UNIVERZITA KARLOVA V PRAZE

Přírodovědecká fakulta

Katedra fyzikální a makromolekulární chemie

Charakterisace proteinu Naaladase L2

Characterisation of Naaladase L2 protein

Diplomová práce studijního oboru Biofyzikální chemie

Praha 2015 Helena Jindrová

Prohlašuji, že jsem tuto diplomovou práci vypracovala samostatně pod vedením školitele doc. RNDr. Jana Konvalinky, CSc. a všechny použité prameny jsem řádně citovala.

V Praze dne 11. 5. 2015 .....

Acknowledgement

First and foremost I would like to thank Jan Konvalinka for accepting me to his research group. I would like thank Pavel Šácha for guiding me through my work and solving any problems that occurred during the process. My thanks belong to Barbora Vorlová and Juliane Proft for careful proof-reading and moral support. I am very grateful to Michal Svoboda, Karolína Šrámková, Jana Starková and Tomáš Knedlík who never hesitated to give me a valuable advice. Last but not least, I would like to thank the whole research group for accepting me and helping me in moments of doubts.

Abstract

N-acetylated alpha linked acidic dipeptidase like 2 protein (Naaladase L2) is a transmembrane protein possessing large extracellular as well as intracellular part. The function of this protein has not been determined yet. It was suggested to be associated with a wide range of diseases such as Kawasaki syndrom, an autoimmune disease causing inflammation of vessels, and others. Its homologue, glutamate carboxypeptidase II (GCPII), also called N-acetylated alpha linked acidic dipeptidase (Naaladase), is a transmembrane protein with large extracellular part possesing protease activity. GCPII was suggested as a diagnostic and prognostic marker of prostate cancer.

According to a recent study, Naaladase L2 mRNA levels correlate with Gleason score used for evaluating the prognosis of prostate cancer. Cells overexpressing Naaladase L2 showed higher invasivity, migration and formed more colonies than the negative controls. Additionally, a difference in mRNA expression between benign hyperplasia and tumour was found. Naaladase L2 is thus believed to have a potential in serving as a diagnostic or prognostic marker of prostate cancer.

Only a few reports have been published concerning investigation of Naaladase L2 and none of them focus on exploration of the protein itself. Therefore, we decided to recombinantly prepare and characterise this protein. We expressed the extracellular part of Naaladase L2 in insect cells and optimised its purification using affinity chromatography and gel filtration. We found that similarly to its homologue GCPII, Naaladase L2 undergoes posttranslational glycosylation. On the other hand, in contrast to GCPII, no proteolytic activity of extracellular part of Naaladase L2 has been observed. We also expressed the intracellular part of Naaladase L2 in HEK cells and investigated its potential interaction partners using pull-down experiments and mass spectrometry. The preliminary data suggest that Naaladase L2 might be associated with cytoskeleton organisation and cytoskeletal changes in cell division by interacting with proteins envolved in these processes. However, more experiments need to be performed to confirm this hypothesis.

Key words: Naaladase L2, GCPII, homologue, protein-protein interaction

Abstrakt

"N-acetylated alpha linked acidic dipeptidase like 2" (Naaladasa L2) je transmembránový protein s velkou extracelulární a intracelulární částí. Jeho funkce není známá. Bývá spojován s celou řadou nemocí včetně Kawasakiho syndromu, autoimunitního zánětlivého onemocnění cév, a mnoha dalšími. Jeho homolog glutamátkarboxypeptidasa II (GCPII), také nazývaná "N-acetylated alpha linked acidic dipeptidasa" (Naaladasa), je transmembránový protein s velkou extracelulární částí s proteasovou aktivitou. Byl navržen jako diagnostický a prognostický marker nádoru prostaty.

Hladiny mRNA Naaladasy L2 podle nedávné studie korelují s Gleasonovým skórem používaným ke klasifikaci nádorů prostaty. Buňky s vysokou expresí tohoto enzymu vykazovaly větší invazivitu, migraci a schopnost tvořit kolonie. Byl nalezen rozdíl v expresi Naaladasy L2 mRNA mezi benigní prostatickou hyperplazií a nádorem prostaty, a proto se o tomto proteinu uvažuje jako o možném markeru nadorů prostaty.

Naaladasa L2 není ještě plně prozkoumána, bylo publikováno jen málo studií o expresi genu kódujícího Naaladasu L2 a žádná z nich se nezabývá proteinem. Z tohoto důvodu jsme se rozhodli se jím zabývat. Připravili jsme jeho extracelulární část v hmyzích buňkách, optimalizovali jeho purifikaci pomocí afinitní chromatografie a gelové filtrace a prozkoumali jeho možnou proteolytickou aktivitu. Zjistili jsme, že stejně jako GCPII , Naaladasa L2 je posttranslačně glykosylována. Na rozdíl od GCPII, u extracelulární části Naaladasy L2 ale nebyla detekována žádná proteasová aktivita. Také jsme exprimovali jeho intracelulární část a hledali jejího interakčního partnera pomocí pull-down experimentu a hmotnostní spektrometrie. Předběžné výsledky ukazují, že funkce tohoto proteinu by mohla být spojená s cytoskeletem a jeho změnami vedoucími k nádorovému bujení. Pro ověření této teorie je ale třeba provést další experimenty.

Klíčová slova: GCPII, Naaladasa L2, homolog, protein-proteinové interakce

List of Abbreviations

AD	activating domain
AMP	adenosin monophosphate
ATP	adenosin triphosphate
AGR2	anterior gradient protein 2 homolog
AviEXST	biotinylated extracellular part of GCPII
AviEXSTL2	biotinylated extracellular part of Naaladase L2
CBP	calmodulin-binding protein
cAMP	cyclic adenosin monophosphate
DNA	deoxyribonucleic acid
DBD	DNA-binding domain
EDTA	ethylen diammin tetraacetate
EGTA	ethylene glycol tetraacetate
FT	flow through
FOLH	folate hydrolase
GPCR	G protein coupled receptor
GCPII	glutamate carboxypeptidase II
GCPIII	glutamate carboxypeptidase III
GST	glutathion-S-transferase
GFP	green fluorescent protein
GDP	guanosin diphosphate
GTP	guanosin triphosphate
HRP	horse radish peroxidase
HEK	human embryonal kidney cells
HILAP	human ileal aminopeptidase
CCD	charge coupled device
IgG	Immunoglobulin G
LC-MS	liquid chromatography mass spectrometry
LNCaP	lymph node carcinoma of prostate
MBP	maltose-binding protein
MS	mass spectrometry
mRNA	messenger ribonucleic acid
Naaladase	N-acetyl-alpha linked acidic dipeptidase
Naaladase L2	N-acetyl-alpha linked acidic dipeptidase like 2 protein

NAAG	N-acetyl-aspartyl glutamate
NEB	New England BioLab
NMR	nuclear magnetic resonance
ORF	open reading frame
PICS	proteomic identication of protease cleavage sites
PNGase F	Peptide -N-Glycosidase F
PBS	phosphate buffer solution
p53	phosphoprotein p53
PAGE	polyacrylamid gel electrophoresis
PVDF	polyvinylidene fluoride
PSA	prostate specific antigen
PSMA	prostate specific membrane antigen
PSMAL	prostate specific membrane antigen like protein
PICS	Proteomic identification of protease cleavage sites
qPCR	real time polymerase chain reaction
RNA	ribonucleic acid
SDS	sodium dodecylsulphate
SPR	surface plasmon resonance
TEV	tobacco etch virus
Tris	tris(hydroxymethyl)aminomethane
TBS	Tris buffer solution
UTR	untranslated region

Contents

Part I

Introduction

1 GCPII

Glutamate carboxypeptidase II (GCPII), prostate-specific membrane antigen (PSMA), N-acetyl-alpha-linked acidic dipeptidase (Naaladase) and folate hydrolase 1 (FOLH1) are all names of one enzyme. This enzyme is encoded by the 62.036 base pairs long FOLH1 gene which is located on chromosome 11 at position 11p11.2 in the human genome. FOLH1 gene contains 18 exons and translates into a 750 amino acids long protein [1]. GCPII is a type II transmembrane glycoprotein spanning the membrane once with its C-terminus exposed to the extracellular space [2]. It is a homodimeric dinuclear zinc metallopeptidase with a molecular weight of 110 kDa [1, 3, 4, 5]. It consists of a large extracellular domain (707 amino acids), a hydrophobic transmembrane domain (24 amino acids) and a short intracellular domain (19 amino acids) [1, 6]. The extracellular domain is highly glycosylated [4]. It belongs to the M28 peptidase family [7] that contains metalloenzymes, aminopeptidases and carboxypeptidases with cocatalytic zinc ions [8]. The names Naaladase and folate hydrolase 1 for GCPII deflect known functions of the enzyme. The first function is the cleavage of the dipeptide N-acetyl aspartyl glutamate (NAAG) into N-acetyl aspartate and free glutamate in the brain; where glutamate is an important neurotransmitter [9, 10]. Second function is hydrolysis of folylpoly-γ-glutamate and facilitation of folate uptake into small intestine [11]. Furthermore, GCPII is expressed in renal tubules and in the prostate where its function remains unknown [12].

The extracellular part of GCPII (figure 1, page 12) consists of three domains: apical (amino acids 117-351), protease (amino acids 56-116 and 352-591) and helical (amino acids 592-750). The apical domain, consisting of three alpha helices and two beta sheets, forms the substrate-binding cavity. The protease domain consists of 11 alpha helices and 8 beta sheets. The helical domain is formed by six alpha helices and connects the two monomers into a dimer [13, 14].

