes, B. E., Ward, C. W., and Wallace, J. C. (2004) Structural determinants for high-affinity binding of insulin-like growth factor II to insulin receptor (IR)-A, the exon 11 minus isoform of the IR. *Mol. Endocrinol.* **18,** 2502–2512
58. Ziegler, A. N., Chidambaram, S., Forbes, B. E., Wood, T. L., and Levison, S. W. (2014) Insulin-like growth factor-II (IGF-II) and IGF-II. Analogs with enhanced insulin receptor-a binding affinity promote neural stem cell expansion. *J. Biol. Chem.* **289,** 4626–4633
59. Henderson, S. T., Brierley, G. V., Surinya, K. H., Priebe, I. K., Catcheside, D. E., Wallace, J. C., Forbes, B. E., and Cosgrove, L. J. (2015) Delineation of the IGF-II C domain elements involved in binding and activation of the IR-A, IR-B and IGF-IR. *Growth Horm. IGF Res.* **25,** 20–27
60. Alvino, C. L., McNeil, K. A., Ong, S. C., Delaine, C., Booker, G. W., Wallace, J. C., Whittaker, J., and Forbes, B. E. (2009) A novel approach to identify two distinct receptor binding surfaces of insulin-like growth factor II. *J. Biol. Chem.* **284,** 7656–7664
61. Delaine, C., Alvino, C. L., McNeil, K. A., Mulhern, T. D., Gauguin, L., De Meyts, P., Jones, E. Y., Brown, J., Wallace, J. C., and Forbes, B. E. (2007) A novel binding site for the human insulin-like growth factor-II (IGF-II)/mannose 6-phosphate receptor on IGF-II. *J. Biol. Chem.* **282,** 18886–18894
62. Sakano, K., Enjoh, T., Numata, F., Fujiwara, H., Marumoto, Y., Higashihashi, N., Sato, Y., Perdue, J. F., and Fujita-Yamaguchi, Y. (1991) The design, expression, and characterization of human insulin-like growth factor-II (IGF-II) mutants specific for either the IGF-II cation-independent mannose 6-phosphate receptor or IGF-I receptor. *J. Biol. Chem.* **266,** 20626–20635
63. Zhou, P., and Wagner, G. (2010) Overcoming the solubility limit with solubility-enhancement tags: successful applications in biomolecular NMR studies. *J. Biomol. NMR* **46,** 23–31
64. Gronenborn, A. M., Filpula, D. R., Essig, N. Z., Achari, A., Whitlow, M., Wingfield, P. T., and Clore, G. M. (1991) A novel, highly stable fold of the immunoglobulin binding domain of streptococcal Protein-G. *Science* **253,** 657–661
65. Williams, C., Hoppe, H. J., Rezgui, D., Strickland, M., Forbes, B. E., Grutzner, F., Frago, S., Ellis, R. Z., Wattana-Amorn, P., Prince, S. N., Zaccheo, O. J., Nolan, C. M., Mungall, A. J., Jones, E. Y., Crump, M. P., *et al.* (2012) An exon splice enhancer primes IGF2:IGF2R binding site structure and function evolution. *Science* **338,** 1209–1213
66. Frasca, F., Pandini, G., Scalia, P., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I. D., Belfiore, A., and Vigneri, R. (1999) Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Mol. Cell. Biol.* **19,** 3278–3288
67. Slaaby, R. (2015) Specific insulin/IGF1 hybrid receptor activation assay reveals IGF1 as a more potent ligand than insulin. *Sci. Rep.* **5,** 7911

68. Cottam, J. M., Scanlon, D. B., Karas, J. A., Calabrese, A. N., Pukala, T. L., Forbes, B. E., Wallace, J. C., and Abell, A. D. (2013) Chemical synthesis of a fluorescent IGF-II analogue. *Int. J. Pept. Res. Ther.* **19,** 61–69
69. King, G. L., Kahn, C. R., Samuels, B., Danho, W., Bullesbach, E. E., and Gattner, H. G. (1982) Synthesis and characterization of molecular hybrids of insulin and insulin-like growth factor-I. The role of the A-chain extension peptide. *J. Biol. Chem.* **257,** 10869–10873
70. Francis, G. L., Ross, M., Ballard, F. J., Milner, S. J., Senn, C., McNeil, K. A., Wallace, J. C., King, R., and Wells, J. R. (1992) Novel recombinant fusion protein analogs of insulin-like growth-factor (IGF)-I indicate the relative importance of IGF-binding protein and receptor-binding for enhanced biological potency. *J. Mol. Endocrinol.* **8,** 213–223
71. Francis, G. L., Aplin, S. E., Milner, S. J., McNeil, K. A., Ballard, F. J., and Wallace, J. C. (1993) Insulin-like growth-factor (IGF)-II binding to IGF-binding proteins and IGF receptors is modified by deletion of the N-terminal hexapeptide or substitution of arginine for glutamate-6 in IGF-II. *Biochem. J.* **293,** 713–719
72. Williams, C., Rezgui, D., Prince, S. N., Zaccheo, O. J., Foulstone, E. J., Forbes, B. E., Norton, R. S., Crosby, J., Hassan, A. B., and Crump, M. P. (2007) Structural insights into the interaction of insulin-like growth factor 2 with IGF2R domain 11. *Structure* **15,** 1065–1078
73. Sohma, Y., Pentelute, B. L., Whittaker, J., Hua, Q. X., Whittaker, L. J., Weiss, M. A., and Kent, S. B. (2008) Comparative properties of insulin-like growth factor 1 (IGF-1) and [Gly7D-Ala]IGF-1 prepared by total chemical synthesis. *Angew. Chem. Int. Ed. Engl.* **47,** 1102–1106
74. Gill, R., Verma, C., Wallach, B., Ursø, B., Pitts, J., Wollmer, A., De Meyts, P., and Wood, S. (1999) Modelling of the disulphide-swapped isomer of human insulin-like growth factor-1: implications for receptor binding. *Protein Eng.* **12,** 297–303
75. Bayne, M. L., Applebaum, J., Underwood, D., Chicchi, G. G., Green, B. G., Hayes, N. S., and Cascieri, M. A. (1989) The C region of human insulin-like growth factor (IGF) I is required for high affinity binding to the type 1 IGF receptor. *J. Biol. Chem.* **264,** 11004–11008
76. Bayne, M. L., Applebaum, J., Chicchi, G. G., Miller, R. E., and Cascieri, M. A. (1990) The roles of tyrosine-24, tyrosine-31, and tyrosine-60 in the high-affinity binding of insulin-like growth factor-I to the type-1 insulin-like growth-factor receptor. *J. Biol. Chem.* **265,** 15648–15652
77. Maly, P., and Lüthi, C. (1988) The binding sites of insulin-like growth factor I (IGF I) to type I IGF receptor and to a monoclonal antibody. Mapping by chemical modification of tyrosine residues. *J. Biol. Chem.* **263,** 7068–7072
78. Keyhanfar, M., Booker, G. W., Whittaker, J., Wallace, J. C., and Forbes, B. E. (2007) Precise mapping of an IGF-I-binding site on the IGF-1R. *Biochem. J.* **401,** 269–277
79. Renshaw, P. S., Veverka, V., Kelly, G., Frenkiel, T. A., Williamson, R. A., Gordon, S. V., Hewinson, R. G., and Carr, M. D. (2004) Sequence-specific assignment and secondary structure determination of the 195-residue complex formed by the *Mycobacterium tuberculosis* proteins CFP-10 and ESAT-6. *J. Biomol. NMR* **30,** 225–226
80. Veverka, V., Lennie, G., Crabbe, T., Bird, I., Taylor, R. J., and Carr, M. D. (2006) NMR assignment of the mTOR domain responsible for rapamycin binding. *J. Biomol. NMR* **36,** Suppl. 1, 3
81. Herrmann, T., Güntert, P., and Wüthrich, K. (2002) Protein NMR structure determination with automated NOE assignment using the new software CANDID and the torsion angle dynamics algorithm DYANA. *J. Mol. Biol.* **319,** 209–227
82. Shen, Y., Delaglio, F., Cornilescu, G., and Bax, A. (2009) TALOS plus: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J. Biomol. NMR* **44,** 213–223
83. Harjes, E., Harjes, S., Wohlgemuth, S., Müller, K. H., Krieger, E., Herrmann, C., and Bayer, P. (2006) GTP-Ras disrupts the intramolecular complex of C1 and RA domains of Nore1. *Structure* **14,** 881–888
84. Koradi, R., Billeter, M., and Wüthrich, K. (1996) MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graph.* **14,** 51–55, 29–32
85. Morcavallo, A., Genua, M., Palummo, A., Kletvikova, E., Jiracek, J., Brzozowski, A. M., Iozzo, R. V., Belfiore, A., and Morrione, A. (2012) Insulin and insulin-like growth factor II differentially regulate endocytic sorting and stability of insulin receptor isoform A. *J. Biol. Chem.* **287,** 11422–11436
86. Kosinová, L., Veverka, V., Novotná, P., Collinsová, M., Urbanová, M., Moody, N. R., Turkenburg, J. P., Jiráček, J., Brzozowski, A. M., and Žáková, L. (2014) Insight into the structural and biological relevance of the T/R transition of the N-terminus of the B-chain in human insulin. *Biochemistry* **53,** 3392–3402
87. Viková, J., Collinsová, M., Kletvíková, E., Buděšínský, M., Kaplan, V., Žáková, L., Veverka, V., Hexnerová, R., Tarazona Aviñó, R. J., Straková, J., Selicharová, I., Vaněk, V., Wright, D. W., Watson, C. J., Turkenburg, J. P., *et al.* (2016) Rational steering of insulin binding specificity by intra-chain chemical crosslinking. *Sci. Rep.* **6,** 19431
88. Slaaby, R., Andersen, A. S., and Brandt, J. (2008) IGF-I binding to the IGF-I receptor is affected by contaminants in commercial BSA: the contaminants are proteins with IGF-I binding properties. *Growth Horm. IGF Res.* **18,** 267–274

Supplementary Information

for

Probing receptor specificity by sampling the conformational space of the insulin-like growth factor II C-domain

Rozálie Hexnerová[‡§#], Květoslava Křížková[‡§#], Milan Fábry[‡§], Irena Sieglová[‡], Kateřina Kedrová[‡§], Michaela Collinsová[‡], Pavlína Ullrichová[†], Pavel Srb[‡], Christopher Williams[£], Matthew P. Crump[£], Zdeněk Tošner[§], Jiří Jiráček[‡], Václav Veverka[‡*] and Lenka Žáková[‡*]

[‡]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i., Flemingovo nám 2, 166 10 Prague 6, Czech Republic

[§]Faculty of Science, Charles University in Prague, Albertov 6, Prague, 128 43, Czech Republic

[†]Department of Analytical Chemistry, University of Chemistry and Technology, Prague, Technická 5, 166 28 Prague 6, Czech Republic

[$]Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, 142 20 Prague 4, Czech Republic

[£]Department of Organic and Biological Chemistry, School of Chemistry, Cantock's Close, University of Bristol, Bristol BS8 1TS, United Kingdom

[#]Joint first authors

Table of Contents

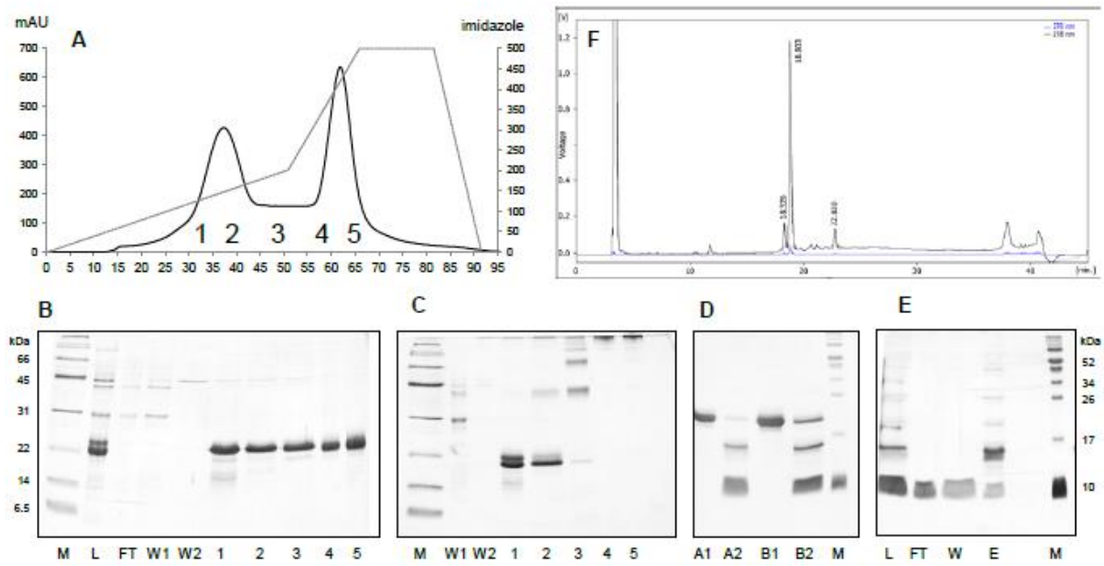


Figure S1. Purification procedure for IGF-II analogs. A. The elution profile from purification of denatured IGF-II in fusion with GB1 protein by IMAC. The material eluted in two major fractions (1-2 and 4-5) at two different imidazole concentrations. SDS-PAGE analysis of collected fractions (1-5) under reducing (**B**) and non-reducing (**C**) conditions revealing the presence of two monomeric isoforms (folded and misfolded) eluting at lower concentration of imidazole (150 mM) and multimeric aggregates eluting at higher imidazole concentration (400 mM). M, molecular weight standard; L, sample load; FT, flow through; W1 and W2, wash; 1-5, eluted fractions. Panel **D** shows reducing SDS-PAGE of the fusion partner cleavage by TEV protease. A1, monomeric fractions before TEV addition; A2, monomeric fractions after 24hrs of TEV digestion; B1, multimeric fraction before TEV addition; B2, multimeric fractions after 24hrs of TEV digestion; M, molecular weight standards. Panel **E** shows reducing SDS-PAGE of cleaved sample after nickel chelating chromatography. The cleaved IGF-II is present in FT and W fraction. L, sample load, FT, flow through; W, wash; E, elution; M, molecular weight standard. Panel **F** shows the final RP-HPLC purification of IGF-II separating forms with differently linked disulfide bonds.

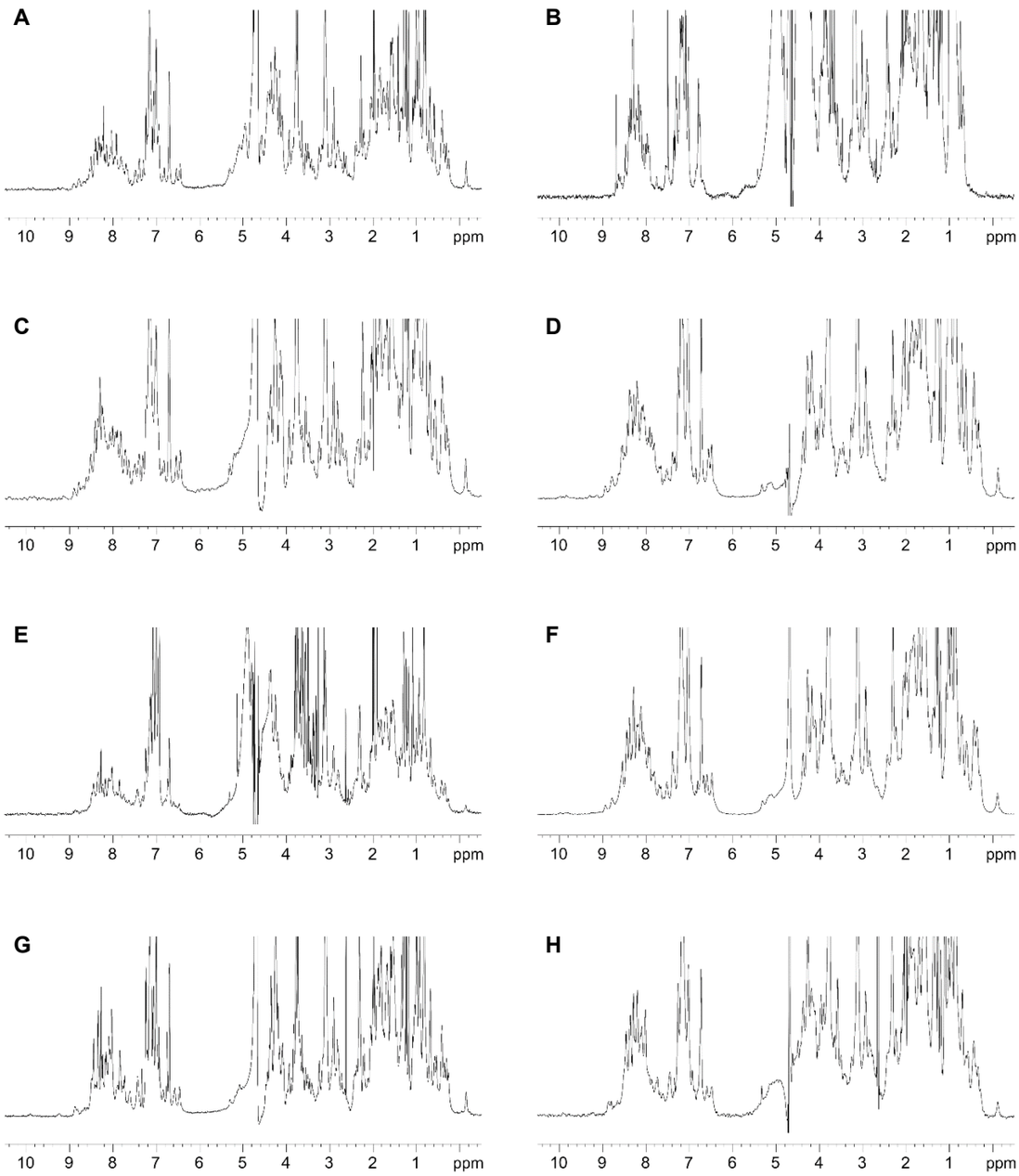


Figure S2. ^{1}H NMR spectra of IGF-II analogues. (**A**) IGF-II, (**B**) misfolded IGF-II, (**C**) [N29]-IGF-II, (**D**) [R34_GS]-IGF-II, (**E**) [S39_PQ]-IGF-II, (**F**) [R34_GS,S39_PQ]-IGF-II, (**G**) [N29, S39_PQ]-IGF-II, (**H**) [N29, R34_GS, S39_PQ]-IGF-II. The difference between correctly folded (A) and misfolded (B) IGF-II spectra was used for verification of correct protein folding of the IGF-II analogs (C-H). In particular, the presence of dispersed aromatic proton signals at 6.5 ppm and upfield shifted methyl signals between 0.5 and -0.2 ppm could be utilized to fingerprint correctly folded IGF-II.

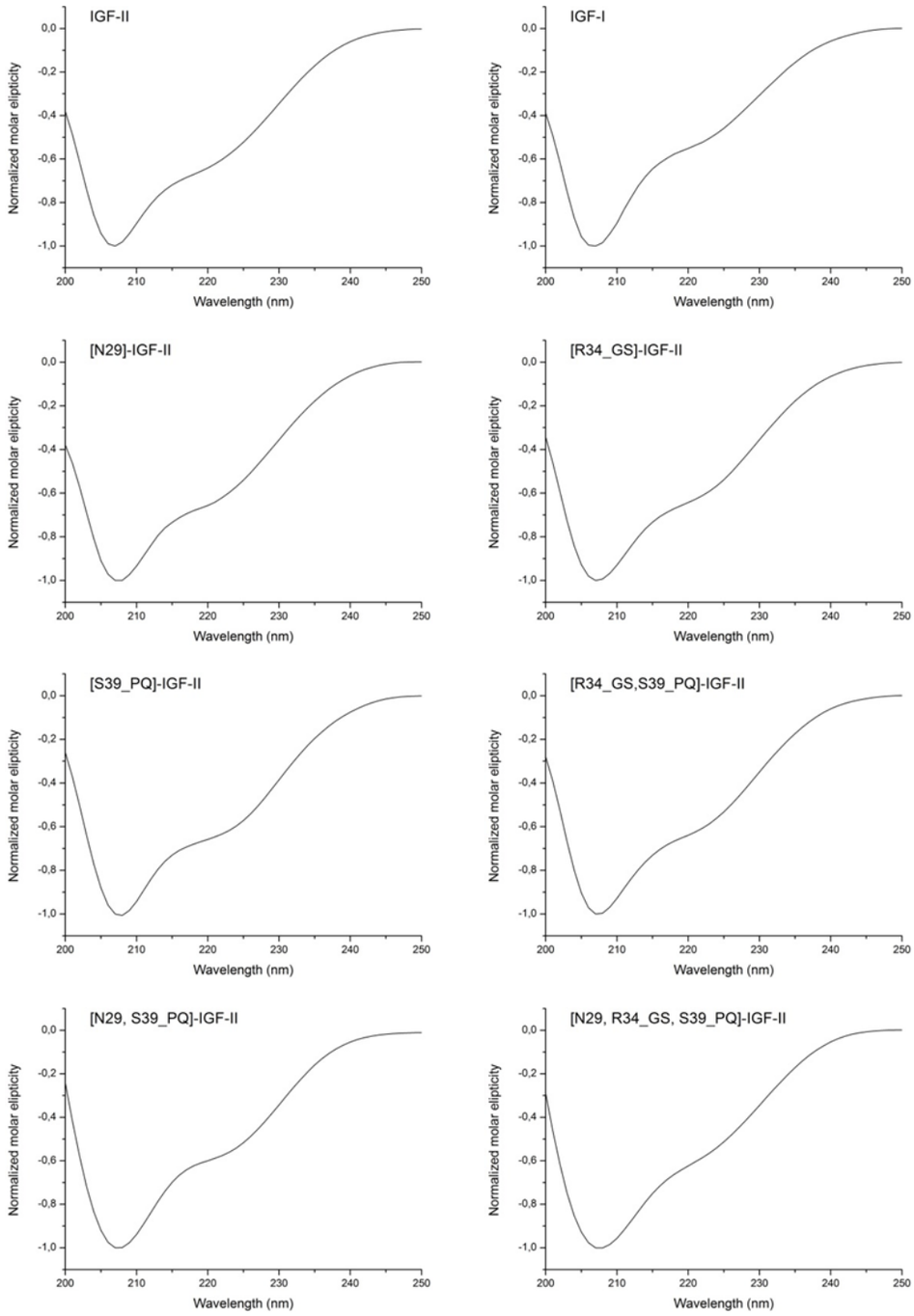


Figure S3. Far UV circular dichroism spectra of IGF-I and studied IGF-II analogs normalized to 207 nm. The curve profiles suggest highly similar presence of the α-helical secondary structure elements in the studied IGF-II analogs.

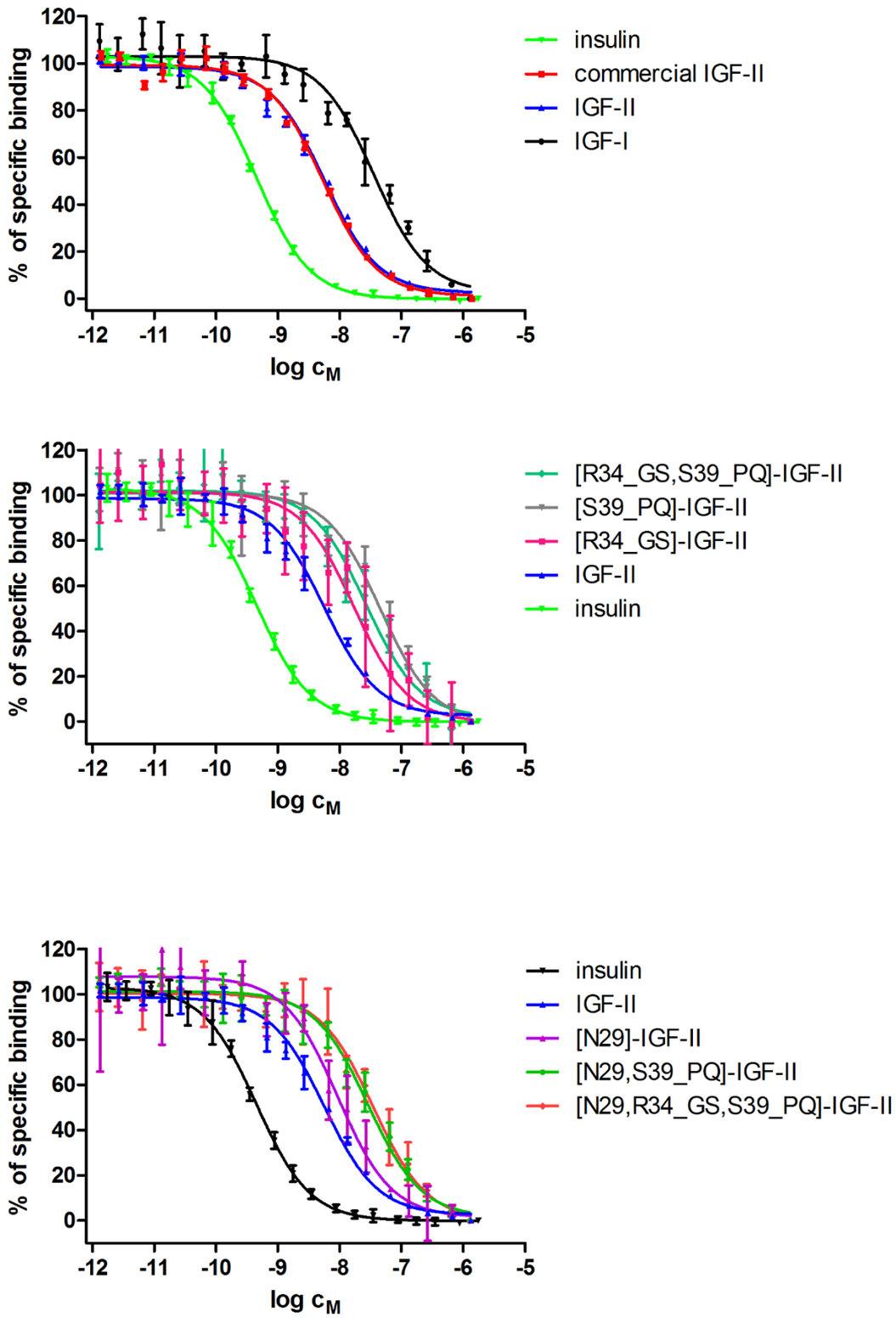
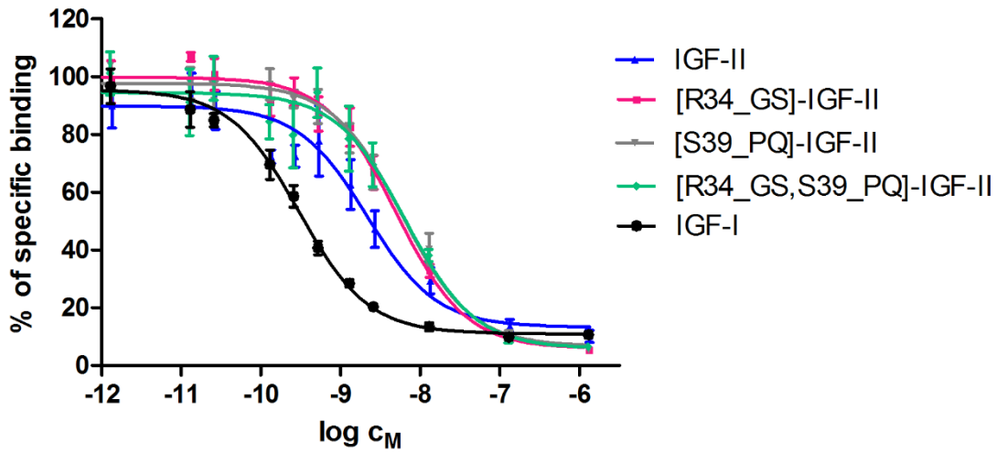
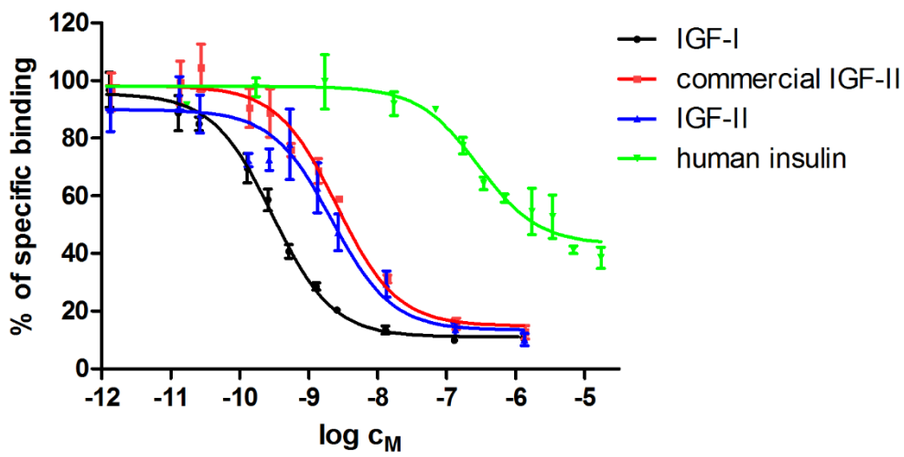


Figure S4. Inhibition of binding of human [^{125}I]-insulin to IR-A in membranes of IM-9 cells by human insulin, IGF-I, IGF-II and IGF-II analogs.





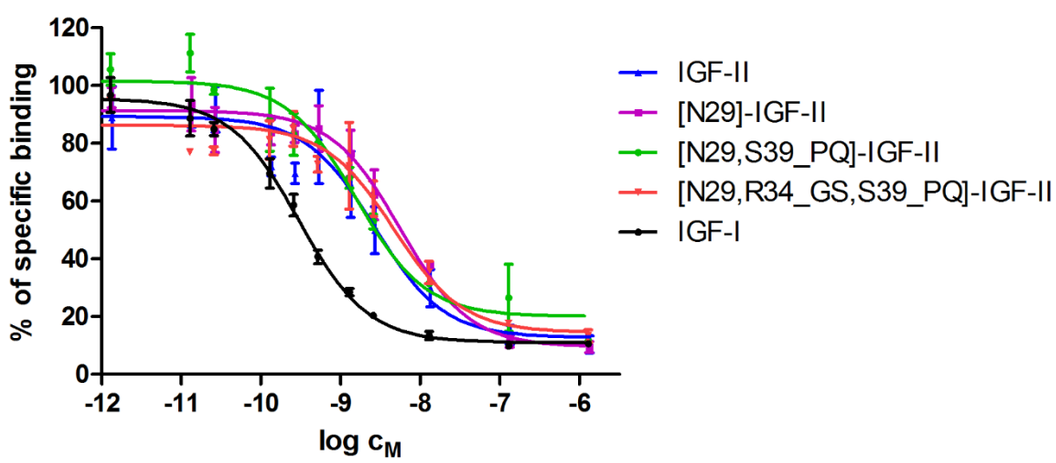


Figure S5. Inhibition of binding of human [^{125}I]-IGF-I to IGF-1R in membranes of mouse fibroblasts by human insulin, IGF-I, IGF-II and IGF-II analogs.

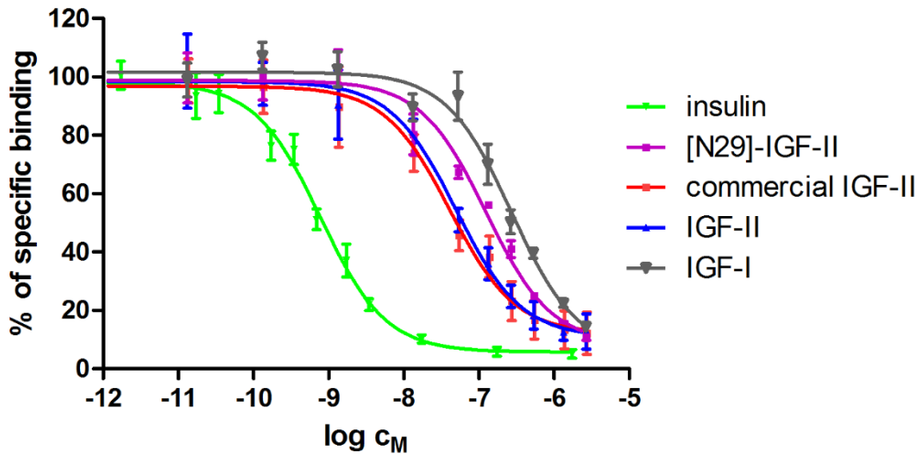


Figure S6. Inhibition of binding of human [^{125}I]-insulin to IR-B in membranes of mouse fibroblasts by human insulin, IGF-I, IGF-II and [N29]-IGF-II analog.

