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Structure and function of C-type lectin NK cell receptors studied by recombinant expression and protein crystallography

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Ph. D. Thesis

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I declare that I have worked on this thesis under the guidance of my supervisor and that all sources of the previous knowledge are properly cited. No part of this work was used and will not be used for obtaining any other academic degree than Ph. D. from Charles University in Prague.

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Contents

Abbreviations

aa	amino acid
Ab	antibody
ADCC	antibody-dependent cellular cytotoxicity
AICL	activation-induced C-type lectin
bp	base pair
CC	cell count (10^6 cells / ml)
CD	Cluster of Differentiation
CHO	Chinese hamster ovary cells
Clrb	C-type lectin related protein b
CRD	carbohydrate recognition domain
CTLD	C-type lectin-like domain
DCDR	drop coating deposition Raman spectroscopy
DTT	dithiothreitol
EBNA1	Epstein-Barr nuclear antigen 1
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionisation
FBS	foetal bovine serum
Fc	fragment crystallizable of immunoglobulin molecule
FPLC	fast protein liquid chromatography
FT-ICR	Fourier transform – ion cyclotron resonance
GF	gel filtration
GFP	green fluorescent protein
HEK293	human embryonic kidney cell line 293
HPLC	high performance liquid chromatography
Ig	immunoglobulin
IPTG	isopropyl-β-D-1-thiogalactopyranoside
ITAM	immunoreceptor tyrosin-based activation motif
ITIM	immunoreceptor tyrosin-based inhibition motif

KIR	killer-cell immunoglobulin-like receptor
LAK	lymphokine-activated killer cells
LB	Luria-Bertani
LRC	leukocyte receptor complex
mAb	monoclonal antibody
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MALDI	matrix-assisted laser desorption ionisation
MS	mass spectrometry
Ni-NTA	nickel-nitrilotriacetic acid
NK	natural killer
NKC	natural killer gene complex
Nkp	natural killer protein
NKR-P1	natural killer receptor – protein 1
NMR	nuclear magnetic resonance
Ocil	osteoclast inhibitory lectin
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEI	polyethylenimine
pNPP	4-nitrophenyl phosphate
SDS	sodium dodecyl sulphate
SEAP	secreted alkaline phosphatase
SHP	Src homology phosphatase
SV40 TAg	large T-antigen of simian virus 40
TGE	transient gene expression
TN1	casein hydrolysate tryptone N1
TOF	time of flight
Tris	Tris(hydroxymethyl)aminomethane
VPA	valproic acid
WPRE	woodchuck hepatitis posttranscriptional regulatory element

1 Introduction

1.1 NK cell receptors

1.1.1 NK cells

Besides the already known B and T lymphocytes, another type of lymphocytes - NK cells - was discovered in 1975 in human peripheral blood. They were found out for their ability to spontaneously kill certain allogeneic tumour cell lines, without any previous sensitization. Thus, they were named natural killer cells [Kiessling R. *et al.*, 1975].

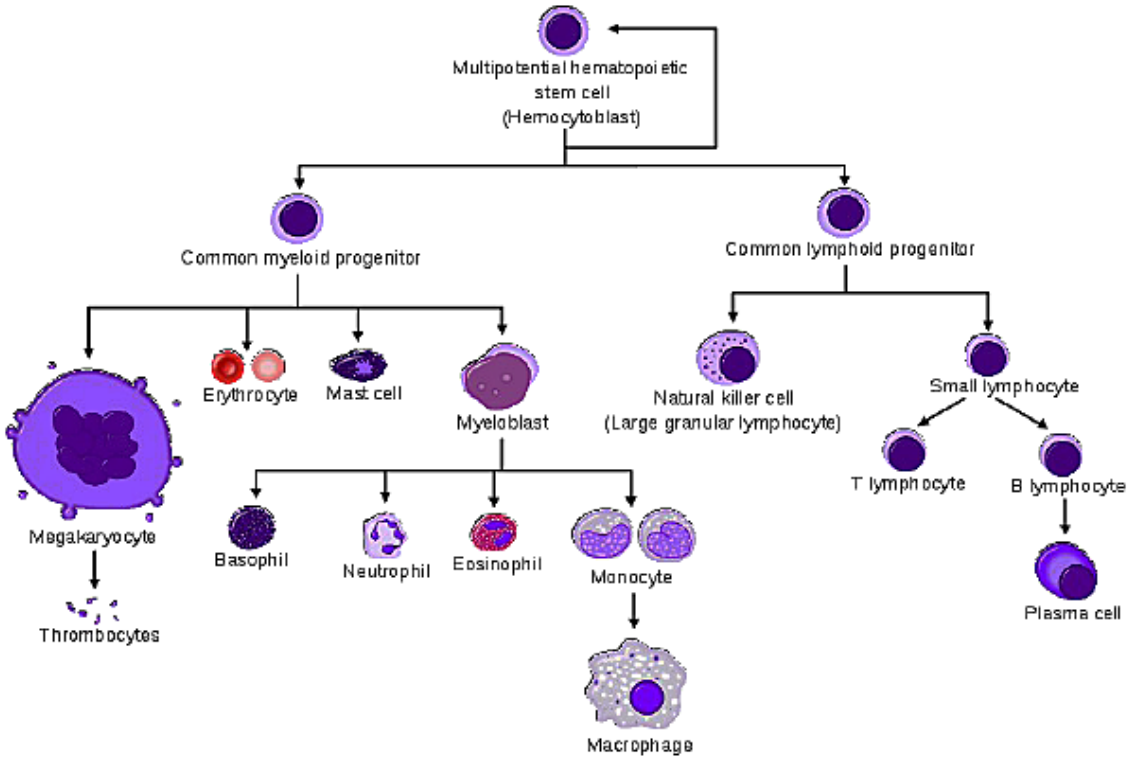

Fig. 1: Haematopoiesis, differentiation of pluripotent cells into various types of blood cells.

NK cells are large granular lymphocytes. They differentiate from pluripotent haematopoietic stem cells, along with B and T lymphocytes (Figure 1). They are characterized by the $CD3^{-}CD16^{+/-}CD56^{+}CD161^{+/-}$

phenotype [Trinchieri G., 1989] or by the presence of NKp46 and Nkp30 activation receptors [Moretta L. *et al.*, 2002]. NK cells lack T or B cell receptors, however, a subpopulation of T cells bearing also typical NK cell surface markers (such as NKR-P1 receptor) was described and hence named NKT cells [Hammond K. J. L. and Godfrey D. I., 2002]. NK cells circulate in peripheral blood where they constitute 5-15 % of mononuclear cells, moreover they can be found in lymphoid organs, especially in spleen, where they can constitute as much as 25 % of all lymphoid cells [Whiteside T. L. *et al.*, 1998].

NK cells are part of non-adaptive immune response with very short reaction time against pathogens such as viruses, intracellular bacteria, parasites, and they are responsible for elimination of certain tumour cells and thus they are able to fight against malignancy and formation of metastasis [Whiteside T. L. and Herberman R. B., 1995]. Besides their role in immunosurveillance, NK cells have also an important regulatory function. They secrete a variety of cytokines, most importantly interferon γ, which then stimulates activation and proliferation of other lymphocytes [Street S. E. *et al.*, 2001, Moretta L. *et al.*, 2002].

Activity of NK cells is regulated through the balance between positive and negative signals mediated by the NK cell surface receptors which are forwarded to the specific signalling pathways [Raulet D. H. *et al.*, 2001, Moretta A. *et al.*, 2001]. There are two functional types of the NK cell surface receptors: activation receptors, specific for non-self ligands, and inhibitory receptors, specific for markers of healthy cells – mainly MHC class I glycoproteins, such as NKG2A-CD94 heterodimer recognizing human MHC class I molecule HLA-E (Figure 2A). In the absence of inhibitory signals, e.g. in the case of abnormally low expression of MHC class I molecules which are often downregulated on tumour cells evading the T cell response, the NK cell may attack and kill the target cell (missing-self recognition) [Ljunggren H. G. and Kärre K., 1990, Medzhitov R. and Janeway C. A. Jr., 2002]. However, the presence

of ligands for activation receptors is also necessary, therefore erythrocytes, cells without MHC class I, are not attacked by NK cells. [Raulet D. H. and Vance R. E., 2006].

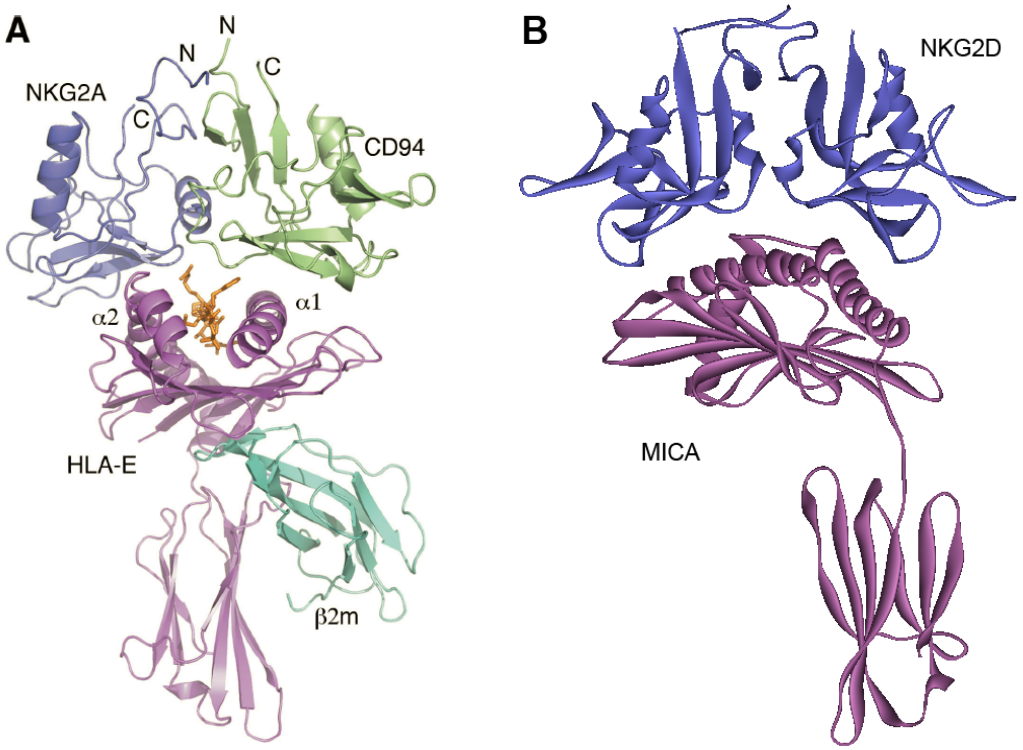

Fig. 2: Missin-self and induced-self ligand recognition. (A) Inhibitory CD94-NKG2A–HLA-E-peptide complex. NKG2A and CD94 are represented as blue and green ribbon structures, respectively. The heavy chain of HLA-E and β_2-microglobulin are shown as violet and cyan ribbons, respectively, with the VMAPRTLFL peptide in orange sticks; PDB ID: 3CDG [Petrie E. J. *et al.*, 2008]. (B) Activation NKG2D–MICA complex. NKG2D and MICA are represented as blue and violet ribbons, respectively; PDB ID: 1HYR [Li P. *et al.*, 2001].

Apart from missing-self recognition relying mostly on inhibitory receptors, NK cells possess at least two other strategies of target recognition using strong activation receptors. Stressed, transformed or infected cells often overexpress some proteins that are otherwise only low abundant in healthy cells. These proteins could be recognized by activating receptors (induced-self recognition), as are the stress-induced MHC class I-like molecules MICA and MICB by NKG2D receptor (Figure

2B) [Bauer S. *et al.*, 1999, Diefenbach A. *et al.*, 2001, Raulet D. H., 2003]. The second strategy is based on low affinity Fc-receptor CD16 (FcγRIII) and ADCC mechanism. When the NK cell meets the target cell opsonized by IgG, CD16 molecules on the NK cell surface bind the Fc fragments of antibodies. In contrast to other activation receptors where multiple signals are required, aggregation of CD16 alone results in NK cells activation, degranulation and target cell elimination [Perussia B., 2000].

1.1.2 NK cell receptors

In contrast to B or T cell, specificity of NK cells is not based only on one type of antigen receptor, but is given by variety of their surface receptors (Figure 3). These receptors could be divided into several groups according to either their functional or structural relationship. From the functional point of view, there are two major groups of NK cell receptors: activation and inhibitory [Lanier L. L., 2005].

Activation receptors have usually rather short cytoplasmic tail rich in charged amino acids that are responsible for interaction with signalling adaptor molecules, such as DAP10, DAP12, FcεRIγ or CD3ζ. These adaptors contain an ITAM signalling sequence which is phosphorylated by protein tyrosine kinases from Src-family (e.g. p56lck or p59fyn) followed by Syk-family kinases activation. Alternatively, signalling is mediated through G proteins associated with the receptors [Vivier E. *et al.*, 2004, Lanier L. L., 2008].

Inhibitory receptors have longer intracellular part containing an ITIM signalling sequence. During signal transmission ITIM recruits protein tyrosine phosphatases (SHP1, SHP2) which then consequently mediate the inhibition of Src-family activating kinases, resulting in inhibition of activation signalling cascades [Long E. O., 2008].