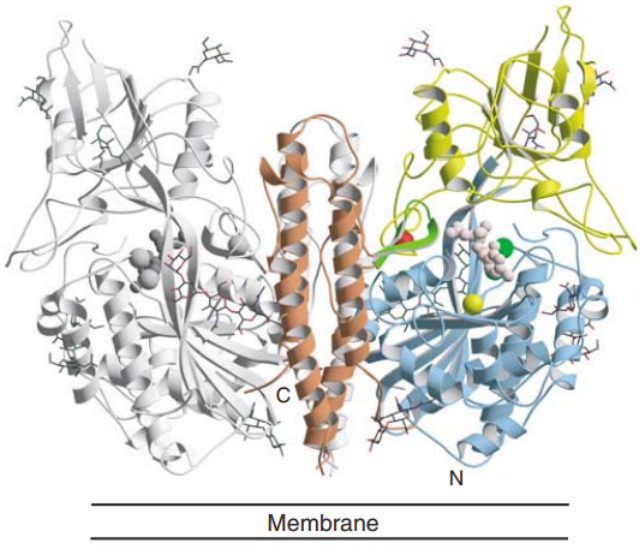

Figure 1: Structure of the extracellular part of GCPII; only one half of the dimer is in colours. Helical domain is in brown, protease domain is in blue and apical domain is in yellow. Zinc ions necessary for the substrate cleavage are indicated by green spheres. Yellow sphere indicates Cl^- and the red sphere stands for Ca^{2+}. The substrate binding cavity is highlighted in darker colours. Adapted from [13].

The highest expression of the enzyme occurs in normal, tumour and metastasizing prostate tissue [15, 16, 17, 18, 19]. The function of GCPII in these tissues is unknown, yet being a transmembrane homodimeric protein gives rise to a speculation about possible receptor function. Furthermore, its similarity to transferrin receptor, regarding both amino acid and domain organisation, supports this hypothesis [20, 14, 21, 13]. As well as receptor proteins, GCPII is internalized through clathrin coated pits, which also supports the receptor

function theory; however, no ligand has been found yet [21]. Interestingly, immunohistochemical experiments have shown a higher expression in metastasizing malignant tissue than in benign tissue or in hyperplasia suggesting its possible role in tumour development [3, 22].

1.1 GCPII Homologues

There are several GCPII homologues in human genome that share 26% to 98% of GCPII sequence - GCPIII, PSMAL, Naaladase L and Naaladase L2.

The closest one is the prostate specific membrane antigen like protein (PSMAL) which was found only in primates. Its gene FOLH1B is located on chromosome 11 at the position 11q14.3 in the human genome [1, 23]. The longest open reading frame (ORF) found leads to a protein consisting of 442 amino acids, sharing 98% identity with GCPII. Its mRNA can be found in human liver and kidney tissue [23]. Protein resulting from expression of the FOLH1B gene is probably proteolytically inactive due to large deletion at the N-terminus [24].

Another known homologue is the glutamate carboxypeptidase III (GCPIII) encoded by NAALAD2 gene. This 740 amino acids long protein folds into a type II transmembrane protein and shares 68% identity with GCPII [25]. Similarly to GCPII, GCPIII is capable of cleaving NAAG [25, 26, 27]. It was suggested that GCPIII can compensate for GCPII in the NAAG hydrolysing activity in GCPII knock-out mice [28, 29]. It also cleaves β-citryl glutamate [30]. GCPIII mRNA was found by Northern blot in testes, spleen and ovary, mild mRNA expression was detected in other tissues like prostate, placenta or heart [25]. Using qPCR, different parts of the brain showed presence of GCPIII mRNA, the highest in cerebellum and hippocampus [31].

N-acetyl-α-linked-acidic dipeptidase like protein (Naaladase L) is another homologue of GCPII. It is a protein encoded by the gene NAALADL1 on chromosome 11q12 [25]. It is 740 amino acids long and shares 37% sequence identity with GCPII. The Naaladase L protein is expressed in the small intestine. An activity similar to the one of GCPII was expected. However, Tykvart et al. recently showed that Naaladase L is an aminopeptidase and does not possess any carboxypeptidase acitivity. It processes a wide range of substrates from short peptides to longer oligopeptides. A new name human ileal aminopeptidase (HILAP) was suggested for the enzyme [32].

2 Naaladase L2

N-acetylated α-linked acidic dipeptidase like 2 protein (Naaladase L2) also belongs to the M28B protein subfamily. It is encoded by the NaaladL2 gene located on chromosome 3 at the position 3q26.31 [33]. It shares 26% sequence identity with GCPII, and around 20% sequence identity with the transferrin receptor. Naaladase L2 is a transmembrane protein with 652 extracellular amino acids, 20 amino acids spanning the transmembrane domain and further 121 intracellular amino acids. There is no published structure yet, though the transmembrane part is predicted to be helical and classifies Naaladase L2 as a type II transmembrane protein with single spanning region and N-terminus exposed to the intracellular side. Theoretical prediction software indicated seven glycosylation sites for Naaladase L2 and one phosphorylation site (figure 2, page 15) [33].

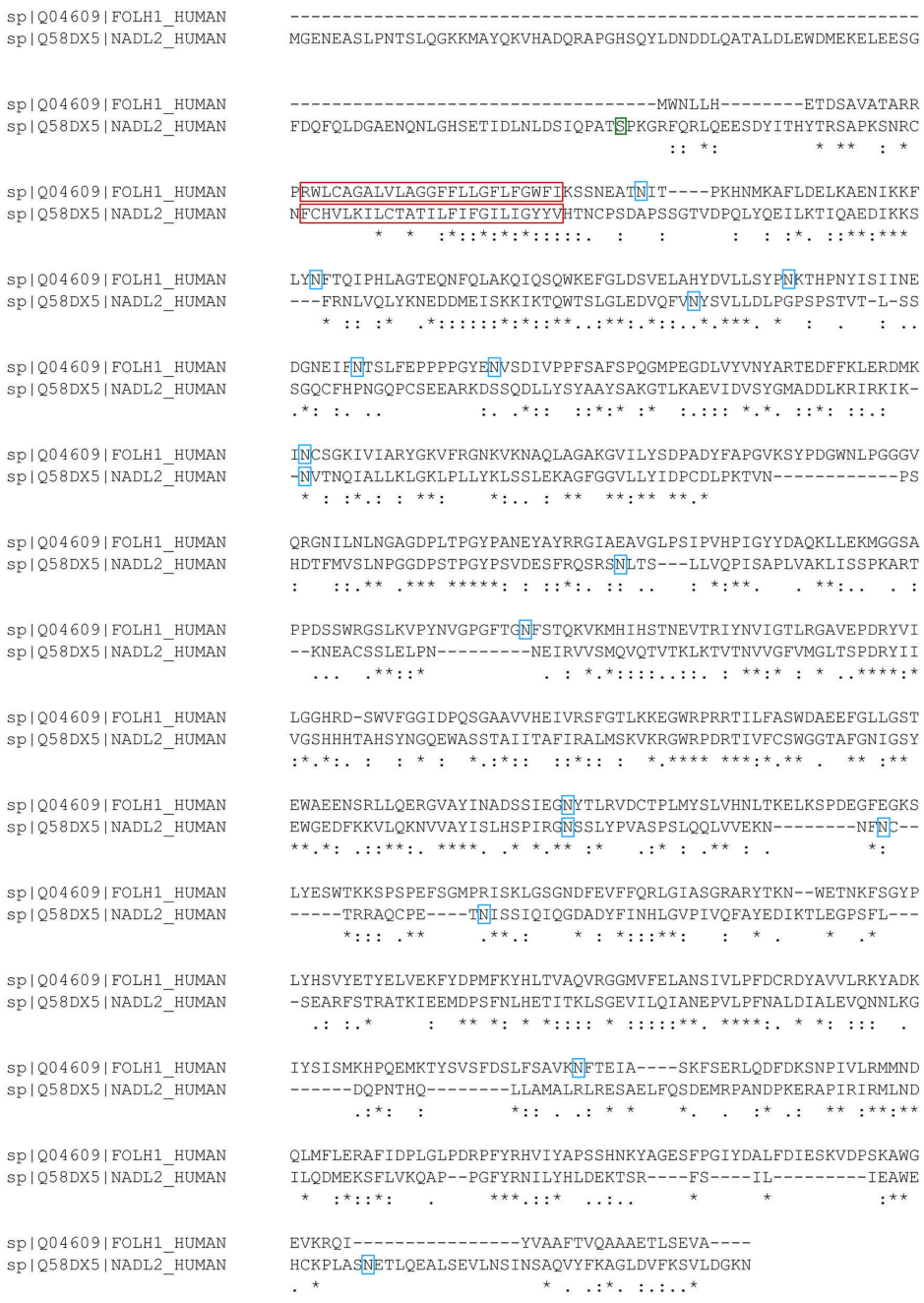

Figure 2: Amino acid alignment of the GCPII and Naaladase L2 sequence. First line corresponds to GCPII, second to Naaladase L2. Red frame highlights the predicted transmembrane domain, blue frame glycosylation sites and the green frame phosphorylation site. Identical amino acids are marked with asterisk. Colon markes a high similarity between amino acids, full stop a weak similarity.

Naaladase L2 was discovered in 2004 in connection with research on the Cornelia de Lange syndrome, a genetic disorder causing physical and cognitive handicaps [34]. The Cornelia de Lange syndrome is a physical and mental disease caused by breakage of chromosome 3 in the same region where NaaladL2 is positioned, however no correlation of disease with Naaladase L2 expression levels was observed [34]. Tonkin et al revealed that the NaaladL2 gene contains 32 exons, spanning 1.37 Mb. The whole region is exon-poor, some of its introns are hundreds of kilo bases (kb) long, the longest intron contains over 230 kb. At least four protein isoforms are formed by extensive alternative splicing at both the 3'UTR and 5'UTR termini. The full-length protein transcribes from the longest open reading frame and has a start codon within the exon 12. Another potential translation start is positioned in exon 14. The full-length protein and some other splice variants contain a transmembrane domain similar to the one of GCPII. In contrast, some other predicted proteins originating from splice variants might lack the hydrophobic region of amino acids 128 - 146 forming the transmembrane domain [34].