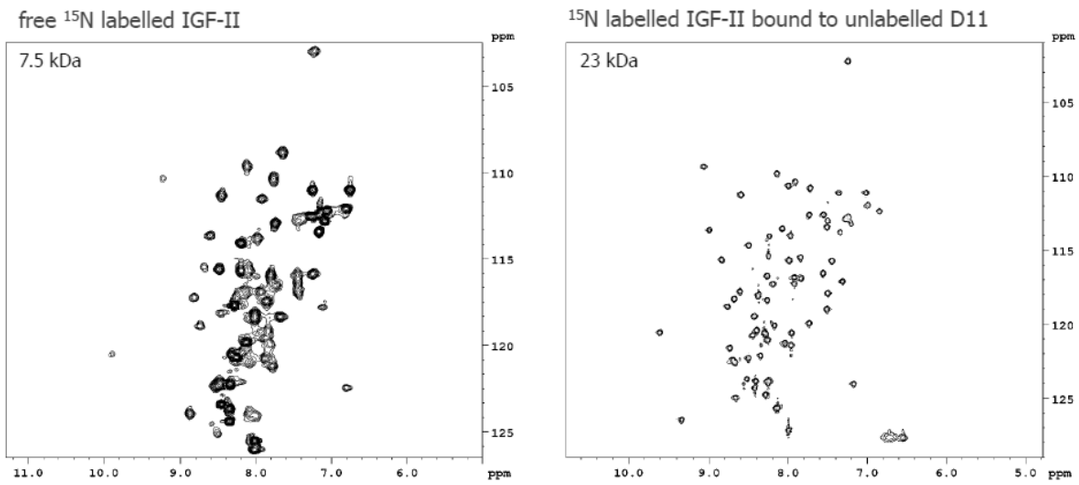


Figure S7. Significant narrowing of IGF-II signals in ^{1}H/^{15}N HSQC spectrum upon binding to IGF-2R Domain 11. A spectrum of free ^{15}N labelled IGF-II is shown on the left panel. Obtained signals do not correspond to the protein mass of 7.5 kDa. The right panel illustrates the signal narrowing observed for IGF-II bound to Domain 11.

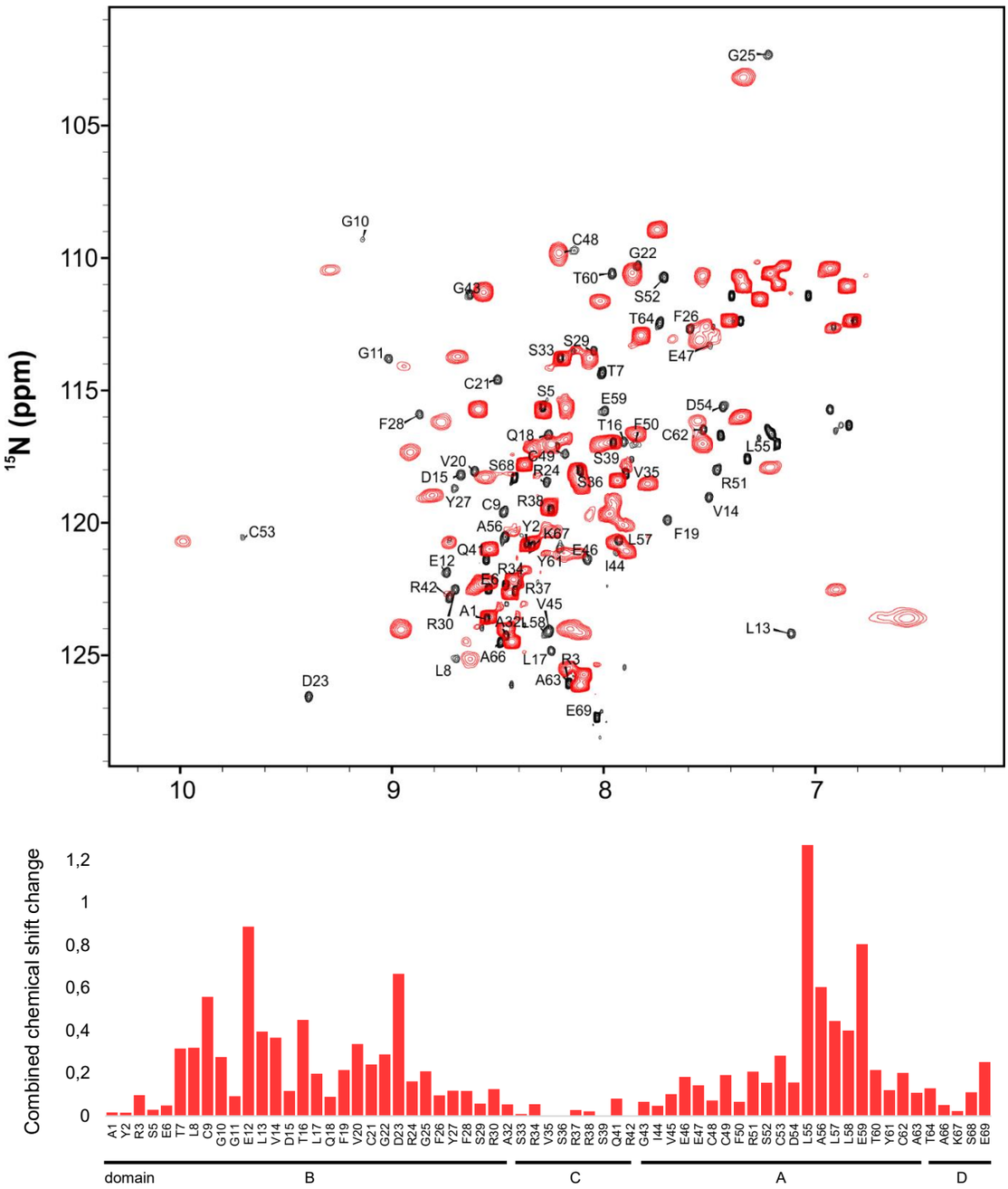


Figure S8. The C-domain of IGF-II is not affected by D11 binding.
(**A**) An overlay of $^{1}H/^{15}N$ HSQC spectra obtained for the free (red) and D11-bound [S39_PQ]-IGF-II (black). (**B**) Values of combined chemical shift changes calculated from the changes of backbone amide signal positions. The major differences upon binding to D11 are distributed across the D11 binding interface, while the signals of the C-domain backbone amides bearing the modifications remain relatively unaffected by the D11 binding.

Table S1. NMR restraints and structural statistics

	IGF-II		*[S39_PQ]-IGF-II*		*[N29, S39_PQ]-IGF-II*	
Non-redundant distance and angle constrains						
Total number of NOE constraints	1039		1116		1395	
Short-range NOEs						
Intra-residue (i = j)	301		315		341	
Sequential (| i - j | = 1)	321		356		406	
Medium-range NOEs (1 < | i - j | < 5)	160		185		281	
Long-range NOEs (| i - j | ≥ 5)	254		257		364	
Torsion angles	46		46		46	
Hydrogen bond restrains	-		-		-	
Total number of restricting constraints	1085		1162		1441	
Total restricting constraints per restrained residue	16.2		16.8		20.9	
Residual constraint violations						
Distance violations per structure						
0.1 – 0.2 Å	5.05		5.85		9	
0.2 – 0.5 Å	2.15		2.3		2.6	
> 0.5 Å	0		0		0	
r.m.s. of distance violation per constraint	0.02 Å		0.02 Å		0.02 Å	
Maximum distance violation	0.45 Å		0.48 Å		0.48 Å	
Dihedral angle violations per structure						
1 – 10 °	1.3		1.2		1.7	
> 10 °	0		0		0	
r.m.s. of dihedral violations per constraint	0.68 °		0.71 °		0.75 °	
Maximum dihedral angle violation	5.00 °		5.00 °		5.00 °	
Ramachandran plot summary from Procheck						
Most favoured regions	94.8%		92.2%		85.9%	
Additionally allowed regions	5.2%		7.8%		13.8%	
Generously allowed regions	0.0%		0.0%		0.1%	
Disallowed regions	0.0%		0.0%		0.1%	
r.m.s.d. to the mean structure	*ordered*[1]	*all*	*ordered*[1]	*all*	*ordered*[1]	*all*
All backbone atoms	0.4 Å	2.9 Å	1.1 Å	2.2 Å	1.0 Å	1.9 Å
All heavy atoms	1.0 Å	3.6 Å	1.7 Å	2.9 Å	1.4 Å	2.5 Å

[1] Residues with sum of phi and psi order parameters > 1.8

Appendix III

Biochemistry

Article

Cite This: Biochemistry 2018, 57, 2373−2382

pubs.acs.org/biochemistry

Converting Insulin-like Growth Factors 1 and 2 into High-Affinity Ligands for Insulin Receptor Isoform A by the Introduction of an Evolutionarily Divergent Mutation

Kateřina Macháčková,[†] Martina Chrudinová,[†] Jelena Radosavljević,[†,∥] Pavlo Potalitsyn,[†] Květoslava Křížková,[†] Milan Fábry,[‡] Irena Selicharová,[†] Michaela Collinsová,[†] Andrzej M. Brzozowski,[§] Lenka Žáková,*[,†] and Jiří Jiráček*[,†]

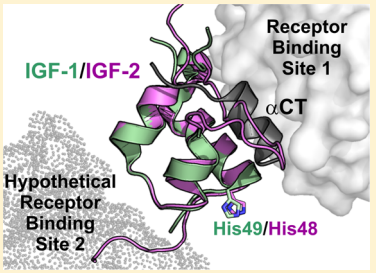
[†]Institute of Organic Chemistry and Biochemistry, The Czech Academy of Sciences, Flemingovo nám 2, 166 10 Prague 6, Czech Republic

[‡]Institute of Molecular Genetics, The Czech Academy of Sciences, Flemingovo n. 2, 166 37 Prague 6, Czech Republic

[§]York Structural Biology Laboratory, Department of Chemistry, The University of York, Heslington, York YO10 5DD, United Kingdom

Supporting Information

ABSTRACT: Insulin-like growth factors 1 and 2 (IGF-1 and -2, respectively) are protein hormones involved not only in normal growth and development but also in life span regulation and cancer. They exert their functions mainly through the IGF-1R or by binding to isoform A of the insulin receptor (IR-A). The development of IGF-1 and IGF-2 antagonists is of great clinical interest. Mutations of A4 and A8 sites of human insulin lead to disproportionate effects on hormone IR binding and activation. Here, we systematically modified IGF-1 sites 45, 46, and 49 and IGF-2 sites 45 and 48, which correspond, or are close, to insulin sites A4 and A8. The IGF-1R and IR-A binding and autophosphorylation potencies of these analogues were characterized. They retained the main IGF-1R-related properties, but the hormones with His49 in IGF-1 and His48 in IGF-2 showed significantly higher affinities for IR-A and for IR-B, being the strongest IGF-1- and IGF-2-like binders of these receptors ever reported. All analogues activated IR-A and IGF-1R without major discrepancies in their binding affinities. This study revealed that IR-A and IGF-1R contain specific sites, likely parts of their so-called sites 2′, which can interact differently with specifically modified IGF analogues. Moreover, a clear importance of IGF-2 site 44 for effective hormone folding was also observed. These findings may facilitate novel and rational engineering of new hormone analogues for IR-A and IGF-1R studies and for potential medical applications.



Two insulin-like growth factors, IGF-1 and IGF-2, together with insulin, are members of a family of small protein hormones that share common evolutionary origins,[1−4] having similar primary (Figure 1) and three-dimensional structures.[5] They regulate a wide spectrum of key physiological events, with insulin being responsible mainly for broad, metabolic control,[6] while IGF-1 and IGF-2 are growth factors involved primarily in the development and growth of mammals.[5] The role of IGF-1 is relatively well studied,[7−9] but physiological functions of IGF-2 are much less understood,[10] despite emerging evidence of its impact on the central nervous system.[11,12]

IGF-1, IGF-2, and insulin exert their activities by binding to different but highly homologous (∼75%), ∼450 kDa $(\alpha\beta)_2$ dimeric tyrosine-kinase receptors: IGF-1 receptor (IGF-1R) and insulin receptor (IR).[13−15] IR exists in two isoforms, IR-A, and IR-B, with distinct biochemical properties[16,17] and a specific tissue distribution. IR-B is a predominant IR form in liver, while muscle and adipose tissues contain both isoforms at different ratios. IR-A is predominant in brain, fetus, and lymphatic tissues and is considered mainly as a "mitogenic" form of the IR, in contrast to IR-B that is considered as the main "metabolic" receptor for insulin.[16−18] A high degree of homology of these receptors results in a significant cross-binding of insulin and both IGFs to IR-A and IGF-1R,[19] and hence some overlapping biological responses to binding of these ligands.[20,21]

The binding of insulin and IGFs to these receptors triggers two major signaling pathways that are initiated by the autophosphorylation of tyrosines within their intracellular tyrosine kinase domains.[22] The phosphoinositide 3-kinase (PI3K)/Akt pathway leads to the metabolic, glycemic responses and effects of the hormone:receptor complex, but it is also important for growth and protein synthesis.[23] The Ras/ERK main pathway involves activation of the Ras/Raf/MAPK/ERK1/2 cascade, which mediates proliferative effects through gene transcription regulation.[21] Whereas insulin signals mainly via both IR isoforms,[24] IGF-1 and IGF-2 promote mitogenic

Received: December 15, 2017
Revised: March 29, 2018
Published: April 2, 2018

DOI: 10.1021/acs.biochem.7b01260
Biochemistry 2018, 57, 2373−2382

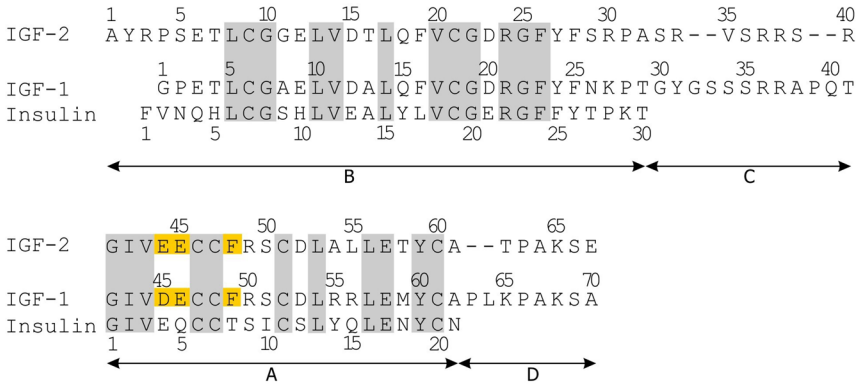


Figure 1. Comparison of the primary sequences of human IGF-1, IGF-2, and insulin. The organization of IGF-1 and IGF-2 into B, C, A, and D domains is shown below the sequences. Insulin A and B chains correspond to IGF A and B domains, respectively. The homologous regions are highlighted in gray, and the residues mutated in this study are highlighted in yellow.

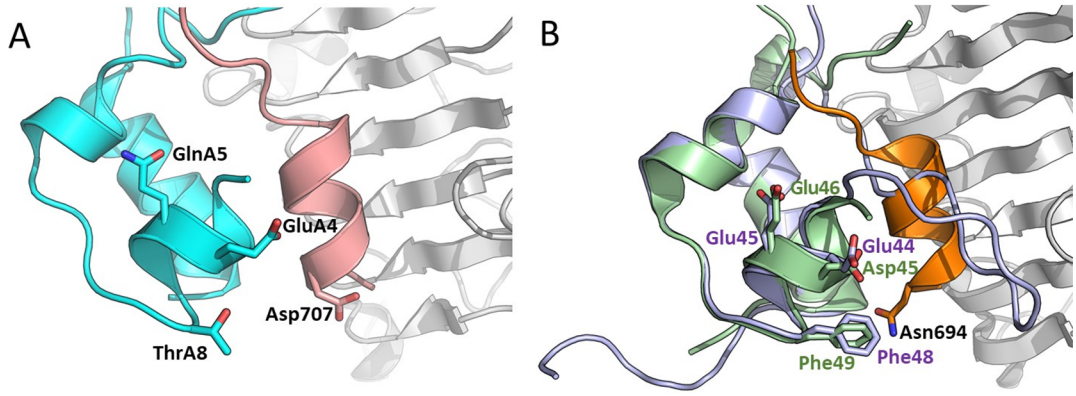


Figure 2. Receptor-bound structures of human insulin and human IGF-1 overlaid with human IGF-2. (A) Complex (4OGA)[38] of human insulin sitting on the IR-L1 domain and IR α-CT peptide. Insulin is colored cyan, the L1 domain light gray, and α-CT pink. Side chains of insulin residues GluA4, GlnA5, ThrA8, and α-CT Asp707 are shown and labeled. (B) Complex (4XSS)[39] of human IGF-1 (violet) bound to the IR-L1 domain (light gray) and the IGF-1R α-CT peptide (orange). Side chains of IGF-1 residues Asp45, Glu46, and Phe49 mutated in this study and α-CT Asn694 are shown and labeled. The complex is overlaid with the NMR structure of human IGF-2 (5L3L),[40] which is colored light green. Mutated IGF-2 residues Glu44, Glu45, and Phe48 and α-CT Asp694 are also shown and labeled.

signaling through IGF-1R, and, importantly, similar mitogenic stimulation may result from binding of IGF-2 to IR-A.[25] The complexity of insulin/IGF signaling is amplified further by the heterodimerization of IGF-1R and IR-A, and the presence of hybrid receptors that can be effectively activated by IGF-1, but not by insulin.[26,27] Moreover, the bioavailability of free IGF-1 and IGF-2 for receptor signaling is modulated by a family of high-affinity IGF binding proteins 1−6 (IGFBP 1−6, respectively),[28,29] and the circulation level of IGF-2 is also affected by a structurally distinct, and presumably nonsignaling, insulin-like growth factor type 2 receptor (IGF-2R), also known as the mannose 6-phosphate receptor.[30] The equilibrium of all individual components and the appropriate function of the entire insulin/IGF system are essential for a proper functioning of the organism.[31]

In recent years, the role of the IGF/insulin system in cancer development and growth has been widely studied.[10,31,32] Substantial efforts have been focused on the development of new anti-IGF-1R-directed therapies, mostly tyrosine-kinase (TK) inhibitors and antireceptor antibodies.[33] However, the results of clinical trials were not satisfactory,[34] because of either the toxicity of the TK-targeting drugs or an increasing overlap and takeover of IGF-1R signaling pathways by the IR. The lack of progress in addressing one of the key hallmarks of cancer underlines the need for new anticancer therapies that would exploit alternative, and specific, targets of the insulin/IGF axis. Here, a high-affinity/no-efficacy IGF-based IGF-1 analogue, i.e., selective antagonist of the IGF-1R, should represent a promising new strategy for combating IGF-1R-related malignancies.

To date, no IGF-like peptide antagonists of the IGF-1R have been identified. However, peptides with good IR/IGF-1R binding and antagonistic properties toward these receptors were discovered by a phage-display technique.[35,36] Whittaker et al.[37] showed that a combination of GluA4His and ThrA8His mutations of human insulin results in insulin analogues with native IR binding affinity but poor efficacy, an impaired ability to stimulate autophosphorylation of IR, and downstream Akt activation. They also proposed that surfaces involving insulin GluA4 and IR Asp707 could be behind the mechanism of receptor activation.

Interestingly, insulin acidic GluA4 is preserved by its equivalent Asp45 and Glu44 in IGF-1 and IGF-2, respectively (Figure 1). However, insulin-neutral Gln A5 is replaced by Glu46, and Glu45 in corresponding sites of IGF-1 and IGF-2. In addition, Asp707 of IR α-CT is replaced by a neutral Asn694 in the IGF-1R α-CT segment (Figure 2).

These correlations and trends between positions A4 and A5 in insulin and their IGFs equivalent 45 and 46 (in IGF-1) and 44 and 55 (in IGF-2) sites prompted us to study (i) the impact

DOI: 10.1021/acs.biochem.7b01260
Biochemistry 2018, 57, 2373−2382

of the mutations of these residues on receptor affinities and potencies of these hormones, and (ii) whether such mutations can generate significant IGF-1R-specific antagonists, with potential anticancer clinical applications. A series of 14 IGF-1 and IGF-2 analogues mutated at these sites have been designed and made, and their binding to IGF-1R and IR-A and abilities to activate the receptors were characterized. Some of these mutations were also combined with the Phe49His mutation in IGF-1 and the Phe48His mutation in IGF-2, as it has been shown that insulin-corresponding mutation ThrA8His significantly increased the IR-A binding potency of this analogue.[37,41−46]

Despite the results for the analogues studied here not showing any antagonism, they revealed interesting properties of new IGF-1 and IGF-2 mutants that can interact differently with receptors for insulin and IGF-1. This could indicate new directions for a rational engineering of these hormones.

MATERIALS AND METHODS

Cloning and Production of IGF-1 and IGF-2 Analogues. As in our previously published research,[40] both human IGF-1 (UniprotKB entry P05019 amino acids 49−118) and human IGF-2 (UniprotKB entry P01344 amino acids 25−91) have been cloned into a modified pRSFDuet-1 expression vector (kindly provided by E. Bouřa from the Institute of Organic Chemistry and Biochemistry in Prague) as a fusion with an N-terminally His6-tagged GB1 protein and TEV protease cleavage site. An additional N-terminal glycine residue (Gly−1) was incorporated into IGF-1 to enable cleavage by TEV protease (Glu-Asn-Leu-Tyr-Phe-Gln↓Gly). In contrast to our previous study,[40] the TEV protease cleavage site for the IGF-2 expression construct was modified to Glu-Asn-Leu-Tyr-Phe-Gln↓Ala, cleavage (↓) of which resulted in a native hormone with an N-terminal alanine. [D45H], [D45N], [D45A], [E46H], [E46Q], and [E46A] mutations in IGF-1 analogues were obtained by the standard site-directed mutagenesis protocol (SDM)[47] using appropriate mutagenic primers (listed in Table S1). After sequence verification, the mutant fragments were reintroduced into the expression vector. Additional [D45N+E46Q], [F49H], [E46H+F49H], [E46Q+F49H], and [D45N+E46Q+F49H] mutations in IGF-1 analogues and in all cloned IGF-2 analogues were introduced either by the overlap-extension polymerase chain reaction (OE strategy in Table S1),[48] using specific primers as flanking master primers and subsequent recloning into expression vector, or by the standard site-directed mutagenesis as mentioned above (SDM strategy in Table S1), using appropriate mutagenic primers.

We succeeded in expressing and purifying all planned IGF-1 analogues. However, only three IGF-2 analogues (with mutations [E45Q], [F48H], and [E45Q+F48H]) were successfully produced. A list of successfully expressed constructs along with primers used for mutagenesis is provided as Table S1.

All successfully constructed analogues were produced, purified, and characterized, using the procedures described by Hexnerova et al.[40] The purity of all tested analogues was >95% (and controlled by reverse phase high-performance liquid chromatography analyses and high-resolution mass spectrometry spectra).

Biological Characterization of IGF-1 and IGF-2 Analogues. Binding affinities for the receptors were determined with receptors in the intact cells. Specifically, binding affinities for IGF-1R were determined with mouse fibroblasts transfected with human IGF-1R and with deleted mouse IGF-1R according to Hexnerova et al.[40] Binding affinities for IR-A were determined with human IR-A in human IM-9 lymphocytes according to Vikova et al.[49] Binding affinities for IR-B were determined with mouse fibroblasts transfected with human IR-B and with deleted mouse IGF-1R according to Zakova et al.[50] Representative binding curves of analogues with the receptors are shown in Figure S1 (IGF-1R), Figure S2 (IR-A), and Figure S3 (IR-B). The binding curve of each analogue was determined in duplicate, and the final dissociation constant (K_d) was calculated from at least three ($n \geq 3$) binding curves (K_d values), determined independently, and compared to binding curves for IGF-1 or for IGF-2, depending on the type of analogue.

The abilities of analogues to induce autophosphorylation of IGF-1R in membranes of mouse fibroblasts transfected with human IGF-1R and with deleted mouse IGF-1R were determined, as described by Machackova et al.[51] The abilities of analogues to induce autophosphorylation of IR-A in mouse fibroblasts transfected with human IR-A and with deleted mouse IGF-1R were determined, as described by Krizkova et al.[52] Briefly, the cells were stimulated in 24-well plates (Schoeller) (4×10^4 cells per well) after being starved for 4 h in serum-free medium. The cells were stimulated with 10 nM ligand (insulin, IGF-1, IGF-2, or analogues) for 10 min. Stimulation was stopped by snap-freezing. Proteins were routinely analyzed, using immunoblotting and horseradish peroxidase-labeled secondary antibodies (Sigma-Aldrich). The membranes were probed with anti-phospho-IGF-1Rβ (Tyr1135/1136)/IRβ (Tyr1150/1151) (Cell Signaling Technology). The blots were developed using the SuperSignal West Femto maximum sensitivity substrate (Pierce) and analyzed using the ChemiDoc MP Imaging System (Bio-Rad). The autophosphorylation signal density generated by each ligand on a Western blot was expressed as the contribution of phosphorylation relative to the IGF-1 (IGF-1R fibroblasts) respective human insulin (IR-A fibroblasts) signal in the same experiment. Mean ± standard deviation (SD) values were calculated from four independent experiments ($n = 4$) and compared to those of native IGF-1 or native IGF-2, depending on the type of analogue. A representative example of an immunoblot used for the evaluation of the abilities of analogues to induce autophosphorylation of receptors is shown in Figure S4.

The dose−response curves for human IGF-1 and [His45]-IGF-1, [Asn45]-IGF-1, [Ala45]-IGF-1, [His46]-IGF-1, and [Gln46]-IGF-1 analogues were also measured to determine their EC_{50} values and their abilities to stimulate the autophosphorylation of IGF-1R; here, the same methodology as for the measurements at a single dose (above) was followed. Log(agonist) versus response (variable slope) curve fitting of data was performed with GraphPad Prism 5. The representative curves are shown in the Figure S5. The EC_{50} values (calculated from at least three independent curves) are shown in the Table S2.

The significance of the changes in binding affinities and in the abilities of analogues to stimulate autophosphorylation was calculated using the two-tailed t test.

RESULTS

Production of IGF-1 and IGF-2 Analogues. The production of IGF-1 and IGF-2 was achieved by their

DOI: 10.1021/acs.biochem.7b01260
Biochemistry 2018, 57, 2373−2382

recombinant expression in *Escherichia coli* as a fusion with an N-terminal and cleavable His6-tagged GB1 protein (immunoglobulin binding domain B1 of streptococcal protein-G), followed by the cleavage of the fusion protein with TEV protease. Recently, we used this strategy for the synthesis of IGF-2 analogues modified in the hormone's C domain and possessing an extra glycine residue at their N-terminus (position −1).[40] Here, we modified the TEV cleavage site (see Materials and Methods) and succeeded in producing native IGF-2 without the additional Gly−1.

However, this strategy was not successful for IGF-1 because of the proline residue at position 2 in IGF-1 (Figure 1), which hampered TEV protease-mediated cleavage. Therefore, all analogues of the hormone produced in this work have an extra glycine residue (Gly−1) at the protein N-terminus that enabled TEV protease cleavage of the precursor. The presence of Gly−1 did not have any significant effect on the binding properties of the IGF-1 derivative for either tested receptor [IGF-1R or IR-A (Table 1 or 2, respectively)], and both native IGF-1 and Gly−1-IGF-1 can be considered as equipotent.

Design of the First Series of Analogues. The first series of analogues was designed with the substitution of IGF-1 Asp45 with "insulin-inspired" His, Asn, and Ala, and a similar strategy was applied for the replacement of IGF-1 Glu46 with His, Gln, and Ala. The Asn45 and Gln46 mutations were also combined. All planned IGF-1 analogues (Tables 1 and 2) were successfully produced in quantities sufficient for their biological and physicochemical characterization.

In parallel, similar mutations were designed for IGF-2 at positions Glu44 and Glu45 that correspond to IGF-1 positions 45 and 46 (Figure 1). However, only one IGF-2 analogue, [Gln45]-IGF-2, was made with a significant yield that allowed its characterization. All other IGF-2 analogues formed insoluble precipitates after the TEV cleavage/folding steps.

IGF-1R Binding and Activation Properties of IGF-1 Analogues Modified at Positions 45 and 46, and the [Gln45]-IGF-2 Analogue. The analogues were tested for their binding to the IGF-1 receptor (IGF-1R) (Table 1), and their binding data were compared with the abilities of the analogues to induce autophosphorylation of the IGF-1R at a concentration of 10 nM (Figure 3A). For human IGF-1 and [His45]-IGF-1, [Asn45]-IGF-1, [Ala45]-IGF-1, [His46]-IGF-1 and [Gln46]-IGF-1 analogues, we also determined EC_{50} values of their abilities to stimulate autophosphorylation of IGF-1R (Figure S5).

In general, the mutations did not dramatically alter the binding characteristics of the analogues in comparison with those of native IGFs. However, it can be noted that analogues with mutations at site 45, [His45]-IGF-1, [Asn45]-IGF-1, and [Ala45]-IGF-1, have significantly reduced (29−60%) binding affinities for IGF-1R, with the lowest values being that of [Ala45]-IGF-1.

The only successfully prepared IGF-2 analogue in this series, [Gln45]-IGF-2, had reduced binding potency for IGF-1R compared to that of native IGF-2 (Table 1) but activated IGF-1R like native IGF-2 did (Figure 3A).

Relative EC_{50} values of IGF-1R stimulation by the selected analogues were in good general agreement with their relative ability to stimulate this receptor performed at a set ligand concentration of 10 nM (Table S2). Hence, it appeared that the autophosphorylation abilities of hormones determined at their 10 nM concentrations were good representations of their properties, and as this approach substantially improved the time and material economy of this extensive methodology, the receptor activation abilities of the rest of the analogues were measured at this set ligand concentration only. In general, no major discrepancies between the IGF-1R binding and activation properties of the analogues mutated at positions 45 and 46 of IGF-1 and [Gln45]-IGF-2 were observed (Table 1 and Figure 3A). Some analogues, e.g., [His46]-IGF-1 and [Gln46]-IGF-1, activated IGF-1R slightly less strongly than human IGF-1 did, but their apparent higher binding affinities for this receptor were not statistically significant. In contrast, [His45]-IGF-1, [Asn45]-IGF-1, and [Ala45]-IGF-1 analogues activated IGF-1R like human IGF-1 did, but their binding affinities were significantly reduced. Therefore, although some minor discrepancies could be observed here, any clear and major antagonism, or receptor overstimulation, was not detected.

IR-A Binding and Activation Properties of IGF-1 Analogues Modified at Sites 45 and 46, and the

Table 1. IGF-1R Binding Affinities of Native Hormones and Analogues Reported in This Work[a]

analogue	K_d ± SD (nM) (n) for the human IGF-1R in mouse fibroblasts	relative binding affinity for human IGF-1R (%) relative to that of human IGF-1
human IGF-1	0.24 ± 0.05[b] (5)	100 ± 21
	0.12 ± 0.01[c] (5)	100 ± 8
	0.34 ± 0.12[d] (4)	100 ± 35
	0.16 ± 0.06[e] (3)	100 ± 37
	0.25 ± 0.03[f] (4)	100 ± 12
first series of IGF-1 analogues		
Gly−1-IGF-1	0.25 ± 0.02[b] (3)	96 ± 8
[His45]-IGF-1	0.39 ± 0.11[c] (4)***	31 ± 9
[Asn45]-IGF-1	0.20 ± 0.07[c] (3)*	60 ± 21
[Ala45]-IGF-1	0.41 ± 0.27[c] (4)*	29 ± 19
[His46]-IGF-1	0.18 ± 0.04[b] (4)	133 ± 29
[Gln46]-IGF-1	0.18 ± 0.01[b] (3)	133 ± 7
[Ala46]-IGF-1	0.26 ± 0.11[b] (3)	92 ± 39
[Asn45,Gln46]-IGF-1	0.70 ± 0.28[d] (4)	49 ± 20
second series of IGF-1 analogues		
[His49]-IGF-1	0.50 ± 0.23[d] (4)	68 ± 31
[His46,His49]-IGF-1	0.29 ± 0.17[e] (4)	55 ± 32
[Gln46,His49]-IGF-1	0.30 ± 0.09[e] (3)	53 ± 16
[Asn45,Gln46,His49]-IGF-1	0.92 ± 0.04[d] (3)***	37 ± 2
human IGF-2	2.3 ± 1.2[f] (3)*	10.9 ± 5.7
first series of IGF-2 analogues		
[Gln45]-IGF-2	2.9 ± 0.3[e] (3)*	5.5 ± 0.6
second series of IGF-2 analogues		
[His48]-IGF-2	0.88 ± 0.23[e] (3)	18.2 ± 4.7
[Gln45,His48]-IGF-2	0.89 ± 0.05[e] (3)	18.0 ± 1.0

[a]The values of K_d and relative binding affinities [relative receptor binding affinity defined as (K_d of human IGF-1)/(K_d of analogue) × 100] of human IGF-1, IGF-2, and analogues were determined for human IGF-1R in mouse fibroblasts. All IGF-1 analogues have an extra glycine residue at the N-terminus (Gly−1). n is the number of replicates. Asterisks indicate that binding of a particular ligand to IGF-1R differs significantly (*$p < 0.05$; ***$p < 0.001$) from the effect of IGF-1 in the case of IGF-1 analogues or differs significantly from the effect of IGF-2 in the case of IGF-2 analogues. Binding of native IGF-2 is related to that of human IGF-1. [b]Relative to the human IGF-1 K_d value of 0.24 ± 0.05 (n = 5). [c]Relative to the human IGF-1 K_d value of 0.12 ± 0.01 (n = 5). [d]Relative to the human IGF-1 K_d value of 0.34 ± 0.12 (n = 4). [e]Relative to the human IGF-1 K_d value of 0.16 ± 0.06 (n = 3). [f]Relative to the human IGF-1 K_d value of 0.25 ± 0.03 (n = 4).