From the structural point of view, the majority of NK cell surface receptors could be classified as the C-type lectin or immunoglobulin-like receptors. Immunoglobulin-like receptors consist of either two or three

immunoglobulin-like extracellular domains. They usually interact with the MHC class I glycoproteins. The most prominent representatives of this group are KIR inhibitory receptors or activation receptors responsible for spontaneous NK cell cytotoxicity - NKp30, NKp44 a NKp46, and also Fc-receptor CD16 responsible for ADCC [Boyington J. C. *et al.*, 2001, Foster C. E. *et al.*, 2003].

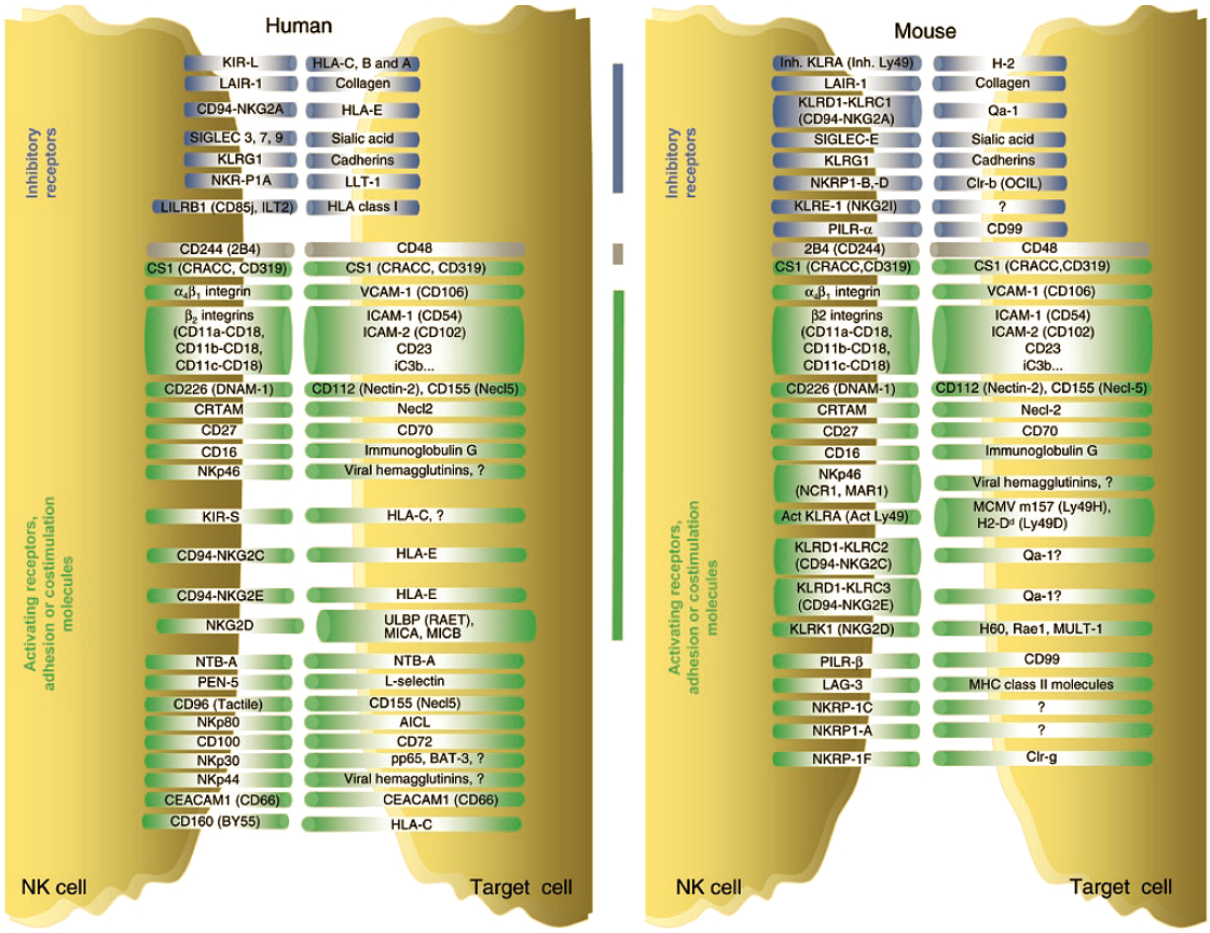

Fig. 3: The NK cell–target cell ‘zipper’. Some human (left) and mouse (right) receptor-ligand interactions are depicted here, to illustrate the combinatorial nature of the NK cell interaction repertoire. Cytokines, chemokines and their receptors are not shown, but are also crucial for the regulation of NK cell functions. Inhibitory receptors are in blue; 2B4, which can act as an activating or an inhibitory molecule, is in gray; other receptors are in green. Vertical lines indicate the receptor-ligand pairs conserved between mice and humans, which consist either of real orthologs (e.g. human and mouse NKp46) or examples of convergent evolution (e.g. KIR and Ly49) [Vivier E. *et al.*, 2008].

1.1.3 C-type lectin receptors

Lectins were defined as proteins able to specifically recognize and reversibly non-covalently bind saccharide structures [Goldstein *et al.*, 1980]. Antibodies and enzymes were excluded from this definition since lectins are structurally distinct from immunoglobulins and they do not alter covalent structure of saccharide molecules. Saccharide recognition is mediated by lectin carbohydrate recognition domains which are often linked together to increase the avidity through multivalent binding of complex (poly)saccharides structures.

Lectins were first discovered more than 100 years ago in plants (ricin, concanavalin A) and later also in many other organisms from viruses to mammals with variety of functions. Lectins can be classified by different criteria but the most useful classification is based on their structural and functional similarities since their specificity might evolve independently several times during evolution. Thus, we can distinguish eight main groups of lectins: calnexin, L-type lectins, P-type lectins, C-type lectins, galectins, I-type lectins, R-type lectins and M-type lectins [Dodd R. B. and Drickamer K., 2001].

C-type lectins were first identified in 1988 [Drickamer K., 1988]. Common feature of this group of animal lectins is the CRD requiring calcium ions for its binding activity. CRD of C-type lectins of around 125 aa is composed of a highly conserved combination of two α-helices and two anti-parallel β-sheets connected by random coils and stabilized by two or three disulphide bonds. In this respect, a typical C-type lectin is represented by the structure of rat mannose-binding protein [Weis W. *et al.*, 1991]. Different binding specificity is provided by the participation of variable loops in binding site formation. During evolution, some members of this group have lost the ability to bind calcium or carbohydrates (or both) and instead gained another substrate specificity, thus the more convenient term for these members is C-type lectin-like proteins [Drickamer K., 1999].

According to amino acid sequence homology, C-type lectins could be divided into seven different subgroups: proteoglycans, type II transmembrane receptors, collectins, selectins, type II lymphocyte receptors, macrophage mannose receptors and free CRDs [Drickamer K., 1993]. Most of them play role in immune recognition, adhesion and cell-cell communication. From these subgroups, mainly type II lymphocyte receptors are expressed on the NK cell surface. These proteins contain one CRD on the extracellular C-terminus. This tertiary structure was initially resolved in case of CD94 molecule [Boyington J. C. *et al.*, 1999]. CRD is usually glycosylated and receptors form homodimers (except for heterodimeric CD94-NKG2 isoforms [Lazetic S. *et al.*, 1996] and murine KLRE-KLRI isoforms [Saether P. C. *et al.*, 2008]), usually covalently linked by one or more disulphide bridges.

Genes encoding these proteins are located in NK gene complex situated on human chromosome 12, rat chromosome 4 and murine chromosome 6 [Hao L. *et al.*, 2006, Carlyle J. R. *et al.*, 2008]. To name the main receptor families within this complex we should mention NKR-P1, NKG2/CD94, Ly-49, KLRF and CLEC2 families, together with stand alone KLRG1 receptor. One peculiarity of this group of NK receptors is that lot of them could be found under at least three different names: CD nomenclature designation, gene name and an acronym(s), reflecting rather historical than functional aspects, and all these names are often used simultaneously without any strict preference (e.g. CD161 = KLRB1 = NKR-P1). Thus, to avoid confusion, one has to be careful while studying the scientific literature.

1.1.4 CD69 receptor

CD69 is an important member of C-type lectin family of NK cell surface receptors. It belongs to C-type lectin domain family 2 (CLEC2 family) which, in humans, comprises KACL (CLEC2A), AICL (CLEC2B), CD69 (CLEC2C) and LLT1 (CLEC2D; rodent orthologs are Clr receptors).

It was originally described in 1986 as early antigen 1 (EA1) on the surface of human T cells after 12-o-tetradecanoyl phorbol-13-acetate treatment [Hara T. *et al.*, 1986] and cloned in 1993 [Hamann J. *et al.*, 1993]. Human CD69 is constitutively expressed on $CD3^+$ thymocytes, monocytes, granulocytes, neutrophils, epidermal Langerhans cells and platelets and the expression is induced very early after NK cells and T cells activation [Testi R. *et al.*, 1994], therefore it has been widely used as the marker of activated lymphocytes. Although the initial *in vitro* studies suggested that CD69 may be one of the activatory molecules triggering the killing of tumour cells [Moretta A. *et al.*, 1991], more recent studies in CD69 deficient knock-out mice have indicated its role in silencing the immune response since the $CD69^{-/-}$ mice had a slightly increased resistance to tumours [Esplugues E. *et al.*, 2003]. Recently, CD69 was shown to inhibit lymphocyte egress from lymphoid organs by specific interaction with membrane helix 4 of sphingosine 1-phosphate receptor-1 ($S1P_1$) [Shiow L. R. *et al.*, 2006, Bankovich A. J. *et al.*, 2010] or play a role in preventing infection-induced immunopathology during listeriosis [Vega-Ramos J. *et al.*, 2010].

CD69 is a type II transmembrane protein composed of short intracellular segment (constitutively phosphorylated; association with G-protein adaptor), single transmembrane helix, stalk region (containing dimerization cysteine C68) and a globular extracellular C-type lectin-like domain whose structure was solved by X-ray crystallography [Natarajan K. *et al.*, 2000, Llera A. S. *et al.*, 2001]. It appears as a disulphide bond linked homodimer of approx. 60 kDa. Detailed biochemical studies of the natural CD69 isolated from leukocytes revealed that their heterogeneity is caused by differences in *N*-glycosylation sites occupation since the extracellular part of CD69 molecule contains one typical and one atypical ($N^{111}AC$) *N*-glycosylation site. Interestingly, this atypical site occurs quite rarely and human CD69 represents only the fourth protein known to utilize this motif [Vance B. A. *et. al.*, 1997, Vance B. A. *et. al.*, 1999].

Apart from $S1P_1$ interaction which is mediated by transmembrane part of CD69 within the same cell, no other natural ligand of CD69 extracellular CTLD has been identified so far. However, a recent study has suggested that this enigmatic ligand might be after all present on certain tumour cell lines [North J. *et al.*, 2007]. Nevertheless, a variety of small molecule ligands was proposed instead, starting with calcium and monosaccharides (GlcNAc, GalNAc) [Pavlíček J. *et al.*, 2003], synthetic oligosaccharides (GlcNAc tetrasaccharide) [Kovalová A. *et al.*, 2010], carboxylated calixarenes [Bezouška K. *et al.*, 2010], up to the synthetic glycoconjugates based on immunoactive LELTE peptide [Renaudet O. *et al.*, 2010].

1.1.5. NKR-P1 receptor

The family of NKR-P1 (KLRB1, CD161) receptors include both activating and inhibitory receptors expressed mostly on NK cells, but also on some T cell subsets, monocytes and dendritic cells [Aust J. G. *et al.*, 2009]. The first member of the NKR-P1 receptor family to be discovered, mouse NKR-P1C, is still generally known by its original name NK1.1 and is widely used as a marker for identifying and defining NK cells in certain strains of mice [Glimcher L. *et al.*, 1997, Koo G. C. and Peppard J. R., 1984]. Cloning of a cDNA for NK1.1 revealed that it belonged to a multigene family [Giorda R. and Trucco M., 1991, Ryan J. C. *et al.*, 1992], later shown to contain at least five members in the C57BL/6 mouse strain (NKR-P1A, C, D, E and F) [Plougastel B. *et al.*, 2001], with a sixth member (NKR-P1G) recently uncovered [Carlyle J. R. *et al.*, 2006]. However, NKR-P1E appears to be a pseudogene in C57BL/6 mice [Plougastel B. *et al.*, 2001]. Curiously, the gene corresponding to the second member of the family to be discovered, NKR-P1B, cannot be found in the C57BL/6 genome, but appears to be present in BALB/c mice, where its position and sequence indicate that it is the BALB/c allele of the C57BL/6 NKR-P1D gene [Carlyle J. R. *et al.*, 2006].

Mouse NKR-P1C contains a positively charged amino acid in its transmembrane domain, associates with the FcεRIγ chain and delivers activatory signals that trigger cytolytic activity and cytokine secretion following cross-linking with appropriate mAbs [Karlhofer F. M. and Yokoyama W. M., 1991, Arase N. *et al.*, 1997]. The sequences of mouse NKR-P1A and F also contain charged transmembrane residues, implying that these receptors may be activatory. By contrast, mouse NKR-P1D and NKR-P1B lack charged transmembrane residues, possess a classical ITIM motif in the cytoplasmic domain, associate with SHP1 and deliver inhibitory signals when cross-linked by mAbs or ligands [Carlyle J. R. *et al.*, 1999, Kung S. K. *et al.*, 1999].