Abundant full-lenght mRNA expression levels have been observed in placenta, kidney, liver and skeletal muscle tissue. Developmental mRNA expression analysis has shown a strong gut expression, hinting a possible role in food and dietary processing [33]. No signal was observed in developing neuronal and limb tissue. Despite high similarity with GCPII, Naaladase L2 lacks crucial highly conserved amino acids enabling the metallopeptidase activity [34]. Naaladase L2 is localized to the plasma membrane, particularly at the basal cell surface in contrast to GCPII which is expressed at the apical part of the cell membrane [35].

Interestingly, the locus of the NaaladL2 gene was found as one of the possible loci affecting human height [36]. The only normal, non-diseased tissues expressing Naaladase L2 protein are kidney and stomach [35]. Several studies

suggested a connection between expression of the NaaladL2 gene and various health issues or phenotypes, such as connections between various stages of mental retardation, psychomotor retardation, dysmorphic features, congenital defects, vascular problems like Kawasaki disease and deletions, nucleotide polymorphism or chromosome breakage in the region of the NaaladL2 gen. However, all studies admit difficulties in correlating chromosome abnormalities and phenotypes that differ greatly among patients [37, 38, 39].

Recently, Whitaker and colleagues have shown that Naaladase L2 is overexpressed in colon and prostate cancer tissue [35]. Mild expression was found in tongue, pancreas and breast cancer. A high increase in protein expression level was observed with increasing Gleason score; which is used for evaluating prostate cancer based on tissue biopsy and microscopy appearance. Patients with overexpressed NaaladL2 gene had a significantly lower survival. The mRNA level of Naaladase L2 in blood is significantly higher in patients with bioptically confirmed prostate cancer. Patients with high prostate specific antigen (PSA, a peptidase secreted by prostate used for prostate cancer screening) levels but negative biopsy showed normal levels of Naaladase L2 [35].

It was shown using knock-down cell lines that cells overexpressing Naaladase L2 showed different adhesion properties than equivalent control cells. LNCaP cells overexpressing Naaladase L2 formed more colonies and showed greater migration and invasion potential than knocked down cells. This might explain why Naaladase L2 mRNA levels correlate with prostate cancer since overexpression of Naaladase L2 might lead to tumour growth and invasion to surrounding tissues. The expression of several androgen-regulated genes was related to the expression of Naaladase L2, the most important is anterior gradient 2 (AGR2) [35]. Androgen regulated genes play a role in almost all cellular functions such as lipid metabolism, differentiation, cellular proliferation

and survival. Hormonal therapy of prostate cancer involves lowering the level of the androgen testosterone resulting in suppressing of the tumour growth [40]. Naaladase L2 expression levels also correlate with downregulation of proapoptotic p53 (also called tumour suppressor p53), cell transformation and migration [41]. These observations led to conclusion that Naaladase L2 may be a potential tumour marker.

3 Protein-Protein interaction detection

There are several methods to study protein-protein interactions. Here is a summary of methods usually used at the very beginning of the search for interacting partner. Most of them are followed by analysis and characterisation of identified molecules by mass spectrometry or nuclear magnetic resonance and other methods.

3.1 Affinity chromatography

Affinity chromatography uses highly specific interactions between two partners, such as receptor and ligand, enzyme and substrate or antibody and antigen to purify molecules from a mixture. It can be used for detection of new interaction partners in a co-purification also called pull-down experiment, which will be described later. Affinity chromatography is a specific, fast, well reproducible and provides a high level of purity. On the other hand, a non-specific adsorbtion cannot be avoided. The yield of purified protein (or its interacting partner) is relatively low. Usually, harsh or even denaturing conditions are needed for the elution and some less stable molecules might be obtained in much lower amounts in native state. Also, expensive materials are used during this experiment. If a tag is used instead of a ligand

(for more information see below), the properties of the protein might change, such as expression levels in the cell, protein folding, binding to the matrix and binding of the putative partner.

First step is binding one of the interaction partners or a ligand of the protein to an insoluble, mechanically and chemically stable matrix. Large surfaces enabling binding of a large quantity of molecules as well as good flow properties are necessary. The matrix is usually used in a form of beads, widely used materials are agarose, glass, cellulose and polyacrylimide [42]. Next step is usually an equilibration of the matrix followed by application of the biological mixture containing the protein/binding partner of interest. Non-specific binding is then washed off with an appropriate washing buffer. The procedure ends with elution step during which the bound protein of interest is separated from its binding partner or both binding partners are separated from the matrix. The process is depicted in the figure 3 on page 20.

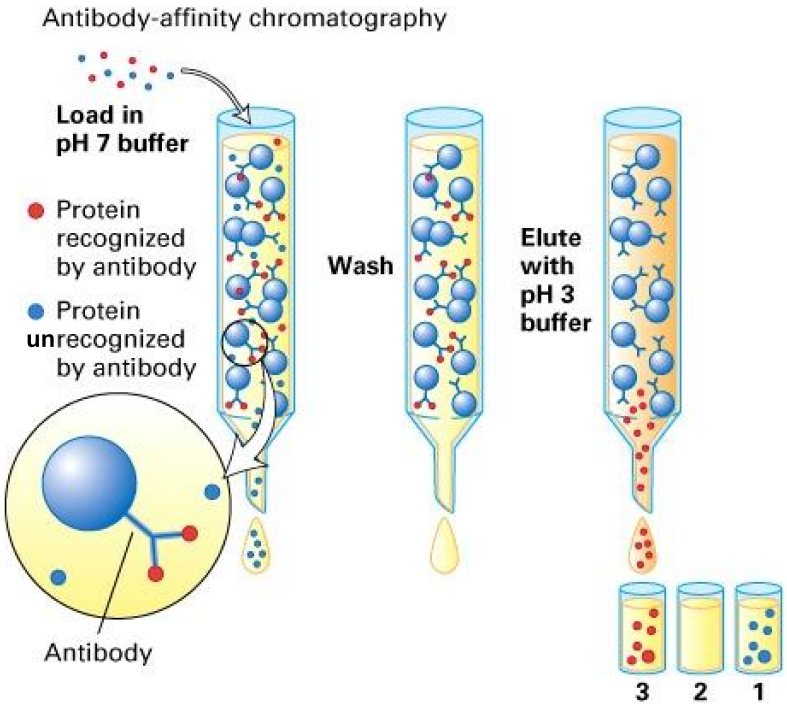

Figure 3: Affinity chromatography; A molecule binding the protein of interest (specific antibody) is bound to the matrix (blue big balls). Mixture of proteins is loaded on the column, proteins recognized by antibody (red) bind to the matrix while the rest of the mixture (blue) is washed out. The elution is performed by changing conditions such as lowering the pH. Adapted from [43].

To simplify binding of the protein or a ligand to the matrix, a tag can be attached. There are several genetically grafted peptide sequences known as protein tags that can be attached to a recombinant protein, for example histidine (His) tag, (FLAG)-tag, Softag, CBP (calmodulin binding peptide), GST-tag (glutathion-S-transferase), Strep-tag or Avi-tag. Histidine forms a coordinate covalent bond to metal ions, therefore a metal containing matrix can be used for proteins with a poly hisitidine tag (His-tag) [44]. FLAG-tag is a peptide consisting of eight amino acids (Asp Tyr Lys Asp Asp Asp Asp Lys) recognized by anti-FLAG antibodies M1, M2 and M5 [45, 46]. Other peptides can be used in combination with a specific antibody as Softags [47]. The calmoduline binding peptide (CBP) consists of 26 amino acids and binds

to the eukaryotic messenger protein calmoduline in the presence of calcium ions [48]. A protein tagged with the tripeptide γ-Glu-Cys-Gly, glutathion, can be purified using a matrix with glutathion-S-transferase [49]. The well known Strep-tag is a peptide bound by streptavidin, a tetrameric protein purified from the bacterium *Streptomyces avidinii*, Avi-tag is a sequence recognised by biotin ligase biotinylating the protein, therefore biotinylated proteins can be purified using modified streptavidin proteins with lower affinity. There are many other tags to be used for purification via affinity chromatography.

Co-immunoprecipitation, or pull down experiments, can be used to investigate a potential protein-protein interaction. The protein of interest is captured from a solution via an antibody or a tag binding to the matrix. After washing out the non-specifically bound proteins so only the protein of interest is bound to the matrix, cell extract with potential binding partners is applied and the protein interacts with its partner. The complex is washed and eluted the same way as was described above. The final product(s) can be analysed by SDS-PAGE, mass spectrometry, NMR or other methods.

3.2 Tandem affinity purification

In case of tandem affinity purification, a multiple tagging of protein is used. It enhances the purity of the product and can lead to high yields. A major disadvantage is the same as mentioned above in the affinity chromatography; a tag has to be added to the protein sequence. It can change the behaviour of the protein and affect protein expression levels. Aditionally, it might hinder the binding of the protein to the matrix [50, 51].

A typical experimental setup is depicted in figure 4, page 22: target protein, calmoduline binding peptide, Tobacco etch virus (TEV) protease cleavage site and protein A. First, a target protein is expressed and bound to a matrix on

which cell extract with interacting partner is applied. The complex of protein and its partner is then purified on matrix with bound IgG via the terminal protein A. After washing, TEV protease is applied and cleaves off the target protein complex which then ends with the calmoduline binding site. This site is used in the second round of purification for binding on calmoduline-coated beads. The remaining TEV protease and other impurities are washed away. Elution is achieved by ethylene glycol tetraacetic acid (EGTA) which binds calcium atoms necessary for calmoduline-CBP bond [52, 50].