DOI: 10.1021/acs.biochem.7b01260
Biochemistry 2018, 57, 2373−2382

Table 2. IR-A Receptor Binding Affinities of Native Hormones and Analogues Reported in This Work[a]

analogue	K_d ± SE (nM) (n) for human IR-A in IM-9 lymphocytes	relative binding affinity for human IR-A (%) relative to that of human insulin
human insulin	0.25 ± 0.05[b] (5)	100 ± 20
	0.27 ± 0.02[c] (5)	100 ± 7
	0.18 ± 0.01[d] (4)	100 ± 6
	0.32 ± 0.09[e] (4)	100 ± 28
	0.30 ± 0.13[f] (5)	100 ± 43
human IGF-1	23.7 ± 11.5[b] (3)***	1.1 ± 0.5
first series of IGF-1 analogues		
Gly−1-IGF-1	35.6 ± 11.9[b] (3)	0.7 ± 0.2
[His45]-IGF-1	20.1 ± 7.8[c] (4)	1.3 ± 0.5
[Asn45]-IGF-1	19.3 ± 9.6[c] (4)	1.4 ± 0.7
[Ala45]-IGF-1	17.6 ± 9.7[c] (3)	1.5 ± 0.8
[His46]-IGF-1	6.6 ± 1.2[d] (3)	2.7 ± 0.5
[Gln46]-IGF-1	18.1 ± 3.6[d] (3)	1.0 ± 0.2
[Ala46]-IGF-1	14.0 ± 1.9[d] (3)	1.3 ± 0.2
[Asn45,Gln46]-IGF-1	17.5 ± 8.4[e] (3)	1.8 ± 0.9
second series of IGF-1 analogues		
[His49]-IGF-1	6.7 ± 2.4[e] (3)*	4.8 ± 1.7
[His46,His49]-IGF-1	3.4 ± 1.7[e] (3)*	9.4 ± 4.7
[Gln46,His49]-IGF-1	7.5 ± 4.1[e] (3)*	4.3 ± 2.3
[Asn45,Gln46,His49]-IGF-1	5.5 ± 2.5[f] (4)***	5.5 ± 2.5
human IGF-2	2.9 ± 0.2[b] (3)***	8.6 ± 0.6
first series of IGF-2 analogues		
[Gln45]-IGF-2	1.6 ± 0.3[e] (3)**	20 ± 3.8
second series of IGF-2 analogues		
[His48]-IGF-2	0.54 ± 0.13[e] (3)***	59.3 ± 14.3
[Gln45,His48]-IGF-2	0.65 ± 0.12[f] (3)***	46.2 ± 8.5

[a]The values of K_d and relative binding affinities [relative receptor binding affinity defined as (K_d of human insulin)/(K_d of analogue) × 100] of human insulin, IGF-1, IGF-2, and analogues were determined for human IR-A in human IM-9 lymphocytes. All IGF-1 analogues have an extra glycine residue at the N-terminus (Gly−1). n is the number of replicates. Asterisks indicate that binding of a particular ligand to IR-A differs significantly (*p < 0.05; **p < 0.01; ***p < 0.001) from the effect of IGF-1 in the case of IGF-1 analogues or differs significantly from the effect of IGF-2 in the case of IGF-2 analogues. Binding of native IGF-2 is related to that of human insulin. [b]Relative to the human insulin K_d value of 0.25 ± 0.05 (n = 5). [c]Relative to the human insulin K_d value of 0.27 ± 0.02 (n = 5). [d]Relative to the human insulin K_d value of 0.18 ± 0.01 (n = 4). [e]Relative to the human insulin K_d value of 0.32 ± 0.09 (n = 4). [f]Relative to the human insulin K_d value of 0.30 ± 0.13 (n = 5).

[Gln45]-IGF-2 Analogue. The IGF-1 analogues of the first series have binding affinities for IR-A similar to that of native IGF-1 (Table 2), and their IR-A activation properties (Figure 3B) are again in general agreement with the properties of native IGF-1.

The [Gln45]-IGF-2 analogue binds IR-A significantly more strongly (≤20% of the binding affinity of native human insulin) than the native IGF-2 that has only 8% of the binding affinity of human insulin. However, the IR-A enhanced affinity of this analogue was not fully translated into its activation potency that is similar to the activation potency of native IGF-2.

Design of the Second Series of IGF-1 and IGF-2 Analogues. The ThrA8His substitution in insulin increases the potency for IR-A;[41−46] hence, it was also used by Whittaker et al.[37] to increase the level of IR binding of A4/A5-modified insulins, without eliminating their antagonistic properties. Therefore, we probed a similar strategy for the IGF-1 and

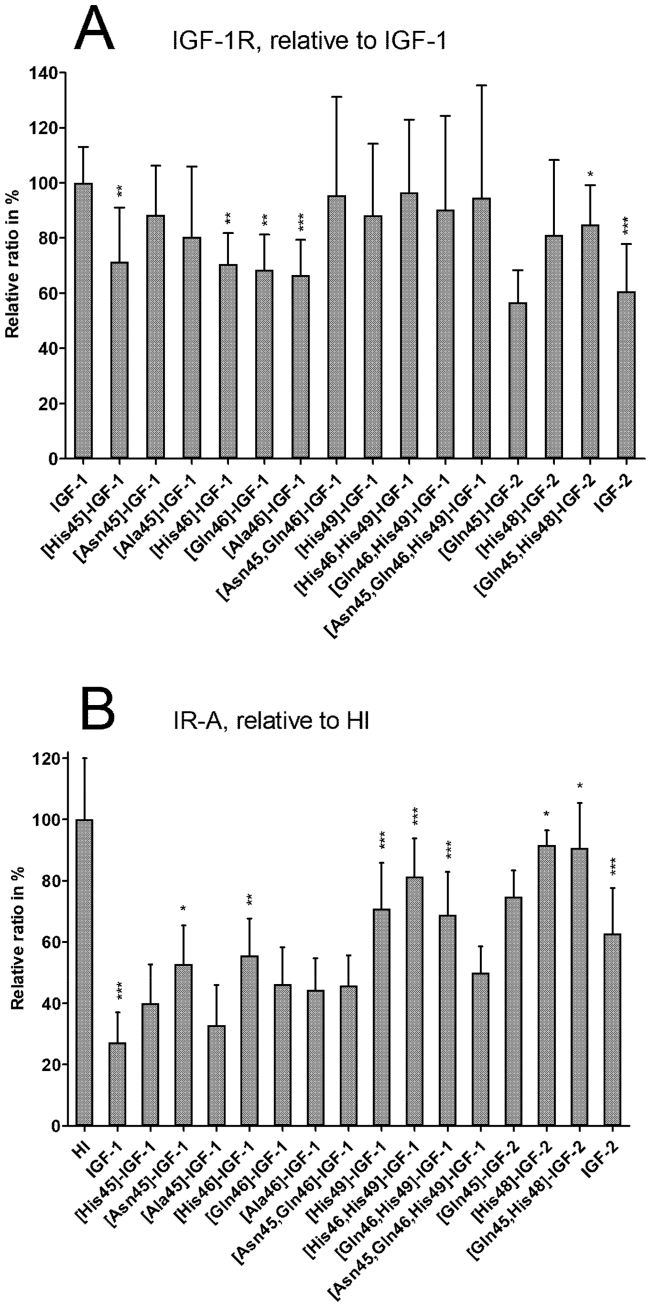


Figure 3. Relative abilities to activate (A) IGF-1R and (B) IR-A of human insulin (HI), human IGF-1, human IGF-2, and IGF-1 and IGF-2 analogues. All IGF-1 analogues contain a glycine residue at position −1. Relative abilities to activate receptors were determined with 10 nM ligands after a 10 min stimulation. Mean ± SD values were calculated from four independent experiments (n = 4). In panel A, the experimental values are related to the biological activity of human IGF-1. In panel B, the experimental values are the biological activity of human insulin (HI). Asterisks indicate that induction of autophosphorylation of a particular receptor induced by a ligand differs significantly (*p < 0.05; **p < 0.01; ***p < 0.001) from the effect of IGF-1 in the case of IGF-1 analogues or differs significantly from the effect of IGF-2 in the case of IGF-2 analogues. In panel A, the significance of the effect of native IGF-2 (asterisks) is related to human IGF-1 and in panel B to human insulin. In panel B, the significance of the effect of native IGF-1 (asterisks) is related to human insulin.

DOI: 10.1021/acs.biochem.7b01260
Biochemistry 2018, 57, 2373−2382

IGF-2 analogues, which both have phenylalanine at the insulin A8 equivalent 49 and 48 sites, respectively (Figure 1).

First, the [His49]-IGF-1 analogue was made to investigate the effect of this single mutation. Subsequently, it was combined with His or Gln single mutations at site 46 and with Asn45/Gln46 double mutations, as well.

In the case of IGF-2, the single mutation [His48]-IGF-2 analogue was made, which was extended for Gln45 mutation, as well, as it was here the only successful substitution of IGF-2 in the first series.

IGF-1R Binding and Activation Properties of the Second Series of IGF-1 and IGF-2 Analogues. All new IGF-1 mutations have fairly minor, or no significant, effects on IGF-1R binding affinities and activation capabilities, with only the [Asn45,Gln46,His49]-IGF-1 triple mutant having significantly less affinity (37%) for the IGF-1R than native IGF-1 has but with native IGF-1-like autophosphorylation activation ability (Table 1 and Figure 3A).

A similar trend was observed for both new IGF-2 analogues, [His48]-IGF-2 and [Gln45,His48]-IGF-2, the IGF-1R binding and activation abilities of which were similar to those of native IGF-2.

IR-A Binding and Activation Properties of the Second Series of IGF-1 and IGF-2 Analogues. The IR-A-related properties of the analogues contrast with their IGF-1R affinities and binding effects. It seems that the presence of His49 strongly enhances (4−9-fold) the IR-A binding affinity of new IGF-1 analogues (Table 2), in comparison to that of native IGF-1. Moreover, His49-containing IGF-1 analogues do not show any IR-A antagonism, as their capabilities to activate this receptor are superior, or similar, to those of native IGF-1 (Figure 3B).

IR-A binding affinities of two new IGF-2 analogues carrying a His48 mutation are also very (5−7-fold) enhanced, in comparison with those of native IGF-2. Remarkably, both of these analogues bind IR-A with subnanomolar affinities, which make them half-equipotent with human insulin with respect to this IR isoform (Table 2). Moreover, the IR-A activation abilities of these two IGF-2 analogues are also very high, with a potency similar to that of human insulin (Figure 3B).

In general, the levels of activation of IR-A by insulin, IGF-1, IGF-2, and analogues determined at 10 nM (Figure 3B) correspond well to their respective binding affinities for this receptor (Table 2), and they are in a good agreement with the levels of IR-A autophosphorylation induced by 10 nM insulin, IGF-1, and IGF-2 in dose−response curves reported recently by Andersen et al.[53]

IR-B Binding Properties of [His49]-IGF-1 and [Gln45,His48]-IGF-2 Analogues. The outstanding enhancement of IR-A binding affinities exhibited by the His49/His48 IGF-1/2 mutants prompted their characterization toward the IR-B isoform, as well. For this purpose, we tested two representative analogues, [His49]-IGF-1 and [Gln45,His48]-IGF-2, for their binding affinity for IR-B (Table 3). Most interestingly, we found that the IR-B binding affinity of [Gln45,His48]-IGF-2 is ∼8 times higher than that of human IGF-2 and that the binding of [His49]-IGF-1 to this IR isoform is ∼3 times stronger than that of native IGF-1. These mutations did not considerably affect the IR isoform specificity of the analogues because their IR-A binding enhancing effects were similar (5 times for [Gln45,His48]-IGF-2 and ∼4 times for [His49]-IGF-1).

Table 3. IR-B Receptor Binding Affinities of Native Hormones and [His49]-IGF-1 and [Gln45,His48]-IGF-2 Analogues Reported in This Work[a]

analogue	K_d ± SE (nM) (n) for human IR-B in mouse fibroblasts	relative binding affinity for human IR-B (%) relative to that of human insulin
human insulin	0.50 ± 0.31 (5)	100 ± 62
human IGF-1	224 ± 16 (4)[b]***	0.22 ± 0.02
[His49]-IGF-1	72.3 ± 12.0 (3)***	0.69 ± 0.11
human IGF-2	35.5 ± 5.6 (4)[b]***	1.4 ± 0.2
[Gln45,His48]-IGF-2	4.3 ± 1.7 (4)**	11.6 ± 4.6

[a]The values of K_d and relative binding affinities [relative receptor binding affinity defined as (K_d of human insulin)/(K_d of analogue) × 100] of human insulin, IGF-1, IGF-2, and analogues were determined for human IR-B in mouse fibroblasts. n is the number of replicates. Asterisks indicate that the binding a particular ligand to IR-B differs significantly (**$p < 0.01$; ***$p < 0.001$) from the effect of IGF-1 in the case of an IGF-1 analogue or differs significantly from the effect of IGF-2 in the case of an IGF-2 analogue. The binding of native IGF-1 and IGF-2 is relative to that of human insulin. [b]From ref 52.

DISCUSSION

All 11 planned IGF-1 analogues were successfully produced, but only three IGF-2 analogues ([Gln45]-IGF-2, [Gln45,His49]-IGF-2, and [His49]-IGF-2) were made, as the other analogues of this hormone formed insoluble precipitates during the folding steps of their purification. As Whittaker et al.[37] successfully prepared HisA4, AlaA4, and HisA5 insulins, the severe aggregation of some IGF-2 analogues indicates different folding mechanisms of these hormones, underlining the importance of IGF-2 Glu44/45 for its efficient assembly. However, a more extensive mutagenesis, especially of the IGF-2 Glu44 site, is needed for an unambiguous confirmation of the folding-related importance of these side chains.

In general, the majority of mutations performed here have a rather minor impact on the IGF-1R binding affinities of IGF-1 and IGF-2 analogues (Table 1). However, different trends may be observed for the His mutation at position 48 of IGF-2 that enhances its IGF-1R affinity, while the His mutation at IGF-1 equivalent site 49 has an opposite effect. This may indicate that the natures of interactions of IGF-1 and IGF-2 with IGF-1R are different, and specific, for IGF-1 and IGF-2 at their sites 49 and 48, respectively; i.e., the equivalent amino acids at these sites do not interact with the IGF-1R in the same fashion.

Interestingly, the mutations of sites 49 and 48 in IGF-1 and IGF-2, respectively, yielded analogues of these hormones with much more significant, and interesting, changes in their IR-A binding affinities (Table 2) than in their IGF-1R binding/activation properties. Here, both IGF-1 and IGF-2 analogues with His at sites 49 and 48, respectively, are much better IR-A binders than their native forms. The data presented here indicate that this effect can be attributed mainly to the isolated impact of His49, or His48, as the simultaneous mutations at these sites and of residues 45 and 46 in IGF-1 (or residue 45 in IGF-2) do not generate any additional, significant positive properties toward IR-A.

It has been shown that the ThrA8His mutation doubles or triples the insulin IR-A binding affinity,[41−45] and Whittaker et al.[37] used such a mutation to restore IR binding affinity of the less active A4 and A5 insulin mutants. Here, similar mutations enhanced 4−9-fold the IR-A binding affinities of the IGF-1 analogues and 5−7-fold the binding affinities of the IGF-2

DOI: 10.1021/acs.biochem.7b01260
Biochemistry 2018, 57, 2373−2382

analogues, compared to the affinities of the native hormones. The positive effect of His49 and His48 mutations on IR-A binding is similar for both IGF-1 and IGF-2, and to the best of our knowledge, these analogues are the strongest IGF-1-like and IGF-2-like binders of the IR-A receptor isoform thus far reported. In particular, the high IR-A binding affinity of [His48]-IGF-2 is remarkable, as only this single mutation was sufficient to generate an analogue with ∼50% insulin-like affinity for the IR-A. Moreover, we found that the IR-A binding affinity enhancing effect of His49 and His48 is manifested with the IR-B, as well, because the [His49]-IGF-1 analogue is an ∼3-fold stronger IR-B binder than native IGF-1 is and, remarkably, [Gln45,His48]-IGF-2 binds IR-B ∼8-fold more strongly than native IGF-2 does, having almost 12% of the binding affinity of human insulin. Therefore, it seems that [His49]-IGF-1 and [Gln45,His48]-IGF-2 analogues are the strongest reported IGF-1-like and IGF-2-like IR-B binders. The exceptional binding "promiscuity" of [His48]-IGF-2 toward IR-A and IR-B is outstanding and reveals how easily IGF-2 can be converted into a high-affinity ligand for IR receptors. This "ubiquitous" hormonal property of IGF-2 may evoke questions about its evolutionary origins, and it could be hypothesized that IGF-2 resembles the hypothetical evolutionary hormonal ancestor of the insulin/IGF axis more closely than insulin or IGF-1 does.

It is generally accepted that insulin and IGFs interact with their receptors through two main binding sites, sites 1 and 2 in the hormones, and sites 1′ and 2′, respectively, in the receptors.[54] The nature of the interactions of site 1 in insulin and IGF-1 with site 1′ in IR-A and IGF-1R is relatively well characterized in the crystal structures of their complexes (Figure 2). However, structural details about hormone site 2−receptor site 2′ interactions are still missing, and insulin amino acids, which determine its site 2, ThrA8, IleA10, SerA12, LeuA13, GluA17, HisB10, GluB13, and LeuA17, have been suggested through extensive mutagenesis studies.[54]

The different effects of His mutations in IGFs on binding affinities for IR-A, IR-B, and IGF-1R could mean that amino acids at positions 49 (IGF-1) and 48 (IGF-2) are engaged with significantly different protein environments in complexes with IR and IGF-1R. Although it is expected that Phe49 in IGF-1, Phe48 in IGF-2, and ThrA8 in insulin (all at site 2 of the hormones) interact with 2′ sites of the receptors, the possibility that the increase in the level of IR-A binding of [His48]-IGF-2 and [His49]-IGF-1 analogues may also result from an enhancement of some contacts with elements of IR site 1′ cannot be excluded (Figure 2). For example, His49 and His48 of IGFs could form double direct, or water-mediated, hydrogen bonds with Asp707 in the IR α-CT segment and with Asn694 in IGF-1R, as well (Figure 2A), while insulin native ThrA8 is too short for such interactions. It is also possible that the native Phe49 and Phe48 in IGFs can contribute only some weak van der Waals intramolecular contacts with the IGF-1 Cys48−Cys6 disulfide bond and Val44 (IGF-2 Cys47−Cys9 and Val43). Hence, His49 and His48 mutations may provide much stronger directional contacts with both receptor sites (1′ and 2′), which seem to be more favorable for IR-A rather than for IGF-1R.

In general, our new IGF-1 and IGF-2 analogues did not show any considerable reversed trends between their affinities and receptor activation abilities (Tables 1 and 2, Figure 3A,B, and Figure S5). Therefore, the previously observed significant disproportionate binding and activation of insulin analogues mutated at A4 and A8 (ref 37) are probably specific and limited for insulin−IR interactions. It is possible that a different receptor activation behavior of insulin and IGF-1 (IGF-2) analogues mutated at positions A4 and A8 and positions 45, 46, and 49 (positions 45 and 48), respectively, may result from different interactions with specific amino acids at 2′ sites of the IR that are currently not yet determined (see the preceding discussion). Such dissimilar natures of hormone−receptor interfaces may lead to non-equivalent receptor binding mechanisms (as previously pertinently mentioned by Sohma et al. in ref 55) and their subsequent different impacts on signal transduction through the receptors and, ultimately, the activation of their tyrosine kinases.

As indicated above, some trends observed in the analogues described here can be also corroborated by the phylogeny of insulin-related hormones. For example, the insulin ThrA8-containing A8−A10 region is considered as a hypervariable part of this hormone because of its significant sequence differences in mammals.[56] However, the A8 site is the most invariable amino acid in the A8−A10 triad. For example, the well IR-A tolerated (87% binding affinity of HI) AlaA8 occurs in cattle, sheep, and goat insulin.[57] Although there is no mammalian insulin with His at site A8, this amino acid is frequently present at that position in fish, frog, and bird insulins.[56] Also, HisA8 was proposed to be responsible for an ∼5-fold higher binding affinity of chicken insulin for IR in human lymphocytes.[41] Herring at al.[46] proposed that it is possible that a "lower-affinity" ThrA8 site in mammals emerged from the evolutionary optimization of the insulin receptor kinetics, which requires a decrease in insulin binding affinity in mammals that is dictated by a different route of insulin delivery. In more ancient vertebrates like birds, insulin is secreted differently, by the kidney, which leads to rapid metabolic changes.[46] However, in mammals, the pancreas-to-liver portal vein-mediated pathway is the primary direction of insulin. In humans, the liver is the main glycemic-response organ with >90% "metabolic" IR-B isoform and where insulin inhibits gluconeogenesis and glycogenolysis before reaching the peripheral tissues. It may be postulated then that to ensure an optimum liver:body distribution of insulin its IR binding affinity needs to be reduced in mammals, to achieve a more systemic glycemic response.[46]

All known naturally occurring variants of IGF-1 and IGF-2 (including avian) maintain Phe at their insulin ThrA8-corresponding sites 49 and 48, respectively. Therefore, it may be assumed that the IR high-affinity inducing HisA8 mutation in insulin selected upon evolution was required for only rapid and immediate metabolic effects of insulin and was not needed for slower and long-lasting growth effects of IGFs. However, a high sensitivity of both IGFs to acquisition of His48 and His49 mutations may reflect their common evolutionary origins with insulin.

It will be interesting to further explore the findings of this report by testing specific amino acid substitutions at the A8 site of insulin, to maintain its potent IR binding but to decrease its affinity for IGF-1R. Such analogues could be useful for the safer treatment of diabetes, as some currently administered insulin derivatives (e.g., glargine) show higher IGF-1R affinity and hence, potentially, an increased risk of cancer.[58−62]

In summary, as we showed that respective positions in insulin and IGFs lead to non-equivalent IR-A and IGF-1R binding mechanisms of these hormones, this work opens new directions for the rational engineering of the hormonal components of the insulin/IGF system.

DOI: 10.1021/acs.biochem.7b01260
Biochemistry 2018, 57, 2373−2382

CONCLUSIONS

We systematically investigated the receptor binding and receptor activation properties of IGF-1 and IGF-2 analogues modified at positions 45, 46, and 49 (IGF-1) and at positions 44, 45, and 48 (IGF-2). These modifications did not significantly affect the IGF-1R binding of these hormones. However, analogues with the Phe49His mutation in IGF-1 and the Phe48His mutation in IGF-2 have remarkably enhanced binding affinities for both IR-A and IR-B isoforms of the insulin receptor. Here, IGF-1 analogues with His at position 49 possess approximately 5−9% of IR-A, and [His49]-IGF-1 possess ∼0.7% of the IR-B binding affinity of human insulin. Moreover, IGF-2 analogues with His at position 48 are approximately half-equipotent to human insulin in binding to IR-A, and [Gln45,His48]-IGF-2 has almost 12% of the insulin binding affinity for this "metabolic" isoform. The binding affinities of all analogues are in general proportionate to their abilities to activate IR and IGF-1R without any important discrepancies. This study revealed that IR and IGF-1R can contain specific sites, probably parts of so-called receptors' sites 2′, which can interact differently with insulin and with IGFs. These findings shed light on new, possible directions of rational engineering of insulin and IGFs toward more selective and receptor-specific analogues with medical applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b01260.

Summary of mutagenesis strategies used for cloning of IGF-1 and IGF-2 analogues (Table S1), binding curves of native hormones and analogues with IGF-1R, IR-A, and IR-B receptors (Figures S1−S3, respectively), representative immunoblot for determination of receptor activation abilities (Figure S4), and dose−response curves for determination of EC_{50} values of IGF-1R activation abilities of selected analogues (Figure S5) and their respective EC_{50} values (Table S2) (PDF)

AUTHOR INFORMATION

Corresponding Authors

*Telephone: +420 220183 441. E-mail: jiracek@uochb.cas.cz.
*E-mail: zakova@uochb.cas.cz.

ORCID

Andrzej M. Brzozowski: 0000-0001-7426-8948
Jiří Jiráček: 0000-0003-3848-2773

Present Address

‖J.R.: Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Studentski trg 12-16, Belgrade, Serbia.

Funding

This work was supported by Czech Science Foundation Grant 15-19018S (to L.Z.), by Medical Research Council Grant MR/K000179/1 (to A.M.B.), and partly also by Medical Research Council Grant MR/R009066/1 (to A.M.B.). Institutional support was provided by Projects RVO 61388963 (for the Institute of Organic Chemistry and Biochemistry) and 68378050 (for the Institute of Molecular Genetics) of the Czech Academy of Sciences.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Chan, S. J., Nagamatsu, S., Cao, Q. P., and Steiner, D. F. (1992) Structure and evolution of Insulin and Insulin-Like Growth-Factors in Chordates. *Prog. Brain Res. 92*, 15−24.
(2) Chan, S. J., and Steiner, D. F. (2000) Insulin through the ages: phylogeny of a growth promoting and metabolic regulatory hormone. *Am. Zool. 40*, 213−222.
(3) McRory, J. E., and Sherwood, N. M. (1997) Ancient divergence of insulin and insulin-like growth factor. *DNA Cell Biol. 16*, 939−949.
(4) LeRoith, D., Kavsan, V. M., Koval, A. P., and Roberts, C. T. (1993) Phylogeny of the insulin-like growth-factors (IGFs) and receptors - a molecular approach. *Mol. Reprod. Dev. 35*, 332−338.
(5) Denley, A., Cosgrove, L. J., Booker, G. W., Wallace, J. C., and Forbes, B. E. (2005) Molecular interactions of the IGF system. *Cytokine Growth Factor Rev. 16*, 421−439.
(6) Mayer, J. P., Zhang, F., and DiMarchi, R. D. (2007) Insulin structure and function. *Biopolymers 88*, 687−713.
(7) Stewart, C. E. H., and Rotwein, P. (1996) Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol. Rev. 76*, 1005−1026.
(8) Clemmons, D. R. (2009) Role of IGF-I in skeletal muscle mass maintenance. *Trends Endocrinol. Metab. 20*, 349−356.
(9) Holly, J. M. P., and Perks, C. M. (2012) Insulin-like growth factor physiology. What we have learned from human studies. *Endocrinol. Metab. Clin. North Am. 41*, 249−263.
(10) Dynkevich, Y., Rother, K. I., Whitford, I., Qureshi, S., Galiveeti, S., Szulc, A. L., Danoff, A., Breen, T. L., Kaviani, N., Shanik, M. H., LeRoith, D., Vigneri, R., Koch, C. A., and Roth, J. (2013) Tumors, IGF-2, and hypoglycemia: insights from the clinic, the laboratory, and the historical archive. *Endocr. Rev. 34*, 798−826.
(11) Alberini, C. M., and Chen, D. Y. (2012) Memory enhancement: consolidation, reconsolidation and insulin-like growth factor 2. *Trends Neurosci. 35*, 274−283.
(12) Chen, D. Y., Stern, S. A., Garcia-Osta, A., Saunier-Rebori, B., Pollonini, G., Bambah-Mukku, D., Blitzer, R. D., and Alberini, C. M. (2011) A critical role for IGF-II in memory consolidation and enhancement,. *Nature 469*, 491−497.
(13) Lawrence, M. C., McKern, N. M., and Ward, C. W. (2007) Insulin receptor structure and its implications for the IGF-1 receptor. *Curr. Opin. Struct. Biol. 17*, 699−705.
(14) De Meyts, P. (2004) Insulin and its receptor: structure, function and evolution. *BioEssays 26*, 1351−1362.
(15) De Meyts, P., and Whittaker, J. (2002) Structure-Function Relationships of Insulin and Insulin-Like Growth Factor-I Receptor Binding. In *Insulin and Related Proteins: Structure to Function and Pharmacology* (Dieken, M. L., Federwisch, M., and De Meyts, P., Eds.) pp 131−149, Kluwer Academic Publishers, Dordrecht, The Netherlands.
(16) Belfiore, A., Frasca, F., Pandini, G., Sciacca, L., and Vigneri, R. (2009) Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. *Endocr. Rev. 30*, 586−623.
(17) Belfiore, A., Malaguarnera, R., Vella, V., Lawrence, M. C., Sciacca, L., Frasca, F., Morrione, A., and Vigneri, R. (2017) Insulin receptor isoforms in physiology and disease: an updated view. *Endocr. Rev. 38*, 379−431.
(18) Vienberg, S. G., Bouman, S. D., Sorensen, H., Stidsen, C. E., Kjeldsen, T., Glendorf, T., Sorensen, A. R., Olsen, G. S., Andersen, B., and Nishimura, E. (2011) Receptor-isoform-selective insulin analogues give tissue-preferential effects. *Biochem. J. 440*, 301−308.
(19) Jiracek, J., and Zakova, L. (2017) Structural perspectives of insulin receptor isoform-slective insulin analogs. *Front. Endocrinol. 8*, 167.
(20) Siddle, K. (2012) Molecular basis of signaling specificity of insulin and IGF receptors: neglected corners and recent advances. *Front. Endocrinol. 3*, 34.
(21) Siddle, K. (2011) Signalling by insulin and IGF receptors: supporting acts and new players. *J. Mol. Endocrinol. 47*, R1−R10.