A major breakthrough in our understanding of NKR-P1 molecules was the discovery that the ligands of at least some NKR-P1s belong to a family of related proteins, designated Clr proteins, encoded by genes interspersed among the NKR-P1 genes themselves [Plougastel B. *et al.*, 2001]. In particular, it was shown that NKR-P1B/D recognizes Clrb and that transfected cells expressing Clrb are partially protected from lysis by NK cells, leading to the suggestion that NKR-P1B/D - Clrb recognition represents a novel form of missing-self recognition designed to monitor cellular levels of Clrb [Iizuka K. *et al.*, 2003, Carlyle J. R. *et al.*, 2004].

While in the rat the organization and function of NKR-P1 family seems quite similar to the mice (although orthologous genes do not have necessarily the same name) [Kveberg L. *et al.*, 2006, Kveberg L. *et al.*, 2009], only one single NKR-P1 gene can be found in human [Lanier L. L. *et al.*, 1994]. Human NKR-P1 is expressed only on a subset of mature NK cells and T cells. The molecule contain an altered ITIM motif in its cytoplasmic tail and is able to deliver inhibitory signals upon ligand interaction. However, natural ligands of human NKR-P1 were found out to be a true orthologs of mouse Clr proteins, namely human LLT1 [Rosen D. B. *et al.*, 2005, Aldemir H. *et al.*, 2005] and recently discovered PILAR (proliferation-induced lymphocyte-associated receptor) [Huarte E. *et al.*,

2008]. Thus, it seems that this alternative missing-self recognition system is conserved in humans, too.

1.1.6 Ligands of C-type lectin NK cell receptors

Ligands of the C-type lectin NK cell receptors can be divided into three major groups (Table 1). The first group consists of receptors recognizing MHC class I or similar molecules. Murine Ly-49 (KLRA1) family is the best examined family of C-type lectin NK cell receptors. It is comprised of approximately 23 highly polymorphic genes (Ly-49A – W) [Dimasi N. and Biassoni R., 2005]. Most Ly-49 receptors are inhibitory and their ligands are classical MHC class I molecules, thereby serving as a self-recognition system. In fact, the missing-self hypothesis was firstly demonstrated on inhibitory Ly-49A (IL-2 activated NK cells were not able to kill target cells with H-2D^{d}) and it was also the first NK cell receptor whose structure in complex with its MHC class I ligand was solved (Table 2) [Tormo J. *et al.*, 1999]. Ly-49 family has evolved in rodents only, the same function in humans is served by KIR immunoglobulin-like receptors.

On the other hand, NKG2/CD94 family is conserved both in human and mice. Single gene of CD94 (KLRD1) is known in all species lying close to NKG2A, -B, -C gene cluster (KLRC1, -2, -3). CD94 and NKG2 molecules form covalent heterodimers where NKG2 is responsible for signalling: A and B is inhibitory, C is activation. Ligands for these heterodimeric receptors are a non-classical MHC class I molecules and their function self-recognition. The last member of the first group is homodimeric activation NKG2D (KLRK1) receptor which recognizes stress-induced MHC class I-like ligands (MICA, MICB) or viral infection associated ligands (ULBP) [Lanier L. L., 2005].

The second group is composed of KLRG1 (MAFA), an inhibitory receptor expressed on a subset of mature T and NK cells. Recently, E-, N-, and R-cadherin, have been identified as ligands for KLRG1 [Li Y. *et al.*, 2009]. Cadherins are a large family of transmembrane or membrane-

Receptor	Ligand	Function
Ly-49 family	MHC class I	+ / -
NKG2/CD94 family	non-classical MHC class I	-
NKG2D	stress-inducible MHC I-like	+
KLRG1	E-, N- and R-cadherins	-
KLRF1	AICL (CLEC2B)	+
KLRF2	KACL=PILAR (CLEC2A)	+
?	CD69 (CLEC2C)	+
NKR-P1 family	Clr family (CLEC2D)	+ / -
mNKR-P1A	?	+
mNKR-P1C	?	+
mNKR-P1B/D	mClrb	-
mNKR-P1F	mClrg	+
mNKR-P1G	?	?
rNKR-P1A	rClr11	+
rNKR-P1B/C	rClr11, RCTL	-
rNKR-P1F	rClr2, 3, 4, 6, 7	+
rNKR-P1G	rClr2, 6, 7	-
hNKR-P1	hLLT1; PILAR=KACL	-
KLRE/I1	?	-
KLRE/I2	?	+

Table 1: Known C-type lectin NK cell receptor-ligand pairs. Receptor families recognizing MHC class I and similar ligands are highlighted in green, those recognizing adhesive molecules in yellow and those recognizing other C-type lectin receptors from CLEC2 family in blue. Detailed analysis of all known NKR-P1 family members identified within mice (m), rat (r) and human (h) is highlighted in gray. Note that RCTL is a rat cytomegalovirus decoy ligand and CLEC2A protein was characterized independently under the two names, KACL or PILAR. Receptor function: + activating, - inhibiting NK cell cytotoxicity; where both signs are given, different family members have different functions.

associated glycoproteins that were thought to only bind specifically to other cadherins to mediate specific cell-cell adhesion in a Ca^{2+}-dependent manner. However, recent study of KLRG1 – E-cadherin

Receptor	PDB	Ligand	PDB	Complex PDB
hCD94	1B6E			
hCD94-hNKG2A	3BDW	HLA-E	3BZE	3CII, 3CDG
hNKG2D	1MPU	MICA	1B3J	1HYR
hNKG2D	1MPU	ULBP3		1KCG
mLy-49A		H-2D^{d}	1DDH	1QO3
mLy-49C	3C8J	H-2K^{b}	2VAA	1P4L, 3C8K
mLy-49G	3CAD			
mLy-49I	1JA3			
mLy-49L	3G8K			
mNKG2D	1HQ8	RAE-1	1JFM	1JSK
hKLRG1		E-cadherin	1O6S	3FF7
mKLRG1	3FF9	E-cadherin	1FF5	3FF8
hCD69	3HUP			

Table 2: Known C-type lectin NK cell receptor-ligand crystal structures. Receptor families recognizing MHC class I and similar ligands are highlighted in green, those recognizing adhesive molecules in yellow and those recognizing other C-type lectin family receptors in blue. PDB accession codes, if available, are given both for receptor and ligand themselves and for their complex, too.

interaction revealed its putative role in regulation of immune response [Banh C. *et al.*, 2009]. Moreover, cadherins are downregulated in metastatic tumours, so this is yet another missing-self system example.

A third group of C-type lectin NK cell receptor-ligand pairs is composed of interesting combination of C-type lectin receptors recognizing another C-type lectin receptors. A common feature of this group is the fact that genes of the receptor-ligand pairs are intermingled with each other in the NKC gene complex and thus are more likely to be conserved during evolution. CD69 is the only orphan CLEC2 family member, however, since receptors for other CLEC2 family members are all from KLR family of NK lectin receptors, it is tempting to speculate who will be the first one to find out the one last KLR missing here. Clr proteins

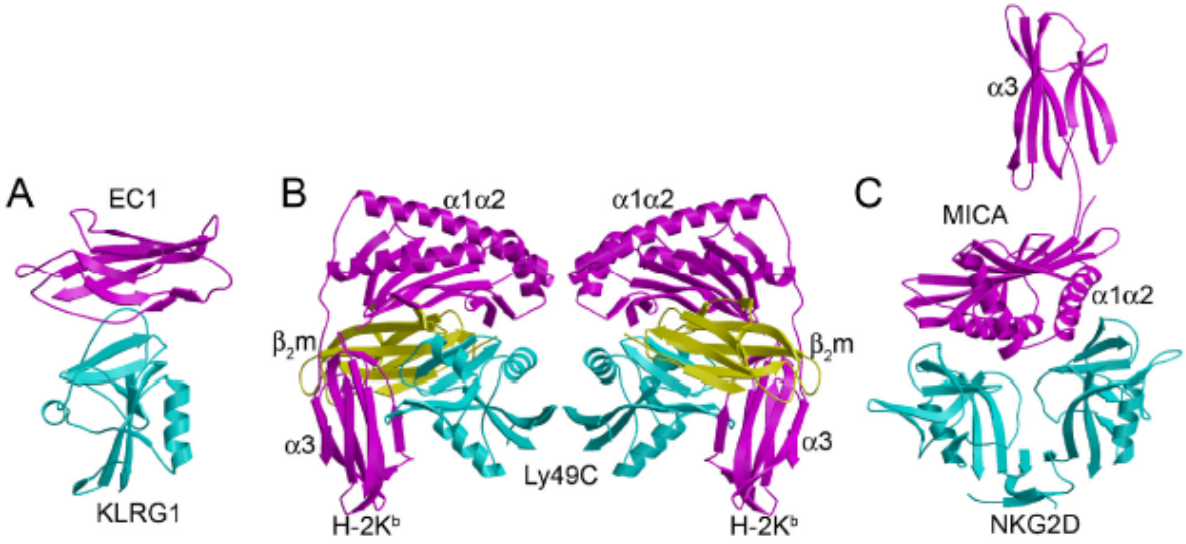

Fig. 4: Comparison of C-type lectin NK cell receptor-ligand binding modes. (A) The mKLRG1–hE-cadherin1 (EC1) complex. One KLRG1 CTLD monomer (cyan) binds one EC1 molecule (purple); PDB ID: 3FF8 [Li Y. *et al.*, 2009]. (B) The Ly49C–H-2K^b complex. Each CTLD of the Ly49C homodimer (cyan) binds one MHC class I molecule (heavy chain, purple; β_2-microblobulin, yellow); PDB ID: 3C8K [Deng L. *et al.*, 2008]. (C) The NKG2D–MICA complex. The two CTLD subunits of the NKG2D homodimer (cyan) bind a single MICA molecule (purple); PDB ID: 1HYR [Li P. *et al.*, 2001].

were originally described as Ocil (osteoclast inhibitory lectin) [Zhou H. *et al.*, 2001] for their role in bone biology but they are widely expressed on all haematopoietic cells (excluding red blood cells) and other tissues. On the other hand expression of KACL/PILAR is restricted primarily to skin and T cells [Spreu J. *et al.*, 2007, Huarte E. *et al.*, 2008].

The first receptor ligand pairs identified in this group were mNKR-P1F – mClrg and mNKR-P1B/D – mClrb [Iizuka K. *et al.*, 2003, Carlyle J. R. *et al.*, 2004]. Based on that observation, hNKR-P1 – hLLT1 pair soon followed [Rosen D. B. *et al.*, 2005, Aldemir H. *et al.*, 2005] and quite recently this system was thoroughly analysed in rat, too [Kveberg L. *et al.*, 2006, Kveberg L. *et al.*, 2009]. Based on all observations, it is now widely accepted that NKR-P1 – Clr interactions represent distinct missing-self recognition system, independent from MHC recognition. Sadly, as seen also for MHC based self-recognition, this system could be

subverted by viral decoy inhibitory ligands [Voigt S. *et al.*, 2007] or by overexpression of native inhibitory ligand in cancer cells [Roth P. *et al.*, 2007]. On the contrary, KLRF1 (Nkp80) – AICL [Welte S. *et al.*, 2006] and the newly described KLRF2 (Nkp65) – KACL [Spreu J. *et al.*, 2010] interactions seem to have rather immunomodulatory then self-recognition role, and this might also be the case for the last orphan NKR-P1 molecules and also for newly described mouse KLRE/I1-2 heterodimers [Saether P. C. *et al.*, 2008]. Interestingly, CLEC2A protein was described independently by two different groups as KACL or PILAR molecule and the last studies suggests that this is the first CLEC2 ligand recognized both by KLRF2 and NKR-P1 (KLRB1), that is by two distinct KLR receptors [Spreu J. *et al.*, 2010, Huarte E. *et al.*, 2008].

From the point of view of protein crystallography (Table 2) it could be immediately seen that while the first and second group of C-type lectin NK cell receptor-ligand pairs is well resolved today, the third group has been surprisingly resilient so far. Apart from orphan CD69, for which five PDB entries are now deposited (only the highest resolution PDB is shown in Table 2), no other crystal structure is publicly available yet.

1.2 Introduction to methods

1.2.1 Recombinant protein expression

Intuitively, a protein will be best expressed in its native cell type under physiological conditions, where a multitude of molecular systems work together for efficient production and quality control at various stages, including synthesis and folding, post-translational modifications and subcellular targeting. However, only a small number of proteins occur naturally in amounts that permit convenient purification of the relatively large quantities required for structural studies. Therefore, various heterologous overexpression systems have been developed to produce recombinant proteins: bacterial (most commonly *Escherichia coli*), yeast (*Pichia pastoris* and *Saccharomyces cerevisiae*), baculovirus-infected insect cells, mammalian cells and, more recently, cell-free systems [Yokoyama S., 2003].

Easy preparation of recombinant DNA was initiated by a discovery of restriction endonucleases, an easy amplification of the desired DNA sequence by polymerase chain reaction (PCR) and by a development of modern sequencing techniques enabling rapid control of prepared DNA. Today we can more or less easily clone or synthesize almost any desired gene or DNA sequence and introduce it to host organism (or simply host cell) by various means. The gene of interest could be inserted into an engineered expression plasmid and transformed into bacterial host strain or transfected into host cell line. Alternatively, the gene might be integrated into viral vector and the host infected. In all cases, the proteosynthetic apparatus of the host is condemned to serve us as a living factory, producing the desired gene product, usually recombinant protein.