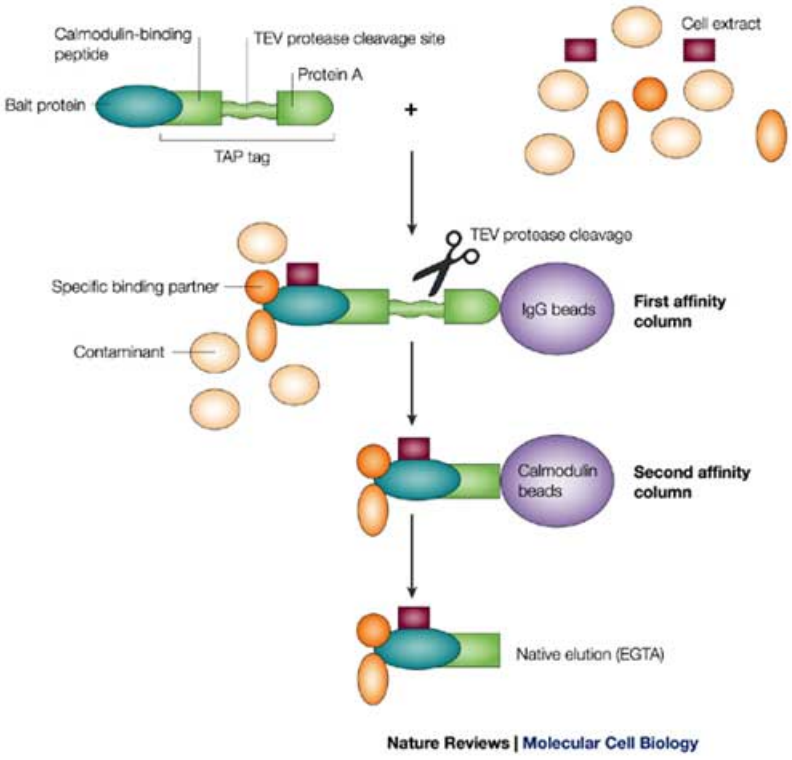

Figure 4: Tandem affinity purification; a bait protein (blue) bound to IgG beads (purple) through CBP (green), TEV protease cleavage site (green) and protein A (green). A cell extract is applied on the column leading to binding of the specific binding partner. After washing the contaminants out, the TEV site is cleaved by TEV protease. The partner-bait complex with CBP is then applied to calmodulin beads. The remaining contaminants are washed out and the final elution is performed by ethylene glycol tetraacetic acid (EGTA). Taken from [53].

Other tags can be used for this procedure; however, this composition has

proved as efficient in recovery when using a low concentration of fusion protein in a complex mixture. The eluate can be analysed by SDS-PAGE, mass spectrometry, NMR or other methods [52, 50].

3.3 Affinity blotting

Affinity blotting can be also used for detecting protein-protein interaction. There is one main disadvantage of this method, the cell extract must be denatured and therefore any interaction requiring native folded structure cannot be detected [54]. A purified prey protein, whose partner is being searched, must be detectable by specific antibody, an antibody recognising a label on the protein must be used or the protein must be radioactively labelled. First, a cell extract with prey proteins is separated by SDS-PAGE and blotted onto a membrane (figure 5, page 23). The membrane is blocked and then the prey protein with a label is applied. The detection is carried out the same way as in conventional blotting. The advantage of this method is that it can be used even for insoluble proteins or proteins difficult to express in cells [55].

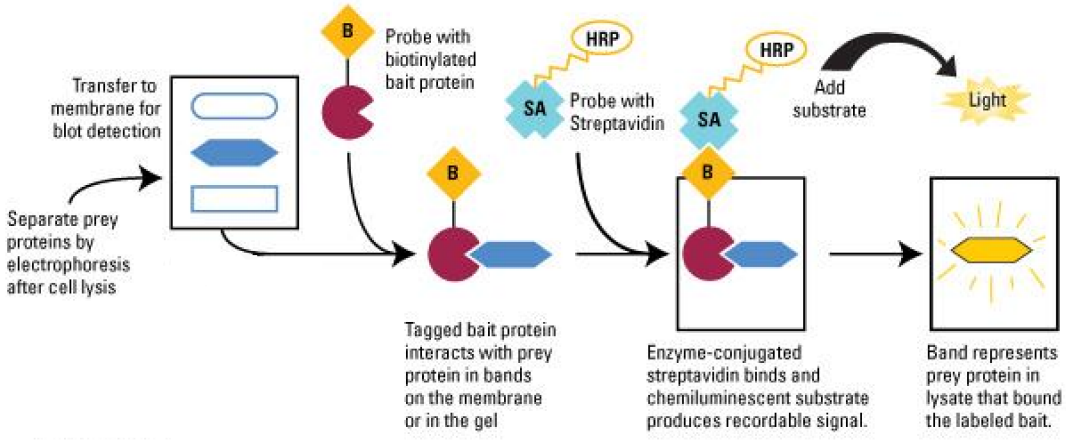

Figure 5: Affinity blotting; A cell extract or other protein mixture is separated on SDS-PAGE followed by Western blot. The membrane is incubated with specific probe containing the protein in question. The bands containing the interaction partner are visualised due to the tag bound to the probe. Adapted from [56].

3.4 Yeast two hybrid

Yeast two hybrid system is a high-throughput method performed in vivo, where the proteins are in their natural environment and can be post-translationally modified.

It was invented by Fields and Song after discovering that transcription activator consists of two parts; the N-terminal domain binding the DNA (DNA binding domain, DBD) and the C-terminal domain activating the transcription (activating domain, AD). After separation of these two domains the N-terminal part still binds to DNA and the C-terminal part does not lose the activation function. However, they work only when put together to the right place.

The principle of this method lies in fusing the target protein (so called bait) with DBD and using a library of potential interacting partners (so called preys) fused with AD. In each yeast cell there must be two plasmids, one is the same for all cells and contains the sequence coding for the bait fused with DBD, the other one contains the sequence of one prey protein fused with AD. These cells are commercially available. If a mixture of cells, therefore a mixture of plasmids coding for different baits is used, there is a possibility that one cell will contain the sequence for the searched partner. When bait meets its prey, DBD and AD interact. DBD is bound to the DNA; therefore AD is in the position allowing RNA polymerase II to start transcription. The final product of transcription can be for example β-galactosidase. The cell then starts cleaving a colorimetric substrate and can be distinguished as a positive hit. These cells can be sequenced and the vector from the prey library is established [57, 58].

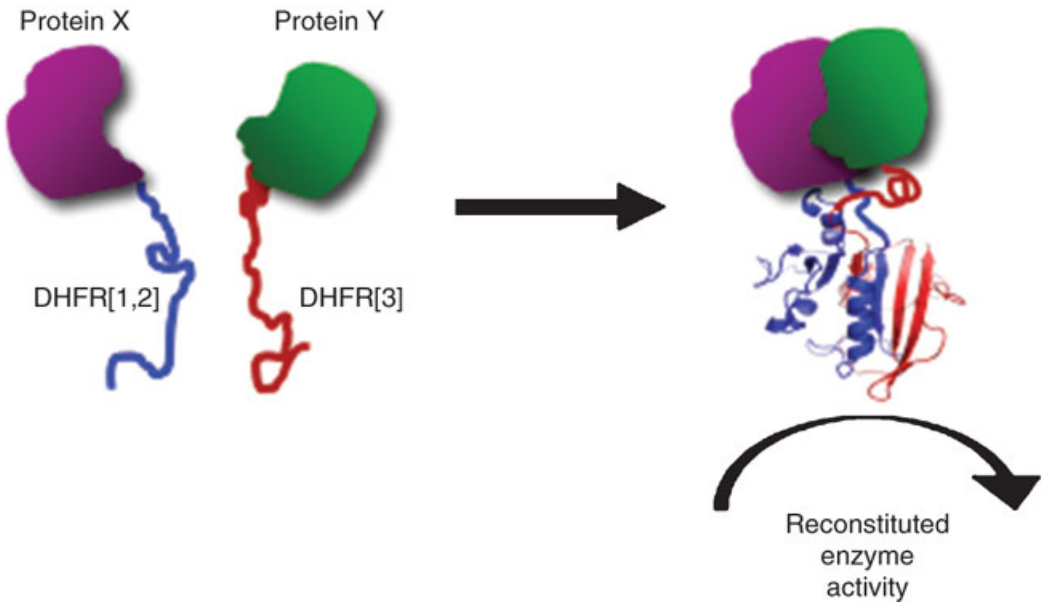

Figure 6: Protein fragment complementation; Protein X serves as a bait, protein Y as a prey. When these two proteins interact, they bring two parts of dihydrofolate reductase (DHFR) to proximity. Cells are detected due to reconstituted DHFR activity and their survival and growth in selective medium. Taken from [59].

A method called protein-fragment complementation is based on the same principle (figure 6, page 25). The reporter can be one protein cut in half and each half is fused to either bait or prey. When put together, a colorimetric product can be produced or some other characteristic as a resistance to an antibiotic. It is necessary that the two parts of protein fold into native structure only when brought together [59, 60].

The drawbacks of this method are that weak interactions might not be detected and the linkage with other molecule might sterically hinder or completely disrupt the folding of the proteins as in all methods using tags. Fusion proteins might also behave differently from the wild-type proteins when it comes to localization or function.

3.5 Display systems

All display methods share the same principle described in the first method, phage display, although they use different media to display the protein or peptide library.

3.5.1 Phage display

Phage display is a useful tool for testing large libraries of peptides or protein domains. It is widely used for antibody search but it can be used for detection of many other interacting molecules such as protein-DNA, protein-RNA, protein-ligand and protein-protein. A library of random peptides must be cloned into a vector containing sequence ensuring that the product will be displayed on the phage surface (figure 7, page 27). These libraries are commercially available. Target protein is bound to surface for example via immobilized antibody. Phage library with exposed potential partners on the surface is added. Only phages containing reacting molecule remain bound to the target. *E. coli* are subsequently infected by the positive phage particles and the whole experiment is performed again in the process called "biopanning" until there are only phages exposing binding partner of the target (usually after three rounds). In the final step, cells are infected by the phages containing the interaction partner. The DNA coding for the partner is extracted and sequenced [61, 62, 63].