DOI: 10.1021/acs.biochem.7b01260
Biochemistry 2018, 57, 2373−2382

(22) Boucher, J., Kleinridders, A., and Kahn, C. R. (2014) Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harbor Perspect. Biol. 6*, a009191.
(23) Hers, I., Vincent, E. E., and Tavare, J. M. (2011) Akt signalling in health and disease. *Cell. Signalling 23*, 1515−1527.
(24) Bedinger, D. H., and Adams, S. H. (2015) Metabolic, anabolic, and mitogenic insulin responses: A tissue-specific perspective for insulin receptor activators,. *Mol. Cell. Endocrinol. 415*, 143−156.
(25) Sacco, A., Morcavallo, A., Pandini, G., Vigneri, R., and Belfiore, A. (2009) Differential signaling activation by insulin and insulin-like growth factors I and II upon binding to insulin receptor isoform A. *Endocrinology 150*, 3594−3602.
(26) Slaaby, R., Schaffer, L., Lautrup-Larsen, I., Andersen, A. S., Shaw, A. C., Mathiasen, I. S., and Brandt, J. (2006) Hybrid receptors formed by insulin receptor (IR) and insulin-like growth factor I receptor (IGF-IR) have low insulin and high IGF-1 affinity irrespective of the IR splice variant. *J. Biol. Chem. 281*, 25869−25874.
(27) Slaaby, R. (2015) Specific insulin/IGF1 hybrid receptor activation assay reveals IGF1 as a more potent ligand than insulin. *Sci. Rep. 5*, 7911.
(28) Clemmons, D. R. (2016) Role of IGF binding proteins in regulating metabolism. *Trends Endocrinol. Metab. 27*, 375−391.
(29) Firth, S. M., and Baxter, R. C. (2002) Cellular actions of the insulin-like growth factor binding proteins. *Endocr. Rev. 23*, 824−854.
(30) Kornfeld, S. (1992) Structure and function of the mannose 6-phosphate insulin-like growth factor-II receptors. *Annu. Rev. Biochem. 61*, 307−330.
(31) Belfiore, A., and Malaguarnera, R. (2011) Insulin receptor and cancer. *Endocr.-Relat. Cancer 18*, R125−R147.
(32) Gallagher, E. J., and LeRoith, D. (2011) Minireview: IGF, insulin, and cancer. *Endocrinology 152*, 2546−2551.
(33) Tao, Y., Pinzi, V., Bourhis, J., and Deutsch, E. (2007) Mechanisms of disease: signaling of the insulin-like growth factor I receptor pathway-therapeutic perspectives in cancer. *Nat. Clin. Pract. Oncol. 4*, 591−602.
(34) Baserga, R. (2013) The decline and fall of the IGF-I receptor,. *J. Cell. Physiol. 228*, 675−679.
(35) Schaffer, L., Brissette, R. E., Spetzler, J. C., Pillutla, R. C., Ostergaard, S., Lennick, M., Brandt, J., Fletcher, P. W., Danielsen, G. M., Hsiao, K. C., Andersen, A. S., Dedova, O., Ribel, U., Hoeg-Jensen, T., Hansen, P. H., Blume, A. J., Markussen, J., and Goldstein, N. I. (2003) Assembly of high-affinity insulin receptor agonists and antagonists from peptide building blocks. *Proc. Natl. Acad. Sci. U. S. A. 100*, 4435−4439.
(36) Pillutla, R., Brissette, R., Blume, A. J., Schaffer, L., Brandt, J., Goldstein, N. I., Spetzler, J., Ostergaard, S., and Hansen, P. H. (2011) Insulin and IGF-1 Receptor Agonists and Antagonists. U.S. Patent 2011/0124556 A1.
(37) Whittaker, J., Whittaker, L. J., Roberts, C. T., Phillips, N. B., Ismail-Beigi, F., Lawrence, M. C., and Weiss, M. A. (2012) α-Helical element at the hormone-binding surface of the insulin receptor functions as a signaling element to activate its tyrosine kinase. *Proc. Natl. Acad. Sci. U. S. A. 109*, 11166−11171.
(38) Menting, J. G., Whittaker, J., Margetts, M. B., Whittaker, L. J., Kong, G. K. W., Smith, B. J., Watson, C. J., Zakova, L., Kletvikova, E., Jiracek, J., Chan, S. J., Steiner, D. F., Dodson, G. G., Brzozowski, A. M., Weiss, M. A., Ward, C. W., and Lawrence, M. C. (2013) How insulin engages its primary binding site on the insulin receptor. *Nature 493*, 241−245.
(39) Menting, J. G., Lawrence, C. F., Kong, G. K. W., Margetts, M. B., Ward, C. W., and Lawrence, M. C. (2015) Structural congruency of ligand binding to the insulin and insulin/type 1 insulin-like growth factor hybrid receptors. *Structure 23*, 1271−1282.
(40) Hexnerova, R., Krizkova, K., Fabry, M., Sieglova, I., Kedrova, K., Collinsova, M., Ullrichova, P., Srb, P., Williams, C., Crump, M. P., Tosner, Z., Jiracek, J., Veverka, V., and Zakova, L. (2016) Probing receptor specificity by sampling the conformational space of the insulin-like growth factor II C-domain. *J. Biol. Chem. 291*, 21234−21245.
(41) Simon, J., Freychet, P., Rosselin, G., and Demeyts, P. (1977) Enhanced binding affinity of chicken insulin in rat-liver membranes and human lymphocytes - relationship to kinetic properties of hormone-receptor interaction. *Endocrinology 100*, 115−121.
(42) Kaarsholm, N. C., Norris, K., Jorgensen, R. J., Mikkelsen, J., Ludvigsen, S., Olsen, O. H., Sorensen, A. R., and Havelund, S. (1993) Engineering stability of the insulin monomer fold with application to structure-activity relationships. *Biochemistry 32*, 10773−10778.
(43) Weiss, M. A., Hua, Q. X., Jia, W., Nakagawa, S. H., Chu, Y. C., Hu, S. Q., and Katsoyannis, P. G. (2001) Activities of monomeric insulin analogs at position A8 are uncorrelated with their thermodynamic stabilities. *J. Biol. Chem. 276*, 40018−40024.
(44) Weiss, M. A., Wan, Z., Zhao, M., Chu, Y. C., Nakagawa, S. H., Burke, G. T., Jia, W., Hellmich, R., and Katsoyannis, P. G. (2002) Non-standard insulin design: structure-activity relationships at the periphery of the insulin receptor. *J. Mol. Biol. 315*, 103−111.
(45) Wan, Z., Xu, B., Huang, K., Chu, Y. C., Li, B., Nakagawa, S. H., Qu, Y., Hu, S. Q., Katsoyannis, P. G., and Weiss, M. A. (2004) Enhancing the activity of insulin at the receptor interface: crystal structure and photo-cross-linking of A8 analogues. *Biochemistry 43*, 16119−16133.
(46) Herring, R., Jones, R. H., and Russell-Jones, D. L. (2014) Hepatoselectivity and the evolution of insulin. *Diabetes, Obes. Metab. 16*, 1−8.
(47) Laible, M., and Boonrod, K. (2009) Homemade site directed mutagenesis of whole plasmids. *J. Visualized Exp. 27*, No. e1135.
(48) Heckman, K. L., and Pease, L. R. (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat. Protoc. 2*, 924−932.
(49) Vikova, J., Collinsova, M., Kletvikova, E., Budesinsky, M., Kaplan, V., Zakova, L., Veverka, V., Hexnerova, R., Avino, R. J. T., Strakova, J., Selicharova, I., Vanek, V., Wright, D. W., Watson, C. J., Turkenburg, J. P., Brzozowski, A. M., and Jiracek, J. (2016) Rational steering of insulin binding specificity by intra-chain chemical crosslinking. *Sci. Rep. 6*, 19431.
(50) Zakova, L., Kletvikova, E., Lepsik, M., Collinsova, M., Watson, C. J., Turkenburg, J. P., Jiracek, J., and Brzozowski, A. M. (2014) Human insulin analogues modified at the B26 site reveal a hormone conformation that is undetected in the receptor complex. *Acta Crystallogr., Sect. D: Biol. Crystallogr. 70*, 2765−2774.
(51) Machackova, K., Collinsova, M., Chrudinova, M., Selicharova, I., Picha, J., Budesinsky, M., Vanek, V., Zakova, L., Brzozowski, A. M., and Jiracek, J. (2017) Insulin-like growth factor 1 analogs clicked in the C domain: chemical synthesis and biological activities. *J. Med. Chem. 60*, 10105−10117.
(52) Krizkova, K., Chrudinova, M., Povalova, A., Selicharova, I., Collinsova, M., Vanek, V., Brzozowski, A. M., Jiracek, J., and Zakova, L. (2016) Insulin-insulin-like growth factors hybrids as molecular probes of hormone:receptor binding specificity. *Biochemistry 55*, 2903−2913.
(53) Andersen, M., Nørgaard-Pedersen, D., Brandt, J., Pettersson, I., and Slaaby, R. (2017) IGF1 and IGF2 specificities to the two insulin receptor isoforms are determined by insulin receptor amino acid 718. *PLoS One 12*, e0178885.
(54) De Meyts, P. (2015) Insulin/receptor binding: the last piece of the puzzle? *BioEssays 37*, 389−397.
(55) Sohma, Y., Pentelute, B. L., Whittaker, J., Hua, Q. X., Whittaker, L. J., Weiss, M. A., and Kent, S. B. H. (2008) Comparative properties of insulin-like growth factor 1 (IGF-1) and [Gly7D-Ala]IGF-1 prepared by total chemical synthesis. *Angew. Chem., Int. Ed. 47*, 1102−1106.
(56) Conlon, J. M. (2001) Evolution of the insulin molecule: insights into structure-activity and phylogenetic relationships. *Peptides 22*, 1183−1193.
(57) Kristensen, C., Kjeldsen, T., Wiberg, F. C., Schaffer, L., Hach, M., Havelund, S., Bass, J., Steiner, D. F., and Andersen, A. S. (1997) Alanine scanning mutagenesis of insulin. *J. Biol. Chem. 272*, 12978−12983.
(58) Kurtzhals, P., Schaffer, L., Sorensen, A., Kristensen, C., Jonassen, I., Schmid, C., and Trub, T. (2000) Correlations of receptor binding

DOI: 10.1021/acs.biochem.7b01260
Biochemistry 2018, 57, 2373−2382

and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes 49*, 999−1005.
(59) Hemkens, L. G., Grouven, U., Bender, R., Gunster, C., Gutschmidt, S., Selke, G. W., and Sawicki, P. T. (2009) Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study. *Diabetologia 52*, 1732−1744.
(60) Jonasson, J. M., Ljung, R., Talback, M., Haglund, B., Gudbjornsdottir, S., and Steineck, G. (2009) Insulin glargine use and short-term incidence of malignancies-a population-based follow-up study in Sweden. *Diabetologia 52*, 1745−1754.
(61) Colhoun, H. M. (2009) Use of insulin glargine and cancer incidence in Scotland: a studyfrom the Scottish Diabetes Research Network Epidemiology Group. *Diabetologia 52*, 1755−1765.
(62) Currie, C. J., Poole, C. D., and Gale, E. A. M. (2009) The influence of glucose-lowering therapies on cancer risk in type 2 diabetes,. *Diabetologia 52*, 1766−1777.

DOI: 10.1021/acs.biochem.7b01260
Biochemistry 2018, 57, 2373−2382

Supporting Information

For

Converting Insulin-like Growth Factors 1 and 2 to High Affinity Ligands for Insulin Receptor Isoform A by the Introduction of an Evolutionarily Divergent Mutation

Kateřina Macháčková[1], Martina Chrudinová[1], Jelena Radosavljević[1], Pavlo Potalitsyn[1], Květoslava Křížková[1], Milan Fábry[2], Irena Selicharová[1], Michaela Collinsová[1], Andrzej M. Brzozowski[3], Lenka Žáková[1,*] and Jiří Jiráček[1,*]

Table of Contents

Table S1. Mutagenesis strategies used for cloning of successfully expressed and produced IGF-1 and IGF-2 analogs.

Analog (expression construct)	Mutation(s)	Forward mutagenic primer (5′-3′)	Reverse mutagenic primer (5′-3′)	Template construct	Applied strategy
Gly-1-IGF-1	-				
[His45]-IGF-1	[D45H]	CCGGCATTGTGCACGAATGCT GCTTTCGC	GCGAAAGCAGCATTCGTGCA CAATGCCGG	Gly-1-IGF-1	SDM
[Asn45]-IGF-1	[D45N]	CCGGCATTGTTAACGAATGCT GCTTTCGC	GCGAAAGCAGCATTCGTTAA CAATGCCGG	Gly-1-IGF-1	SDM
[Ala45]-IGF-1	[D45A]	CCGGCATTGTGGCGGAATGCT GCTTTCGC	GCGAAAGCAGCATTCCGCCA CAATGCCGG	Gly-1-IGF-1	SDM
[His46]-IGF-1	[E46H]	CCGGCATTGTGGATCACTGCT GCTTTCGC	GCGAAAGCAGCAGTGATCCA CAATGCCGG	Gly-1-IGF-1	SDM
[Gln46]-IGF-1	[E46Q]	CCGGCATTGTGGATCAATGCT GCTTTCGC	GCGAAAGCAGCATTGATCCA CAATGCCGG	Gly-1-IGF-1	SDM
[Ala46]-IGF-1	[E46A]	CCGGCATTGTGGATGCATGCT GCTTTCGC	GCGAAAGCAGCATGCATCCA CAATGCCGG	Gly-1-IGF-1	SDM
[Asn45,Gln46]-IGF-1	[D45N+E46Q]	ACCGGCATTGTTAACCAGTGC TGCTTTCGCAGC	GCTGCGAAAGCAGCACTGGT TAACAATGCCGGT	Asn45-IGF-1	OE
[His49]-IGF-1	[F49H]	GTGGATGAATGCTGCCATCGC AGCTGCGATCTG	CAGATCGCAGCTGCGATGGC AGCATTCATCCAC	Gly-1-IGF-1	OE
His46,His49]-IGF-1	[E46H+F49H]	GTGGATCACTGCTGCCACCGC AGCTGCGATCTG	CAGATCGCAGCTGCGGTGGC AGCAGTGATCCAC	His46-IGF-1	SDM
[Gln46,His49]-IGF-1	[E46Q+F49H]	GTGGATCAATGCTGCCATCGC AGCTGCGATCTG	CAGATCGCAGCTGCGATGGC AGCATTGATCCAC	Gln46-IGF-1	OE
[Asn45,Gln46,His49]-IGF-1	[D45N+E46Q+F49H]	ATTGTTAACCAGTGCTGCCATC GCAGCTGCGATCT	AGATCGCAGCTGCGATGGCA GCACTGGTTAACAAT	Asn45-IGF-1	OE
IGF-2	-				
[Gln45]-IGF-2	[E45Q]	CGCGGCATTGTGGAACAGTGC TGCTTTCGCAGC	GCTGCGAAAGCAGCACTGTT CCACAATGCCGCG	IGF-2	SDM
[His48]-IGF-2	[F48H]	GTGGAAGAATGCTGCCATCGC AGCTGCGATCTG	CAGATCGCAGCTGCGATGGC AGCATTCTTCCAC	IGF-2	OE
[Gln45,His48]-IGF-2	[E45H+F48H]	CGCGGCATTGTGGAACAGTGC TGCCATCGCAGC	GCTGCGATGGCAGCACTGTT CCACAATGCCGCG	His48-IGF-2	SDM

OE - overlap-extension PCR (*1*), SDM - site-directed mutagenesis of the whole plasmid (*2*).

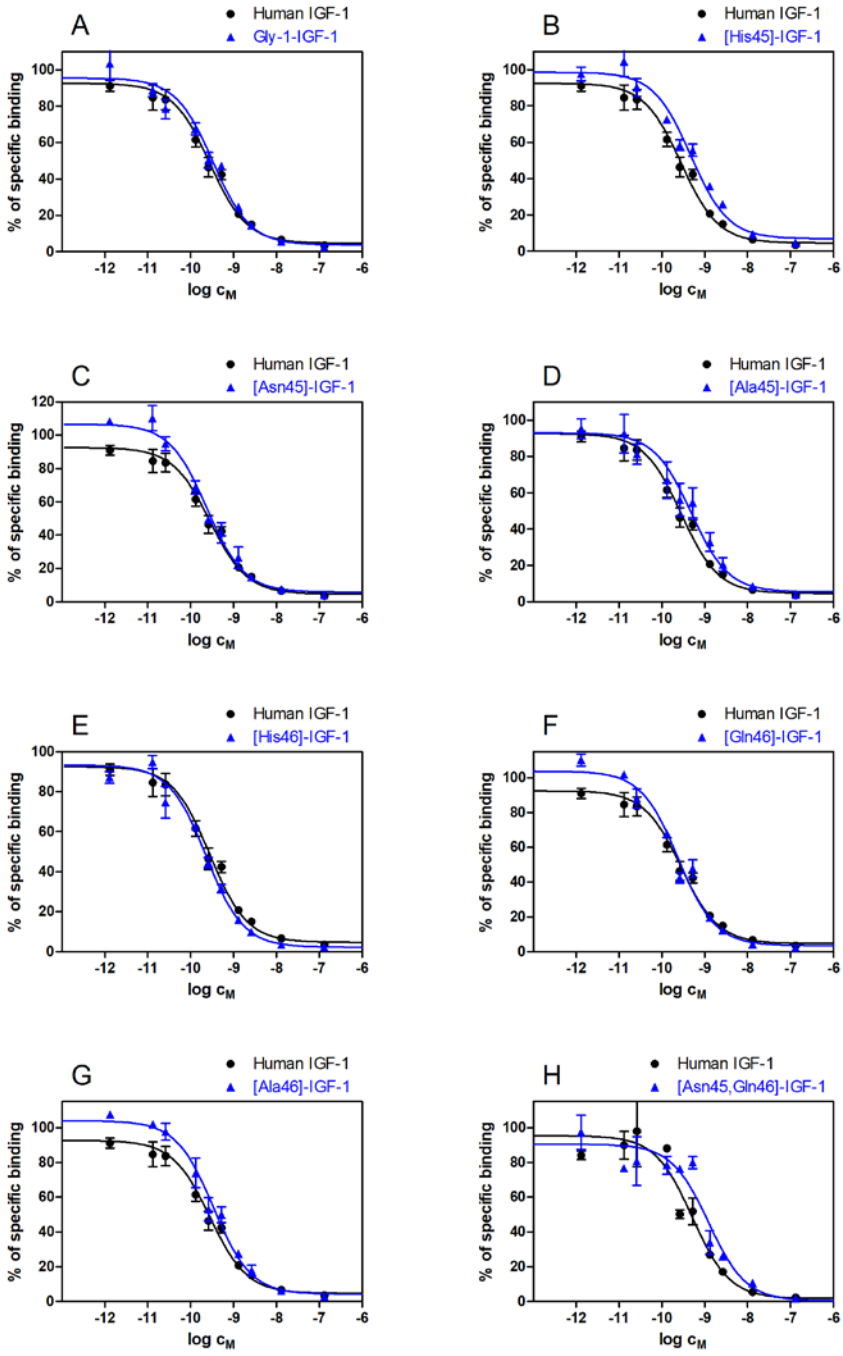


Figure S1. Inhibition of binding of human [^{125}I]monoiodotyrosyl-IGF-1 to IGF-1R in membranes of mouse embryonic fibroblasts by Gly-1-IGF-1 (**A**), [His45]-IGF-1 (**B**), [Asn45]-IGF-1 (**C**), [Ala45]-IGF-1 (**D**), [His46]-IGF-1 (**E**), [Gln46]-IGF-1 (**F**), [Ala46]-IGF-1 (**G**), [Asn45,Gln46]-IGF-1 (**H**) and by human IGF-1 (in all panels). All IGF-1 analogs have Gly at the position -1. The representative binding curves are shown.

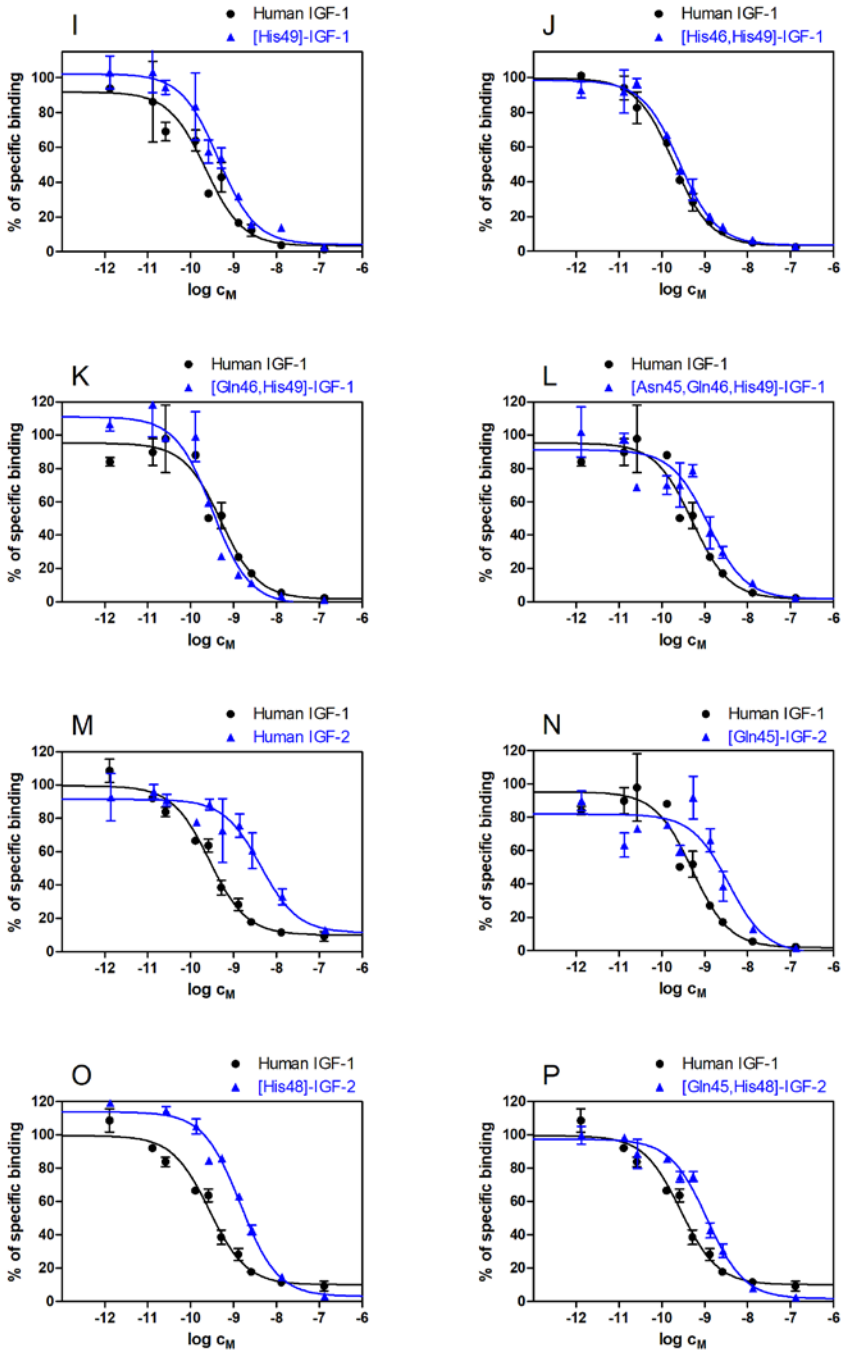


Figure S1 (Continued). Inhibition of binding of human [^{125}I]monoiodotyrosyl-IGF-1 to IGF-1R in membranes of mouse embryonic fibroblasts by [His49]-IGF-1 (**I**), [His46,His49]-IGF-1 (**J**), [Gln46,His49]-IGF-1 (**K**), [Asn45,Gln46,His49]-IGF-1 (**L**), human IGF-2 (**M**), [Gln45]-IGF-2 (**N**), [His48]-IGF-2 (**O**), [Gln45,His48]-IGF-2 (**P**) and by human IGF-1 (in all panels). All IGF-1 analogs have Gly at the position -1. The representative binding curves are shown.

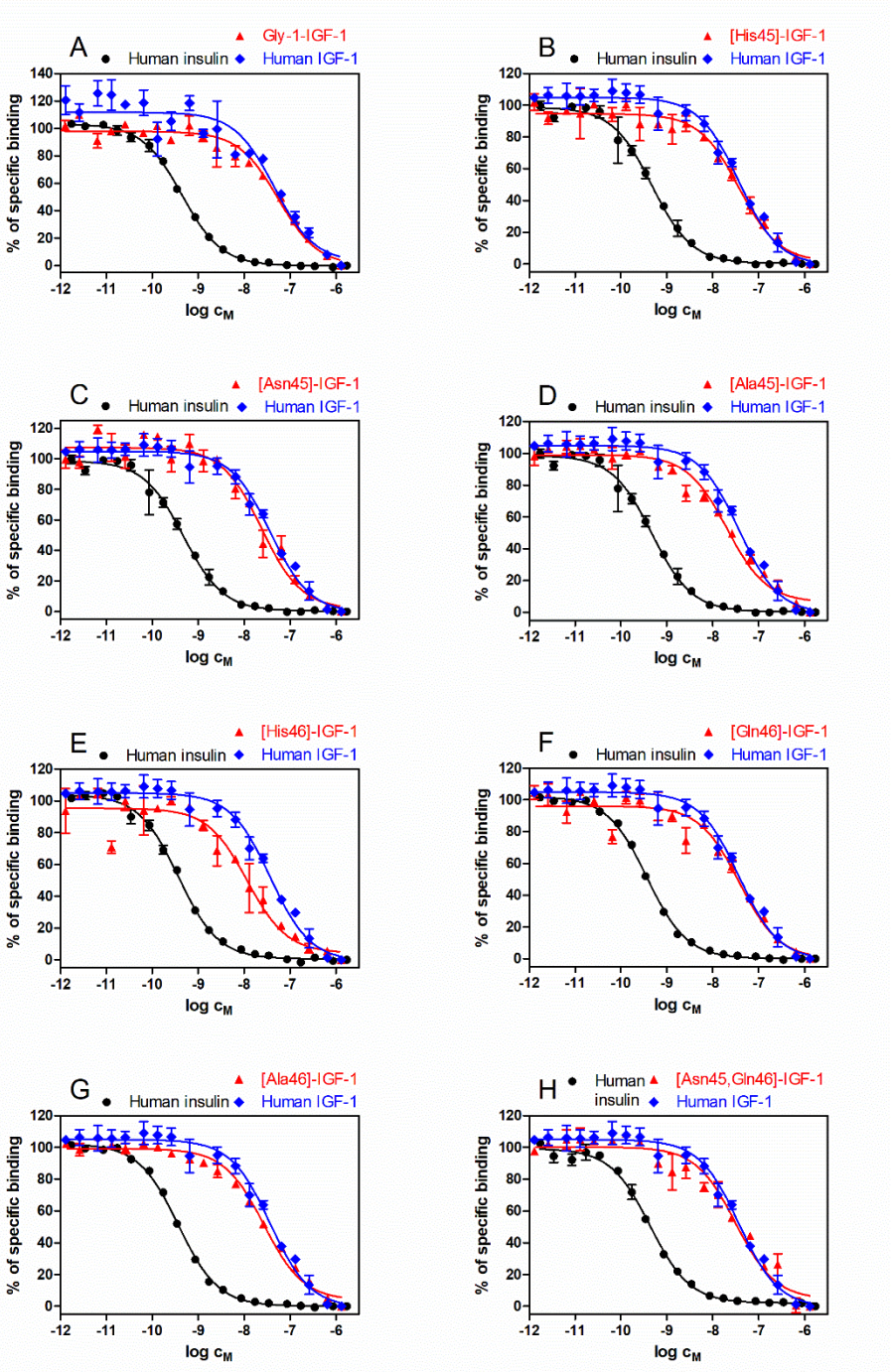


Figure S2. Inhibition of binding of human [^{125}I]monoiodotyrosyl-insulin to IR-A in membranes of human IM-9 lymphocytes by Gly-1-IGF-1 (**A**), [His45]-IGF-1 (**B**), [Asn45]-IGF-1 (**C**), [Ala45]-IGF-1 (**D**), [His46]-IGF-1 (**E**), [Gln46]-IGF-1 (**F**), [Ala46]-IGF-1 (**G**), [Asn45,Gln46]-IGF-1 (**H**) and by human insulin and human IGF-1 (in all panels). All IGF-1 analogs have Gly at the position -1. The representative binding curves are shown.

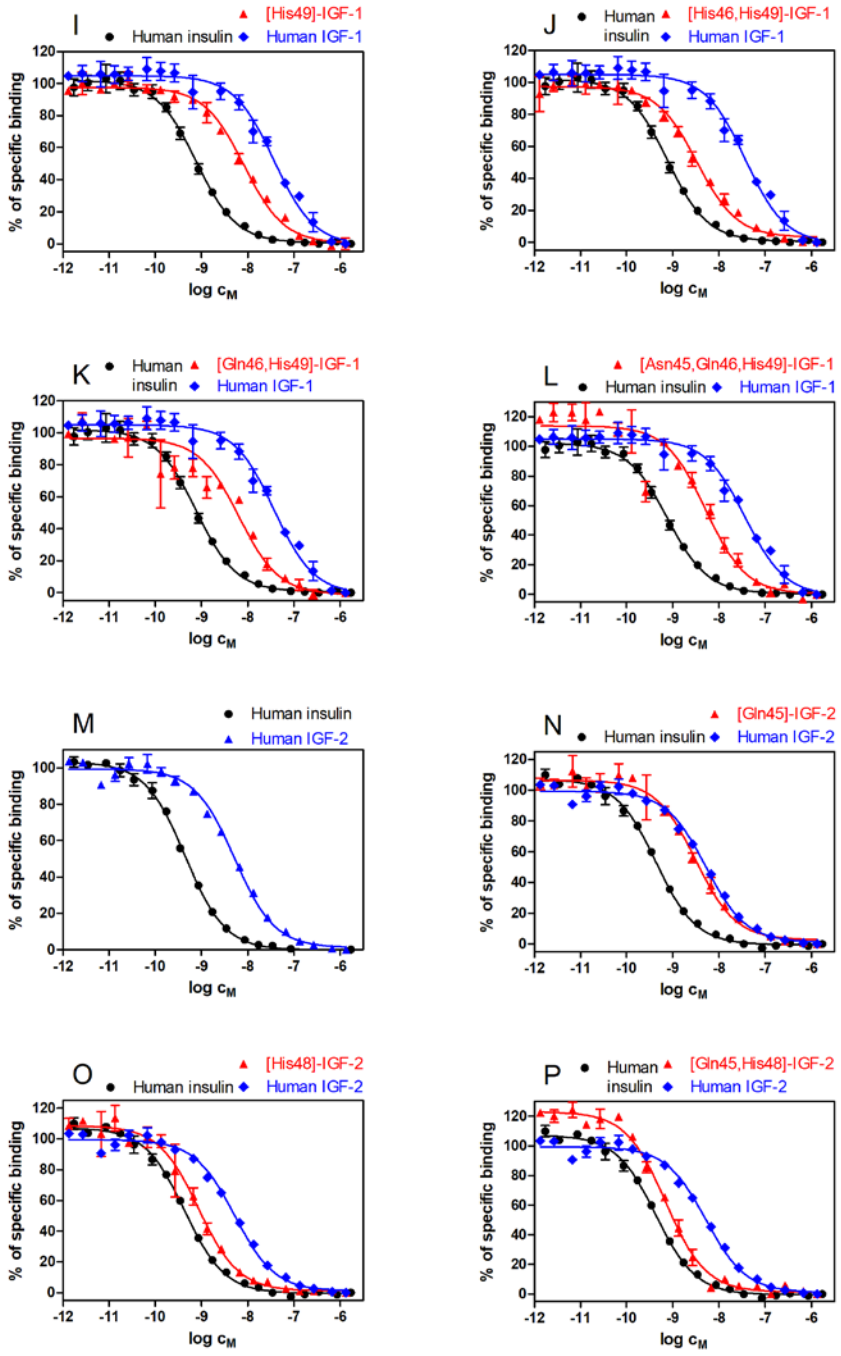


Figure S2 (Continued). Inhibition of binding of human [^{125}I]monoiodotyrosyl-insulin to IR-A in membranes of human IM-9 lymphocytes by [His49]-IGF-1 (**I**), [His46,His49]-IGF-1 (**J**), [Gln46,His49]-IGF-1 (**K**), [Asn45,Gln46,His49]-IGF-1 (**L**), human IGF-2 (**M**), [Gln45]-IGF-2 (**N**), [His48]-IGF-2 (**O**), [Gln45,His48]-IGF-2 (**P**) and by human insulin (in all panels), human IGF-1 (in panels **I**-**L**) and human IGF-2 (in panels **M**-**P**). All IGF-1 analogs have Gly at the position -1. The representative binding curves are shown.

References:

1. Heckman, K. L., and Pease, L. R. (2007) Gene splicing and mutagenesis by PCR-driven overlap extension, *Nat. Protoc. 2*, 924-932.
2. Laible, M., and Boonrod, K. (2009) Homemade site directed mutagenesis of whole plasmids, *J. Vis. Exp. 27*, e1135, doi:10.3791/1135.

Appendix IV

JBC Papers in Press. Published on September 26, 2019 as Manuscript RA119.010072
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.RA119.010072

Mutations at hypothetical binding site 2 in insulin and insulin-like growth factors 1 and 2 result in receptor- and hormone-specific responses

Kateřina Macháčková[#1], Květoslava Mlčochová[#1], Pavlo Potalitsyn[#], Kateřina Hanková[#], Ondřej Socha[#], Miloš Buděšínský[#], Anja Muždalo[#], Martin Lepšík[#], Michaela Černeková[#], Jelena Radosavljević[#2], Milan Fábry[§], Katarína Mitrová[#], Martina Chrudinová[#], Jingjing Lin[#], Yevgen Yurenko[#], Pavel Hobza[#], Irena Selicharová[#], Lenka Žáková[#] and Jiří Jiráček[3]

From [#]Institute of Organic Chemistry and Biochemistry, The Czech Academy of Sciences, Flemingovo nám. 2, 166 10 Prague 6, Czech Republic and [§]Institute of Molecular Genetics, The Czech Academy of Sciences, Flemingovo nám. 2, 166 37 Prague 6, Czech Republic

Running title: Insulin and IGFs respond to equivalent mutations differently

[1]These two authors contributed equally to this work

[2]Present address: University of Belgrade, Faculty of Chemistry, Department of Biochemistry, Studentski trg 12-16, Belgrade, Serbia

[3]To whom correspondence should be addressed: Tel. +420220183441; E-mail: jiracek@uochb.cas.cz

Keywords: insulin, insulin-like growth factor (IGF), receptor tyrosine kinase, receptor binding, hormone analog, NMR structure, molecular dynamics, peptide hormone, receptor autophosphorylation

Information on how insulin and insulin-like growth factors 1 and 2 (IGF-1 and -2) activate insulin receptors (IR-A and -B) and the IGF-1 receptor (IGF-1R) is crucial for understanding the difference in the biological activities of these peptide hormones. Cryo-EM studies have revealed that insulin uses its binding sites 1 and 2 to interact with IR-A and have identified several critical residues in binding site 2. However, mutagenesis studies suggest that IleA10, SerA12, LeuA13, and GluA17 also belong to insulin's site 2. Here, to resolve this discrepancy, we mutated these insulin residues and the equivalent residues in IGFs. Our findings revealed that equivalent mutations in the hormones can result in differential biological effects and that these effects can be receptor-specific. We noted that the insulin positions A10 and A17 are important for its binding to IR-A and IR-B and IGF-1R and that A13 is important only for IR-A and IR-B binding. The IGF-1/IGF-2 positions 51/50 and 54/53 did not appear to play critical roles in receptor binding, but mutations at IGF-1 position 58 and IGF-2 position 57 affected the binding. We propose that IGF-1 Glu58 interacts with IGF-1R Arg704 and belongs to IGF-1 site 1, a finding supported by the NMR structure of the less active Asp58-IGF-1 variant. Computational analyses indicated that the aforementioned mutations can affect internal insulin dynamics and inhibit adoption of a receptor-bound conformation, important for binding to receptor site 1. We provide a molecular model and alternative hypotheses for how the mutated insulin residues affect activity.