Choosing the right host or expression system is the key to success. Among the main characteristics which one has to take into account are the degree and closeness of the host posttranslational modifications,

cultivation requirements of chosen host organism and its cost with respect to expected production yield and product demand. Since the market with therapeutic recombinant proteins is ever-increasing, this issue has also very practical economical aspects. One good example of successful therapeutic recombinant protein is erythropoietin (EPO), a haematopoietic growth factor which induces proliferation of red blood cells and is often used in the treatment of anaemia [Navarro M. *et al.*, 1991]. EPO has three *N*-glycans and one *O*-glycan and the *N*-glycans are critical for its biological activity [Takeuchi M. *et al.*, 1989, Yuen C. T. *et al.*, 2005]. All recombinant EPOs on the market are produced by mammalian cell lines and generated sales of more than $10 billion in 2005 [Walsh G., 2006]. Of course, this is just a penny in comparison with the market of recombinant mAbs and vaccines. There are slightly over 165 recombinant pharmaceuticals currently approved for human use. Another 500 protein candidates are in preclinical and clinical development, about 7 0% of these being glycosylated proteins. The need for expression systems allowing the efficient manufacturing of high quality glycoproteins is thus becoming imperative [Durocher Y. and Butler M., 2009].

1.2.2 Prokaryotic expression systems

Prokaryotic organisms were the first and are still the most widely used hosts for recombinant protein expression. If we look into the Brookhaven Protein Data Bank of protein structures, we could see that approximately 90 % of all solved structures still originate from recombinant proteins produced in *Escherichia coli*. This bacteria is a very attractive host because of its ability to grow rapidly in inexpensive media. In addition, *E. coli* is a well-characterized microorganism both genetically and physiologically [Baneyx F., 1999]. Due to the ease of genetic manipulation, short doubling time, capacity for high-level protein expression, and low cost, *E. coli* is by far the most widely used host for

recombinant protein production [Dai X. *et al.*, 2005], another commonly used bacteria is *Lactococcus lactis*. The main advantage of bacterial expression is that it is very cheap, it has only modest instrumentation requirements and it is also very fast when compared to most of eukaryotic expression systems.

The simplicity of bacterial organism facilitating its easy use turns out to be a disadvantage when it comes to recombinant expression of eukaryotic, especially human, proteins in this host. Bacteria lack most of posttranslational modifications that are common in proteins from higher eukaryotes, moreover their proteosynthetic apparatus is quite different, too. The lack of specific chaperones and oxidoreductases often results in recombinant protein misfolding and formation of insoluble protein aggregates – inclusion bodies [Clark E. D., 2001]. Inclusion bodies might be easily harvested, solubilized in chaotropic agents and in some cases successfully refolded *in vitro* [Singh S. M. and Panda A. K., 2005], but the refolding yields are often low, making the whole process cost-ineffective, especially from the point of view of industrial applications. However, regarding C-type lectin receptors of NK cells, this is the most common strategy of their recombinant production. Up to date, all their structures were solved using soluble constructs consisting of C-type lectin-like domain only that were expressed in *E. coli* and refolded *in vitro*.

The workflow of bacterial expression systems is usually as follows: firstly, the desired gene of interest (or its part – expression construct) is inserted under the control of lactose operon into a suitable expression plasmid bearing also an antibiotic resistance which is then transformed into host bacterial strain and transformants are selected against that antibiotic. The culture is expanded in liquid medium until a desired volume and cell density are reached, then the production is induced by addition of IPTG as a lactose operon activator. After several hours the cells are harvested and frozen or the target protein is immediately purified (in case of inclusion bodies or native protein production).

1.2.3 Eukaryotic expression systems

Unlike prokaryotes, eukaryotes are able to perform a number of posttranslational modifications including disulfide bridge formation, proteolytic cleavage and glycosylation, yielding stable and functional mature proteins. Eukaryotic expression systems can largely be grouped into categories based on the nature of the cellular system used; namely, yeast, insect cells (the basis for baculovirus expression), plant cells and mammalian cells.

1.2.3.1 Yeast

Yeasts are single-cell eukaryotic hosts which combine some of the advantages of prokaryotic and eukaryotic based expression systems; for example, they are physically robust and amenable to high-density fermentation but possess the necessary cellular machinery to carry out posttranslational modifications. The methylotrophic yeast *Pichia pastoris* gives high yields of recombinant proteins [Cereghino G. P. and Cregg J. M., 1999], can be grown to high cell densities using defined minimal media and offers a cost-effective method for ^{13}C-labelled protein production for NMR-based structural analyses. Typically, genes of interest are expressed under the control of the strong and tightly regulated *P. pastoris* alcohol oxidase 1 (AOX1) promoter. Baker's yeast, *Saccharomyces cerevisiae*, provides an alternative to *P. pastoris*, but with genes of interest expressed under the control of a different promoter; for example, the copper inducible metallothionein (CUP1) promoter. Yeast offer fast, economical growth and is easy to genetically manipulate compared to other eukaryotic hosts. Many approved non-glycosylated therapeutic proteins are currently produced in yeast, including insulin (and analogues), growth hormone, hirudin (a leech-derived anticoagulant) and albumin. However, as they provide *N*- and *O*-linked high-mannose-type glycans that could be immunogenic in humans [Dasgupta S. *et al.*, 2007], they are not well suited for the production of glycosylated

proteins. Additionally, yeasts cannot perform tyrosine *O*-sulfation, a posttranslational modification that occurs in higher eukaryotes and in mammals [Moore K. L., 2003]. Recently, the successful engineering of the glycosylation pathways in *P. pastoris* has allowed expression of recombinant proteins with human-type glycans [Hamilton S. R. and Gerngross T. U., 2007] and high expression level of a glycoengineered monoclonal antibody was achieved [Potgieter T. I., 2009].

1.2.3.2 Insect cells

The insect cell-baculovirus expression system is a powerful platform to rapidly produce high level of recombinant proteins. The main insect cell lines used are *Spodoptera frugiperda* SF9 or SF21 and *Trichoplusia ni* BTI 5B1-4 (High Five™). One advantage is that they can be easily cultivated at very high cell densities, at room temperature and without CO_2 atmosphere. Owing to the high-mannose and paucimannose type of glycosylation that is obtained in insect cells, no therapeutic protein is currently produced in this system as this would compromise *in vivo* bioactivity and potentially induce allergenic reactions. Engineering those cells with glycosyltransferases allows the production of proteins with mammalian-type glycans [Harrison R. L. and Jarvis D. L., 2006]. Insect cells also offer an interesting platform to produce vaccine antigens or virus-like particles [Hu Y. C. *et al.*, 2008], while engineered baculoviruses containing mammalian promoters (BacMam) show great potential as genetic vectors for mammalian cells transduction [Condreay J. P. and Kost T. A., 2007, Dukkipati A. *et al.*, 2008]. In structural biology, baculovirus expression had also become a well established method for many proteins that are difficult to express in *E. coli*.

1.2.3.3 Plant cells

Plant cell suspension cultures especially those from tobacco and genetically modified plants are being used for recombinant protein expression. The major advantages associated with *in vitro* plant systems

compared to transgenic plants include the rapidity of protein production and the ability to manipulate environmental conditions for better control over protein production and quality. The drawbacks are the relatively slow growth of plant cells and differences in glycosylation compared to mammalian cells [Doran P. M., 2000]. Genetically modified plants are especially well suited for the production of edible oral vaccines. However, issues such as field containment (especially for crop plants) and public acceptance need to be addressed. There are currently a few plant-derived therapeutics approved in Europe for topical use in human and several products are currently in clinical trials, including interferon α produced in *Lemna minor* and glucocerebrosidase manufactured in carrot cells, making it obvious that plant-based expression systems are gaining acceptance to manufacture protein therapeutics [Karg S. R. and Kallio P. T., 2009].

1.2.3.4 Mammalian cells

Mammalian cells can produce the most therapeutically effective recombinant proteins because they can perform the necessary posttranslational modifications most accurately among the various hosts mentioned here. Theoretically, any cell which can be maintained in culture can also be manipulated to express foreign genes. Unfortunately, mammalian cells could be also expensive to maintain in culture and may grow more slowly in comparison to the other hosts mentioned above. Finally, it has to be mentioned that mammalian cells are susceptible to infection with mammalian viruses, potentially leading to a contamination of the protein product.

The main approach to the expression of recombinant proteins in mammalian cells remains the establishment of a cell line in which the recombinant gene is integrated into the host genome and stably expressed over time, but the identification and characterization of stable cell lines is a costly and time consuming process. This labour intensive

process is based on the isolation, characterization and expansion of a highly productive clonal cell line from a pool of transfected cells and it can take from several weeks up to many months to complete. Nevertheless, for industrial applications this is the only reasonable way.

However, it may frequently be necessary to rapidly evaluate many candidate proteins or several variants of a single target protein for their expression yield and correct biological activity. This is foreseeable for applications in structural biology such as optimization of crystallizable construct of the target protein, addition and removal of different tags or site-directed mutagenesis. For these purposes rapid approaches to the expression of milligram to gram quantities of a recombinant protein are needed. One promising method is transient gene expression (TGE) in transiently transfected mammalian cells [Baldi L. *et al.*, 2007]. TGE does not require stable DNA integration. After the DNA enters the cell's nucleus, transcription of the transgene starts and recombinant protein synthesis begins. Usually the protein can be detected within a few hours after transfection. Using low cost DNA delivery agents such as calcium phosphate (CaPi coprecipitation) [Jordan M. *et al.*, 1996, Jordan M. and Wurm F., 2004] or calcium chloride (calfection) [Lindell J. *et al.*, 2004] and foremost polyethylenimine (PEI, polyplex formation) [Boussif O. *et al.*, 1995, Akinc A. *et al.*, 2005], suspension cultures of mammalian cells at volumes ranging from 1 ml to 100 l have been transiently transfected to produce recombinant proteins in mg/l to g/l range in 5-10 days after transfection [Schlaeger E. J. and Christensen K., 1999, Girard P. *et al.*, 2002, Durocher Y. *et al.*, 2002, Backliwal G. *et al.*, 2008].

Monoclonal antibodies and Fc-fusion proteins, a major class of licensed therapeutics, are mainly produced in Chinese hamster ovary cells (CHO), mouse myeloma cells (NS0, SP2/0) and hybridomas [Beck A. *et al.*, 2008]. Most of the recombinant protein therapeutics currently under development are also expressed in CHO cells because of their extensive characterization and ability for human-like glycosylation. Other

animal cell lines used for production include baby hamster kidney (BHK21), human fibrosarcoma (HT1080), human lymphoma (Namalwa) and human embryonic kidney 293 (HEK293) [Durocher Y. and Butler M., 2009].

1.2.3.5 Transient gene expression in HEK293 cell line

Several cell lines have been used for transient expression of recombinant proteins, but only a few of them present the advantages of (1) high transfectability with common methods, (2) ease of adaptation to suspension cultivation in serum-free conditions and (3) cost-effective scalability. HEK293 is the most commonly used cell line for large-scale transient transfection. It was created 33 years ago by transformation of primary human embryonic kidney cells with sheared fragments of adenoviral DNA (Ad5) [Graham F. L. *et al.*, 1977], and they constitutively express the adenovirus E1A and E1B genes that has been shown to significantly enhance transcription from the cytomegalovirus (CMV) promoter. In order to support large-scale adenovirus production in bioreactor cultures, this cell line was adapted to suspension growth and the resulting cell line was named 293N3S. Yet another cell line (293S) was generated through gradual adaptation to serum-free medium. A subclone of this cell line (293SF-3F6) has been further selected for its capacity to grow at high density (8×10^6 cell/ml) in serum-free suspension.

In the attempt to specifically improve TGE through promoting episomal replication of the exogenous plasmid DNA, two other HEK293 derivatives have been generated: HEK293E, expressing the EBNA1 and HEK293T, featuring the SV40 TAg. These cell lines sustain episomal replication or amplification of plasmids containing the EBV oriP (HEK293E) or SV40 ori (HEK293T) origins of replication, respectively. Although the SV40 ori/TAg system has been shown to allow very high levels of plasmid replication and to significantly improve expression in COS cells, this system does not appear to increase transient recombinant

protein expression levels in HEK293T cells [Durocher Y. *et al.*, 2002]. This is likely the result of a competition between gene replication and expression that specifically occurs in HEK293 cells [Lewis E. D. and Manley J. L., 1985]. In contrast, the use of EBV oriP-bearing plasmids consistently provides higher expression in HEK293E compared to HEK293 cells [Schlaeger E. J. and Christensen K., 1999, Durocher Y. *et al.*, 2002]. Because of this enhanced productivity, HEK293E is now the most widely used cell line for large-scale TGE [Baldi L. *et al.*, 2005, Baldi L. *et al.*, 2007, Durocher Y. and Butler M., 2009].