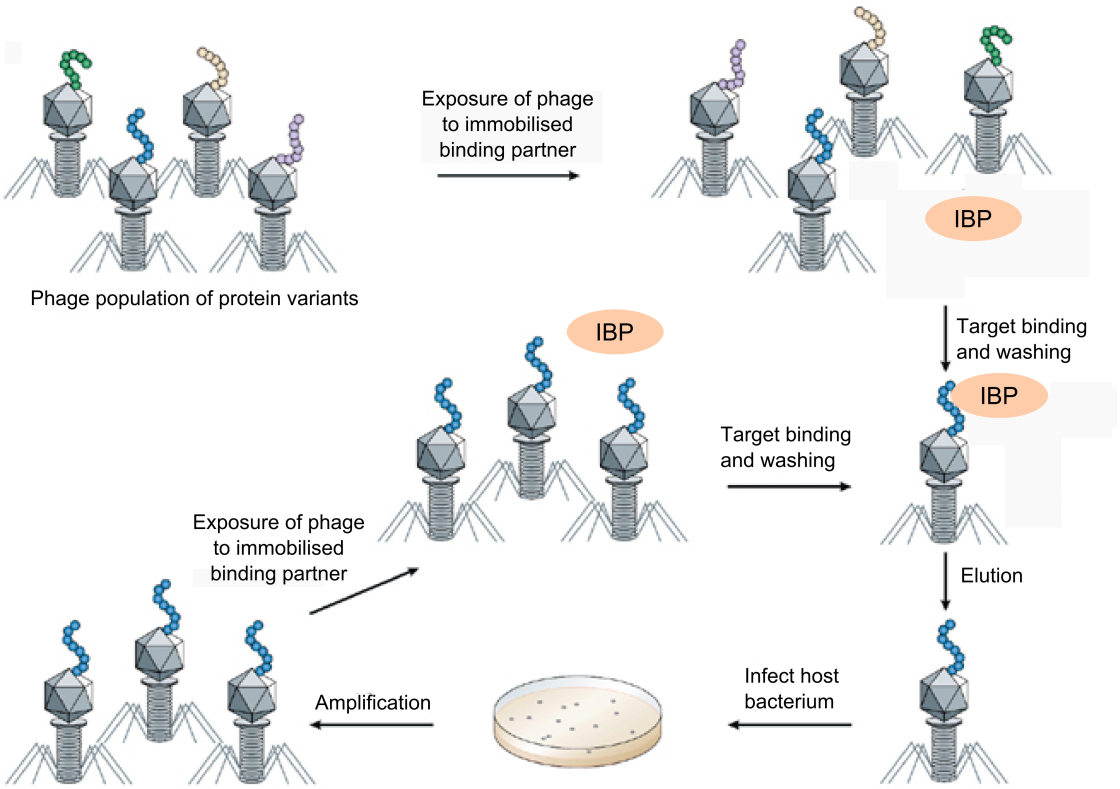

Figure 7: Phage display; phage particles containing different proteins exposed on the surface are incubated with immobilized binding partner (IBP). Only the phage with interacting protein remains bound to the molecule after washing. After elution bacteria are infected with these phages in an amplification step. The incubation with partner is done again and the cycle is performed until only the phages with the interacting parter are present, usually after three rounds. This process is called panning. Adapted from [64].

Phage display libraries are cheap, commercially available, easy to amplify and it is possible to store them for a long time. Libraries contain a large number of peptides (up to 10^9) and are tolerant to harsh conditions [65]. On the other hand, only simple linear or cyclic peptides consisting mostly of naturally occuring amino acids can be used [66], although it is possible to incorporate even D-amino acids [67].

3.5.2 Bacterial display

In bacterial display the library of prey proteins is fused with a scaffold protein expressed on the cell surface. The procedure is identical to the one in

phage display, only instead of phages bacteria are used. Using Gram-positive bacteria is advantageous since they do not contain the periplasmic membrane and the translocation of the fusion protein to the cell surface is thus easier [68, 69, 70].

This method is often coupled with fluorescence-activated cell sorting or magnetic-activated cell sorting system where cells are sorted based on their epitopes. In the first case, the bait protein is fluorescently tagged and only those bacteria which are displaying the prey have a flourescently labeled bait protein bound. The analysis then proceeds the same way as in phage display. Using fluorescence-activated cell sorter lowers the amount of false positive hits caused by non-specific binding to the surface. It can also be used for quantification [71]. If using a magnetic cell sorter, bacteria are incubated with magnetic beads to which the bait protein is bound. After washing only the positive cells remain bound to beads [68, 51]. However, display of some proteins might be problematic due to the complex bacterial surface and negative effects on cell growth and viability [71].

3.5.3 Other display methods

Other display methods, such as mRNA display, ribosomal display, eukaryotic virus display and yeast cell surface display are also used. The first two methods mentioned are based on *in-vitro* protein evolution. Ribosomal display envolves a cDNA library coding for peptides. They lack a stop-codon in their sequence therefore the translation complex remains associated. After a bait protein binds, the sequence coding for the peptide can be easily identified and amplified from mRNA sequence [72].

mRNA display uses a puromycine linkage and affinity chromatography. An mRNA library is synthetised from a cDNA library (figure 8, page 29). In

the second step, the mRNA is ligated to a DNA spacer linker containing puromycin, a protein synthesis inhibitor used as an antibiotic. The mRNA is then *in-vitro* translated into proteins that remain bound to the mRNA because puromycin ends the translation process prematurely. The mRNA is then reverse transcribed to DNA for easier manipulation and to avoid forming of RNA secondary structures. The selection is performed as previously on an immobilised bait. Once partners are bound, the resin is washed and a PCR can be performed starting the whole cycle again. The advantage of this method is that cell manipulation is avoided. The drawback is that *in-vitro* translation is a complicated process [73, 74].

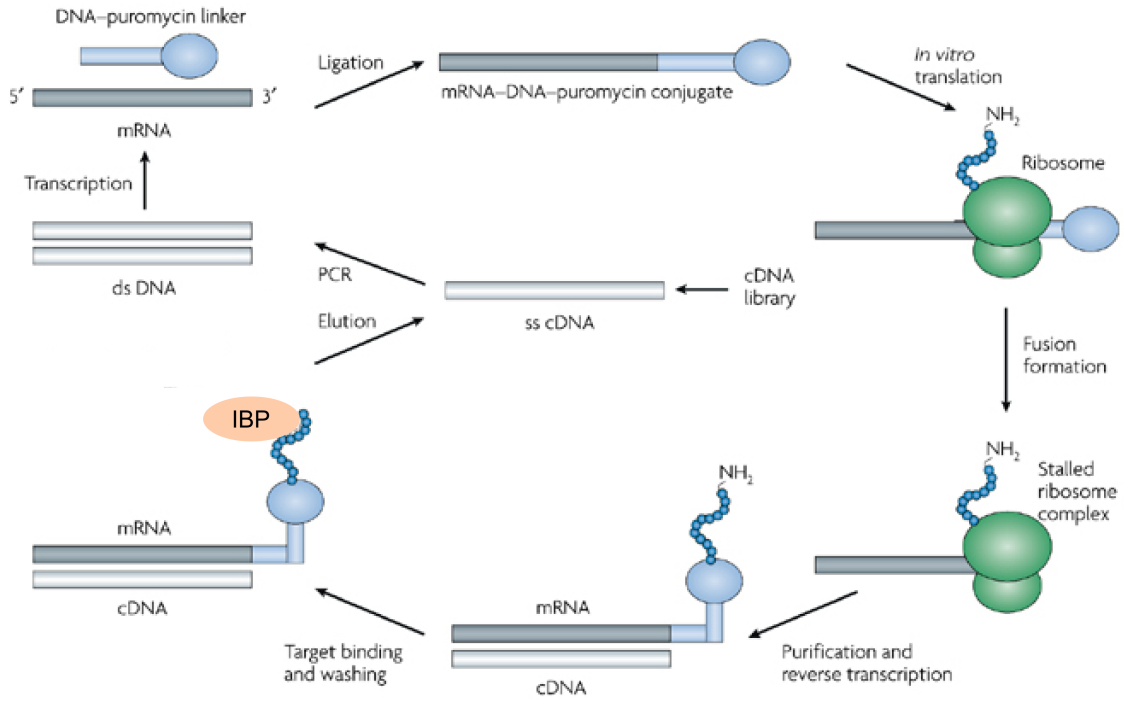

Figure 8: mRNA display; mRNA is ligated with DNA-puromycin linker. The mRNA is translated into protein and the ribosome complex is stalled due to puromycine. The mRNA is then reverse transcribed to cDNA to avoid forming of RNA secondary structures. The complex is applied to immobilised binding partner (IBP). After washing out the non-specificities, the complex is eluted and disrupted so a free single strand cDNA (ss cDNA) is formed. A second strand is then formed in a polymerase chain reaction (PCR) and another cycle can begin. Adapted from [64].

Yeast cell surface display has the advantage of eukaryotic protein folding system and codon usage similar to mammalian cells, fluorescence-activated cell sorter can also be used [75]. One disadvantage is different glycosylation. However, this problem is overcome in eukaryotic virus display that uses various types of cells and baculoviruses [76].