Insulin, insulin-like growth factors 1 and 2 (IGF-1 and 2) are hormones with similar primary sequences (Figure 1), 3D-structures and common evolutionary origins. They elicit different but partly overlapping biological functions, primarily metabolic for insulin and mainly mitogenic for both IGFs (1,2).

These hormones trigger their functions by binding to three highly homologous (50-85 % sequence homology) transmembrane glycoprotein receptors for insulin and IGF-1 (3), which belong to a large family of receptor tyrosine-kinases (4). The receptors are formed by two extracellular α-subunits and two membrane-spanning β-subunits. The receptor subunits are interconnected by several disulphide bridges to form the $(\alpha\beta)_2$ heterodimer. There are two isoforms of the insulin receptor, IR-A and IR-B, which differ only by a 12-amino acid segment at the C-terminus of the extracellular α-subunit (called the α-CT peptide) (5). The receptor for IGF-1 (IGF-1R) contains, similarly to IR-A, a

shorter version of the α-CT peptide (3). Moreover, IR-A, IR-B and IGF-1R can form what are known as hybrid receptors, composed of one pair of αβ subunits from one receptor and the second αβ pair from another receptor (6). The IR-A, IR-B and IGF-1R receptors have different tissue distributions and can bind individual hormones with different affinities (7). Furthermore, the availability of IGFs is modulated by a family of six IGF-binding proteins (8) and IGF-2 also binds to a distinct receptor for IGF-2 (IGF-2R) (9,10). All these factors contribute to the different physiological functions of the hormones. Defects in the functioning of the insulin-IGF network can have severe consequences that can result in two types of diabetes (type I and type II), growth disorders, cancer, or Alzheimer disease (11-13).

It is broadly accepted that the insulin molecule interacts with the receptor by an interplay of two binding sites: the primary binding site 1, which binds receptors with a high affinity (~6 nM), and the secondary binding site 2, which binds receptors with a lower affinity (~400 nM) (14,15). Binding of insulin sites 1 and 2 to respective sites 1' and 2' of the receptor creates a high-affinity complex (~0.2 nM), which induces a structural change in the extracellular domains of the receptor, transmission of the signal through the cell membrane and activation of intracellular tyrosine-kinase subunits. Structural information is still incomplete on how the interaction of insulin and IGFs with the receptors activates intracellular signalling.

Crystallographic studies provided structural details about interactions of site 1 of insulin (16,17) or IGF-1 (18,19) with site 1' formed by L1 and α-CT-domains of IR-A or IGF-1R. Interactions of insulin and IGF-1 with site 1' of the receptors are similar (Figs 2A and 2B). The results confirmed the conclusions of previous mutational studies with site 1 of insulin (reviewed in (20)). To date, no structural information is available for a complex of IGF-2 (Fig. 2C) with either insulin or IGF-1 receptor, but it can be expected that at least site 1-site 1' interactions will be similar to insulin and IGF-1.

Characterization of site 2-site 2' contacts has resisted all attempts in the long term for structural analyses, probably due to the highly dynamic character of the interaction. Recently, two studies revealed the character of site 2-site 2' binding of insulin with IR-A. Scapin *et al*. (21) published a Cryo-EM structure of the insulin receptor extracellular ectodomain in a complex with insulin. The results showed that site 2 in insulin is structurally restricted to ThrA8, CysA7, GlnB4–GlyB8 and HisB10 residues, interacting with the receptor's site 2' FnIII-1 domain. Shortly thereafter, Weis *et al*. (22) confirmed the findings of Scapin *et al*. by solving another Cryo-EM structure of insulin bound to the IR-A receptor soluble ectodomain construct. This study extended insulin's site 2 for GluB13. However, these structural results do not fully match the results of mutagenesis and kinetic studies with insulin, which indicated that amino acids IleA10, SerA12, LeuA13 and GluA17 should form insulin's site 2 as well (15,20).

Therefore, we focused on insulin residues IleA10, SerA12, LeuA13, and GluA17 and prepared a series of mutants to study their interactions with the receptors. Each hormone residue investigated in this study was mutated with two different amino acids. Firstly, all mutated positions were substituted for His. Histidine has a relatively large side chain and its imidazole group with a partially aromatic character and ability of hydrogen bonding can participate in different protein-protein interactions. Hence, a rather significant impact on the binding and activation properties of analogs can be expected in the case of His mutations. Secondly, each of the modified residues was also mutated with a similar amino acid (e.g. Ser to Thr, etc.). Here, a subtler modulation of the properties of hormones can be expected. In parallel, similar mutations were prepared in homologous positions of IGF-1 (positions Ser51, Asp53, Leu54 and Glu58) and IGF-2 (positions Ser50, Asp52, Leu53 and Glu57) (Figure 1). The 3D structures of the hormones with highlighted residues modified in this study are shown in Figure 2.

We determined the binding affinities of prepared mutants for IR-A, IR-B and IGF-1R and the abilities of mutants to activate these receptors. Computational simulations provided information about the roles of mutations in insulin structure. We compared the receptor-bound structure of native IGF-1 with the NMR structure of a less active Asp58-IGF-1 mutant. The results allowed direct comparison of equivalent sites in the

hormones and provided an unusually complex view of the roles of mutated residues in binding and activation of the receptors.

Results

Design and production of analogs

Three series of hormone analogs with single mutations were planned: insulin analogs modified at the positions IleA10, SerA12, LeuA13 and GluA17, IGF-1 analogs modified at the positions Ser51, Asp53, Leu54, and Glu58, and IGF-2 analogs modified at the positions Ser50, Asp52, Leu53 and Glu57. These positions are structurally equivalent in all three hormones (Figures 1 and 2) and, based on the results of several mutagenesis studies with insulin ((20) and the references herein), IGF-1 (23) and IGF-2 (24,25), they were considered as parts of hormones' sites 2. We intended to mutate each position in two ways. We either introduced a homologous exchange; i.e. Ser→Thr, Leu(Ile)→Val, Glu→Asp and Asp→Glu or exchanged the wild-type amino acid for His. The planned analogs are listed in Table 1.

Insulin analogs were prepared by the solid-phase peptide synthesis of A and B chains and biomimetic recombination of their disulfide bridges (27-29). IGF-1 and IGF-2 analogs were prepared in *E. coli* cells as previously (26,30). All IGF-1 analogs have an extra glycine at the N-terminus, which allowed cleavage of the fusion protein by TEV protease. We have already shown (30) that the extra Gly residue at -1 position of the hormone does not affect the receptor-binding properties of analogs.

It seems that positions A12/53/52 are important for folding of all three hormones, as only ThrA12-insulin was prepared. In addition, modifications of the A17 position in insulin and the equivalent 57 position in IGF-2 (but not 58 in IGF-1) also did not produce all the planned analogs, which indicates some roles in insulin/IGF-2 folding. On the other hand, all analogs modified at positions A10/51/50 and A13/54/53 were produced, although yields of ValA10-insulin and ValA13 insulin were not sufficient for all biological experiments (Table 1). Table 1 shows production yields of insulin and IGF analogs related to native hormones. The typical yield for standard chemical synthesis of insulin (starting with 100 μmol of resin) was about 1 mg. The typical yield for IGF-1 or IGF-2 production from 1 l of media was about 0.4 mg or 0.3 mg, respectively. IGF-1 and IGF-2 analogs were prepared in at least two independent experiments. More laborious chemical synthesis of insulin analogs was performed only once in each case.

Receptor-binding affinities and receptor activation abilities of analogs

All produced hormone analogs were tested for their binding to IR-A, IR-B and IGF-1R receptors and for their ability to induce phosphorylation of these receptors. The binding data are summarized in Table 1, the detailed results of biological experiments are provided in Tables S1-S3, representative binding curves of analogs for receptors are shown in Figures S1-S3 and representative Western blots for relative abilities of analogs to stimulate receptors' phosphorylation are shown in Figures S4-S6.

Some general trends in binding affinities are clearly visible, despite the fact that not all planned analogs were produced and tested with all the receptors. *Positions A10/51/50:* Binding of HisA10-insulin is severely compromised for all three receptors. On the contrary, all IGF-1 and IGF-2 analogs modified at these positions are tolerated. *Positions A12/53/52:* The only successfully prepared ThrA12-insulin with homologous Ser-to-Thr mutation has binding affinities similar to native insulin for IR-A and IGF-1R, but statistically significantly decreased for IR-B receptor. *Positions A13/54/53:* Similarly to A10 position, HisA13 significantly reduces binding affinity of the analog for both isoforms of IR and all IGF-1 and IGF-2 analogs with mutations at equivalent positions have binding properties generally similar to the native hormones. However, on the contrary to HisA10-insulin, HisA13-insulin has a native-like affinity for IGF-1R. *Positions A17/58/57:* Mutations at these positions provided a more complicated picture of the binding affinities of analogs. HisA17-insulin, similarly to A10 and A13 positions, has a very low binding affinity for IR-A. No binding data are available for IR-B and IGF-1R due to the low amount of the analog prepared, but we were able to show HisA17-insulin is inactive in inducing IGF-1R autophosphorylation (Table S3), which indicates that its IGF-1R binding is severely impaired as

well. Interestingly, homologous Glu-to-Asp mutations at position 58 in IGF-1 and position 57 in IGF-2 resulted in different biological effects. Asp57-IGF-2 binds in a similar manner as native IGF-2 to IR-A/IGF-1R or slightly better to IR-B. On the other hand, Glu-to-Asp change at position 58 of IGF-1 had a rather reducing effect on the analog's binding affinity for IGF-1R. This trend was confirmed by the very low binding affinity of His58-IGF-1 for IGF-1R and by reduced affinities for IR-A and IR-B.

Activation of receptors: For all prepared analogs, we did not observe any major discrepancies between their binding (K_d values) and trends in receptor activation abilities (Tables S1-S3). We did not detect any important signs of partial or complete antagonism or receptor over-activation.

NMR structure of Asp58-IGF-1 helped to explain analog's reduced binding affinity

We determined the NMR structure of ^{15}N and ^{13}C-labeled Asp58-IGF-1 to investigate the interesting effects of Glu-to-Asp substitution at the 58 position of IGF-1. The representative (lowest energy) NMR structure of Asp58-IGF-1 analog (pdb 6RVA, this work) was compared to the IGF-1R receptor-bound crystal structure of native IGF-1 (pdb 5U8Q from Ref. (19)). Figure 3 shows that the structures of the Asp58-IGF-1 analog and receptor-bound native IGF-1 are very similar. The structure similarity of Asp58-IGF-1 and native IGF-1 is supported by the observed small differences of NMR chemical shifts NH, ^{15}N and Hα (Supplemental Information). The only marked differences are in the C-domain that is known to be flexible and thus only partly visible in the complex and is probably rearranged during binding to the receptor.

The structure of the IGF-1:IGF-1R complex (pdb 5U8Q) shows that α-CT's Arg704 side chain points to Glu58 carboxylate of complexed IGF-1 and that these residues could possibly form a salt bridge. Some further potential intramolecular stabilization of receptor-complexed native IGF-1 Glu58 residue could also be deduced from the positions of two other Arg21 and Arg55 residues from IGF-1. Locking of Glu58 in place to enable this salt bridge could be maintained by two intramolecular salt bridges from Arg21 and Arg55 residues from IGF-1 (Figure 3). These latter interactions are expected to be weak and transient, as the electron densities for the two Arg side chains are missing in pdb 5U8Q. Moreover, the crystallographic resolution of the IGF-1R:IGF-1 structure is 3.27 Å (pdb 5U8Q) (19), which means that interpretations should be made carefully.

The Asp58 side chain of the less active Asp58-IGF-1 analog is one methylene shorter than the Glu58 side chain. Consequently, Asp58 may have difficulties to maintain all three salt bridges possibly present in the complex. Indeed, modeling of the complex (not shown) suggests that a rearrangement of the IGF-1 and/or α–CT helix would be needed to enable a potential Asp58-Arg704 salt bridge. The stabilization of Asp58 in IGF-1 by intramolecular contacts with Arg21 and Arg55 is improbable (at least in solution) as it is not present in any of 20 available NMR structures (pdb 6RVA).

Insulin analogs' metadynamics reveals different free energy profiles that can affect site 1 binding

As the insulin residues Ile10, SerA12, LeuA13 and GluA17 were not found to be directly involved in binding the insulin receptor (21,22), we tested the possibility that mutations at these sites can affect the dynamics of insulin analogs, especially the crucial detachment of the B-chain C-terminus of insulin (17,31), that could in turn modulate binding to the receptor.

The native human insulin along with the HisA10, HisA13 and HisA17 analogs with impaired affinity and the native-like affinity ThrA12 analog (Table 1) were subjected to enhanced-sampling molecular dynamics (MD) simulation in explicit solvent along two collective variables, dwo_1 (GlyB8-ProB28) and dwo_2 (ValB12-TyrB26), described before (32), defining the B-chain C-terminus opening. Figure 4 shows that the dynamics of the low-affinity His-mutants are qualitatively different in that they are less likely to assume the conformation with the detached B-chain C-terminus compatible with IR-binding.

The wild-type insulin is likely to assume a wide range of open conformations, with the global minimum exceeding the defined threshold for the wide-open state observed by (32). In comparison, the minimal energy ensembles of the HisA10- and the HisA13-insulin are partially

collapsed, the B-chain α-helix unwinds locally and the coil-like structure results in open states. This loss of secondary structure, however, is not compatible with IR binding. On the other hand, the HisA17-insulin is stabilized in a closed conformation, with both N- and C-termini of the B chain remaining close to the insulin core. Here, no detachment of the B-chain C-terminus occurs and thus no IR binding can be expected. Interestingly, the native-like affinity ThrA12-insulin shows a free energy profile similar to the native insulin where both, a broad range of wide-open conformations are possible and the structural integrity of the B-chain α-helix is not as compromised.

Since all mutated sites are distant from the B-chain C-terminus segment, we were interested in identifying the specific inter-residue contacts that form in the course of biasing the B-chain C-terminus opening and destabilizing the protein core. We might expect the mutations at apolar sites IleA10 and LeuA13 to a polar hydrogen-bond donor/acceptor histidine to be disruptive.

It is apparent from the minimal energy ensemble (in Figure 4), the HisA10-insulin mutant underwent the most pronounced collapse, resulting in the partial disintegration of the B-chain α-helix. Similarly, the destabilization of the His13-insulin mutant was mostly restricted to the B chain, though not as pronounced. The difference in contact lifetimes between the mutant and wild-type insulin revealed that contacts between a range of B-chain N-terminal residues and residues in the α-helix were established to maintain these partially collapsed states (Figure S7). The low-affinity HisA17-insulin was stabilized in the closed conformation with the N-terminus of the B-chain placed parallel to the B-chain α-helix but also close to the A-chain termini (Figure 4). On the other hand, although the native-like affinity ThrA12 mutant was stabilized in open conformations, contact analysis revealed that hydrophobic contacts at the A/B chains' interface of insulin more frequently formed than in the wild type. This compaction of the hydrophobic core was observed for all other mutants as well, except for the His10-insulin which collapsed to more extended states (Figure S8A). Connected to the extent of the hydrophobic collapse, we observed a change in protein-water hydrogen bonding local to the point of mutation. The dynamics of H-bond formation were more stable in the closed compact state of HisA17-mutant (Figure S8B). On the other hand, the bulkier ThrA12 was buried inside the core and formed fewer H-bonds with water than the wild-type SerA12.

Having considered the conformational diversity of the minimal energy ensembles of insulin mutants, we might expect the conformational strain necessary to assume the insulin-IR-bound form to contribute to the mutation-induced changes in affinity. Consequently, we estimated the free energy of strain upon binding as the relative free energy going from insulin free in solution, taken as the global minimum in F (dwo_1, dwo_2) in Figure 4, to insulin bound to the IR, corresponding to the black dots in Figure 4 obtained from molecular dynamics of insulin-IR complexes. The insulin mutant-IR- bound complexes were produced starting from the Cryo-EM structure of the wild-type human insulin bound to the IR-A isoform of the receptor (22) and mutagenesis of the respective sites in the A chain (see Methods). The resulting free energies of strain of HisA10-, HisA13-, HisA17- and ThrA12 mutants relative to native insulin were 11.5 ± 1.8, 23.7 ± 1.8, 21.2 ± 1.9, 8.0 ± 3.8 kJ mol^{-1}, respectively (the absolute value for the free energy of the strain of native insulin was 16.1 ± 2.8 kJ mol^{-1}). The experimentally measured relative binding free energies between insulin mutants and native insulin evaluated using $\Delta\Delta G_{exp} = RT \ln (K_d \text{mutant} / K_d \text{ insulin})$ were 4.6 ± 1.2, 4.7 ± 0.1, 6.6 ± 0.4 and -0.2 ± 1.4 kJ mol^{-1}, respectively (relative affinities are reported in Table S1). The largest calculated strains of HisA13 and HisA17 and the low strain of ThrA12 are in qualitative agreement with the total experimentally determined affinities of insulin mutants. We can thus attribute part of the changed IR-binding affinity for the insulin mutants to the change in conformational strain upon IR binding.

Discussion

Each hormone residue investigated in this study was mutated with two different amino acids; with His and with a similar amino acid (e.g. Ser to Thr, etc.). For His mutations, a rather significant impact on the binding and activation properties of analogs was expected. On the other

hand, a subtler modulation of the properties of hormones was expected for similar mutations. Interestingly, both these presumptions were confirmed in this study: His-insulin mutants have important effects on binding affinities and Glu-to-Asp mutation provided differential results in IGF-1 and IGF-2 (Table 1).

Our results indicate that mutations at the positions A12/53/52 in all 3 hormones, at the position A17 in insulin and at the position 57 in IGF-2 (but not at equivalent 58 position in IGF-1) can affect conformation of the premature polypeptides and consequently their folding. Our results are relatively surprising, because others were more successful in modifications of A12 and A17 positions in insulin (33), position 53 in IGF-1 (34) and positions 52 and 57 in IGF-2 (24,25). The reasons for these different production yields could be in different production strategies, e.g. yeast *vs E. coli*, different fusion protein partners, etc.

Other studies (cited in (20)) showed that mutations of insulin positions A10, A12, A13 and A17 can negatively affect binding of analogs to IR and the residues were proposed to belong to the hypothetical site 2 of insulin. Some analogs were prepared for equivalent positions in IGF-1 and IGF-2 as well. A summary of available literature data on receptor-binding affinities of insulin and IGFs is provided in Table S4**,** but the listed do not provide complete information about binding affinities of mutants with all three receptors, i.e. IR-A, IR-B and IGF-1R. In this respect, our study offers a unique, complex, and comprehensive picture of the involvements of mutated residues in hormone-IR-A/IR-B/IGF-1R interactions.

We prepared only ThrA12-insulin in the A12/53/52 series. Hence, it is not possible to deduce important conclusions about the roles of these positions in receptor binding. Binding affinities of ThrA12-insulin were similar to the native hormone for all 3 receptors (Table 1). Taken together, our data and the data of others (Table S4) rather do not indicate any crucial roles of A12/53/52 positions in binding to the receptors.

On the other hand, the results with other mutants revealed interesting differences in the receptor- binding behavior of the hormones. While positions 51/50 and 54/53 in IGF-1/IGF-2 are relatively tolerant to modifications, insulin binding to all 3 receptors is severely impaired by modifications at A10. However, Leu-to-His mutation of insulin's A13 negatively affects only binding to IR-A and IR-B and not to IGF-1R. This finding is in full agreement with data published by Schäffer (15) (Table S4). Firstly, these data could indicate that insulin and both IGFs on the other hand do not interact with the receptors by the same mechanisms. Secondly, the difference in IR and IGF-1R responses to mutation at the insulin A13 position could mean that respective sites 2' in IR and IGF-1R are different. Such a possibility has already been mentioned by others (35-37) and by our team as well (30).

Insulin binding to IR-A and IGF-1R (according to IGF-1R activation, Table S3) was impaired by mutation at A17. Similar results were found for mutations in IGF-1, but not in IGF-2. Notably, the difference in effects of homologous Glu-to-Asp mutations in IGF-1 and IGF-2 is interesting. The closer look at the crystal structure of IGF-1-IGF-1R complex (pdb 5U8Q) recently reported by Xu *et al*. (19) reveals that Glu58 could create close contact (2.7-3.0 Å, probably a salt bridge) with Arg704 of α-CT peptide (Figure 3). We are aware that this interaction should be considered with caution because of the resolution of the complex (3.27 Å). Furthermore, a different orientation of Asp58 side in the NMR structure of Asp58-IGF-1 compared to the position of Glu58 in complexed-IGF-1 (Figure 3) may be caused by the low pH of the NMR experiment, which would result in a higher probability of protonation of Asp58. But it seems logical that the lower binding affinity of Asp58-IGF-1 could be explained by the inability of the analog's shorter Asp side chain to form these salt bridges, and the very low affinity of His58-IGF-1 could be explained by a mutual repulsion of His58 and Arg704. Interestingly, similar stabilization of homologous insulin's GluA17 is not visible in available insulin-IR-A complexes, pdb 4OGA (16) or pdb 6HN5 (22), or in the complex of IGF-1 with IR L1 and IGF-1R α-CT (pdb 4XSS) (18).

There are no structural data showing a complex of IGF-2 with any of the receptors, which could explain the enhancing effect of Asp57 in IGF-2 in binding affinity. Hence, the mechanisms of how Glu at A17/58/57 positions in insulin and both IGFs affect hormone binding to

receptors are not fully clear. Nevertheless, our data indicate that at least Glu58 in IGF-1 can interact with α-CT Arg704, and that would classify Glu58 as a part of hormones' site 1.

It cannot be excluded that mutations at insulin A10/A12/A13/A17 sites can affect the structure of the hormone and consequently its ability to interact with receptors' Site 1'. To investigate this possibility, we initiated a series of computational experiments with insulin mutants and with native insulin. Native insulin receptor-bound conformation is characterized by a partial detachment of B25-B30 residues from the core of the insulin molecule that is essential for potent insulin binding to receptor site 1' (17,38,39). The data summarized in Figure 4 show that His mutations at A10/A13/A17 (but not Ser-to-Thr mutation at A12) could have an impact on insulin conformational dynamics and negatively affect the ability of mutants to adopt what are called "active" (open) conformation at insulin's site 1. The theoretical data are in good general agreement with the binding data of analogs. Therefore, as an alternative to the hypothesis that insulin residues A10, A13 and A17 are involved in direct interaction with the receptor site 2, we suggest that they may be important for the structural integrity of the hormone at its site 1.

It is important to bear in mind the advantages and limitations of the computational protocol employed. The benefit of metadynamics (40) as opposed to classical molecular dynamics is its efficiency in accelerating slow conformational transitions along the selected collective variables (CVs), while having control over statistical convergence. On the other hand, the added bias may induce unnatural conformational states. Balancing these opposing effects constitutes a demanding project, which is beyond the current study. For the sake of very rough error boundaries, we have however carried out several calculations differing in simulation length, biasing criteria and the initial structure and found uncertainties in the strain-free energies of 10-20 $kJ.mol^{-1}$. This effect is of a greater order of magnitude than the statistical error bounds presented above. With that in mind, the current computational results allow for putting forward an alternative hypothesis of the effect of insulin mutations in the A chain, which will warrant a further study.

Weis *et al*. (22) proposed that insulin A12, A13, A17 and B17 residues, predicted by mutagenesis studies to belong to hormone site 2, but not found in contacts with the receptor site 2' in Cryo-EM insulin-IR complexes, may be involved in the initial docking of insulin to the receptor, an event postulated to precede the relaxation of insulin's induced fit to its primary binding site (19). This hypothesis could also explain our experimental data showing that mutations at insulin positions A10, A13 and A17 can cause important changes in receptor-binding affinities that can even be receptor-specific (for the A13 position). Interestingly, insulin analogs mutated at LeuA13 were shown to have slow association rates, which supports the hypothesis that A13 residue is involved in some first contacts with the receptor (15,41). The data also indicate that such hypothetical initial docking interactions of insulin and both IGFs with the receptors could be different, as only 58/57 positions and not 51/50 and 54/53 in IGF-1/IGF-2 were sensitive to mutations. It is not excluded that future advances in X-ray crystallography or Cryo-EM methodology will decipher structures of such hypothetical transient protein hormone complexes and reveal complex mechanisms of receptor activation by the hormones. In this context, during the preparation and revision of this manuscript, two studies (42,43) were published showing Cryo-EM maps of the IR extracellular domains with four insulins bound. Two of these insulins are positioned as shown previously by Scapin et al. (21) but the binding site for the other two insulins is located in the FnIII-1 (or FnIII-1') domain and was not detected previously. This newly identified binding site was proposed as a new site 2 and should interact with insulin residues studied in this work. These findings support the hypothesis of initial docking and transient interactions between insulin and IR that are followed by structural rearrangements of the complex. These new findings are not contradictory to our results as mutations of insulin residues A12, A13 and A17 could affect both insulin dynamics and site 1 interactions as well as new site 2 interactions.

Summary

Deciphering molecular mechanisms by which hormones insulin, IGF-1 and IGF-2 bind their

cognate receptors and elicit different biological effects has been a central problem for biologists for decades. Two Cryo-EM studies (21,22) showed how insulin binds the insulin receptor through its binding sites 1 and 2. However, these findings do not fully match the results of mutagenesis studies, which predicted that insulin residues IleA10, SerA12, LeuA13 and GluA17 should belong to hormone site 2 as well. Therefore, we systematically mutated these hypothetical insulin site 2 residues and equivalent residues in IGF-1 and IGF-2. Comparison of the biological properties of insulin, IGF-1 and IGF-2 site 2 mutants on three transmembrane receptors (IR-A, IR-B and IGF-1R) revealed that the hormones respond to equivalent mutations differently and that responses can be receptor-specific. Specifically, we showed that insulin sites A10 and A17 are important for binding to all tested receptors, but A13 is only important for IR-A and IR-B. In IGF-1/IGF-2, the positions 51/50 and 54/53 probably do not play any important role in receptor binding. On the other hand, we propose that Glu58 of IGF-1 can interact with IGF-1R site 1 Arg704 residue and hence Glu58 could belong to IGF-1's Site 1. The results of computational metadynamics show that mutations can affect the internal dynamics of insulin and inhibit its ability to adopt receptor-bound conformation, which is important for binding to receptor site 1. This indicates that studied insulin residues might not be involved in direct interactions with site 2 of receptors. Recently, two studies (42,43) were published showing Cryo-EM maps of the IR extracellular domains with insulin bound to a newly identified binding site that was proposed as a new site 2 and should interact with insulin residues studied in this work. These new findings support the hypothesis of initial docking and transient interactions between insulin and IR.

Experimental procedures

Synthesis of insulin analogs

Insulin analogs were prepared by the solid-phase chemical synthesis of A- and B-chains in their S-sulfonate forms, followed by a biomimetic recombination of disulfide bridges according to previously published protocol (27,29). The peptide synthesis was performed on a Spyder Mark II® automatic peptide synthesizer (a prototype developed by a team in the Developmental workshops in the Institute of Organic Chemistry and Biochemistry, headed by Dr. Michal Lebl, the European patent application number is EP 17206537.7).

Cloning and Production of IGF-1 and IGF-2 Analogs

IGF-1 and IGF-2 analogs were produced according to our previously published methodology (26,30). Briefly, both human IGF-1 (UniprotKB entry P05019 amino acids 49-118) and human IGF-2 (UniprotKB entry P01344 amino acids 25-91) were cloned into a modified pRSFDuet-1 expression vector as a fusion with an N-terminally His6 tagged-GB1 protein and TEV protease cleavage site. An additional N-terminal glycine residue (Gly^{-1}) was incorporated into IGF-1 to enable cleavage by TEV protease (sequence Glu-Asn-Leu-Tyr-Phe-Gln↓Gly^{-1}), but Glu-Asn-Leu-Tyr-Phe-Gln↓Ala^{1} sequence yielding a native hormone was used for TEV protease cleavage site (↓) in the IGF-2 expression constructs. Constructs were transformed into *E. coli* BL21(λDE3), cultivated by using LB medium or minimal medium containing ^{15}N-ammonium sulphate and ^{13}C-D-glucose, and hormones were purified as described previously (26).

All successfully produced analogs were purified by RP-HPLC. The purity of all tested analogs was higher than 95 % (and controlled by RP-HPLC analyses and HR-MS spectra).

Binding affinities for the receptors

Binding affinities of analogs were determined with receptors in the intact cells. Specifically, binding affinities for IGF-1R were determined with mouse fibroblasts transfected with human IGF-1R and with deleted mouse IGF-1R, according to Hexnerova *et al*. (26). Binding affinities for IR-A were determined with human IR-A in human IM-9 lymphocytes, according to Vikova *et al*. (44). Binding affinities for IR-B were determined with mouse fibroblasts transfected with human IR-B and with deleted mouse IGF-1R, according to Zakova *et al*. (31). The binding curve of each analog was determined in duplicate and the final dissociation constant (K_d) was calculated from at least three ($n \geq 3$)

binding curves (each curve giving a single K_d values), determined independently and compared to binding curves for insulin, IGF-1 or for IGF-2, depending on the type of analog.

The abilities of analogs to induce autophosphorylation of the receptors

The abilities of analogs to induce autophosphorylation of IGF-1R in membranes of mouse fibroblast transfected with human IGF-1R and with deleted mouse IGF-1R were determined, as described by Machackova *et al*. (29). The abilities of analogs to induce autophosphorylation of IR-A or IR-B in mouse fibroblast transfected with human IR-A or IR-B and with deleted mouse IGF-1R were determined, as described by Krizkova *et al*. (45). Briefly, the cells were stimulated in 24-well plates (Schoeller) ($4x10^4$ cells per well) after 4 hours' starving in serum-free medium. The cells were stimulated with 10 nM concentration of the ligands (insulin, IGF-1, IGF-2 or analogs) for 10 min. Stimulation was stopped by snap-freezing. Proteins were routinely analyzed, using immunoblotting and horseradish peroxidase-labeled secondary antibodies (Sigma-Aldrich). The membranes were probed with anti-phospho-IGF-1Rβ (Tyr1135/1136)/IRβ (Tyr1150/1151), Cell Signaling Technology. The blots were developed, using the SuperSignal West Femto maximum sensitivity substrate (Pierce), and analyzed using the ChemiDoc MP Imaging System (Bio-Rad). The autophosphorylation signal density generated by each ligand on western blot was expressed as the contribution of phosphorylation relatively to the IGF-1 (IGF-1R fibroblasts) respective human insulin (IR-A and IR-B fibroblasts) signal in the same experiment. Mean ± SD values were calculated from four independent experiments (n=4) and compared to native insulin, IGF-1 or IGF-2, depending on the type of analog.

Significance of the changes in binding affinities was calculated using the two-tailed t test. Significance of changes in abilities of analogs to stimulate autophosphorylation was calculated using one-way analysis of variance with Dunnett's test, comparing all analogs *vs* control (insulin or IGFs, depending on the type of the analogs).

NMR spectroscopy

All NMR data for Asp58-IGF-1 and native IGF-1 were acquired on 600 MHz Bruker Avance II spectrometer equipped with 5 mm $^1H/^{13}C/^{15}N$ cryoprobe. The NMR spectra were collected at 40 °C using 450 µl samples of protein dissolved in 50 mM solution of CD_3COOD (pH 3.0) in water (95 % H_2O + 5 % D_2O) with 0.01 % NaN_3.

Proton NMR data of both proteins were obtained from homonuclear 2D-TOCSY and 2D-NOESY spectra of non-labeled samples: native IGF-1 (0.44 mg; 0.11 mM) and Asp58-IGF-1 (0.70 mg; 0.18 mM). Isotopically ^{15}N-labeled native IGF-1 (0.25 mg; 0.06 mM) and Asp58-IGF-1 (0.07 mg; 0.02 mM) were used for determination of ^{15}N chemical shifts from ^2D-^1H,^{15}N-HSQC experiments. Doubly ^{13}C,^{15}N-labeled Asp58-IGF-1 (0.18 mg; 0.05 mM) dissolved in D_2O provided also ^{13}C chemical shifts from 2D-^1H,^{13}C-HSQC and 2D-^1H,^{13}C-HMBC experiments. Structurally assigned ^1H, ^{15}N and ^{13}C NMR data (Supplemental Information, Tables S5, S6 and S7) were then used for 3D structure calculations.