With respect to the protein crystallography, it is important to note that two other HEK293 derivatives displaying simplified glycosylation are available. Firstly, an HEK293S cell line resistant to ricin (Lec1) was prepared by mutagenesis by using ethyl methanesulfonate [Reeves P. J. *et al.*, 2002]. It was shown to lack *N*-acetylglucosaminyltransferase I (GnTI) activity and consequently unable to synthesize complex *N*-glycans. Analysis of the *N*-glycan in rhodopsin expressed by the HEK293S GnTI$^-$ stable cell line showed it to be homogeneous $Man_5GlcNAc_2$. Secondly, the same method was recently applied to HEK293T cells to create Lec36 cell line deficient in Golgi α-mannosidase II activity which produces deglycosylatable hybrid-type glycans [Crispin M. *et al.*, 2009]. Moreover, the use of *N*-glycosylation processing inhibitors kifunensine and swainsonine for HEK293T cells leading to homogeneous and cleavable *N*-glycans was thoroughly described [Chang V. T. *et al.*, 2007] as well as selenomethione labelling for easy phase problem solving [Aricescu A. R. *et al.*, 2006].

1.2.4 Affinity purification

For more efficient recombinant protein manipulation, it is often genetically fused to the peptide or protein sequence - a tag. The primary role of a tag is to simplify and unify purification and subsequent characterization of the target protein. Purification via a tag is based on a

specific and sufficiently strong affinity of tag peptide/protein sequence and its binding partner. Such tag is therefore called an affinity tag. Except for this utilization, the tags have been used for more diverse purposes, such as increasing yield of recombinant proteins production, enabling their detection or investigating their potential protein partners [Nilsson J. *et al.*, 1997].

There are several reasons why to prefer affinity purification approach. Firstly, an affinity purification protocol is more versatile than a standard purification protocol which is mostly worked out just for a particular protein. Secondly, the effort which has to be invested into establishing standard purification protocol is considerably higher than in the case of affinity purification. Thirdly, affinity purification procedure mostly consists of only one or two purification steps in contrast to multiple-step purification which has to be commonly performed in case of standard purification. Finally, affinity purification generally tends to have higher yields than standard purification [Lichty J. J. *et al.*, 2005].

Tags might have negative effect on crystallization of fusion protein. Generally, it is assumed that large tags (proteins such as GST or MBP) have a negative impact on the ability of protein to crystallize. On the other hand, it is believed that small tags (peptides such as His-tag, FLAG-tag or Arg-tag) do not influence the crystallization of the fusion protein significantly. However, it was shown that even small peptide might influence the formation and quality of the protein crystals [Bucher M. H. *et al.*, 2002]. Therefore, the cleavage of the tag prior the crystallization will probably be the most often used approach.

The tag may influence the behaviour of target protein in undesired fashion. Hence, it is often cleaved off after the purification. In some cases, e.g. production of recombinant proteins for pharmaceutical purposes, it is necessary to produce the protein in its wild-type form to avoid unexpected reaction of the patient during administration. The most common way to cleave the tag off is by application of endopeptidases.

This approach involves endopeptidases with highly specific cleavage sites, such as TEV or 3C proteases, enabling efficient tag cleavage at neutral pH and low temperature [Arnau J. *et al.*, 2006].

1.2.4.1 His-tag

His-tag is by far the most used affinity tag. More than 60 % of the proteins produced for structural studies include His-tag [Derewenda Z. S., 2004]. It may be composed of 2-10 histidine residues, while the most common form of His-tag is His_6-tag which consists of six histidine residues. These residues mediate the non-covalent interaction (chelation) of this tag to the transition metal cations (Ni^{2+}, Co^{2+}, Zn^{2+} or Cu^{2+}). The idea of immobilized metal-affinity chromatography (IMAC) dates back to 1975 [Porath J. *et al.*, 1975]. However, it fully advances just after development of His-tag. In this experimental set-up the transition metal ions are immobilized on a matrix through special chemical groups. Ni^{2+}-NTA, nickel ions adsorbed through nitrilotriacetic acid (NTA) and Co^{2+}-CMA (Talon), cobalt ions adsorbed through carboxylmethyl aspartate (CMA), are currently the most used IMAC chromatography resins.

Purification via His-tag can be performed in both native and denaturing conditions. Bound protein can be eluted in two ways - either by chelating competitors (imidazole) or by low pH (under 5.0), which causes protonation of histidine imidazole rings and abandons their chelation to the transition metal ions. The first approach tends to be milder for purified protein and hence it is often used.

More than 100 structures of His-tagged proteins have been deposited in the Protein Data Bank in 2003 and the number has probably increased since then [Terpe K., 2003]. The tag is believed to have minor effect on the crystallization of target protein as supported by the recent comparative study [Bucher M. H. *et al.*, 2002]. The influence on protein biological activity cannot be excluded completely but due to the size of tag the probability of such intervention is quite low. Nevertheless, if this

is the case, it can be often solved by moving the tag to the opposite terminus of the target protein [Goel A. *et al.*, 2000].

1.2.5 Protein crystallography

History of protein crystallography stretch back to 19th century. The first protein crystal (haemoglobin) was observed as early as in 1830. In 1895 X-rays were discovered by W. C. Röntgen and the first crystal diffraction was observed 17 years later. In 1913 the first crystal structures of small inorganic molecules (KCl, NaCl, KBr, KI; W. L. Bragg) were already solved. The first diffraction on protein crystal was observed in 1934 and the first protein structure (sperm whale myoglobin) was solved in 1959 by Max Perutz and Sir John Cowdery Kendrew, for which they were awarded the Nobel Prize in Chemistry in 1962. Since that success, over 58321 X-ray crystal structures of proteins, nucleic acids and other biological molecules have been determined (as retrieved from Protein Data Bank statistics on 17th August 2010). For comparison, the nearest competing method in terms of structures analysed is nuclear magnetic resonance (NMR) spectroscopy, which has resolved 8533 chemical structures. Moreover, crystallography can solve structures of arbitrarily large molecules (as large as whole ribosome or viruses), whereas solution-state NMR is restricted to relatively small ones (less than approx. 100 kDa). X-ray crystallography is now used routinely by scientists to determine how a pharmaceutical drug interacts with its protein target and what changes might improve it [Scapin G., 2006]. However, intrinsic membrane proteins remain challenging to crystallize because they require detergents or other means to solubilize them in isolation, and such detergents often interfere with crystallization. Such membrane proteins are a large component of the genome and include many proteins of great physiological importance, such as ion channels and receptors [Lundstrom K., 2006].

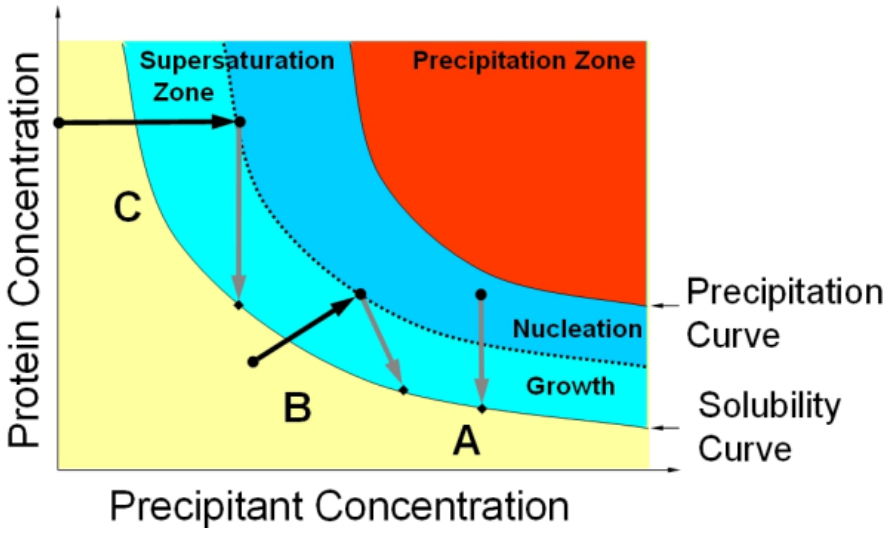

Fig. 5: Protein crystallization phase diagram schema. Crystal nucleation is a critical phenomenon that occurs only in an area of the supersaturation zone. Crystals grow under conditions of supersaturation only once nuclei have formed. Yellow: unsaturated solution; red: too high supersaturation; dark-blue: crystallization nuclei may form; light-blue: crystal growth. Pathway A: batch-type crystallization; pathway B: vapour diffusion-type crystallization; pathway C: dialysis-type crystallization.

Protein crystallization can be seen as three distinct events: nucleation, growth and stop of growth. Crystallization occurs only from supersaturated solutions where the protein concentration exceeds its solubility in a given solution (Figure 5). The state of this supersaturation depends on many factors and tweaking these factors is what makes protein crystallization more an art than a science. Trial and error is still the only available method for screening the crystallization conditions, although robotics made life a lot easier and the whole process much faster, allowing for high-throughput structural genomics studies [Chayen N. E., 2004, Bolanos-Garcia V. M. and Chayen N. E., 2009, Joachimiak A., 2009]. Three basic methods used in protein crystallization are batch method, vapour diffusion method and microdialysis. In batch method, protein and precipitant solutions are directly mixed, usually in a microbatch setting where a small drop of resultant mixture is covered with layer of oil preventing evaporation (Figure 5A). In vapour diffusion

method, a small drop of resultant mixture is placed either on a cover slide of the crystallization plate (hanging drop method) or at the prepared support in the plate well (sitting drop method) containing reservoir of significantly larger volume of precipitant solution. In both cases, an equilibrium between the drop and the precipitant reservoir is attained through vapour phase (hence the name), the drop gets concentrated and the crystallization might occur (Figure 5B). In microdialysis method, protein is placed directly into the precipitant reservoir enclosed in a chamber covered with dialysis membrane (Figure 5C) [McPherson A., 2004, Chayen N. E. and Saridakis E., 2008].

Once the appropriate crystallization conditions have been found and the diffraction quality of the crystals tested (usually involving optimization of cryoprotectant, too), frozen crystals are stored in liquid nitrogen waiting for high-resolution data collection at synchrotron (Figure 6). Firstly, the crystal symmetry, the unit cell parameters, the crystal orientation and the resolution limit need to be ascertained. Armed with this information, a data collection strategy which will maximize both

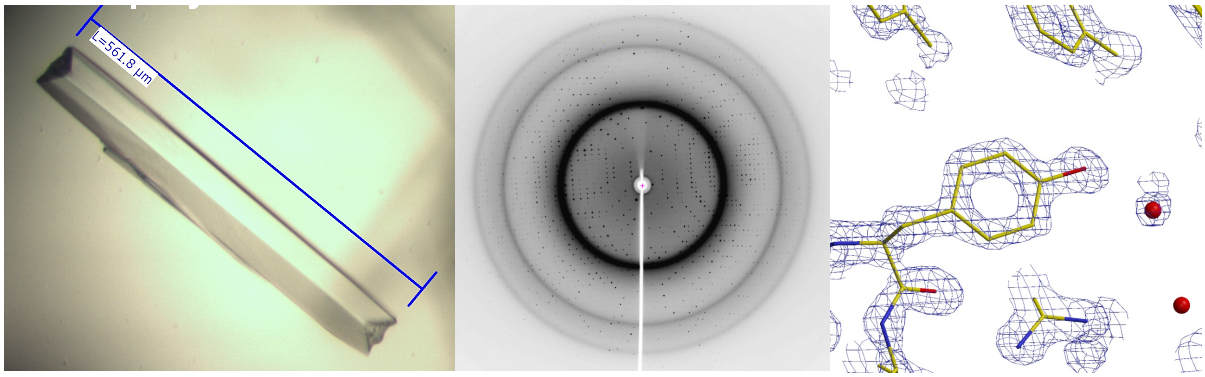

Fig. 6: Protein crystallography is a three step process. First, protein crystal has to be grown, then the diffraction data are collected and the phase problem has to be solved to obtain electron density maps into which a model of the molecule might be build. Finally, refinement of the molecular model is performed by iteratively cycling between the raw diffraction data and the calculated model to yield a single, unambiguous structure solution. Human CD69 crystal, its diffraction pattern and the structure solved to 1.37 Å resolution (PDB ID: 3HUP).

the resolution and completeness of the data set might be derived. In order to visualize our structure the phase problem has to be solved. For protein structure determinations it could be done in one of several ways. If the structure of a similar protein is already known it is possible to try to solve the structure using a process called molecular replacement which involves taking the known structure as a model and rotating and translating it into our new crystal system until a good match to our experimental data is obtained. If successful, then the amplitudes and phases can be calculated from this solution which can then be combined with our data to produce an electron density map. The X-rays are diffracted by the electrons in the structure and consequently the result of an X-ray experiment is a 3-dimensional map showing the distribution of electrons in the structure [Taylor G., 2003].

If there is no starting model available, then an isomorphous replacement methods can be used whereby one or more heavy atoms are introduced into specific sites within the unit cell without perturbing the crystal lattice. However, this is again trial-and-error procedure and often it is not apparent whether it has worked until more X-ray data have been collected. Heavy atoms are electron dense and give rise to measurable differences in the intensities of the spots in the diffraction pattern. By measuring these differences for each reflection, it is possible to derive some estimate of the phase angle using vector summation methods. In practice data from one or more heavy atom derivatives is required to get good enough phases - hence multiple isomorphous replacement (MIR).