3.6 Protein microarray

Protein microarray is a fast high-throughput method to identify an interaction partner. Commercially available chips contain a library of bound proteins. The preparation of the chip is described in the figure 9, page 31. A fluorescently labelled target protein is applied to the chip and read by laser scanner. Positive hits are then analysed usually by MS. Immobilising proteins to a surface can lead to a loss of their native state and folded structure. However, binding of the ligands often demands the part of native protein that is necessary for the protein-protein interaction. For that reason, protein chip storage capacity is limited. The extraction of the protein from the chip might be difficult. This promising method is still in its beginning [77].

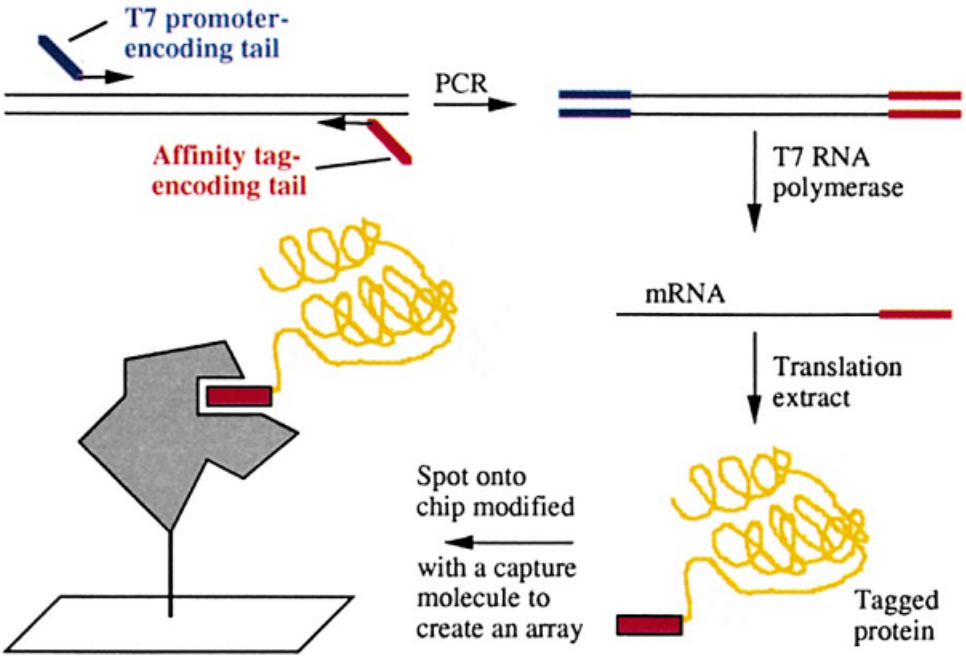

Figure 9: Protein microarray preparation; Affinity tag and T7 promoter are ligated to the cDNAs coding for a wide variety of proteins. These cDNAs are transcribed to mRNA and translated so a library of tagged proteins is created. Proteins are immobilised to the chip in the final step. Taken from [77].

3.7 Cross-linking

The advantage of this method is stabilizing weak or fast interactions. It is an *in vitro* method using a wide scale of more or less specific chemical cross-linkers. For example, cells containing the target protein are grown in a medium with photoreactive leucine and methionine analogues. These amino acids are activated by UV light and they covalently crosslink the nearest proteins after activation. This crosslinked complex is then extracted and analysed by usual methods such as MS [78, 79]. This method can also be used for studying protein interaction or a structure of protein complex, therefore a binding site can be determined as well as the binding partner can be identified. The disadvantage of this method is in the step of extraction of the cross-linked complex from the cell where the solubilizing agent might damage the bond.

Bonds are usually created non-specifically and anything in the proximity will react resulting in false positive results. Therefore evaluating the data is time-consuming [80].

3.8 Surface plasmon resonance (SPR)

SPR can be used for capturing the partner from a cell lysate or a medium. First, a target protein is bound to a sensor chip. Then a cell extract is applied. Binding of a partner changes the signal measured. When light beam hits metal surface at a given angle, surface plasmons resonate with the light. The resonance causes absorption of the light. This gives the information whether there is some molecule bound because binding of a molecule changes the intensity of light reflected at a given angle. If there is some molecule bound to the surface, the experiment can proceed with washing and recovery of proteins bound to the chip. These molecules can be analysed by MS. SPR also gives many valuable information about the interaction such as the strength, the equilibrium constant, binding affinity and others [81, 82, 83].

The main advantage of this method is that no labels are necessary. The disadvantage is that it cannot distinguish between specific and non-specific interaction because even after several washing steps there are still some non-specific contaminations [84]. Also, binding of lower molecular weight particles is more difficult to detect [85].

Part II

Objectives

To optimise purification of extracellular part of recombinant Naaladase L2 (AviEXSTL2)

To characterise AviEXSTL2 in terms of its putative proteolytic activity

To establish expression of intracellular part of Naaladase L2 in HEK cells and compare transient transfection versus stable transfection

To search for putative interaction partner of intracellular part of Naaladase L2

Part III

Materials and Methods

4 Materials

4.1 Chemicals

Bio-rad, California, USA
Bradford solution
Duchefa, Haarlem, Netherlands
glycine
Gibco, California, USA
SF900II media, 10% fetal bovine serum (FBS), 100x Defined Lipid Concentrate, 50x Yeastolate Ultrafiltrate
GE Healthcare, Little Chalfont, UK
Gel filtration calibration kit LMW, Gel filtration kit HMW
Hirschmann Gerätte, Eberstadt, Germany
Pasteur pipettes
Invitrogen, California, USA
Drosophila S2 Schneider cells
Koh-i-noor Hardmuth, České Budějovice, CZ
transparent 96-well microplate with flat bottom
Lachema, Brno, CZ
potassium dihydrogenphosphate, sodium hydrogenphosphate, magnesium chloride, ammonium acetate
Lach-Ner, Neratovice, CZ
hydrochloric acid, sodium acetate, sodium carbonate, sodium hydroxide, sodium chloride, calcium chloride, silver nitrate, ammonium sulphate, sodium thiosulphate, urea
Millipore, Massachusetts, USA
0.5 ml Microcon Centrifugal Filter Unit (10 kDa c/o), Immobilon-P transfer membrane

New England BioLabs (NEB), Ipswich (USA)
10% NP40, PNGase F, 10X Glycoprotein denaturing buffer,
10X G7 Reaction buffer
Penta, Prague, CZ
MOPS, methanol, acetic acid, acetone, formaldehyde, isopropanol,
formic acid, potassium hydroxide, ethyleneglycol, ethanol
Pierce, Illinois, USA
D-biotin, Neu-HRP (1 mg/ml), Casein Blocker,
SuperSignal West Dura/Pico Chemoluminescence substrate,
antimouse-IgG antibody conjugated with HRP (0.8 mg/ml)
Roche, Basel, Switzerland
Streptavidin mutein matrix
Solulink, California, USA
Streptavidine agarose
Serva, Heidelberg, Germany
Coomassie Brilliant Blue G250, bromphenol blue, BSA, ammonium persulfate,
Triton X-100
Sigma-Aldrich, Buchs, Switzerland
EDTA, PEG 3350, SDS, 2-mercaptoethanol, glycerol, Tris, acrylamide, TEMED,
N,N'-bisacrylamide, Gly-Pro-AMC, boric acid, sucrose, SYPRO orange,
HEPES, potassium chloride, sodium citrate,
phosphoric acid, MES, imidazole, bicin
Thermo Fisher Scientific
Nitrocellulose membrane 0.45 micron
USB, Cleveland, USA
Tween 20

4.2 Instruments

Electrophoresis apparatus, AP Czech, CZ
NanoDrop ND-1000, Thermo Fisher Scientific, USA
ÄKTA explorer, Amersham Pharmacia Biotech, Sweden
GENios microplate reader, Tecan, Switzerland
ChemiDoc-It 600 Imaging System, UVP, Germany
Ultra 0.5ml Centrifugal Filters MWCO 10,000, Amicon, USA
Wet transfer blot, Bio-rad, USA
LightCycler 480 System, Roche, Switzerland
MLS-3020U autoclave, Sanyo, Japan
Centrifuges: Megafuge 2.0R, Heraeus Instruments, Germany;
Biofuge pico, Heraeus Instrument, Germany;
Eppendorf 5414R, Eppendorf, Germany
Transparent 96-well microplate with flat bottom, P-Lab, CZ
Plastic disposable column, Pierce
Innova 44R rotate incubator, New Brunswick Scientific, Germany
TFF Millipore Large Scale, Millipore, USA
MLS-3020U autoclave, Sanyo, Japan
9450 pH meter, Unicam, UK
Superdex 200/10 300 GL, GE Healthcare Life Sciences, UK
Laboratory balances: HL-400, A&D Engineering, Inc., USA;
EK-400H, A&D Engineering, Inc., USA; Radwag XA 110/X, Merci, CZ

5 Methods

5.1 Extracellular part of Naaladase L2 (AviEXSTL2): Protein expression, purification and the analysis of the protein

For expression of the extracellular part of Naaladase L2 the Drosophila Schneider's S2 cell line containing biotin ligase from E.coli (BirA) in endoplasmatic reticulum was transfected with a plasmid pMTBipAviEXSTL2 (made previously in our laboratory) containing sequence coding for the biotinylated extracellular part of Naaladase L2. Transfection was done by Jana Starková. The cells were cultured in a 35 mm plate in SF900II media (Gibco) supplemented with 10% new fetal bovine serum (Gibco) at 22-24°C until they reached a density of 2 to 4x10^6 cells/ml. Solution A (36 µl of 2 M CaCl2, 9 µg of recombinant DNA, 0.5 µg of pCoBLAST, tissue culture sterile water to the final volume of 300 µl) was added dropwise to Solution B (300 µl of 2x HEPES-Buffered Saline (HBS; HBS 2x: 50 mM HEPES, 1.5 mM Na_2HPO_4, 280 mM NaCl, pH 7.14)) with continuous vortexing and the final mixture was incubated at room temperature for 40 min. Subsequently, the solution was mixed and added dropwise to the cells. The cells were then incubated for 16-24 hours at 22-24°C. The transfected cells were then transferred into a 15 ml sterile falcon tube and centrifuged at 500 g for 2 min. The medium was aspired, 3 ml of fresh SF900II medium with 10% new fetal bovine serum (complete media) were added and the cells were replaced into the new plate. After one-day incubation at 22-24°C, the medium was carefully aspired and 3 ml of fresh complete medium containing either blasticidin (final concentration 5 µg/ml) or hygromycin (final concentration 300 µg/ml) were added. The selective medium was replaced every 4-5 days until resistant cells started growing out (usually 3-4 weeks).