Structure elucidation

Structures were calculated, using XPLOR-NIH (46) with implicit solvent and default force field. Distance constraints (with tolerances +20 %, -50 %) were derived from manually picked NOESY cross-peaks using CcpNmr Analysis (47). TALOS-N(48) was used to generate backbone dihedral angle restraints from H, C, N chemical shifts, whereas only predictions classified as "strong" were used. Also, a few *J*(NH,Hα) based restraints were applied for residues with coupling value > 8 Hz. There were no explicitly enforced hydrogen bonds. After the first rounds of structure calculation, several Cα–Cβ dihedral angle restraints were added, which were based on a combination of preliminary structure, NOE contacts and *J*-coupling values.

Starting with 100 randomly generated extended structures, the simulation protocol consisted of two rounds of simulated annealing. The first round of simulated annealing began with short molecular dynamics at 3 500 °C (variable integration time step, 1000 steps or 100 ps, whatever was met first), followed by slow cooling to 25 °C with the 1 °C step (at every temperature:

variable integration time step, 100 steps or 0.2 ps, whatever was met first).

The structure with the lowest constraint violation count was subsequently selected as the starting structure for the next round of simulated annealing. Distance restraint was considered as violated when the difference between the calculated and the experimental distance was more than 0.3 Å. The starting structure was simulated from 3 000 °C to 25 °C with 0.5 °C step (otherwise the same annealing protocol as the previous round). This was repeated with random starting velocities, yielding another 100 different protein conformations, from which 20 structures with no constraint violations were selected and sorted with respect to the force field energy combined with the energy of NOE term.

The atomic coordinates of Asp58-IGF-1 analog (pdb 6RVA) have been deposited in the Protein Data Bank (http://www.pdb.org/).

Structural modeling of the IR-insulin complex and equilibration of the system

Starting from the Cryo-EM structure (22), we remodeled the missing loops in the original structure, using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC). The remodeled loops contained residues 160-168 of the CR domain, 447-455 of the L2 domain, 824-843 of the FnIII-1' domain and the B-chain residues in insulin, N-terminal B1-B6 and the C-terminal B27-B30. Molecular dynamics simulations were carried out using Gromacs 5.1.2 (49) and the AMBER ff14SB force field (50). We continued with solvating the system with 32072 OPC3 waters (51) and adding Na, Cl ions to the 0.15 M concentration, with extra 10 Na ions added to neutralize the system. The system was minimized in 2 000 steps with steepest descent. We then heated only the remodeled part of the structure with the rest of the protein kept frozen, with simulated annealing with time increments of 10 ps and temperature increments of 50 K. The sampling of the remodeled loops was enhanced in this way by heating up to 600 K and cooling back to 300 K. The whole system was then equilibrated in an NpT ensemble at 300 K for 500 ps and the pressure of 1 bar. The cutoff distance for the non-bonded interactions was 1.4 nm, with electrostatics treated with PME and the van der Waals interactions with a simple cutoff. The ending structure of the wild-type insulin-IR complex was used as a starting structure for a 20 ns production run. For the production of insulin mutant-IR complexes, we used the mutagenesis plugin in PyMOL to introduce His residues (HIE, histidine with hydrogen on the epsilon nitrogen) at A-chain wild-type sites Ile10, Leu13 and Glu17 and the Thr residue at site Ser12. These complexes were then minimized and equilibrated for 500 ps, first in the NVT ensemble at 300 K, and then in the NpT ensemble to reach the pressure of 1 bar.

Metadynamics of insulin mutants to enhance sampling of the B-chain C-terminus detachment

The structures of insulin monomers were extracted from the minimized and equilibrated structures of insulin mutant-IR complexes described in the previous sections. The monomers were then neutralized by adding Na atoms (+1 for HisA17 mutant and +2 for all other neutral mutants and wild-type), solvating with OPC3 waters and adding Na, Cl ions up to 0.15 M concentration. We minimized the system with steepest descent and equilibrated for 500 ps in an NpT ensemble by gradually increasing the temperature in 50 K temperature and 10 ps time increments. The cutoff for non-bonded interactions was 1.4 nm, with PME and a simple cutoff treatment of the electrostatics and van der Waals interaction, respectively. The metadynamics production runs were performed using the PLUMED plugin (52) to Gromacs 5.1.2 (49). As collective variables to describe the B-chain C-terminus detachment, we chose the residues GlyB8-ProB28 and ValB12-TyrB26, which we denote as dwo_1 and dwo_2 respectively. These two distances were characterized as the most indicative of switching from the closed to wide-open state in a previous molecular dynamics study of wild-type insulin(32). In metadynamics, a history dependent potential $V_G(s,t)$ of Gaussian functions of the form $V_G(s,t) = \int_0^t dt' W \exp\left(-\sum_{i=1}^{d} \frac{(s_i(R)-s_i(R(t')))^2}{2\sigma_i^2}\right)$ is added to selected collected variables $s(R)$, where W is the energy rate and σ_i the width of the Gaussian potential for the i-th collective variable. We used the adaptive approach of determining the width based on the space for the CV covered in time (ADDAPTIVE=DIFF keyword for

PLUMED, from (40). The Gaussian potentials of 2.5 kJ mol^{-1} height were added every 150 steps. The free energy is reconstructed by summing the added Gaussian potentials, assuming $\lim_{t\to\infty} V_G(s,t) \sim -F(s)$. The metadynamics simulation was run for a total of 80 ns.

Estimation of free energy of strain upon binding for insulin from metadynamics free energy plots

The free energy of strain upon binding ΔF_{strain} is defined as the difference in free energies of insulin in the insulin-IR-bound state and free insulin in solution, $\Delta F_{strain} = F_{ins,bound} - F_{ins,free}$. The strain for insulin in the IR-bound state $F_{ins,bound}$ was estimated based on the projection of the (d_{wo1}, d_{wo2}) distances assumed in the 20 ns molecular dynamics run of the complex on to the free energy map $F(d_{wo1}, d_{wo2})$ obtained from metadynamics (see Figure 4). The strain for insulin free in solution was taken as the $F(d_{wo1}, d_{wo2})$ global minimum, resulting in $F_{ins,free} = 0$.

Differential contact map calculation

The frequency of inter-residue contact formation, defined for pairs of residues as the fraction of time spent in contact during the metadynamics run, was calculated using the CONAN plugin (53). We defined a contact between residues if their centers of mass were within 0.6 nm distance. To evaluate which contacts are formed more or less frequently in mutants compared to the wild-type, we calculated the difference between total interaction times $\Delta f_{ij} = f_{ij,\ wt} - f_{ij,\ mut}$, for the (i, j) residue pair. These are reported as differential contact maps in Figure S7.

Hydrogen Bond Analysis

The number of water-protein hydrogen bonds local to the point of mutation was determined for a shell of the radius 1 nm around the Cα (CA) atom of a mutated residue. With OH and NH groups regarded as donors and O and N atoms as acceptors, the donor-acceptor cutoff distance was set to 0.35 nm and the angle hydrogen-donor-acceptor to 30 °.

Supporting information - This article contains Tables S1-S7 and Figures S1-S8.

Funding - The research was supported by Medical Research Council Grants MR/K000179/1 and MR/R009066/1 and by European Regional Development Fund; OP RDE; Project: "Chemical biology for drugging undruggable targets (ChemBioDrug)" (No. CZ.02.1.01/0.0/0.0/16_019/0000729). Institutional support was provided by projects RVO 61388963 (for the Institute of Organic Chemistry and Biochemistry) and 68378050 (for the Institute of Molecular Genetics) of the Czech Academy of Sciences.

Acknowledgements - K.Ma., K.Ml., P.P., O.S. and M.Ch. thank the Charles University in Prague for Ph.D. scholarships.

Author contributions - J.J., L.Z., I.S., M.L. and P.H. conceived the study. J.J. and P.H. supervised the project. J.J., L.Z., I.S., M.L., P.H. and M.B. participated in research design. K.Ma., K.Ml., P.P., K.H., O.S., M.B., A.M., M.Ce., J.R., M.F., K.Mi., M.Ch., J.L., Y.Y., I.S. and L.Z. conducted the experiments. L.Z., I.S., J.J., M.L. and M.B. performed the data analysis. J.J., I.S., A.M., M.L. and M.B. wrote and contributed to the writing of the manuscript.

Conflict of Interest - The authors declare that they have no conflict of interest with the contents of this article.

References

1. De Meyts, P. (2004) Insulin and its receptor: structure, function and evolution. *Bioessays* **26**, 1351-1362
2. Le Roith, D. (2003) The insulin-like growth factor system. *Exp. Diabesity Res.* **4**, 205-212
3. Ward, C. W., and Lawrence, M. C. (2012) Similar but different: ligand-induced activation of the insulin and epidermal growth factor receptor families. *Curr. Opin. Struct. Biol.* **22**, 360-366
4. Lemmon, M. A., and Schlessinger, J. (2010) Cell signaling by receptor tyrosine kinases. *Cell* **141**, 1117-1134
5. Belfiore, A., Malaguarnera, R., Vella, V., Lawrence, M. C., Sciacca, L., Frasca, F., Morrione, A., and Vigneri, R. (2017) Insulin receptor isoforms in physiology and disease: An updated view. *Endocr. Rev.* **38**, 379-431
6. Slaaby, R. (2015) Specific insulin/IGF1 hybrid receptor activation assay reveals IGF1 as a more potent ligand than insulin. *Sci. Rep.* **5**, 7911
7. Jiracek, J., and Zakova, L. (2017) Structural perspectives of insulin receptor isoform-selective insulin analogs. *Front. Endocrinol.* **8**, 167
8. Clemmons, D. R. (2016) Role of IGF Binding proteins in regulating metabolism. *Trends Endocrin. Met.* **27**, 375-391
9. Kornfeld, S. (1992) Structure and function of the mannose 6-phosphate insulin-like growth factor-II receptors. *Ann. Rev. Biochem.* **61**, 307-330
10. Williams, C., Hoppe, H. J., Rezgui, D., Strickland, M., Forbes, B. E., Grutzner, F., Frago, S., Ellis, R. Z., Wattana-Amorn, P., Prince, S. N., Zaccheo, O. J., Nolan, C. M., Mungall, A. J., Jones, E. Y., Crump, M. P., and Hassan, A. B. (2012) An exon splice enhancer primes IGF2:IGF2R binding site structure and function evolution. *Science* **338**, 1209-1213
11. Brody, H. D., S.; Greenbaum, S.; Gravitz, L.; Mandrup-Poulsen, T.; Scully, T.; Dolgin, E.; Jonietz, E.; Shetty, P.; Edwards, S.P. (2012) Diabetes. *Nature* **485** *Suppl. to 17 May*, S1-S19
12. Gallagher, E. J., and LeRoith, D. (2010) Insulin, insulin resistance, obesity, and cancer. *Curr. Diabetes Rep.* **10**, 93-100
13. Craft, S. (2012) Insulin resistance and AD-extending the translational path. *Nat. Rev. Neurol.* **8**, 360-362
14. Kiselyov, V. V., Versteyhe, S., Gauguin, L., and De Meyts, P. (2009) Harmonic oscillator model of the insulin and IGF1 receptors' allosteric binding and activation. *Mol. Syst. Biol.* **5**, 243
15. Schaffer, L. (1994) A model for insulin binding to the insulin receptor. *Eur. J. Biochem.* **221**, 1127-1132
16. Menting, J. G., Whittaker, J., Margetts, M. B., Whittaker, L. J., Kong, G. K. W., Smith, B. J., Watson, C. J., Zakova, L., Kletvikova, E., Jiracek, J., Chan, S. J., Steiner, D. F., Dodson, G. G., Brzozowski, A. M., Weiss, M. A., Ward, C. W., and Lawrence, M. C. (2013) How insulin engages its primary binding site on the insulin receptor. *Nature* **493**, 241-245
17. Menting, J. G., Yang, Y. W., Chan, S. J., Phillips, N. B., Smith, B. J., Whittaker, J., Wickramasinghe, N. P., Whittaker, L. J., Pandyarajan, V., Wan, Z. L., Yadav, S. P., Carroll, J. M., Strokes, N., Roberts, C. T., Ismail-Beigi, F., Milewski, W., Steiner, D. F., Chauhan, V. S., Ward, C. W., Weiss, M. A., and Lawrence, M. C. (2014) Protective hinge in insulin opens to enable its receptor engagement. *Proc. Natl. Acad. Sc.i USA* **111**, E3395-E3404
18. Menting, J. G., Lawrence, C. F., Kong, G. K. W., Margetts, M. B., Ward, C. W., and Lawrence, M. C. (2015) Structural congruency of ligand binding to the insulin and insulin/Type 1 insulin-like growth factor hybrid receptors. *Structure* **23**, 1271-1282
19. Xu, Y. B., Kong, G. K. W., Menting, J. G., Margetts, M. B., Delaine, C. A., Jenkin, L. M., Kiselyov, V. V., De Meyts, P., Forbes, B. E., and Lawrence, M. C. (2018) How ligand binds to the type 1 insulin-like growth factor receptor. *Nat. Commun.* **9**, 821
20. De Meyts, P. (2015) Insulin/receptor binding: The last piece of the puzzle? *Bioessays* **37**, 389-397

21. Scapin, G., Dandey, V. P., Zhang, Z., Prosise, W., Hruza, A., Kelly, T., Mayhood, T., Strickland, C., Potter, C. S., and Carragher, B. (2018) Structure of the insulin receptor-insulin complex by single-particle cryo-EM analysis. *Nature* **556**, 122-125
22. Weis, F., Menting, J. G., Margetts, M. B., Chan, S. J., Xu, Y., Tennagels, N., Wohlfart, P., Langer, T., Muller, C. W., Dreyer, M. K., and Lawrence, M. C. (2018) The signalling conformation of the insulin receptor ectodomain. *Nat. Commun.* **9**, 4420
23. Gauguin, L., Klaproth, B., Sajid, W., Andersen, A. S., Mcneil, K. A., Forbes, B. E., and De Meyts, P. (2008) Structural basis for the lower affinity of the insulin-like growth factors for the insulin receptor. *J. Biol. Chem.* **283**, 2604-2613
24. Alvino, C. L., McNeil, K. A., Ong, S. C., Delaine, C., Booker, G. W., Wallace, J. C., Whittaker, J., and Forbes, B. E. (2009) A novel approach to identify two distinct receptor binding surfaces of insulin-like growth factor II. *J. Biol. Chem.* **284**, 7656-7664
25. Delaine, C., Alvino, C. L., McNeil, K. A., Mulhern, T. D., Gauguin, L., De Meyts, P., Jones, E. Y., Brown, J., Wallace, J. C., and Forbes, B. E. (2007) A novel binding site for the human insulin-like growth factor-II (IGF-II)/mannose 6-phosphate receptor on IGF-II. *J. Biol. Chem.* **282**, 18886-18894
26. Hexnerova, R., Krizkova, K., Fabry, M., Sieglova, I., Kedrova, K., Collinsova, M., Ullrichova, P., Srb, P., Williams, C., Crump, M. P., Tosner, Z., Jiracek, J., Veverka, V., and Zakova, L. (2016) Probing receptor specificity by sampling the conformational space of the insulin-like growth factor II C-domain. *J. Biol. Chem.* **291**, 21234-21245
27. Kosinova, L., Veverka, V., Novotna, P., Collinsova, M., Urbanova, M., Moody, N. R., Turkenburg, J. P., Jiracek, J., Brzozowski, A. M., and Zakova, L. (2014) Insight into the structural and biological relevance of the T/R transition of the N-terminus of the B-chain in human insulin. *Biochemistry* **53**, 3392-3402
28. Krizkova, K., Veverka, V., Maletinska, L., Hexnerova, R., Brzozowski, A. M., Jiracek, J., and Zakova, L. (2014) Structural and functional study of the GlnB22-insulin mutant responsible for maturity-onset diabetes of the young. *Plos One* **9**, 11, e112883
29. Machackova, K., Collinsova, M., Chrudinova, M., Selicharova, I., Picha, J., Budesinsky, M., Vanek, V., Zakova, L., Brzozowski, A. M., and Jiracek, J. (2017) Insulin-like growth factor 1 analogs clicked in the C domain: chemical synthesis and biological activities. *J. Med. Chem.* **60**, 10105–10117
30. Machackova, K., Chrudinova, M., Radosavljevic, J., Potalitsyn, P., Krizkova, K., Fabry, M., Selicharova, I., Collinsova, M., Brzozowski, A. M., Zakova, L., and Jiracek, J. (2018) Converting insulin-like growth factors 1 and 2 into high-affinity ligands for insulin receptor isoform A by the introduction of an evolutionarily divergent mutation. *Biochemistry* **57**, 2373-2382
31. Zakova, L., Kletvikova, E., Lepsik, M., Collinsova, M., Watson, C. J., Turkenburg, J. P., Jiracek, J., and Brzozowski, A. M. (2014) Human insulin analogues modified at the B26 site reveal a hormone conformation that is undetected in the receptor complex. *Acta Crystallogr. .D* **70**, 2765-2774
32. Papaioannou, A., Kuyucak, S., and Kuncic, Z. (2015) Molecular dynamics simulations of insulin: elucidating the conformational changes that enable its binding. *Plos One* **10**, 12, e0144058
33. Kristensen, C., Kjeldsen, T., Wiberg, F. C., Schaffer, L., Hach, M., Havelund, S., Bass, J., Steiner, D. F., and Andersen, A. S. (1997) Alanine scanning mutagenesis of insulin. *J. Biol. Chem.* **272**, 12978-12983
34. Gauguin, L., Delaine, C., Alvino, C. L., Mcneil, K. A., Wallace, J. C., Forbes, B. E., and De Meyts, P. (2008) Alanine scanning of a putative receptor binding surface of insulin-like growth factor-I. *J. Biol. Chem.* **283**, 20821-20829
35. Mynarcik, D. C., Williams, P. F., Schaffer, L., Yu, G. Q., and Whittaker, J. (1997) Analog binding properties of insulin receptor mutants. Identification of amino acids interacting with the COOH terminus of the B-chain of the insulin molecule. *J. Biol. Chem.* **272**, 2077-2081
36. Lawrence, M. C., McKern, N. M., and Ward, C. W. (2007) Insulin receptor structure and its implications for the IGF-1 receptor. *Curr. Opin. Struct. Biol.* **17**, 699-705

37. Ward, C. W., Menting, J. G., and Lawrence, M. C. (2013) The insulin receptor changes conformation in unforeseen ways on ligand binding: Sharpening the picture of insulin receptor activation. *Bioessays* **35**, 945-954
38. Jiracek, J., Zakova, L., Antolikova, E., Watson, C. J., Turkenburg, J. P., Dodson, G. G., and Brzozowski, A. M. (2010) Implications for the active form of human insulin based on the structural convergence of highly active hormone analogues. *Proc. Natl. Acad. Sci. USA* **107**, 1966-1970
39. Zakova, L., Kletvikova, E., Veverka, V., Lepsik, M., Watson, C. J., Turkenburg, J. P., Jiracek, J., and Brzozowski, A. M. (2013) Structural integrity of the B24 site in human insulin is important for hormone functionality. *J. Biol. Chem.* **288**, 10230-10240
40. Branduardi, D., Bussi, G., and Parrinello, M. (2012) Metadynamics with adaptive Gaussians. *J. Chem. Theory Comput.* **8**, 2247-2254
41. De Meyts, P. (1994) The structural basis of insulin and insulin-like growth factor-I receptor binding and negative co-operativity, and its relevance to mitogenic versus metabolic signalling. *Diabetologia* **37** *Suppl. 2*, S135-S148
42. Gutmann, T., Schaeffer, I. B., C., P., Brankatschk, B., Vattulainen, I., Strauss, M., and Coskun, U. (2019) Cryo-EM structure of the complete and ligand-saturated insulin receptor ectodomain. bioRxiv 679233; doi: https://doi.org/10.1101/679233
43. Uchikawa, E., Choi, E., Shang, G. J., Yu, H. T., and Bai, X. C. (2019) Activation mechanism of the insulin receptor revealed by cryo-EM structure of the fully liganded receptor-ligand complex. *eLife* **8**, e48630
44. Vikova, J., Collinsova, M., Kletvikova, E., Budesinsky, M., Kaplan, V., Zakova, L., Veverka, V., Hexnerova, R., Avino, R. J. T., Strakova, J., Selicharova, I., Vanek, V., Wright, D. W., Watson, C. J., Turkenburg, J. P., Brzozowski, A. M., and Jiracek, J. (2016) Rational steering of insulin binding specificity by intra-chain chemical crosslinking. *Sci. Rep.* **6**, 19431
45. Krizkova, K., Chrudinova, M., Povalova, A., Selicharova, I., Collinsova, M., Vanek, V., Brzozowski, A. M., Jiracek, J., and Zakova, L. (2016) Insulin-insulin-like growth factors hybrids as molecular probes of hormone:receptor binding specificity. *Biochemistry* **55**, 2903-2913
46. Schwieters, C. D., Kuszewski, J. J., Tjandra, N., and Clore, G. M. (2003) The Xplor-NIH NMR molecular structure determination package. *J. Magn. Reson.* **160**, 65-73
47. Vranken, W. F., Boucher, W., Stevens, T. J., Fogh, R. H., Pajon, A., Llinas, P., Ulrich, E. L., Markley, J. L., Ionides, J., and Laue, E. D. (2005) The CCPN data model for NMR spectroscopy: Development of a software pipeline. *Proteins* **59**, 687-696
48. S Shen, Y., and Bax, A. (2013) Protein backbone and sidechain torsion angles predicted from NMR chemical shifts using artificial neural networks. *J. Biomol. NMR* **56**, 227-241
49. Van der Spoel, D., Lindahl, E., Hess, B., Groenhof, G., Mark, A. E., and Berendsen, H. J. C. (2005) GROMACS: Fast, flexible, and free. *J. Comput. Chem.* **26**, 1701-1718
50. Maier, J. A., Martinez, C., Kasavajhala, K., Wickstrom, L., Hauser, K. E., and Simmerling, C. (2015) ff14SB: Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *J. Chem. Theory Comput.* **11**, 3696-3713
51. Izadi, S., Anandakrishnan, R., and Onufriev, A. V. (2014) Building Water Models: A Different Approach. *J. Phys. Chem. Lett.* **5**, 3863-3871
52. Bonomi, M., Branduardi, D., Bussi, G., Camilloni, C., Provasi, D., Raiteri, P., Donadio, D., Marinelli, F., Pietrucci, F., Broglia, R. A., and Parrinello, M. (2009) PLUMED: A portable plugin for free-energy calculations with molecular dynamics. *Comput. Phys. Commun.* **180**, 1961-1972
53. Mercadante, D., Grater, F., and Daday, C. (2018) CONAN: A Tool to Decode Dynamical Information from Molecular Interaction Maps. *Biophys. J.* **114**, 1267-1273

Table 1. Simplified overview of relative receptor-binding affinities of insulin, IGF-1 and IGF-2 analogs. The relative binding affinities are shown in % of the native hormone, which has 100 % binding affinity for the specific receptor (i.e. insulin analogs are related to human insulin, IGF-1 analogs to IGF-1, etc.). The approximate major trends in binding affinities of the analogs are indicated by arrows: the symbol ↑ means >170 %, ↗ 170-130%, → 130-70 %, ↘ 70-30 %, and ↓ < 30 % of binding affinity of the native hormone (100 %). Numbers in parentheses show mean K_d values, nd is not determined. Asterisks indicate that binding of the ligand to the receptor by the ligand differs significantly from that of the native hormone (*p<0.05; **p<0.01; ***p< 0.001). Details are provided in Tables S1-S3. The results of production are related (in %) to native hormones. The typical approximate yield for standard chemical synthesis of insulin (starting with 100 µmol of resin) was about 1 mg. The typical yield for IGF-1 or IGF-2 production from 1 l of media was about 0.4 mg or 0.3 mg, respectively.

Position in the native hormone	Analog	Result of production (%)	Binding affinity (in % of the native hormone) for		
			IR-A	IR-B	IGF-1R
IleA10-insulin	ValA10-insulin	30	→ (73)	↘ (53)	nd
	HisA10-insulin	100	↓ **(16)	↓ ***(26)	↓ *(25)
Ser51-IGF-1	Thr51-IGF-1	100	↑ (220)	↘ (67)	→ (120)
	His51-IGF-1	100	→ (70)	↘ (53)	→ (109)
Ser50-IGF-2	Thr50-IGF-2	100	→ (78)	↗ (153)	↑ (264)
	His50-IGF-2	100	→ (100)	↘ (68)	↗ (136)
SerA12-insulin	ThrA12-insulin	80	→ (107)	↘ **(31)	↘ (63)
	HisA12-insulin	0	nd	nd	nd
Asp53-IGF-1	Glu53-IGF-1	0	nd	nd	nd
	His53-IGF-1	0	nd	nd	nd
Asp52-IGF-2	Glu52-IGF-2	0	nd	nd	nd
	His52-IGF-2	0	nd	nd	nd
LeuA13-insulin	ValA13-insulin	30	→ (91)	↘ **(40)	nd
	HisA13-insulin	80	↓ **(15)	↓ ***(14)	↗ (138)
Leu54-IGF-1	Val54-IGF-1	100	↗ (160)	↗ (167)	→ (104)
	His54-IGF-1	100	↗ (150)	→ (100)	↘ (63)
Leu53-IGF-2	Val53-IGF-2	100	→ (72)	↘ *(37)	↗ (145)
	His53-IGF-2	100	↘ *(51)	→ (84)	↑ (182)
GluA17-insulin	AspA17-insulin	0	nd	nd	nd
	HisA17-insulin	10	↓ ***(7)	nd	nd
Glu58-IGF-1	Asp58-IGF-1	100	→ (75)	↘ (53)	↘ **(31)
	His58-IGF-1	100	↓ **(20)	↘ (43)	↓ *(7)
Glu57-IGF-2	Asp57-IGF-2	100	→ (106)	↗ *(166)	↑ (200)
	His57-IGF-2	0	nd	nd	nd

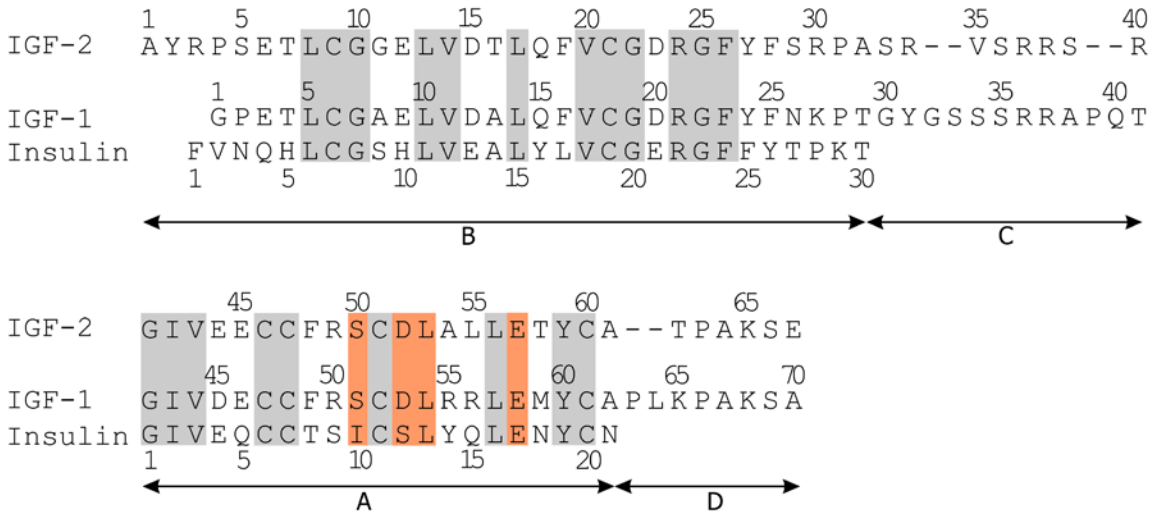


Figure 1. **Primary sequences of human insulin, IGF-1 and IGF-2.** The residues mutated in this study are highlighted with an orange background and homologous residues with a gray background.

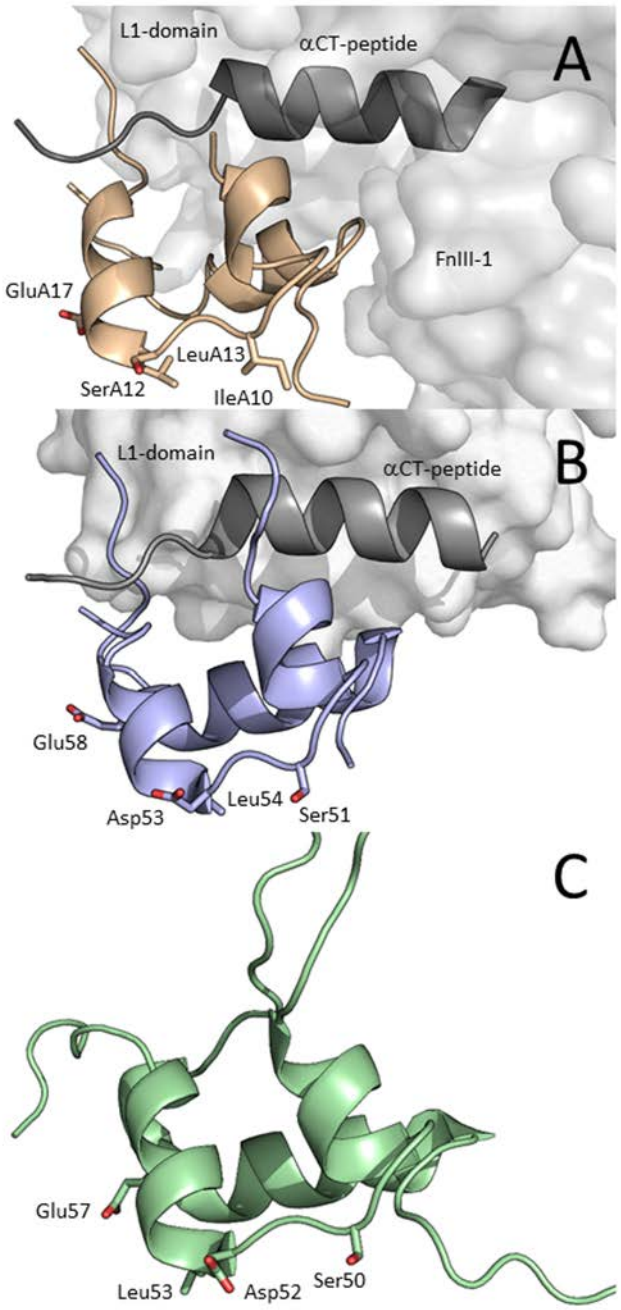


Figure 2. Receptor-bound structures of insulin and IGF-1 and NMR structure of IGF-2. A. CryoEM structure of IR-A-bound insulin (pdb 6HN5 from (22), in light brown). Receptor site 1' is represented by L1-domain (light gray) and αCT-peptide (dark gray) and receptor site 2' by FnIII-1 domain (light gray). **B.** Crystal structure of IGF-1 (pdb 5U8Q from (19), in violet) bound to L1 domain (in light gray) and αCT (in dark gray) representing site 1' of IGF-1R. **C.** NMR structure of human IGF-2 (pdb 5L3L from (26), in green). The side chains of residues modified in this study are shown as sticks and are numbered.

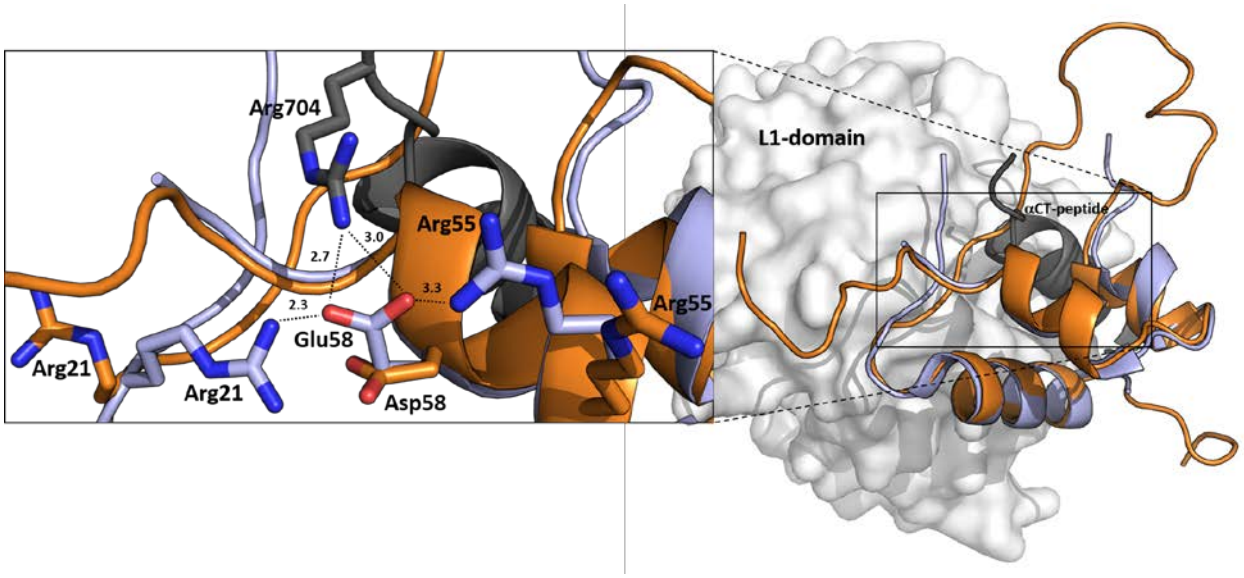


Figure 3. An overlay of IGF-1R-bound human IGF-1 with Asp58-IGF-1. Human IGF-1 is in light blue (pdb 5U8Q, from (19)) and a representative (lowest energy) NMR structure of Asp58-IGF-1 is in orange (pdb 6RVA). The receptor site 1' is represented by L1 domain (in gray) and α-CT peptide (in black). The enlarged window on the left shows side chains of hormones' Glu58 or Asp58, Arg704 (from α-CT) and two other IGF-1 arginines (Arg21 and Arg55) as sticks with nitrogen atoms in blue and oxygen atoms in red. Some possible interactions of Glu58 and Arg704, Arg21 and Arg55 residues identified in the complex are indicated by dashed lines with distances in Å.