In some cases it is possible to make use of the anomalous scattering behaviour of certain atoms at or near their X-ray absorption edges to gain useful phase information. Many of the atoms used in isomorphous replacement are also useful in this respect. This additional information can enhance the structure solution. Multiwavelength anomalous dispersion (MAD) is an elegant and often very effective method that relies entirely on the measurement of the anomalous

differences produced by one or more anomalously scattering atoms in the crystal. In practice three or more consecutive data sets are recorded from the same crystal at different wavelengths around the X-ray absorption edge of the anomalous scatterer. As this method requires a tuneable X-ray source, it can only be performed at a synchrotron. The resultant phase information can often produce very high quality electron density maps, thereby simplifying the subsequent interpretation. Selenium is a particularly good anomalous scatterer and it can be incorporated into proteins by overexpressing them in host cells grown on minimal media supplemented with amino acids using selenomethionine in place of methionine [Yokoyama S., 2003, Aricescu A. R. *et al.*, 2006].

Once the phase problem has been solved, model building is the next step. This is the process where the electron density map is interpreted in terms of a set of atomic coordinates. This is more straightforward in the molecular replacement case because one already has a coordinate set to work with. In the case of isomorphous replacement there is only the map. It is essentially a 3-dimensional jigsaw puzzle with the pieces being the amino acid residues. The normal procedure is to fit a protein backbone first then if the resolution permits, the sequence is inserted. The amount of detail that is visible is dependent on the resolution and the quality of the phases. Often regions of high flexibility are not visible at all due to static disorder, where the structure varies from one molecule to the next within the crystal, or dynamic disorder, where the region is mobile within the crystal. The latter type of disorder is eradicated in cryogenic data collection [Taylor G. L., 2010].

Once the preliminary model has been built it could be refined against our data. This will have the effect of improving the phases which results in clearer maps and therefore better models. Typically this cycle is performed several times until little or no further improvements could be achieved. At this stage a crystallographic R-factor of below 25 % is expected. This is a measure of the agreement between the model and the

data – the lower the value the better the model. Nevertheless, the final model must make chemical sense and there must be no large regions of electron density unaccounted for. Finally, the crystal structure is checked for wrong amino acid geometry and deposited in Protein Data Bank [Adams P. D. *et al.*, 2009].

1.2.6 Analytical ultracentrifugation

Analytical ultracentrifugation is an extremely versatile and powerful technique for characterizing the solution-state behaviour of macromolecules. When coupled with contemporary data analysis methods, experiments performed in the analytical ultracentrifuge are capable of rigorously determining sample purity, characterizing assembly and disassembly mechanisms of biomolecular complexes, determining subunit stoichiometries, detecting and characterizing macromolecular conformational changes, and measuring equilibrium constants and thermodynamic parameters for self- and hetero-associating systems. After a lengthy hiatus, in which the instrumentation largely disappeared from laboratories throughout the world and the principles of analytical ultracentrifugation disappeared from classrooms a modern version of the analytical ultracentrifuge has again become available to the academic and industrial biomedical research communities [Cole J. L. and Hansen J. C., 1999].

Analytical ultracentrifugation often is confused with more familiar types of procedures performed in preparative ultracentrifuges (e.g. sucrose/glycerol gradient centrifugation, buoyant density centrifugation) However, analytical ultracentrifuge experiments are fundamentally different in several key aspects. Most importantly, the sample is visualized in real time during sedimentation, allowing extremely accurate determination of hydrodynamic and thermodynamic parameters. In addition, the purpose of the experiment is to characterize key sample properties rather than prepare or purify a sample for subsequent use. In

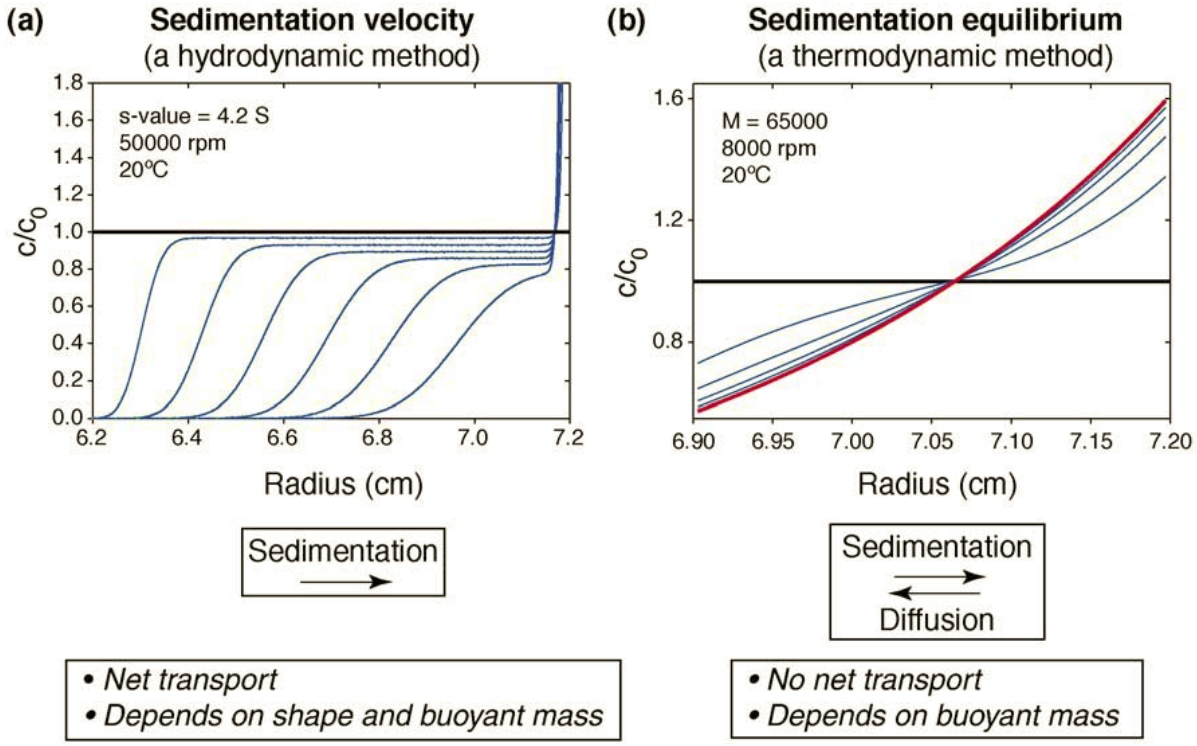

Fig. 7.: Types of analytical ultracentrifugation experiments. Simulation of the sedimentation of a 65 kDa single species at 20 °C with sedimentation coefficient of 4.2 S. c/c_0 is the ratio of the protein concentration at a given radial position and time to the loading concentration before centrifugation (black). (a) A sedimentation velocity (high-speed) experiment. Successive gradients correspond to elapsed time intervals of 5 min. (b) An approach to equilibrium (low-speed) experiment. Successive gradients correspond to elapsed time intervals of 4 h. The final gradient (red) represents a close approximation to sedimentation equilibrium [Howlett G. J. *et al.*, 2006].

contrast to many biophysical techniques, biomolecules are characterized during analytical ultracentrifugation in their native state under biologically relevant solution conditions. Because the experiments are performed in free solution, there are no complications caused by interactions with matrices or surfaces that can obscure interpretation of certain types of commonly used experiments, such as gel filtration.

An analytical ultracentrifuge can be used to perform two types of experiments, referred to as sedimentation velocity and sedimentation equilibrium (Figure 7). Sedimentation velocity is a hydrodynamic

technique and is sensitive to the mass and shape of the macromolecular species. In a sedimentation velocity experiment, a moving boundary is formed on application of a strong centrifugal field (Figure 7A). A series of scans (i.e. measurements of sample concentration, c, as a function of radial distance, r) are recorded at regular intervals to determine the rate of movement and broadening of the boundary as a function of time. In contrast, sedimentation equilibrium is a thermodynamic technique that is sensitive to the mass but not the shape of the macromolecular species. These experiments are performed at lower speeds and measure the equilibrium concentration distribution of macromolecules that eventually forms when sedimentation is balanced by diffusion (Figure 7B).

The sedimentation velocity and equilibrium measurements provide complementary information and it is often useful to apply both techniques to a given problem. Although the detailed mathematical theory that underlies sedimentation velocity and sedimentation equilibrium experiments can be complex, many user-friendly data analysis programs allow a non-expert to analyse analytical ultracentrifuge data with confidence [Lebowitz J. *et al.*, 2002].

2 Aims of the thesis

• To develop the refolding and purification protocol providing sufficient amount of human CD69 for structural and functional studies

• To develop the refolding and purification protocol providing sufficient amount of rat NKR-P1A and B for structural and functional studies

• To study the structure of CD69 and NKR-P1 proteins by protein crystallography

• To develop eukaryotic expression system suitable for recombinant expression of native NK cell lectin receptors

• To develop suitable affinity purification protocol for easy purification of secreted recombinant proteins from conditioned culture medium

• To manage sedimentation analysis of recombinant proteins in analytical ultracentrifuge

3 Methods

3.1 Molecular biology methods

3.1.1 DNA cloning

DNA cloning is a process of obtaining multiple copies of desired DNA (usually a gene of interest) *in vitro*. In this thesis DNA cloning usually followed this workflow: RNA isolation using TRI REAGENT (MRC Inc.), reverse transcription (SuperScript III, Invitrogen), PCR, restriction digestions and ligation. pBlueScript SK+ (Stratagene) or pCR2.1-TOPO (Invitrogen) was used for routine cloning in XL-1 MRF' Blue (Stratagene) or DH5α competent cells (Invitrogen) and pRSETB (Invitrogen), pET30a+ or pET28b+ (both Merck) were used as expression vectors for *E. coli* expression, while pHLsec and pHLsecFcHis plasmids were used for transient transfection of HEK293T cells [Aricescu A. R. *et al.*, 2006].

The cloning procedure for pHLsec plasmids developed during this thesis is simple and robust, starting with PCR amplification of the gene of interest with DeepVent DNA polymerase, PCR product digestion with AgeI and KpnI restriction endonucleases which both create 4 nucleotide single strand overhangs, digest purification on JETQUICK spin columns (Genomed) and ligation with T4 DNA ligase into pre-prepared linearised plasmid (all enzymes from New England Biolabs). Positive clones were selected by colony PCR screening using PPP Master Mix (Top-Bio) with a combination of vector and insert specific primers. The overall cloning efficiency was about 80 % for all constructs cloned.

All expression vectors allow to add a His-tag on the N- or C-terminus of the protein, however, it was utilized just in case of production of covalent dimer of human CD69 molecule in *E. coli*. On the other hand, a C-terminal six histidine tag was present on all proteins expressed in HEK293T cells for the ease of purification.

3.1.2 Plasmid DNA preparation

Expression plasmids containing the gene of interest were sequenced and a stock solutions of purified DNA were prepared using JETSTAR 2.0 Maxi kit (Genomed). In case of plasmids used for HEK293T cells transfection we used endotoxin-free version of the same kit. High quality DNA is essential for successful transfection, therefore only plasmid preparations with an A_{260}/A_{280} ratio of 1.8-2.0 were used. The pHLsec and derivative plasmids are all very high copy number, therefore one can expect about 4 mg of pure plasmid DNA from a 500 ml overnight bacterial culture (in our experience, cultures grown for more than 12–14 h give poor yields, probably due to a loss of ampicillin resistance in prolonged bacterial culture). The DNA samples must be sterile; therefore, the DNA precipitates were carefully washed with 70 % ethanol before dissolving them in sterile 10 mM Tris, pH 8, and the DNA solution was usually sterile filtered once again just before the transfection.

3.2 Protein expression and purification

3.2.1 Choice of expression system

Almost all proteins considered in this thesis were produced in prokaryotic expression system due to the fact that it is a classical expression system for this group of proteins and that in most cases optimized refolding conditions could be found. However, some other C-type lectin NK cell receptors, that are not desribed here in great detail, were pretty resilient to this strategy, especially the human NKR-P1, for which we were not able to find suitable refolding conditions, or human KLRF1 (Nkp80) which was not expressed in bacteria at all. Thus, it appeared necessary to utilize some kind of eukaryotic expression system for these troublemakers, a system not too demanding instrumentally and yet robust and modular at the same time.

While we had some initial success with human CD69 production in the yeast *Pichia pastoris*, later attempts to produce rat NKR-P1A and B receptors in this host were not so positive. Research in the literature revealed only few notions of a successful production in insect cell based expression systems [Rosen D. B. *et al.*, 2005], but on the contrary, mammalian cell lines seemed to be a good choice. Especially the HEK293 cell line was often used in immunological studies for transfection with various C-type lectin receptor constructs [Kveberg L. *et al.*, 2006, Kveberg L. *et al.*, 2009] and importantly, even for their recombinant production [Aldemir H. *et al.*, 2005, Welte S. *et al.*, 2006].

3.2.2 Recombinant expression in *E. coli*

In this thesis proteins were produced in *E. coli* BL-21 (DE3) Gold or *E. coli* BL-21 (DE3) RIPL competent cells (Stratagene) in the form of aggregated inclusion bodies as prokaryotic expression systems promising soluble protein productions (low temperature growth optimized cell lines from ArcticExpress system with cold-active chaperones (Stratagene), periplasmic production with pMal-p2 plasmid (NEB) or Rosetta-gami 2 host strain with oxidoreductases and rare codon plasmids (Novagen)) provided unsatisfactory results. From other works concerning prokaryotic expression of CD69 and other C-type lectin NK cell receptors we can conclude that it is a general finding [Boyington J. C. *et al.*, 1999, Natarajan K. *et al.*, 2000, Iizuka K. *et al.*, 2003].