5.1.1 Affinity purification on Streptavidin mutein resin

Medium from cells was spinned down at 5,250 g and 4°C for 20 minutes. The supernatant was then concentrated on 100kDa membrane (Millipore) approximately 25x using TFF Millipore Large Scale system. The retenate was spinned again at 5,250 g and 4°C for 20 minutes. The supernatant was mixed in 1:2 with 3x equillibrium buffer. For sample in buffer A the equilibration buffer A (411mM NaCl, 8.1mM KCl, 38.1mM phosphate, pH 7.2) and for buffer B equilibration buffer B (450mM phosphate, 1350 mM NaCl, pH 7.4) were used. 2 ml of Streptavidin mutein resin were washed two times with wash buffer (wash buffer A: 137mM NaCl, 2.7mM KCl, 12.7mM phosphate, pH 7.2; wash buffer B: 150mM phosphate, 450 mM NaCl, pH 7.4). The mixtrue of medium and equilibration buffer was then applied to the resin and incubated over night at 4°C in a 50 ml falcon tube. A plastic disposable column (Pierce) was washed with 1 ml of wash buffer and the decanted medium was carefully poured onto the column without whirling the resin. Flow through fraction (FT) was collected. Then, the resin was transferred to the column using several mililitres of flow through fraction. 50 ml of wash buffer was applied in two 20 ml and one last 10 ml step onto the column to wash out the remaining medium and non-specifically bound proteins. In the next step, 1 ml of elution buffer A (137mM NaCl, 2.7mM KCl, 12.7mM phosphate, 2mM D-biotin, pH 7.2) or elution buffer B (150mM phosphate, 450 mM NaCl, 2mM D-biotin, pH 7.4) was applied and when the buffer soaked into the resin the column lid was closed so that no liquid was leaking. After one hour incubation the elution 1 fraction was collected. Four more 1ml elution fractions were susequently collected (elution 2, elution 3, elution 4 and elution 5). The whole experiment was performed at 4°C. All the samples were analysed by SDS-PAGE (see chapter 5.1.2).

5.1.2 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for the separation of the sample. A gel consisted of 11% resolving gel and 5% stacking gel. The mixture for 11% resolving gel contained 375 mM Tris-HCl (pH 8.8), 11% (v/v) acrylamide mixture (acrylamide with N,N'-bisacrylamide in the ratio 35.7:1), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS) and 0.01% (v/v) TEMED. The mixture for 5% stacking gel contained 250 mM Tris-HCl (pH 6.8), 5% (v/v) acrylamide mixture (acrylamide with N,N'-bisacrylamide in a ratio 35.7:1), 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS) and 0.02% (v/v) TEMED. Gels were let to polymerise and put into electrophoresis apparatus (AP Czech) which was filled with SDS Running buffer (25 mM Tris, pH 8.8, 250 mM glycine, 0.1% SDS). Samples were denatured in 1x loading buffer (6x buffer: 350 mM Tris, pH 6.8, 30% glycerol, 10% SDS, 0.6% 2- mercaptoethanol, 1.2% bromphenol blue) by heating for 10 minutes to 95°C and then loaded into the wells of the gel. Electrophoresis was run at 140 V for 1 hour.

5.1.3 Silver staining of polyacrylamide gels

The gel was fixed for 15 minutes in a solution of 10% (v/v) acetic acid, 10% (v/v) methanol and 0.5% (v/v) formaldehyde and washed thoroughly in 50% (v/v) methanol. In the next step the gel was exposed for 1 minute in the solution of sodium thiosulfate pentahydrate (0.2 g/l) and washed three times for 20 seconds in water. After 20 minute incubation in the solution of silver nitrate (2 g/l) and 0.5 % (v/v) formaldehyde the gel was washed three times in water and incubated in a solution containing sodium carbonate (60 g/l), sodium thiosulfate (4 mg/l) and 0.5% (v/v) formaldehyde until protein bands were clearly visible. The gel was then washed again three times with water

and as finally washed for ten minutes with 12% (v/v) acetic acid and 50% (v/v) methanol solution. Stained gel was scanned on scanner (Canon).

5.1.4 Concentration determination on NanoDrop

Concentration of proteins in elution fractions was measured on NanoDrop 1000 (Thermo Fisher Scientific) using absorbance at 280 nm. 1 µl of sample was used. 1 ul of dH_2O was used as a blank sample.

5.1.5 Thermofluor (Differential scanning fluorimetry)

Sample of AviEXSTL2 protein was analysed for folding by the thermofluor method. Several dye (50x, 25x and 12.5x) and protein concentrations (0.1, 0.25, 0.5, 1, 2 and 5 µg) were used in optimisation of this method [86]. When temperature rises to a certain level (melting temperature, Tm), the protein unfolds and reveals its hydrophobic core. A dye present in the solution binds to hydrophobic regions and a fluorescent signal can be measured. Dilutions were made in Tris buffer. The experiment for measuring of the protein folding was performed in a real-time PCR light cycler (Roche). The appropriate amount of protein sample was mixed with a appropriate buffer (Tris buffer: 20mM Tris, 40mM NaCl; PBS: 137mM NaCl, 2.7mM KCl, pH 7.2; phosphate buffer: 150mM NaCl, 50 mM phosphate, pH 7.4) and a dye (SYPRO Orange, diluted 25x). 1µg of AviEXST was used as a standard. The reaction was performed in a 96 well plate (Roche) final volume in each well was 25 µg. The temperature scheme was following: target temperature 20°C, 1 s, 4.4°C/s; target temperature 20°C, 1 min, 4.4°C/s; target temperature 95°C, 0.01°C/s, 50 acquisitions/°C; target temperature 95°C, 10 s, 4.4°C/s; target temperature 20°C, 10 s, 2.2°C/s.

5.1.6 Buffer screen

A set of buffers was screened to determine in which buffer AviEXSTL2 is the most stable. The screening was done by thermofluor. The most suitable buffer was then used for further manipulations. Following 100mM buffers were tried out: sodium acetate (pH 4.5, 5.0), sodium citrate (pH 4.7, 5.5), potassium phosphate (pH 5.0, 6.0, 7.0), sodium phosphate (pH 5.5, 6.5, 7.5), MES (pH 6.2, 6.5), HEPES (pH 7.0, 8.0), ammonium acetate (pH 7.3), Tris (pH 7.5, 8.0, 8.5), imidazole (pH 8.0), bicin (pH 8.0, 9.0), water. The concentration of all buffers was 100mM and all buffers were used both as water solutions and as a water solutions containing 200mM NaCl.

5.2 Gel filtration

The sample was analysed by gel filtration followed by measurement of absorbance. A superdex column S 200 10/300 GL was first calibrated using a set of calibration proteins. First, it was washed by 1 column volume of distilled water, 1 column volume of 1M NaOH and again by 2 column volumes of distilled water. Then, it was equilibrated by 2 column volumes of chromatography buffer. Two different phosphate buffers were used; buffer A (137mM NaCl, 2.7mM KCl, 12.7mM phosphate, pH 7.2) and buffer B (450mM NaCl, 150mM phosphate, pH 7.4). Following parameters were set: volume of the injection valve 500 µl, flow rate 0.5 ml/min, dead volume 9 ml. Two sets of standard proteins were used: ferritin (0.2 mg/ml, Mr 440,000), conalbumin (2 mg/ml, Mr 75,000), carbonic anhydrase (2 mg/ml, Mr 29,000) and ribonuclease A (2 mg/ml, Mr 13,700) in the first set and aldolase (2.67 mg/ml, Mr 158,000), ovalbumine (2.67 mg/ml, Mr 43,000), ribonuclease A (2 mg/ml, Mr 13,700), aprotinine (2 mg/ml, Mr 6,500) in the second set. The partition coefficient Kav was plotted against the log Mr. Partition coefficient is a constant

calculated from the elution volume of protein and column parameters:

$$Kav = \frac{V_e - V_0}{V_c - V_0},$$

where V_e is elution volume of the protein, V_0 is column void volume and V_c is geometric column volume.

Samples of elution fraction from affinity chromatography (chapter 5.1.1) with the highest amount of AviEXSTL2 (elution 2 from the sample in buffer B, elution 3 from the sample in buffer A) was applied to the column and elution fractions were collected by the volume of 250 µl in the total amount of 96 fractions. The absorbance at 280 nm of each fraction was plotted into a graph against the volume of the mobile phase. Selected fractions with high absorption were analysed on SDS-PAGE (see chapter 5.1.2).

5.3 Protein concentration

Selected samples from gel filtration were concentrated on an Amicon Ultra-0.5 ml Centrifugal Filters MWCO 10,000. Fractions from B5 to B9 were joined to one sample A, fractions from B4 to C1 were joined as a sample B and fractions from C2 to C6 were joined as a sample C. In the sample A there were both bands of AviEXSTL2, in the sample B was only the upper band and in the sample C was only the lower band. Samples were applied onto the filters and spinned at 5,000 g for 15 minutes at 4°C, mixed by pipeting after each minute. The recovery was performed by reverse spinning at 1,000 g for 2 minutes according to manual. Final concentration of all samples was measured on NanoDrop (see chapter 5.1.4).