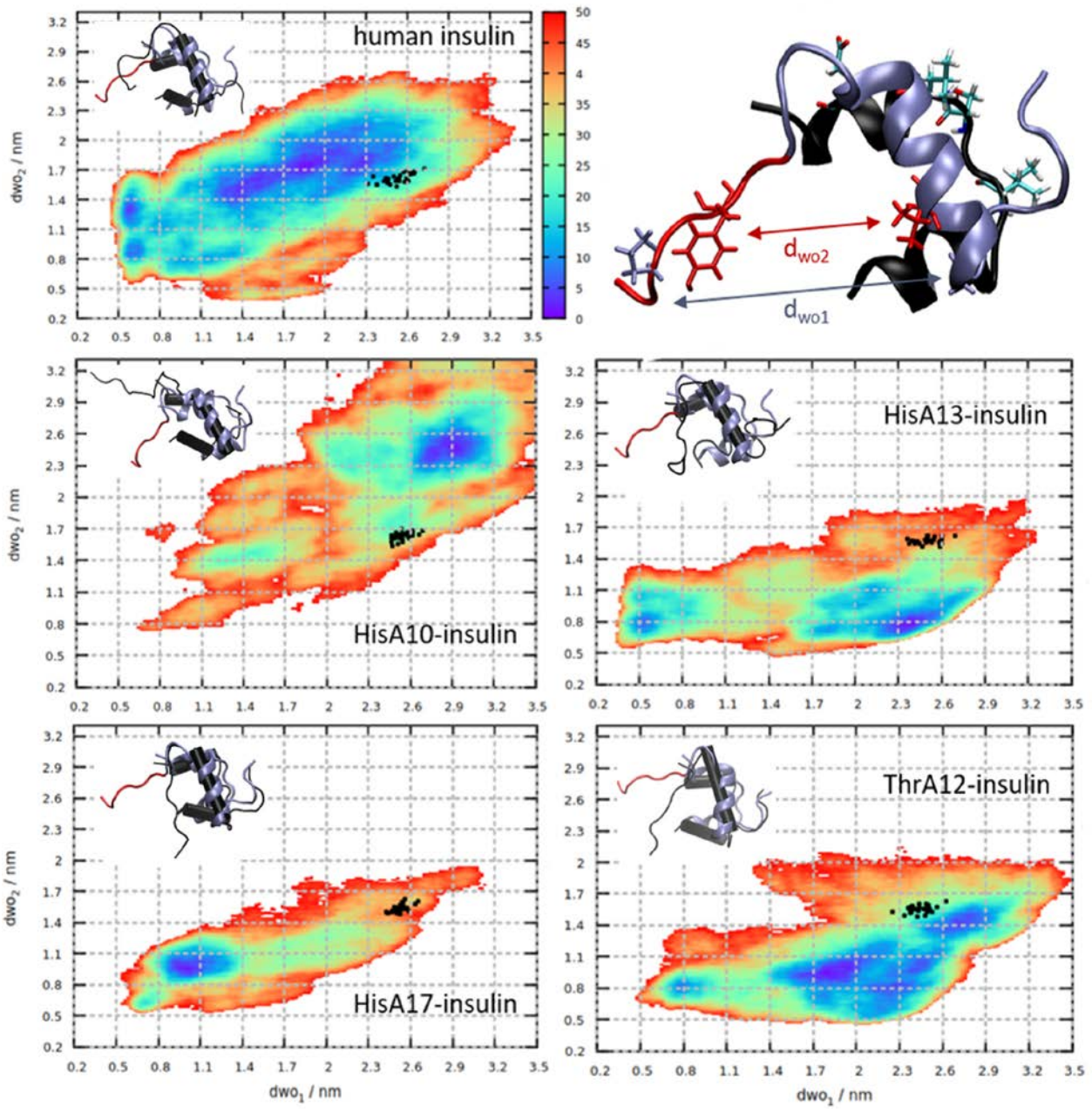


Figure 4. Free energy profiles (color scale in kJ/mol) of human insulin and analogs obtained from metadynamics. Black dots represent insulin conformations from molecular dynamics simulations in complex with IR. The B-chain of representative minimal energy conformers of insulin mutants (in black) aligned to the IR-bound conformation of human insulin (ice blue, C-terminus in red) are depicted in the insets. The IR-bound conformation of human insulin is shown on the upper right, with residues defining the dwo_1 (GlyB8-ProB28) and dwo_2 (ValB12-TyrB26) distances, represented as ice blue and red licorice, respectively. The A-chain is in black, with the mutated residues (IleA10, SerA12, LeuA13, GluA17) shown in licorice and colored by atom type.

SUPPORTING INFORMATION

Mutations at hypothetical binding Site 2 in insulin and insulin-like growth factors 1 and 2 elicit receptor- and hormone-specific responses

Macháčková *et al*.

Table of contents

Table S1. Receptor-binding affinities of human insulin, IGF-1, IGF-2 and analogs for human IR-A in membranes of IM-9 lymphocytes and relative abilities of the hormones/analogs to stimulate phosphorylation of human IR-A in membranes of transfected mouse fibroblasts (details are provided in Methods). The K_d values or relative stimulations were obtained from at least three measurements. (n) is number of replicates. Asterisks indicate that binding of the ligand to the receptor or activation of the receptor by the ligand differs significantly from that of the native hormone (*p<0.05; **p<0.01; ***p< 0.001).

Native hormone or analog	K_d ± S.D. for human IR-A [nM] (n)	Relative binding affinity [a] for human IR-A [%]		Relative stimulation of human IR-A [%]	
Insulin	0.12 ± 0.04 (3)¶	100 ± 33 [b]		100 ± 8	
	0.32 ± 0.10 (4)"	100 ± 31			
	0.30 ± 0.13 (5)€	100 ± 43			
	0.27 ± 0.01 (5)#	100 ± 4			
	0.18 ± 0.01 (4)$	100 ± 6			
	0.25 ± 0.05 (5)^	100 ± 20			
IGF-1	24.0 ± 11.5 (3)^	1.0 ± 0.5 [c]	100 ± 48 [d]	27 ± 10	100 ± 37 [d]
IGF-2	2.9 ± 0.2 (3)^	8.6 ± 1.8	100 ± 7	63 ± 15	100 ± 24
ValA10-insulin	0.44 ± 0.06 (3)"	73 ± 25		74 ± 9*	
HisA10-insulin	0.74 ± 0.24 (4)¶	16 ± 7.5 **		41 ± 11***	
Thr51-IGF-1	8.1 ± 4.8 (4)$	2.2 ± 1.3	220 ± 170	33 ± 10	122 ± 58
His51-IGF-1	38.7 ± 2.75 (3)#	0.7 ± 0.06	70 ± 35	36 ± 12	133 ± 66
Thr50-IGF-2	2.7 ± 0.2 (3)$	6.7 ± 0.6	78 ± 18	68 ± 19	108 ± 40
His50-IGF-2	2.1 ± 0.2 (3)$	8.6 ± 0.9	100 ± 23	70 ± 17	111 ± 38
ThrA12-insulin	0.28 ± 0.11 (3)€	107 ± 62		93 ± 17	
ValA13-insulin	0.35 ± 0.07 (4)"	91 ± 34		71 ± 7*	
HisA13-insulin	2.0 ± 1.0 (3)€	15 ± 9**		33 ± 9***	
Val54-IGF-1	11.2 ± 5.4 (3)$	1.6 ± 0.8	160 ± 113	27 ± 11	100 ± 55
His54-IGF-1	12.2 ± 1.5 (3)$	1.5 ± 0.2	150 ± 78	19 ± 4	70 ± 30
Val53-IGF-2	2.9 ± 0.2 (3)$	6.2 ± 0.5	72 ± 16	40 ± 16	63 ± 29*
His53-IGF-2	4.1 ± 1.5 (3)$	4.4 ± 1.6	51 ± 21*	33 ± 11	52 ± 21**
HisA17-insulin	3.8 ± 0.3 (3)#	7.1±0.6***		0.2 ± 0.2***	
Asp58-IGF-1	24.1 ± 2.8 (3)$	0.75 ± 0.1	75 ± 39	30 ± 16	111 ± 72
His58-IGF-1	91 ± 40 (3)$	0.2 ± 0.09	20 ± 13**	4.8 ± 2.3	18±11***
Asp57-IGF-2	2.96 ± 0.28 (3)#	9.1 ± 0.9	106 ± 24	72 ± 17	115 ± 39

[a] Relative binding affinity is defined as (K_d of the native hormone / K_d of analog) x 100 (%).

[b] The K_d of human insulin for IR-A was determined in six independent series of measurements (indexed with ¶, ", €, #, $ and ^).

[c] The individual K_d values of ligands in this column are relative to a corresponding native insulin K_d value (e.g. $ to $, etc.)

[d] In these columns for each IGF analogue their values relative to insulin are then expressed relative to native IGF-1 (100%) for IGF-1 analogs or relative to native IGF-2 (100%) for IGF2 analogs.

Table S2. Receptor-binding affinities of human insulin, IGF-1, IGF-2 and analogs for human IR-B and relative abilities of the hormones/analogs to stimulate phosphorylation of this receptor in membranes of transfected mouse fibroblasts (details are provided in Methods). The K_d values or relative stimulations were obtained from at least three measurements. (n) is number of replicates. Asterisks indicate that binding of the ligand to the receptor or activation of the receptor by the ligand differs significantly from that of the native hormone (*p<0.05; **p<0.01; *** p<0.001).

Native hormone or analog	K_d ± S.D. for human IR-B [nM] (n)	Relative binding affinity [a] for human IR-B [%]		Relative stimulation of human IR-B [%]	
Insulin	0.31 ± 0.08 (4)€ 0.35 ± 0.06 (3)" 0.39 ± 0.23 (6)$ 0.38 ± 0.10 (4)# 0.68 ± 0.28 (5)^	100 ± 26 [b] 100 ± 17 100 ± 59 100 ± 26 100 ± 41		100 ± 4	
IGF-1	224 ± 33 (4)^	0.3 ± 0.13 [c]	100 ± 43 [d]	11 ± 5.2	100 ± 47 [d]
IGF-2	35.4 ± 11.2 (4)	1.9 ± 1	100 ± 52	20 ± 7.4	100 ± 37
ValA10-insulin	0.66 ± 0.37 (4)"	53 ± 31		93 ± 15	
HisA10-insulin	1.2 ± 0.3 (3)€	26 ± 9***		46 ± 13***	
Thr51-IGF-1	173 ± 18 (3)$	0.2 ± 0.12	67 ± 49	nd	
His51-IGF-1	245 ± 130 (3)$	0.16 ± 0.13	53 ± 49	10 ± 6	91 ± 69
Thr50-IGF-2	13.3 ± 4.3 (3)$	2.9 ± 2	153 ± 132	19 ± 4	96 ± 49
His50-IGF-2	28.9 ± 14.7 (3)$	1.3 ± 1	68 ± 63	25 ± 5	123 ± 52
ThrA12-insulin	1.22 ± 0.30 (3)#	31 ± 11**		63 ± 17*	
ValA13-insulin	0.94 ± 0.15 (3)#	40 ± 12**		60 ± 6**	
HisA13-insulin	2.64 ± 0.61 (3)#	14 ± 4.9***		29 ± 10***	
Val54-IGF-1	78 ± 37 (3)$	0.5 ± 0.38	167 ± 146	13 ± 7	116 ± 83
His54-IGF-1	143 ± 93 (4)$	0.3 ± 0.26	100 ± 97	12 ± 4	111 ± 64
Val53-IGF-2	58 ± 25 (3)$	0.7 ± 0.5	37 ± 33*	14 ± 2	69 ± 27
His53-IGF-2	24.9 ± 9.9 (3)$	1.6 ± 1.1	84 ± 73	6.7 ± 1	33 ± 13**
HisA17-insulin	nd			nd	
Asp58-IGF-1	250 ± 223 (2)$	0.16 ± 0.17	53 ± 61	8 ± 4	73 ± 50
His58-IGF-1	311 ± 74 (3)$	0.13 ± 0.08	43 ± 32	7.8 ± 2.9	71 ± 43
Asp57-IGF-2	9.8 ± 2.8 (6)€	3.16 ± 1.2	166 ± 107*	27 ± 11	135 ± 74

[a] Relative binding affinity is defined as (K_d of the native hormone / K_d of analog) x 100 (%).

[b] The K_d of human insulin for IR-B was determined in five independent series of measurements (indexed with €, ", $, # and ^).

[c] The individual K_d values of ligands in this column are relative to a corresponding native insulin K_d value (e.g. € to €, etc.).

[d] In these columns for each IGF analogue their values relative to insulin are then expressed relative to native IGF-1 (100%) for IGF-1 analogs or relative to native IGF-2 (100%) for IGF2 analogs.

Table S3. Receptor-binding affinities of human insulin, IGF-1, IGF-2 and analogs for human IGF-1R and relative abilities of the hormones/analogs to stimulate phosphorylation of this receptor in membranes of transfected mouse fibroblasts (details are provided in Methods). The K_d values or relative stimulations were obtained from at least three measurements. (n) is number of replicates. Asterisks indicate that binding of the ligand to the receptor or activation of the receptor by the ligand differs significantly from that of the native hormone (*p<0.05; **p<0.01; *** p<0.001).

Native hormone or analog	K_d ± S.D. for human IGF-1R [nM] (n)	Relative binding affinity [a] for human IGF-1R [%]		Relative stimulation of human IGF-1R [%]	
IGF-1	0.11 ± 0.05 (5)#	100 ± 45 [b]		100 ± 21	
	0.24 ± 0.11 (5)$	100 ± 46			
	0.24 ± 0.10 (5)^	100 ± 42			
	0.25 ± 0.09 (4)€	100 ± 36			
	0.19 ± 0.08 (5)"	100 ± 42			
insulin	292 ± 54 (3)^	0.08 ± 0.036 [c]	100 ± 45 [d]	2 ± 0.1***	100 ± 5 [d]
IGF-2	2.3 ± 1.2 (3)€	11 ± 7	100 ± 63	61 ± 17**	100 ± 28
ValA10-insulin	nd			1.6 ± 0.2	80 ± 11
HisA10-insulin	451 ± 53 (3)#	0.02 ± 0.009	25 ± 16*	1.0 ± 0.3	50 ± 15
Thr51-IGF-1	0.20 ± 0.05 (4)$	120 ± 63		83 ± 15	
His51-IGF-1	0.22 ± 0.01 (4)€	109 ± 50		98 ± 30	
Thr50-IGF-2	0.84 ± 0.17 (4)$	29 ± 14	264 ± 210	60 ± 17	98 ± 39
His50-IGF-2	1.6 ± 0.8 (4)$	15 ± 10	136 ± 125	64 ± 21	105 ± 45
ThrA12-insulin	372 ± 174 (4)"	0.05 ± 0.03	63 ± 47	1.6 ± 0.4	80 ± 20
ValA13-insulin	nd			2.0 ± 0.4	100 ± 21
HisA13-insulin	167 ± 44 (4)"	0.11 ± 0.05	138 ± 88	1.4 ± 0.6	70 ± 30
Val54-IGF-1	0.23 ± 0.04 (3)$	104 ± 51		66 ± 16*	
His54-IGF-1	0.38 ± 0.08 (3)$	63 ± 32		73 ± 14	
Val53-IGF-2	1.5 ± 0.3 (3)$	16 ± 8	145 ± 117	45 ± 15	74 ± 32
His53-IGF-2	1.2 ± 0.4 (4)$	20 ± 11	182 ± 152	46 ± 20	75 ± 39
HisA17-insulin	nd			0	0
Asp58-IGF-1	0.80 ± 0.26 (3)€	31.3 ± 15**		99 ± 32	
His58-IGF-1	3.3 ± 0.2 (3)$	7.3 ± 3.4*		60 ± 15**	
Asp57-IGF-2	0.5 ± 0.2 (4)#	22 ± 13	200 ± 173	73 ± 23	120 ± 50

[a] Relative binding affinity is defined as (K_d of the native hormone / K_d of analog) x 100 (%).

[b] The K_d of human IGF-1 for IR-IGF-1R was determined in five independent series of measurements (indexed as #, $, ^, € and ").

[c] The individual K_d values of ligands in this column are relative to a corresponding native IGF-1 K_d value (e.g. # to #, etc.).

[d] In these columns for each IGF analogue their values relative to insulin are then expressed relative to native IGF-1 (100%) for IGF-1 analogs or relative to native IGF-2 (100%) for IGF2 analogs.

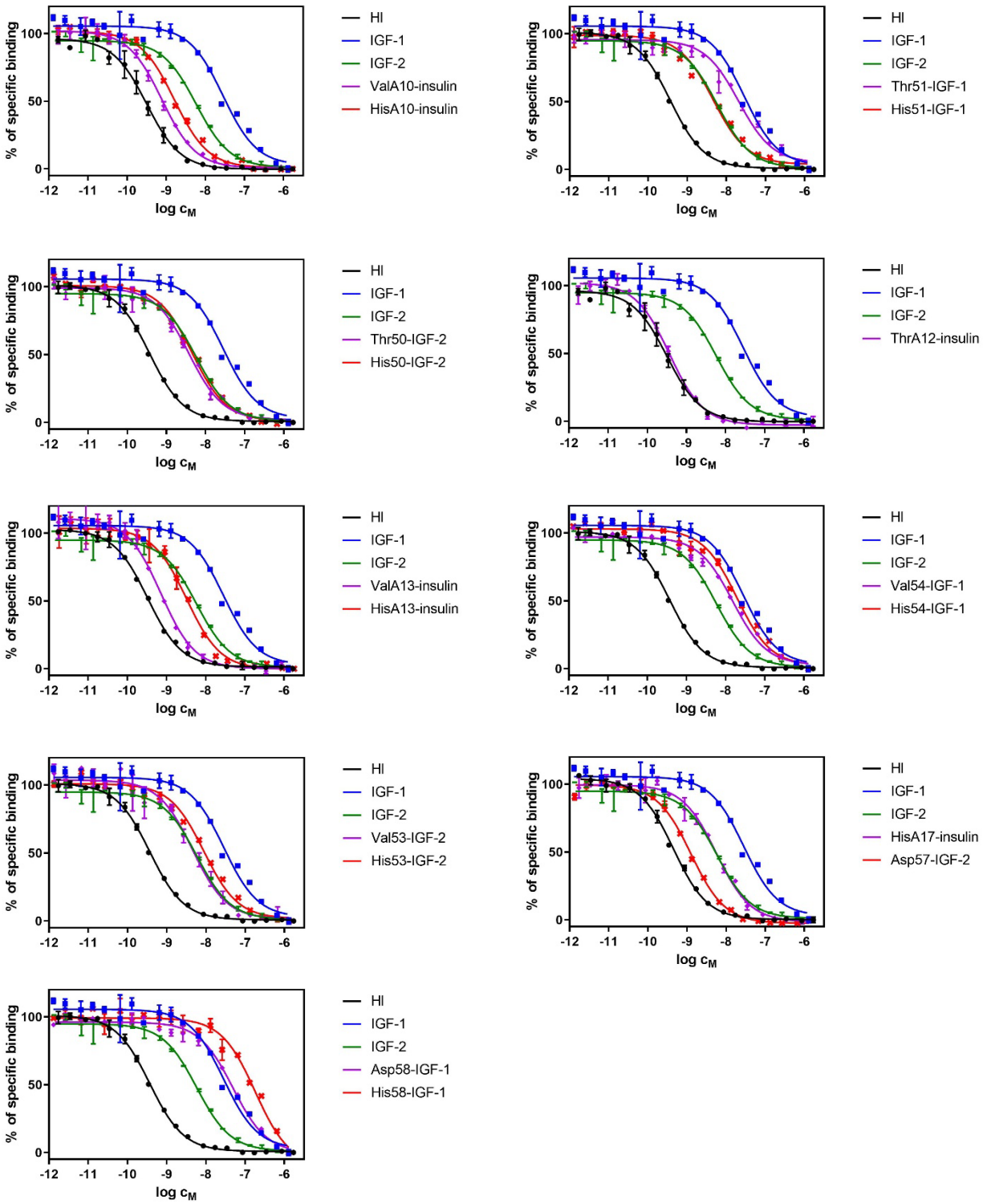


Figure S1. Binding curves for IR-A. Inhibition of binding of human [^{125}I]-monoiodotyrosyl-insulin to IR-A by human insulin (HI), IGF-1, IGF-2 and analogs. Representative binding curve for each hormone or analog is shown.

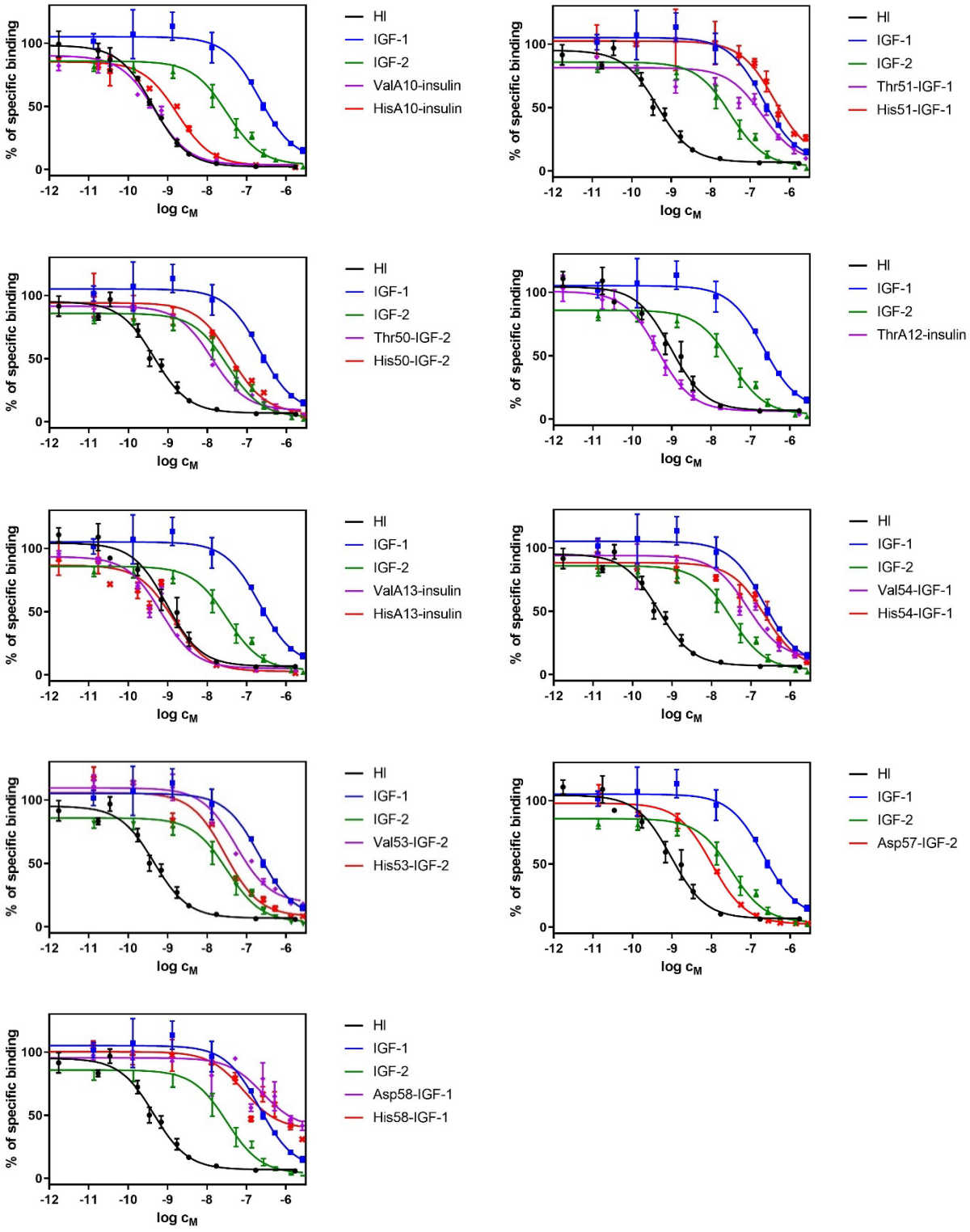


Figure S2. Binding curves for IR-B. Inhibition of binding of human [^{125}I]-monoiodotyrosyl-insulin to IR-B by human insulin (HI), IGF-1, IGF-2 and analogs. Representative binding curve for each hormone or analog is shown.

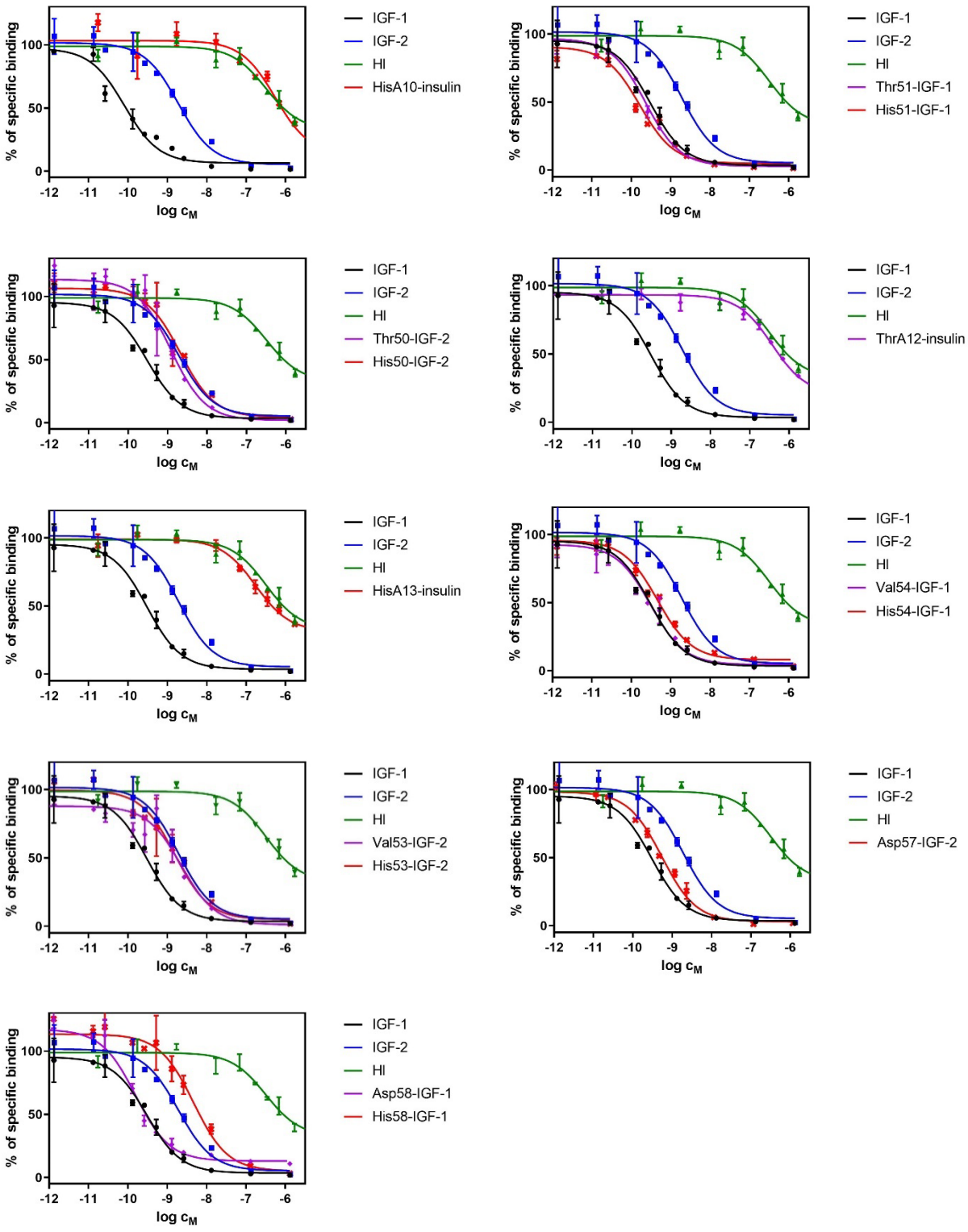


Figure S3. Binding curves for IGF-1R. Inhibition of binding of human [^{125}I]-iodotyrosyl-IGF-1 to IGF-1R by human insulin (HI), IGF-1, IGF-2 and analogs. Representative binding curve for each hormone or analog is shown.

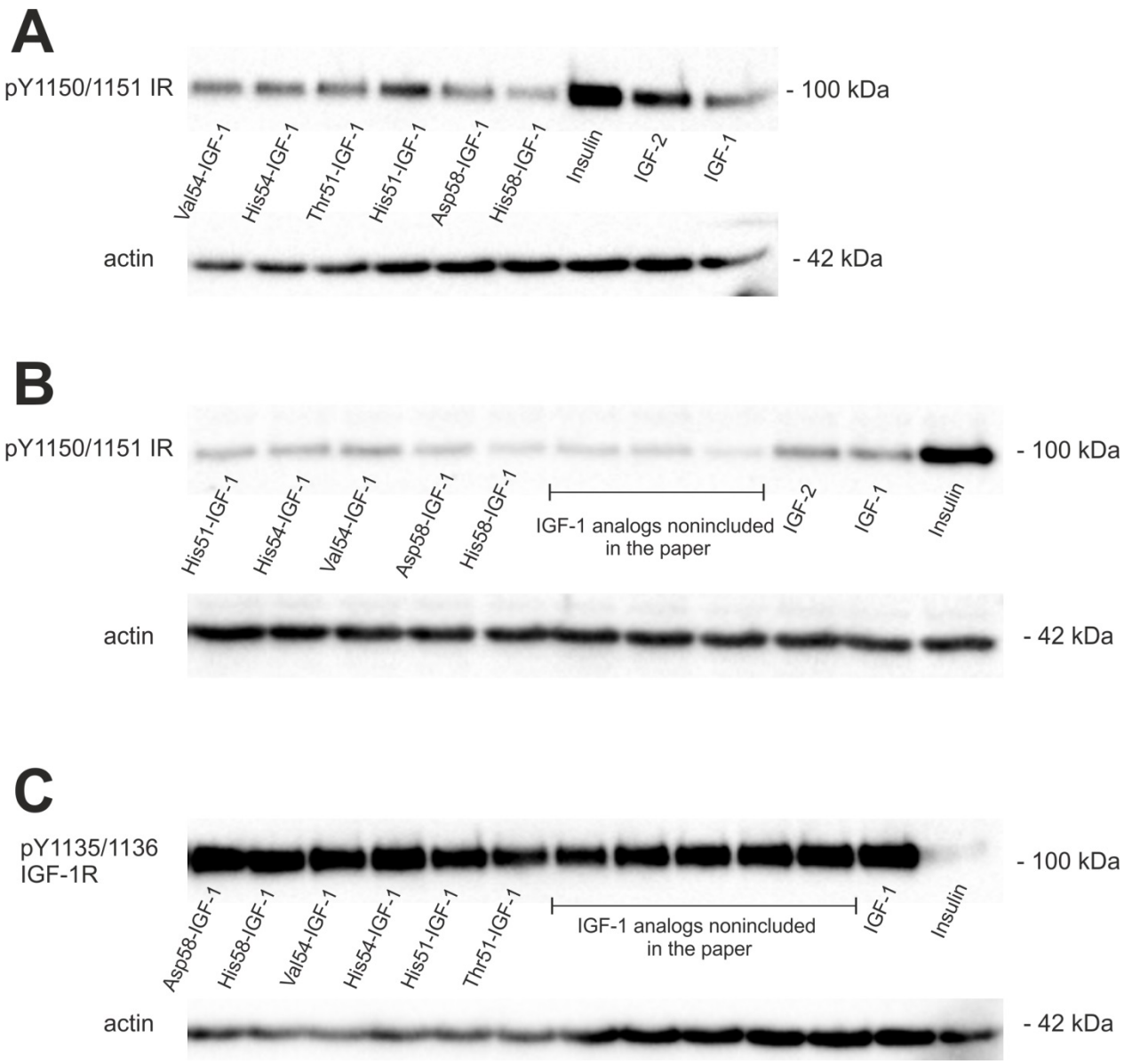


Figure S4. Representative Western blots for relative abilities of IGF-1 analogs to stimulate receptors' phosphorylation. A. IR-A transfected fibroblasts. **B.** IR-B transfected fibroblasts. **C.** IGF-1R transfected fibroblasts. Cells were stimulated with 10 nM ligands for 10 min. Membranes were cut at 75 kDa and 50 kDa standards and respective parts were developed with anti-phospho-IGF-1Rβ (Tyr1135/1136)/IRβ (Tyr1150/1151) antibody (Mr above 75 kDa) and with anti-actin antibody (Mr bellow 50 kDa). In some cases, cells were also stimulated with analogs that were not discussed in the manuscript.