Transformed competent cells were cultivated in LB medium in Erlenmeyer flasks at 37 °C until the optical density of 0.6-0.8 (measured as absorbance at 550 nm), then the production was induced by adding 0.1-0.5 mM IPTG. After 4-8 h of production the bacteria were harvested and lyzed by several freeze – thaw – sonicate cycles in 25 % sucrose buffer. After centrifugation at 5000× g the pellet was resuspended in 0.1 % Triton X-100 and centrifuged. The inclusion bodies were washed in 50 mM Tris, 150 mM NaCl, pH 7.4.

3.2.3 Protein refolding *in vitro*

All C-type lectin NK cell receptors produced in *E. coli* that are considered in this thesis were refolded using the fast dilution method. An exception is the covalent dimer of human CD69 which was refolded while immobilized on Ni-NTA Sepharose column.

Isolated inclusion bodies were dissolved in denaturating buffer. In most cases 6 M guanidinium hydrochloride, pH 8, with addition of 100 mM DTT was used. The refolding itself was done by adding dissolved inclusions to appropriate pre-chilled refolding buffer that was slightly different for each protein, but in all cases contained 0.4-1 M L-arginine hydrochloride as stabilizing agent and cysteamine/cystamine redox pair.

The dimeric CD69 was solubilized in 8 M urea, slowly dropped into 2 M urea with redox buffer and loaded onto Ni-NTA column, where it was consecutively washed with 2 M urea without redox buffer, Tris buffer and finally eluted using 50 mM EDTA [Childs R. A. *et al.*, 1999].

3.2.4 Transient transfection of HEK293 cells

Being a partner in the EU funded SPINE2: Complexes project, the easiest way seemed to be to try and test HEK293T expression system developed by Radu Aricescu in Oxford – one of the project partners [Aricescu A. R. *et al.*, 2006]. Initially this expression system was tested on six constructs of three C-type lectin NK cell receptors cloned into pHLsec plasmid. Small-scale transfection and immunodetection with PentaHis mAb (Qiagen) were kindly performed by Radu Aricescu in Oxford. Out of the six constructs tested four could be expressed in HEK293T cells and we later reproduced this promising result in Prague, too. However, the original protocol used in Oxford rely on an adherent culture of HEK293T cells and scale-up is done in expanded-surface roller bottles (Greiner Bio-One). Even though we were able to reproduce the protocol technically, the scale-up in roller bottles was time consuming and offer only poor yields in our hands.

Therefore we decided to switch to suspension culture. Firstly, we adapted the originally adherent HEK293T cell line to growth in serum-free chemically defined medium EXCELL293 (Sigma Aldrich) supplemented with 4 mM L-glutamine. Secondly, the cells were adapted to suspension growth, initially in roller bottles and later in a square shaped glass bottles with vented caps (P-lab) fitted by adhesive tape on an orbital shaker (Labnet) set on 135 rpm (rotational diameter 19 mm) placed inside a 37 °C, 5 % CO_2 incubator [Muller N. *et al.*, 2005]. The cells were routinely split twice or thrice a week in the range of 0.2-4×10^6 cell/ml cell densities, initially in EXCELL293 medium alone and later in a 1:1 mixture of EXCELL293 medium and F17 medium (Invitrogen) supplemented with 4 mM L-glutamine and 0.1 % Pluronic F68.

Transient transfection was performed with 25 kDa linear PEI (Polysciences), the ratio of PEI to DNA and other transfection parameters were optimized using pTT vectors kindly provided by Yves Durocher [Durocher Y. *et al.*, 2002]. One of the pTT vectors contains secreted alkaline phosphatase (SEAP), allowing for easy enzymatic assay of recombinant protein production level. Diluted samples of conditioned media were mixed with pNPP substrate and the reaction was monitored at 410 nm. The second pTT vector contains GFP, allowing to monitor transfection efficiency by flow cytometry. Briefly, the desired amount of sterile solutions of plasmid DNA and PEI were mixed in standard cell culture grade of sterile PBS buffer or in F17 medium, incubated for 10-30 min and added to the cell suspension in appropriate volume of F17 medium. After 3-4 hours, the same volume of EXCELL293 media was added and the production was boosted by addition of valproic acid (VPA, Sigma Aldrich) [Backliwal G. *et al.*, 2008] or later by feeding with casein hydrolysate Tryptone N1 (TN1, OrganoTechnie) [Pham P. L. *et al.*, 2005].

3.2.5 Affinity purification

All proteins expressed in HEK293T cells were purified using cobalt

charged TALON metal affinity resin (Clontech). After 4-6 days of batch culture, the cells were spun down at 25000× g and the conditioned medium was filtered, diluted twice with PBS buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM NaN_3, pH 7.0) and batch incubated with TALON resin for 30 min while shaking in an Erlenmeyer flask (110 rpm). The resin was collected on 1×30 cm Econo column (BioRad), washed with PBS and bound proteins eluted with 250 mM imidazole in PBS. The eluted fractions containing the protein of interest, as assessed by SDS-PAGE, were pooled, concentrated using Amicon device (Millipore) and subjected to gel filtration on Superdex 200 HR 10/30 column (GE Healthcare).

3.3 Protein crystallization

Recombinant C-type lectin NK cell receptors were crystallized by hanging drop vapour diffusion method using 24-well Linbro plates (Hampton Research) or EasyXtal plates (Qiagen) or by sitting drop method using 96-well Intelli plate (Art Robins Instruments). The drops were set up by hand or by Crystal Gryphon crystallization robot (Art Robins Instruments). Initial crystallization conditions were established at 18 °C using selected precipitants from JBScreen kits (Jena Bioscience), Crystal screen (Hampton Research) or JCSG+ screen (Qiagen). Each drop was prepared by mixing equal volumes of the protein and the precipitant solution (1 µl or 0.2 µl in case of hanging drops or sitting drops, respectively) and each drop was equilibrated against a reservoir of precipitant solution (1 ml or 50 µl in case of hanging drops or sitting drops, respectively). After optimization of crystallization conditions, crystals were tested for diffraction. For X-ray data collection, crystals were mounted in nylon loops and cryoprotected by soaking in the reservoir solution with suitable cryoprotectant and then flash frozen in liquid nitrogen. Diffraction data collection and structure determination was performed by RNDr. Jiří Brynda, Csc., Mgr. Pavlína Řezáčová, Ph.D.,

Ing. Petr Kolenko, Ph.D. or Ing. Jan Dohnálek, Ph.D.

3.4 Sedimentation analysis

Sedimentation velocity and sedimentation equilibrium experiments were performed using a ProteomeLabXL-I analytical ultracentrifuge (Beckman Coulter) equipped with an An50Ti rotor and dual absorbance and laser interference optics. Before the experiment, 0.5 ml sample of protein diluted to suitable concentration (A_{280} between 0.2-0.8 – usually corresponding to 0.1-0.4 mg/ml) was dialyzed at 4 °C for 20 h against desired buffer and the dialysis buffer was used then as a reference and sample dilution buffer. Sedimentation velocity experiments were typically carried out at 42000-48000 rpm and 20 °C using an epon double-sector cell (Beckman Coulter). Sample and dialysate (both 400 µl) were loaded into the sample and reference sectors, respectively. Absorbance scans were recorded at 280 nm in 5 min intervals with 30 µm spatial resolution in continuous scan mode. Based on buffer composition and amino acid sequence, buffer density and protein's partial specific volume were estimated using the program SEDNTERP (www.jphilo.mailway.com). Data were analyzed with the program SEDFIT [Schuck P., 2000]. A continuous sedimentation coefficient distribution was calculated and the sedimentation coefficient value was determined by integration. Sedimentation equilibrium experiments were performed in a six-sector epon cell at 4 °C with 110 and 130 µl of sample and reference, respectively. Absorbance data was collected in step scan mode at 280 nm by averaging 20 scans with 10 µm spatial resolution after 20 h of achieving equilibrium. The sedimentation equilibrium experiments were globally analysed with the program SEDPHAT [Schuck P., 2003] using non-interacting discrete species model. The size and shape of sedimenting species was predicted using the Teller method in SEDNTERP.

4 Results

4.1 Soluble recombinant CD69 receptors optimized to have an exceptional physical and chemical stability display prolonged circulation and remain intact in the blood of mice

4.2 The high-resolution structure of the extracellular domain of human CD69 using a novel polymer

4.3 Cooperation between subunits is essential for high-affinity binding of N-Acetyl-D-hexosamines to dimeric soluble and dimeric cellular forms of human CD69

4.4 Structural analysis of natural killer cell receptor protein 1 (NKR-P1) extracellular domains suggests a conserved long loop region involved in ligand specificity

4.5 Structure of NKR-P1 family of receptors of natural killer cells: crystal structure of mouse NKR-P1A at 1.7 Å resolution

5 Discussion

The main focus of this thesis was to develop suitable techniques for recombinant expression of NK cell C-type lectin-like receptors, thus enabling their further biochemical, structural and functional studies. One of the most studied receptor, with long research tradition within the research group of Prof. Bezouška is human leukocyte antigen CD69. Initially it was suggested to be an activation receptor [Moretta A. *et al.*, 1991] whose expression is induced very early after NK cells and T cells activation [Testi R. *et al.*, 1994], therefore it has been and still is widely used as the marker of activated lymphocytes. However, since CD69 knockout mice were of rather healthy phenotype and they even displayed increased tumour resistance [Esplugues E. *et al.*, 2003], the role of CD69 is more likely in immune response downregulation and it seems that some tumours are able to trigger apoptosis of NK cells by stimulating them through this surface receptor, which might be an important pathway of autoimmunity regulation. Interestingly, the authors have found that the enhanced anti-tumour response was NK cell and T lymphocyte-mediated, and was due, at least in part, to an increase in local, tumour infiltrating, lymphocytes. Furthermore, anti-CD69 antibody treatment in WT mice induced a specific down-regulation in CD69 expression that resulted in augmented anti-tumour response. Thereby a novel role for CD69 as a negative regulator of anti-tumour responses was identified together with the possibility of a novel approach for the therapy of tumours.

Coincidentally, in about the same time and by opposite approach, a potential role for CD69 in thymocyte emigration was proposed by the characterization of mice that constitutively express high levels of surface CD69 on immature and mature T cells throughout development [Feng C. *et al.*, 2002]. Constitutive surface expression of CD69 did not affect T cell maturation, signaling through the TCR or thymocyte selection. However,

phenotypically and functionally mature thymocytes accumulated in the medulla of CD69 transgenic mice and failed to be exported from the thymus. The retention of mature thymocytes correlated with transgene dose and CD69 surface levels, suggesting a potential role for CD69 in controlling thymocyte export.

These two studies thus independently pointed out for a regulatory role of CD69 in lymphocytes trafficking (increase in CD69 knockout mice vs. decrease in CD69 transgene mice). Naive lymphocytes continually enter and exit lymphoid organs in a recirculation process that is essential for immune surveillance. During immune responses, the egress process can be shut down transiently. When this occurs locally it increases lymphocyte numbers in the responding lymphoid organ; when it occurs systemically it can lead to immunosuppression as a result of the depletion of recirculating lymphocytes. Lymphocyte egress requires a GPCR sphingosine 1-phosphate receptor-1 ($S1P_1$) signaling and several years later, CD69 was shown to negatively regulate $S1P_1$ signaling [Shiow L. R. *et al.*, 2006]. In co-expression experiments, CD69 inhibited $S1P_1$ chemotactic function and led to downmodulation of $S1P_1$. Moreover, CD69 also co-immunoprecipitated with $S1P_1$ but not the related receptor, $S1P_3$. These observations indicated that CD69 forms a specific complex with and negatively regulates $S1P_1$ to promote lymphocyte retention in lymphoid organs. These finding have been quite recently corroborated by a detailed immunoprecipitation study of series of deletion and domain swapped mutants within CD69 and $S1P_1$ molecules [Bankovich A. J. *et al.*, 2010]. CD69 interacts with transmembrane helix 4 of S1P1 through its own transmembrane part and both intracellular or extracellular parts of CD69 do not contribute to this strong interaction. Thus, the biological role of CD69, or at least one part of it, has been identified.

On the other hand, very little is still known about the biological role of CD69 extracellular C-type lectin-like domain, including the native physiological receptor ligand. In the beginning, based on sequence

similarity with classical C-type lectins, a calcium dependent carbohydrate binding activity was expected. The carbohydrate binding was initially questioned [Childs R. A. *et al.*, 1999], however it was later characterized through site directed-mutagenesis and molecular modeling to be calcium dependent with the strongest affinity to GlcNAc and GalNAc monosaccharides in micromolar range [Pavlíček J. *et al.*, 2003].

These findings prompted us to investigate CD69 binding properties also on a structural level. While the crystal structure of CD69 was already known at that time [Natarajan K. *et al.*, 2000, Llera A. S. *et al.*, 2001], the acidic crystallization conditions used in both of these studies precluded presence of calcium or any other ligand, since CD69 ligand binding was shown to be pH-dependent [Pavlíček J. *et al.*, 2003]. While we were able to crystallize and solve the structure of CD69 in several neutral pH conditions, with the addition of calcium or saccharides (direct addition or crystal soaking), no interpretable electron density corresponding to bound ligand could be observed [Vaněk O. *et al.*, 2008, Kolenko P. *et al.*, 2009]. Possible explanation might be the fact that binding of a single monosaccharide itself is too weak and unstable to be visible by protein X-ray crystallography. Therefore other techniques were employed, such as NMR titration experiments, equilibrium dialysis and tryptophan fluorescence quenching titration, that were able to at least biochemically analyze CD69 binding properties [Kavan D. *et al.*, 2010]. Difference between the work of Pavlíček J. *et al.* (2003) and Kavan D. *et al.* (2010) is in the CD69 receptor expression construct: while in the former study a short construct containing only two disulphide bridges was used, in the latter study the full-length C-type lectin-like domain containing three disulphide bridges was utilized. This recombinant form of the receptor is now considered the most stable form that is suitable even for *in vivo* applications [Vaněk O. *et al.*, 2008] and the latter study thus confirm the previous results and further broadens our understanding of CD69 lectin-like domain.