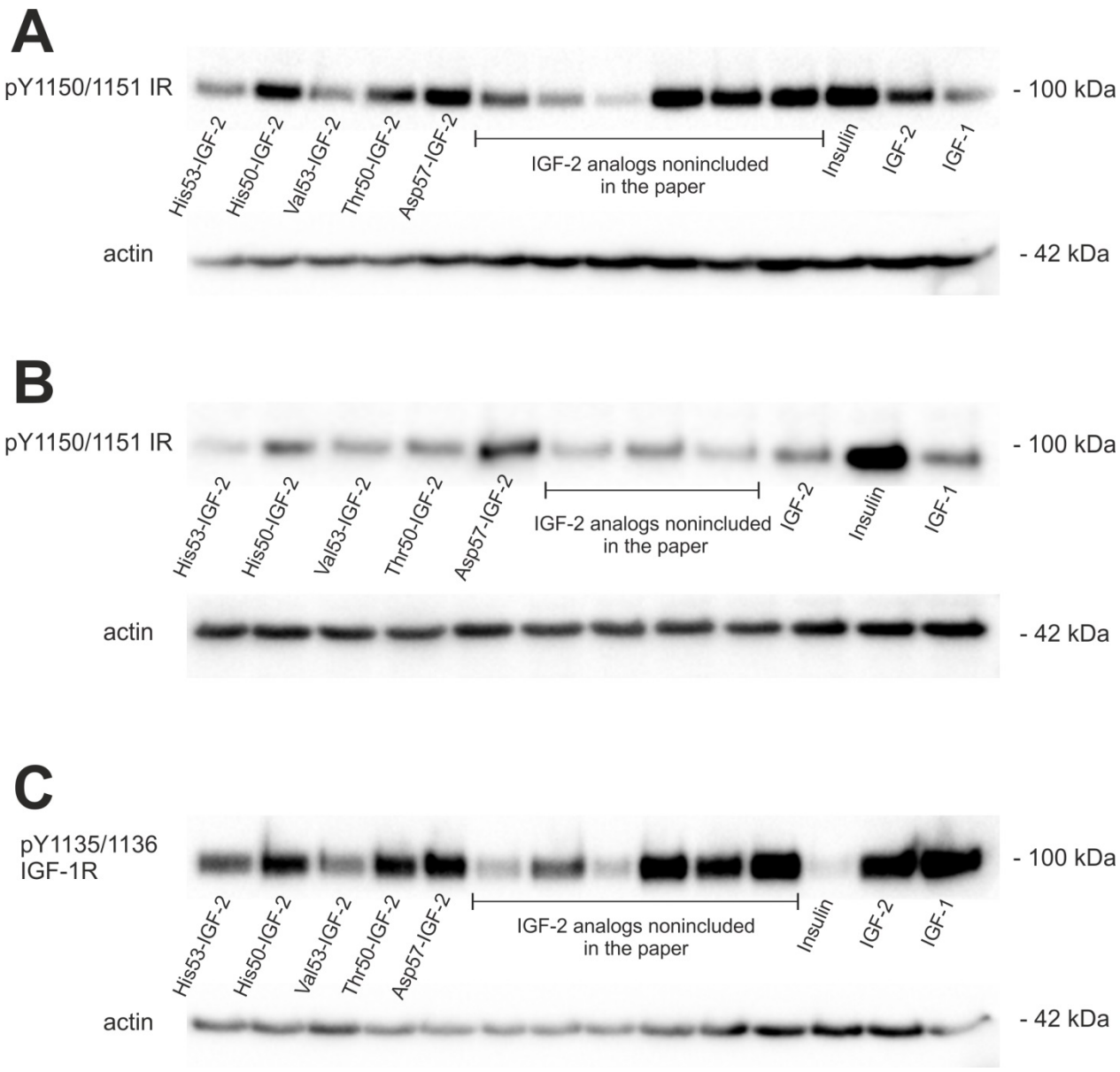


Figure S5. Representative Western blots for relative abilities of IGF-2 analogs to stimulate receptors' phosphorylation. A. IR-A transfected fibroblasts. **B.** IR-B transfected fibroblasts. **C.** IGF-1R transfected fibroblasts. Cells were stimulated with 10 nM ligands for 10 min. Membranes were cut at 75 kDa and 50 kDa standards and respective parts were developed with anti-phospho-IGF-1Rβ (Tyr1135/1136)/IRβ (Tyr1150/1151) antibody (Mr above 75 kDa) and with anti-actin antibody (Mr bellow 50 kDa). In some cases, cells were also stimulated with analogs that were not discussed in the manuscript.

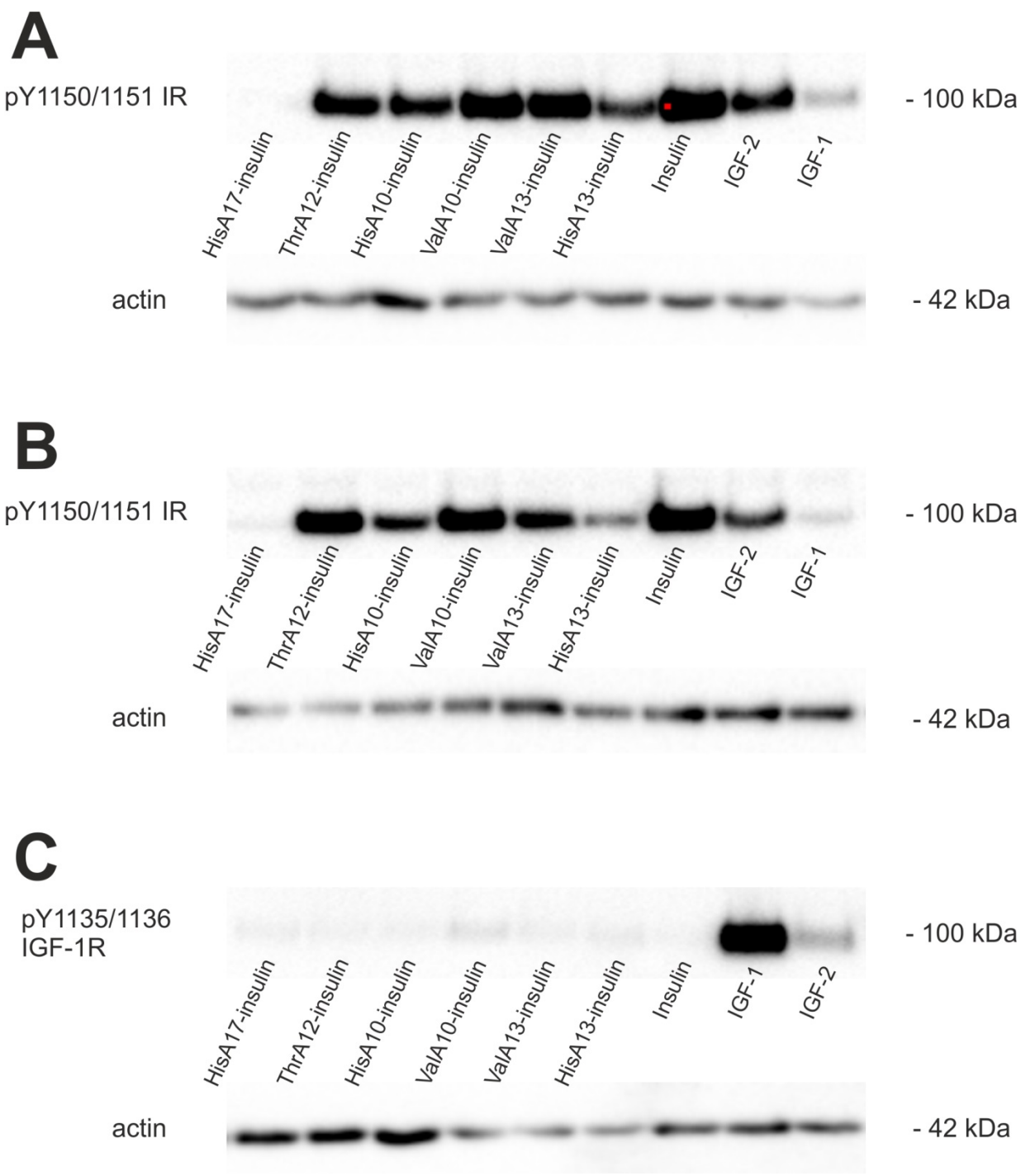


Figure S6. Representative Western blots for relative abilities of insulin analogs to stimulate receptors' phosphorylation. A. IR-A transfected fibroblasts. **B.** IR-B transfected fibroblasts. **C.** IGF-1R transfected fibroblasts. Cells were stimulated with 10 nM ligands for 10 min. Membranes were cut at 75 kDa and 50 kDa standards and respective parts were developed with anti-phospho-IGF-1Rβ (Tyr1135/1136)/IRβ (Tyr1150/1151) antibody (Mr above 75 kDa) and with anti-actin antibody (Mr bellow 50 kDa).

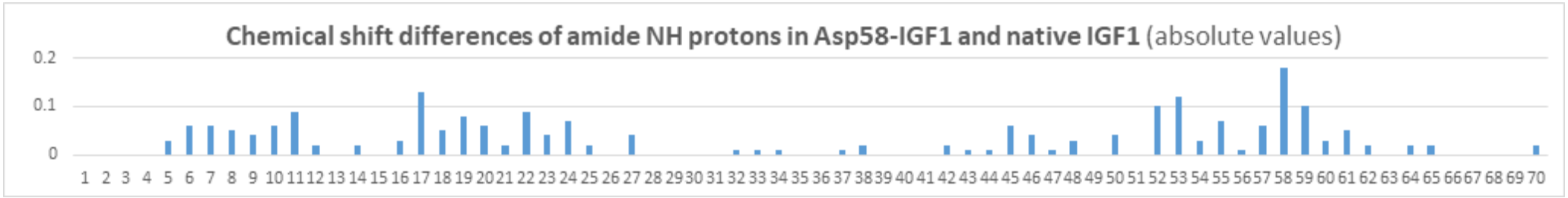
Table S4. Relative binding affinities of insulin analogs mutated at positions A10/A12/A3/A17, IGF-1 analogs mutated at 51/53/54/58 positions and IGF-2 analogs mutated at 52/53/57 sites. We have not found any available data on IGF-2 mutated at the position 50. Relative binding affinity is expressed in % of binding affinity of the native hormone.

Position in the native hormone	Mutation	Binding affinity (in % of the native hormone) for			References
		IR-A	IR-B	IGF-1R	
IleA10-insulin	Ser	20			Ref. (1)
Ser51-IGF-1	Ile	61			Ref. (1)
SerA12-insulin	Ala	108 (soluble receptor)			Ref. (2)
		36 (cell receptor)			A.-M. Jensen thesis referenced in Ref. (3)
Asp53-IGF-1	Ala			66	Ref. (4)
Asp52-IGF-2	Ala	133	134	87	Ref. (5)
	Asn			170	Ref. (6)
	Lys			60	Ref. (6)
	Glu			30	Ref. (6)
LeuA13-insulin	Ala	30			Ref. (2)
	Glu	20		163	Ref. (7)
Leu54-IGF-1	Ala			24	Ref. (4)
Leu53-IGF-2	Ala	25	43	40	Ref. (5)
				50	Ref. (6)
GluA17-insulin	Ala	56 (soluble receptor)			Ref. (2)
		35 (cell receptor)			A.-M. Jensen thesis referenced in Ref. (3)
Glu58-IGF-1	Ala			15	Ref. (4)
Glu57-IGF-2	Ala	17	17	34	Ref. (5)

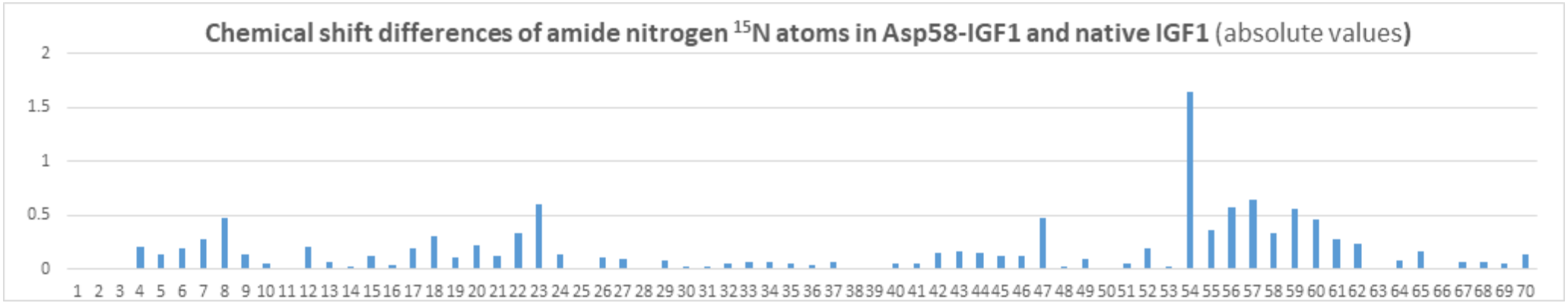
Table S5. Chemical shifts of amino acid residues in Asp58-IGF-1 and native IGF-1. (The ^{15}N signals of prolines were not detected).

Asp58-IGF-1								native IGF-1							
	NH	^{15}N	Hα		NH	^{15}N	Hα		NH	^{15}N	Hα		NH	^{15}N	Hα
Gly1	8.42	108.82	4.17,4.19	Arg36	8.13	122.14	4.33	Gly1	8.42	108.83	4.16,4.21	Arg36	8.13	122.18	4.33
Pro2	-	*	4.42	Arg37	8.13	121.29	4.33	Pro2	-	*	4.41	Arg37	8.12	121.22	4.32
Glu3	8.57	120.09	4.47	Ala38	8.13	126.00	4.57	Glu3	8.57	120.08	4.48	Ala38	8.11	126.00	4.57
Thr4	8.00	114.44	4.50	Pro39	-	*	4.41	Thr4	8.00	114.65	4.49	Pro39	-	*	4.41
Leu5	8.24	124.29	4.44	Gln40	8.42	119.90	4.37	Leu5	8.27	124.43	4.45	Gln40	8.42	119.96	4.38
Cys6	8.18	120.48	4.68	Thr41	8.03	114.07	4.34	Cys6	8.24	120.28	4.69	Thr41	8.03	114.12	4.36
Gly7	8.65	110.54	3.80,3.93	Gly42	8.45	110.83	4.08,4.06	Gly7	8.71	110.82	3.92,3.76	Gly42	8.47	110.98	4.07,4.13
Ala8	8.57	126.73	4.09	Ile43	7.85	120.24	4.00	Ala8	8.62	127.2	4.07	Ile43	7.84	120.40	3.96
Glu9	8.09	115.84	4.14	Val44	7.89	122.07	3.73	Glu9	8.05	115.7	4.12	Val44	7.88	122.22	3.70
Leu10	7.39	121.37	4.03	Asp45	7.98	120.61	4.51	Leu10	7.33	121.32	3.99	Asp45	7.92	120.49	4.50
Val11	7.50	117.90	3.40	Glu46	7.99	118.50	4.16	Val11	7.41	117.89	3.33	Glu46	7.95	118.38	4.14
Asp12	8.09	118.14	4.41	Cys47	8.22	114.34	4.81	Asp12	8.07	117.93	4.39	Cys47	8.23	113.87	4.86
Ala13	7.75	122.95	4.22	Cys48	7.98	116.85	4.55	Ala13	7.75	123.02	4.23	Cys48	7.95	116.82	4.55
Leu14	8.10	118.70	3.87	Phe49	7.84	116.68	4.67	Leu14	8.12	118.72	3.82	Phe49	7.84	116.58	4.67
Gln15	8.13	118.45	4.12	Arg50	7.78	118.58	4.44	Gln15	8.13	118.58	4.12	Arg50	7.74	118.57	4.46
Phe16	7.69	118.69	4.44	Ser51	7.88	112.78	4.47	Phe16	7.66	118.65	4.42	Ser51	7.88	112.73	4.47
Val17	8.29	118.22	3.74	Cys52	8.83	121.20	4.83	Val17	8.42	118.02	3.69	Cys52	8.93	121.39	4.86
Cys18	8.54	116.08	4.79	Asp53	8.28	121.77	4.69	Cys18	8.59	115.77	4.80	Asp53	8.16	121.80	4.69
Gly19	7.81	109.31	3.95,3.98	Leu54	8.42	124.70	4.02	Gly19	7.73	109.2	3.96,3.99	Leu54	8.39	123.05	3.99
Asp20	8.68	122.51	4.55	Arg55	8.13	117.08	4.12	Asp20	8.74	122.73	4.54	Arg55	8.06	116.72	4.03
Arg21	8.07	118.63	4.19	Arg56	7.81	118.05	4.34	Arg21	8.05	118.5	4.17	Arg56	7.82	118.63	4.20
Gly22	7.55	104.57	3.77,3.98	Leu57	7.80	119.18	4.20	Gly22	7.46	104.24	4.01,3.77	Leu57	7.86	118.53	4.15
Phe23	7.58	115.20	5.01	**Asp58**	8.05	115.18	4.56	Phe23	7.54	114.6	5.07	**Gln58**	7.87	114.85	4.22
Tyr24	8.44	119.68	4.73	Met59	7.67	117.74	4.24	Tyr24	8.51	119.54	4.70	Met59	7.57	117.18	4.21
Phe25	8.19	118.04	4.67	Tyr60	7.93	117.65	4.56	Phe25	8.17	118.03	4.69	Tyr60	7.90	117.19	4.52
Asn26	8.12	119.61	4.73	Cys61	7.46	116.20	5.02	Asn26	8.12	119.5	4.77	Cys61	7.41	115.92	5.04
Lys27	8.11	122.14	4.47	Ala62	8.24	126.53	4.43	Lys27	8.15	122.23	4.48	Ala62	8.26	126.77	4.42
Pro28	-	*	4.47	Pro63	-	*	4.40	Pro28	-	*	4.46	Pro63	-	*	4.40
Thr29	8.11	113.54	4.31	Leu64	8.18	121.97	4.23	Thr29	8.11	113.62	4.31	Leu64	8.20	122.05	4.22
Gly30	8.23	110.34	3.89,3.95	Lys65	8.24	123.48	4.61	Gly30	8.23	110.36	3.95,3.89	Lys65	8.26	123.65	4.60
Tyr31	8.07	120.06	4.55	Pro66	-	*	4.38	Tyr31	8.07	120.08	4.56	Pro66	-	*	4.38
Gly32	8.34	110.86	3.97,3.88	Ala67	8.31	124.65	4.29	Gly32	8.35	110.92	3.97,3.88	Ala67	8.31	124.72	4.29
Ser33	8.18	115.69	4.47	Lys68	8.18	120.10	4.35	Ser33	8.19	115.76	4.47	Lys68	8.18	120.17	4.35
Ser34	8.34	117.25	4.49	Ser69	8.26	117.74	4.44	Ser34	8.35	117.32	4.49	Ser69	8.26	117.79	4.43
Ser35	8.16	117.07	4.44	Ala70	8.02	129.97	-	Ser35	8.16	117.12	4.44	Ala70	8.04	129.83	4.20

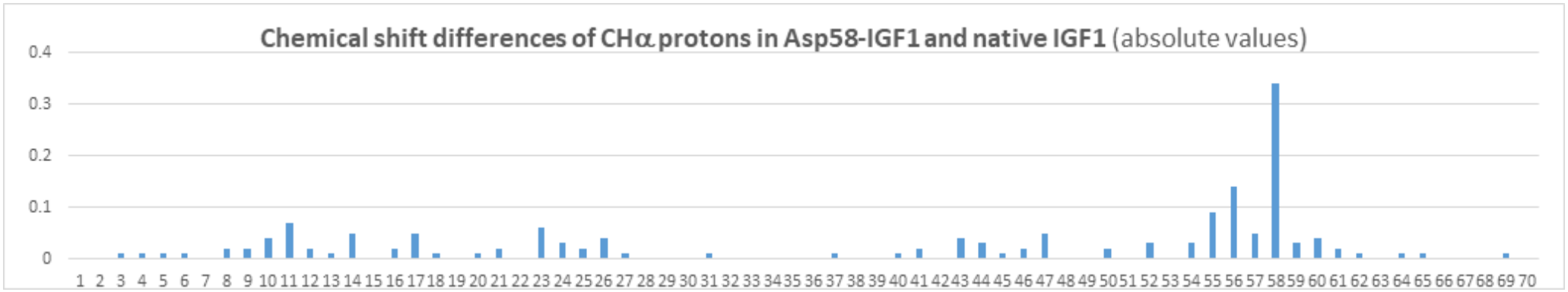
Table S6. Chemical shift differences between Asp51-IGF-1 and native IGF-1.



residue number



residue number



residue number

Table S7. NOEs statistics

Distance restraints (NOEs)	
Intra-residue	330
Sequential (|*i-j*| = 1)	159
Medium range (|*i-j*|≤ 4)	48
Long-range (|*i-j*| ≥ 5)	57
Ambiguous	147
Total	741
Dihedral angle restraints	
ϕ	18
Ψ	18
χ	3
Total	39
J-coupling restraints	
Total	10
*Ensemble statistics**	
Backbone (Å)	0.46
Heavy atoms (Å)	0.92
All atom (Å)	1.09

---

*only well-defined parts of the structure: Ala8-Cys18, Ile43-Tyr60

Differential Contact Maps calculated from metadynamics of the insulin B-chain C-terminus opening

The inter-residue contacts with a negative difference in contact lifetime between the wild-type and mutants are those that are more frequently present in mutant compared to wild-type insulin (colored in orange in **Figure S7**). A range of hydrophobic contacts between the B-chain N-terminus (PheB1, ValB2) and the A chain (LeuA13, LeuA16) were established to maintain the collapsed state for HisA10 and HisA13 mutants, as well as the closed state for the HisA17 mutant. Contacts between the B-chain N-terminus and residues in the B-chain α-helix were more frequently present in the collapsed/closed states of the His-insulin mutants. For example, the contacts in HisA13 mutant between residues HisB5-GlyB8, GlnB4-HisB10, GlnB4-GluB13 contributed to the partial collapse of the B-chain α-helix. For the native-like affinity ThrA12 contacts that were formed more frequently than in the wild type (for example IleA2, ValA3 with LeuB15, LeuB11) maintained the hydrophobic interface between A/B chains in a more compact state.

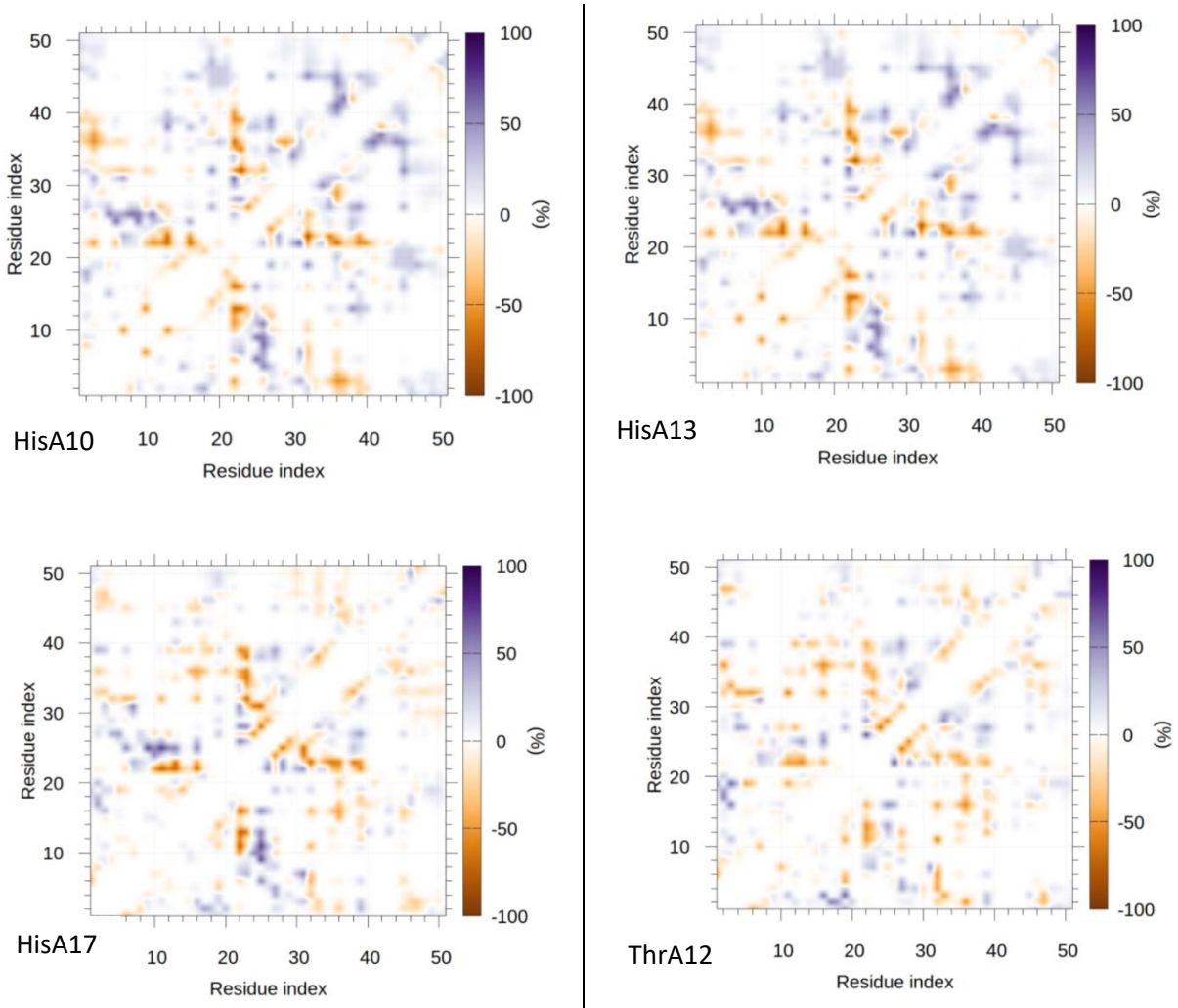
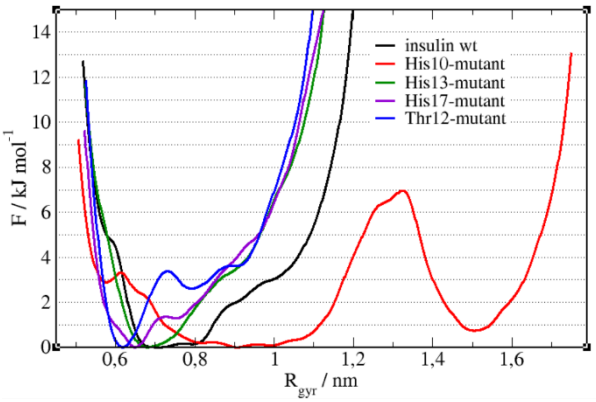


Figure S7. Change in total interaction time for insulin mutants calculated as the difference in fraction of time contacts are present between residues for the wild type and the mutant (a negative difference signifies a contact less frequently present in wild type or a contact gained in mutant). The residue index goes from 1-21 for A chain and 22-51 for B chain of insulin. HisA10-insulin, Left upper panel; HisA13-insulin, right upper panel; HisA17-insulin, left lower panel; ThrA12-insulin, right lower panel.

Structural characterization of the metadynamics ensembles

The insulin hydrophobic core comprises residues IleA2, ValA3, GlyB8, LeuB11, ValB12, LeuB15, PheB24 and TyrB26 (8). We calculated its size in terms of the radius of gyration R_{gyr} comprising all atoms for the respective residues. The hydrophobic core is more compact for the mutants compared to the wild-type insulin, except for the HisA10-mutant which collapses to extended states (**Figure S8A**). Connected to the extent of the hydrophobic collapse, we observed different protein-water hydrogen bonding local to the point of mutation. The number of water-protein hydrogen bonds was determined for a shell of the radius 1 nm around the Cα (CA) atom of a mutated residue. With OH and NH groups regarded as donors and O and N atoms as acceptors, the donor-acceptor cutoff distance was set to 0.35 nm and the angle hydrogen-donor-acceptor to 30°. The number of water-protein H-bonds in a sphere of a 1 nm radius around the Cα atom of a mutated/wild-type residue is plotted in **Figure S8B** comparing the HisA17-mutant and ThrA12-mutant in red to wild-type native insulin in black. The dynamics of H-bond formation is more stable in the closed compact state of HisA17-mutant. On the other hand, the bulkier ThrA12 is buried inside the core and forms less H-bonds with water than the wild-type SerA12.

A



B

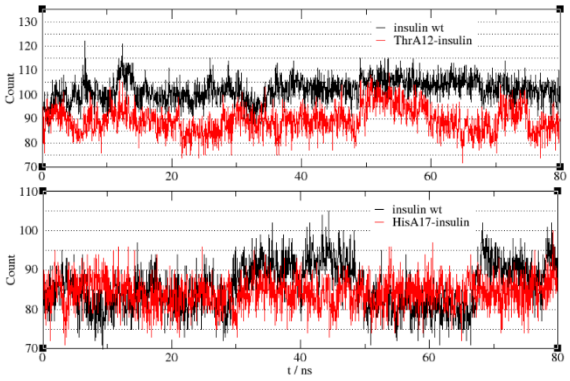


Figure S8. A. Free energy profiles with respect to the radius of gyration of residues defining the hydrophobic core. **B.** The number of protein-solvent hydrogen bonds in the sphere of 1 nm around Cα atoms of mutated residues: Cα (ThrA12) in ThrA12-insulin mutant and Cα (SerA12) in wild-type insulin (up), Cα (HisA17) in HisA17-insulin mutant and Cα (GluA17) in native insulin (down). Insulin wt is a native wild-type insulin.

References

1. Gauguin, L., Klaproth, B., Sajid, W., Andersen, A. S., Mcneil, K. A., Forbes, B. E., and De Meyts, P. (2008) Structural basis for the lower affinity of the insulin-like growth factors for the insulin receptor. *J. Biol. Chem.* **283**, 2604-2613

2. Kristensen, C., Kjeldsen, T., Wiberg, F. C., Schaffer, L., Hach, M., Havelund, S., Bass, J., Steiner, D. F., and Andersen, A. S. (1997) Alanine scanning mutagenesis of insulin. *J. Biol. Chem.* **272**, 12978-12983

3. De Meyts, P. (2015) Insulin/receptor binding: The last piece of the puzzle? *Bioessays* **37**, 389-397

4. Gauguin, L., Delaine, C., Alvino, C. L., McNeil, K. A., Wallace, J. C., Forbes, B. E., and De Meyts, P. (2008) Alanine scanning of a putative receptor binding surface of insulin-like growth factor-I. *J. Biol. Chem.* **283**, 20821-20829

5. Alvino, C. L., McNeil, K. A., Ong, S. C., Delaine, C., Booker, G. W., Wallace, J. C., Whittaker, J., and Forbes, B. E. (2009) A Novel approach to identify two distinct receptor binding surfaces of insulin-like growth factor II. *J. Biol. Chem.* **284**, 7656-7664

6. Delaine, C., Alvino, C. L., McNeil, K. A., Mulhern, T. D., Gauguin, L., De Meyts, P., Jones, E. Y., Brown, J., Wallace, J. C., and Forbes, B. E. (2007) A novel binding site for the human insulin-like growth factor-II (IGF-II)/mannose 6-phosphate receptor on IGF-II. *J. Biol. Chem.* **282**, 18886-18894

7. Schaffer, L. (1994) A model for insulin binding to the insulin receptor. *Eur. J. Biochem.* **221**, 1127-1132

8. Papaioannou, A., Kuyucak, S., and Kuncic, Z. (2015) Molecular dynamics simulations of insulin: elucidating the conformational changes that enable its binding. *Plos One* **10**