While the results with CD69 and monosaccharides binding might not seem too impressive because of their low affinity, this work pave the way to generation of more complex artificial glycomimetic or other immunoactive ligands targeted to the CD69 receptor. These ligands are based on a presumption that natural saccharide structures recognized by the CD69 receptor are polyvalent branched structures and that the low affinity interaction of individual carbohydrates is overcome by high avidity leading to effective receptor aggregation and lymphocyte stimulation. Several groups of these high-affinity ligands are now available, such as synthetic oligosaccharides based on GlcNAc (e.g. GlcNAc tetrasaccharide) [Kovalová A. *et al.*, 2010], carboxylated calixarenes [Bezouška K. *et al.*, 2010], or the synthetic glycoconjugates based on an immunoactive LELTE peptide [Renaudet O. *et al.*, 2010]. Some of these substances have shown interesting anti-tumour properties both *in vitro* and *in vivo* and even though their mode of action is not entirely understood yet, they certainly possess some therapeutic potential worth of further investigations.

Regarding the native CD69 physiological ligand, an interesting study has recently suggested that this enigmatic ligand might be after all present on certain tumour cell lines [North J. et al., 2007]. The authors have shown that CD69 expressed on the activated NK cell caps at the immunological synapse with RAJI tumour cells, implying that a CD69 ligand is expressed on these NK-susceptible tumour cells. The expression of CD69 ligand on several tumour cell lines was demonstrated using confocal microscopy and also by flow cytometry with fluorescently labeled soluble recombinant CD69 receptors. Moreover, NK cell lysis could be blocked with the recombinant CD69 protein. As the authors used only the extracellular lectin part of the CD69 molecule, this effect could be attributed solely to its C-type lectin-like domain and not to its transmembrane part, as was the case with $S1P_1$ interaction. The CD69 ligand was not expressed on normal peripheral blood lymphocytes and

the identity of this ligand is currently unknown. Since tumour cells often display aberrant glycosylation profile, the CD69 carbohydrate binding might potentially play role in its tumour-restricted ligand recognition.

The exact sequence of the CD69 protein that is chosen as an expression construct proved to be a very important aspect. Many various constructs were cloned and produced in our laboratory throughout years. There were significant differences in *in vitro* refolding protocols as well as in long term stability of protein constructs differing just slightly from each other. The construct G70-K199 showed to be the most stable, it could be concentrated up to 20 mg/ml and was stable for several days at room temperature, while several others precipitated at concentrations as low as 5 mg/ml and a degradation visible on SDS gel electrophoresis occurred even after 24 hours at room temperature. The flexible N-terminal part of the protein seemed to be the most crucial aspect. While too short N-terminal part resulted in protein instability (although all three conserved disulphide bridges were formed correctly), the longest construct tried refolded very inefficiently due to a presence of the odd dimerization cysteine. This might not be the problem now with *Pichia pastoris* and HEK293 eukaryotic expression system in place and truly, covalent dimers of CD69 longest construct are expressed quite easily in these hosts.

Human CD69 as well as all other C-type lectin-like receptors produced in prokaryotic expression systems in our laboratory is precipitated into the inclusion bodies during production. We have tried the periplasmic production in pMAL-p2x system, the production with assistance of low-temperature active chaperons in Arctic Express cells, the production in Origami 2 host cells with thioredoxin reductase and glutathione reductase mutations which should greatly enhance the cytoplasmic disulphide bonds formation or the production in pET-32 vectors with thioredoxin fusion tag. Nevertheless none of this system produced reasonable amount of soluble protein, possibly because of the

three disulphide bonds that are absolutely required for stable receptor molecule and bacteria are simply not able to close them correctly. However, the majority of proteins produced in our laboratory could be refolded *in vitro* with better or worse results using fast dilution method. Some of them, like mouse NKR-P1A, seem to refold well in almost any condition. Nevertheless, we mainly use at least 0.4 M L-arginine hydrochloride as a stabilizing agent (so-called “low molecular weight chaperone”), Tris as a buffer at pH 8 – 9 and cysteamine / cystamine as a redox pair enhancing disulphide bond formation. CD69 belongs to medium-difficult group of proteins that are not very sensitive to L-arginine hydrochloride concentration.

On the other hand, proteins from the NKR-P1 family, being another group of the NK cell receptors much studied within our laboratory, are usually on the worse side. Apart from mouse NKR-P1A and C receptors, that are easily refolded – and that is the main reason why the crystal structure of the A isoform was already solved and the C isoform was recently crystallized – the other family members are not prepared so easily and the research here is still ongoing. Especially with the human NKR-P1, an attractive target from the point of view of human NK cell biology, we were not able to refold this protein at all, in spite of several expression constructs and strategies were tried. This was the main reason to seek an eukaryotic expression system able to produce native NK cell receptors. Two of such systems were employed in our laboratory, the yeast *Pichia pastoris* and HEK293 based expression of secreted proteins, the latter being also one of the major focus of this thesis.

As to the crystal structure of mouse NKR-P1A, this is the result of long lasting efforts of several generations of researchers and students within our research group. The work on this receptor family has started already twenty years ago in early 90s by proposing oligosaccharides being high-affinity ligands for NKR-P1 receptors [Bezouška K. *et al.*, 1994]. Similarly to CD69, this binding specificity was later questioned,

however, some promising results with in vivo cancer treatment were also obtained [Pospíšil M. *et al.*, 2000]. In this study ganglioside GM2 and heparin related-IS oligosaccharides, representing the high affinity ligands for NKR-P1 receptor, increased the sensitivity of tumour targets for killing by the rat effectors isolated from blood and spleen *in vitro* and this positive effect was further confirmed *in vivo* by GM2 and IS carried by liposomes during induced rat colorectal carcinogenesis, where the reduction of cancer incidence versus the controls was greatly reduced. Although these compounds showed possible therapeutic effects, the preparation of liposomes was difficult, therefore the research was later redirected to glycomimetic ligands, as in the case of CD69 protein.

On the structural level, apart from several homology models, nothing was known about NKR-P1 family until now. For historical reasons, the main NKR-P1 target within our laboratory was the rat NKR-P1A isoform. This particular protein could be produced in *E. coli* and refolded *in vitro*, although with a lower yield when compared to CD69. We have tried very hard to crystallize this protein for several years, and also the rat NKR-P1B isoform, but the key to success was to change the source organism. While refolding and purification of rat NKR-P1A protein was never straightforward, the mouse ortholog could be refolded in very high yield and it also displayed very good long-term stability. Its crystal structure was solved by molecular replacement with CD69 structure and finally refined to resolution that do not permit any ambiguity.

The most intriguing part of mouse NKR-P1A crystal structure are the two extended loops and their domain swapping dimerization. These loops are something that was never before observed within C-type lectin-like family of NK cell receptors. Although these loops occur in some C-type lectins outside the NK cell receptor group, we did not find any support for correctness and potential function of these structures. By the time the crystal structure was prepared for publication, a deletion mutant of mouse NKR-P1A was prepared where the whole extended loop is

replaced by two alanines joining the flanking amino acid chains. From yet unpublished observations it seems that the loop is folded back onto the core of the protein in diluted solution. This finding further supports the hypothesis that these loops might be involved in ligand binding and that the extended conformation might represent ligand-bound state of the receptor. Meanwhile the 3D NMR data were collected and the solution structure of mouse NKR-P1A will be hopefully solved soon.

By the same time, the recombinant mouse and rat NKR-P1 isoforms were independently examined by precise Raman spectroscopic techniques coupled to homology modeling. While the homology model of mouse NKR-P1A is not the same as its crystal structure with respect to the loop position, it may be the correct model for the solution state of the receptor. More importantly, bioinformatic analysis of NKR-P1 family sequences in comparison with other C-type lectin-like NK cell receptors revealed that the loop sequences are uniquely conserved feature of the NKR-P1 family that are likely to be involved in ligand binding specificity. The spectroscopic thermal denaturation data and molecular dynamics simulation both suggested that the loop may change its position with regard to the rest of the molecule, although the precision of these techniques is not high enough to provide more details about this process.

Another biophysical technique that was often used throughout this thesis was sedimentation analysis in an analytical ultracentrifuge. Albeit it is a rather old method discovered by Nobel laureate Theodore Svedberg about ninety years ago, modern instrumentation is still capable of providing results that cannot be obtained by any other faster or more high-throughput technique. It is especially useful when comes to analysis of a solution behavior of recombinant proteins. It was routinely used to assess monomeric / dimeric state of soluble recombinant receptors prepared within this thesis. This again proved crucial in case of mouse NKR-P1A: whereas in crystal structure a tetramer (or a dimer of dimers) is observed, the solution state is unequivocally monomeric, at least in

diluted solutions measured. However, the native state of not only mouse NKR-P1A but also other C-type lectin-like NK cell receptors is dimeric, although not many experimental data are available on this subject. The recombinant production of covalent dimer of CD69 was not easy and it was clear that an alternative expression strategy was required. The best option that might seem obvious would be to overexpress these receptors directly in their natural source, that is in NK cells. Although this approach is certainly technically possible, it would require cultivation of large number of NK cells to achieve reasonable yield of desired recombinant protein and this is the stumbling block. NK cells are relatively hard to cultivate, they divide slowly and require expensive media supplementation. Moreover, their chemical transfectability is also rather low when compared to other mammalian cell lines, though they can be successfully transduced by lentiviral vectors for therapeutic purposes [Tran J. and Kung S. K. P., 2007].

Nowadays many relatively cheap and simple eukaryotic expression systems are available. The research in this biotechnological field is driven mainly by pharmaceutical industry where the market with therapeutic recombinant proteins is ever increasing. Each system has its own advantages and disadvantages, but from the point of view of structural biology and especially protein crystallography, several key aspects have to be taken into account. Besides the production yield, which does not necessarily have to be so high as for the market purposes, a suitable recombinant protein production protocol should enable some control over protein glycosylation state, an easy purification with preferably only one or two steps, and also a possibility of selenomethionine labeling would be highly appreciated. The transient transfection of HEK293 cell line and its derivatives fulfills all these needs, therefore it is widely used in structural biology community, though still not as widely as insect cell based expression systems. The advantage of HEK293 cells over the insect cell lines is the glycosylation which is of complex, human type, and this was

one of the reasons why it was chosen for this study. We tried to develop simple and robust protocol for this expression system. The cloning could be easily completed within two days, expression tests within two weeks and the scale-up is straightforward. Recently, we have acquired two derivative HEK293 cell lines, the EBNA modified 293-6E cell line from Yves Durocher [Durocher Y. *et al.*, 2002] and the glycosylation deficient *N*-acetylglucosaminyltransferase I-negative HEK293S cell line [Reeves P. J. *et al.*, 2002]. The former cell line has already proven its good performance when used with a accompanying suite of pTT5 expression vectors containing oriP, an EBNA replication site, showing approximately two-fold increase in SEAP activity when compared to our standard HEK293T cell line. In fact, the first crystals of rat Clrb^{WAG} were observed during writing of this thesis. The protein used for crystallization was produced in 293-6E cell line in the presence of *N*-glycosylation processing inhibitor kifunensine [Chang V. T. *et al.*, 2007]. The second GnTI^{-} cell line might provide the same result without the need to buy an expensive inhibitor. The future improvement of this expression system may include development of cheap culture medium prepared in-house, with possibility of easy selenomethionine labeling, when necessary. The culture productivity might be improved by co-transfection with plasmids bearing genes coding for cell cycle regulatory proteins or anti-apoptotic factors [Backliwal G. *et al.*, 2008]. Alternatively, stable cell line generation might be considered for most interesting targets, possibly by a recombination cloning, where a master cell line with a reporter gene is selected for high productivity and then the reporter gene is replaced by the gene of interest by enzyme mediated site specific recombination [Wilke S. *et al.*, 2010]. Thus, in combination with today’s technology of enzyme synthesis and carbohydrate engineering, it might be soon possible to produce fully functional and appropriately glycosylated forms of dimeric soluble NK cell receptors invaluable for structural and biochemical studies, as well for practical applications in tumor therapies.

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Appendices

Recombinant production of soluble dimeric C-type lectin-like receptors of natural killer cells suitable for structural and functional studies

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Vaněk O., Nálezková M., Kavan D., Borovičková I., Pompach P., Novák P., Kumar V., Vannucci L., Hudeček J., Hofbauerová K., Kopecký V. Jr., Brynda J., Kolenko P., Dohnálek J., Kadeřávek P., Chmelík J., Gorčík L., Žídek L., Sklenář V., Bezouška K. (2008): Soluble recombinant CD69 receptors optimized to have an exceptional physical and chemical stability display prolonged circulation and remain intact in the blood of mice. FEBS J, 275, 5589-5606.

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