5.4 Protein deglycosylation

Deglycosylation of selected fractions was performed in the buffer containing urea which composition was following: 2.5M urea, 50mM phosphate, 150mM NaCl, pH 7.4. The whole 250µl of the fraction was used. Samples were incubated at 37°C over night with 0.5 µl of proteoglycan N-linked glycosidase F (PNGase F, NEB). The samples were separated by SDS-PAGE (see chapter 5.1.2) and then analysed by Western blot (see chapter 5.6) or by mass spectrometry (see chapter 5.5).

5.5 Polyacrylamide gel staining for MS

Samples were separated by SDS-PAGE (see chapter 5.1.2). The gel was handled with maximum cautiousness to avoid contamination. The gel was then fixed in a fixing solution (10% acetic acid, 40% methanol in dH_2O) for 1 hour, washed three times in distilled water and stained by Silver blue dye (1.78mM Coomassie G-250, 24.5% (v/v) ml methanol, 92.9mM ammonium sulfate, 14% (v/v) ml phosphoric acid, filtered) at room temperature. After an hour the gel was washed over night in distilled water and sent to MS analysis of stained bands.

5.6 Western blot

Samples of AviEXSTL2 were separated on SDS-PAGE (see chapter 5.1.2) and transferred using wet transfer apparatus (Biorad). Nitrocellulose membrane was used, the tank was filled with blotting solution (10% (v/v) methanol, 25mM Tris-HCl, 192mM glycine, 0.1% SDS, pH 8.2). The blotting was performed for 60 minutes at the constant voltage of 100 V. Once the transfer was finished, the membrane was blocked by 5 ml of Casein blocker (Thermo

Scientific) diluted in ratio 1:20 in PBS+0,05% Tween in a Petri dish for 1 hour at room temperature. The membrane was then incubated with 1 µl of Neutravidin-HRP conjugate (Pierce, 1 mg/ml) diluted in 5 ml of PBS+0,05% Tween at room temperature. After washing three times for 5 minutes with PBS+0,05% Tween the membrane was visualised by adding 600 µl of Super-Signal West Pico chemiluminiscent solution (Thermo Scientific). The membrane was then dried and the signal was detected on a CCD camera (UVP, Analytik Jena).

If a set of antibodies was used instead of Neutravidin-HRP, the membrane was incubated in 1 µl of αNaaladL2 antibody diluted 1:5,000 in 5 ml of PBS+0,05% Tween for 1 hour at room temperature after the blocking step. The membrane was washed three times with PBS+0,05% Tween and incubated for 1 hour with goat-α-mouse IgG-HRP secondary antibody (0.8 mg/ml, Pierce) diluted in ratio 1:20,000. After washing three times again, appropriate chemiluminiscent solution was applied, SuperSignal West Pico/Femto (Thermo Scientific) and the signal was detected as previously.

5.7 Preparing samples for N-terminal sequencing

If preparing a sample fo N-terminal sequencing, the sample was separated by SDS-PAGE (see chapter 5.1.2), transferred to PVDF membrane by Western blotting at 100 V for 1 hour in a transfer buffer (10% (v/v) methanol, 25mM Tris-HCl, 192mM glycine, 0.1% SDS, pH 8.2). The membrane was then stained with Coomassie Blue Brilliant G-25 and destained by 10% acetic acid, dried and sent to sequencing.

5.8 Intracellular part of Naaladase L2: expression and analysis of the protein

For expression of the intracellular part of Naaladase L2 HEK cells (a cell line derived from human embryonal kidney cells) were transiently transfected with a plasmid coding for the intracellular and intracellular plus transmembrane part of Naaladase L2. All the experiments were performed by Jana Starková. HEKoffA2BA17 cells, which are stably transfected with pTetOffAdvanced plasmid (TetOff system; enabling to switch off the expression from pTRE plasmids in presence of doxycycline) and pcDNA4BirA plasmid coding for biotin ligase (localised in cytoplasm), were cultured in a 2x100 mm plates in x medium IMDM complete (+10% FBS, + glycine) until they were confluent to 90 %. 21 µg of plasmid pTREnaalL2 1-124Avi coding for the intracellular part of Naaladase L2 (first 124 amino acids) was used for the transient transfection. DNA was diluted into 750 µl of Optimem solution (Invitrogen) and 81 µl polyethylenimine (Sigma) was added. Prepared solutions were incubated at room temperature for 15 minutes until lipid-DNA complexes were formed. Then this solution was carefully added to the cells and gently mixed by shaking. Cells were incubated at 37°C and 5 % CO_2. In the next step, the cells were resuspended 40 hours after transfection, transferred to plastic tubes and spinned down for 3 minutes at 500x g and 4°C. Supernatant was discarded and the pellet was washed thoroughly by TBS (50mM Tris, 150mM NaCl), mixed thoroughly and spinned again. After discarding the supernatant, the pellet was resuspended in 100 µl of RIPA buffer (100mM Tris HCl, pH 7.4; 150mM NaCl; 1% (v/v) TritonX-100; 0.1% (v/v) NP-40) and kept for 1 hour at 4°C. The solution was then spinned down for 10 minutes at 16,000x g. Supernatant was collected to a new tube. As a negative control, cells were treated exactly the same way, only the plasmid was omitted in the transfection solution.

The same experiment to prepare intracellular and transmembrane part of Naaladase L2 was prepared by transfection of HEKoffA2 cells with plasmids pTREnaalL2 1-154Avi, coding for the first 154 amino acids of Naaladase L2, and pSecBirAKDEL, coding for a secreted biotin ligase. Signal sequence KDEL retains biotin ligase in endoplasmatic reticulum. All the constructs were made previously in our laboratory by colleagues. Also, stable clones expressing the first 124 amino acids of Naaladase L2 (biotinylated) were prepared previously by colleagues. Lysates from these cells were made the same way as previously.

5.8.1 Bradford Protein Assay

The amount of protein in cell lysates was measured according to Bradford protein Assay. Bovine serume albumine was used as a standard according to the Bio-Rad protocol [87]. The measurement was done in transparent 96 well plate (P-Lab). Bovine serum albumine was used in concentrations of 0, 0.25, 0.5, 1, 2 and 4 mg/ml in well. All samples were measured in duplicates. 140 µl of Bradford solution (Bio-Rad) was pipetted into each well, then a sample of standard or cell lysate was added. In the last step, water was added to the final volume of 200 µl. Each well was mixed thoroughly by pipetting. The absorbance was measured at 595 nm by the GENios microplate reader (Tecan).

5.8.2 Affinity purification on Streptavidin resin

To obtain the intracellular part of Naaladase L2, the cell lysate was purified on Streptavidin agarose (Solulink) in a batch setup in a PCR tube. First, 100 µl of the resin was washed twice with 100 µl of TBS. Then, 100 µl of lysate was applied and the mixture was incubated over night at 4°C on a

rotator. After spinning down the resin, the supernatant was put aside as a flow through fraction (FT). Then, the resine was washed three times with 100 µl of TBS+0.05 % Tween (fractions wash 1, wash 2 and wash 3). The sample was eluted from the resin by adding 40 µl of 1x sample buffer for SDS-PAGE (see chapter 6.1.2) and heated at 95°C for 10 minutes. The sample was then analysed by SDS-PAGE followed by either Silver staining or Western blot (see chapters 5.1.2, 5.1.3 and 5.6).

5.9 Co-precipitation of the intracellular part of Naaladase L2 and its putative interacting partner

Putative binding partner of the intracellular part of Naaladase L2 was investigated by co-precipitation via Streptavidin agarose (Solulink). First, 100 µl of the resin was washed twice with 100 µl of TBS. Then, 100 µl of lysate was applied and the mixture was incubated over night at 4°C on a rotator. After spinning down the resin, the supernatant was put aside as a flow through fraction (FT). Then, the resine was washed three times with 100 µl of TBS+0.05 % Tween (fractions wash 1, wash 2 and wash 3). 100 µl sample of lysate of prostate cancer (1 mg/ml of total protein) was applied to the resin and incubated over night at 4°C on a rotator. After spinning down the resin, the supernatant was put aside as a flow through fraction 2 (FT2). Then, the resin was washed three times with 100 µl of TBS+0.05 % Tween (fractions wash 4, wash 5 and wash 6). The sample was eluted from the resin by adding 40 µl of 1x sample buffer for SDS-PAGE (see chapter 5.1.2) and heated to 95°C for 10 minutes. The whole elution fraction was then separated by SDS-PAGE (see chapter 5.1.2), stained by Silver blue (see chapter 5.5) and analysed by MS.

The same experiment was performed with not transfected cells as a negative control.

Part IV

Results

6 Expression and purification of the extracellular part of Naaladase L2 (AviEXSTL2)

Recombinant biotinylated extracellular part of Naaladase L2 (AviEXSTL2) was prepared using the Drosophila Schneider's S2 cell line (see chapter 5.1). The protein was purified on Streptavidin mutein resin routinely used in our laboratory for purification of the extracellular part of GCPII. Purification of AviEXSTL2 was the performed using Streptavidin mutein resin and two phosphate buffers recommended for gel filtration (buffer A: 137mM NaCl, 2.7mM KCl, 12.7mM phosphate, pH 7.2; buffer B: 150mM NaCl, 50mM phosphate, pH 7.4) as described in chapter 5.1.1. Medium with the recombinant protein was concentrated (fraction medium after concentration) and mixed with equilibration buffer. The solution was incubated with the resin and decanted. The flow through fraction is the supernatant. The resin was then washed several times using wash buffer (fractions wash) and eluted with elution buffer (fractions elution). A sample of the resin was then resuspended in 10 µl of sample buffer for SDS-PAGE. All fractions from the purification were analysed by SDS-PAGE (see chapter 5.1.2) and visualised by silver staining (see chapter 5.1.3) (Figure 9, page 49).

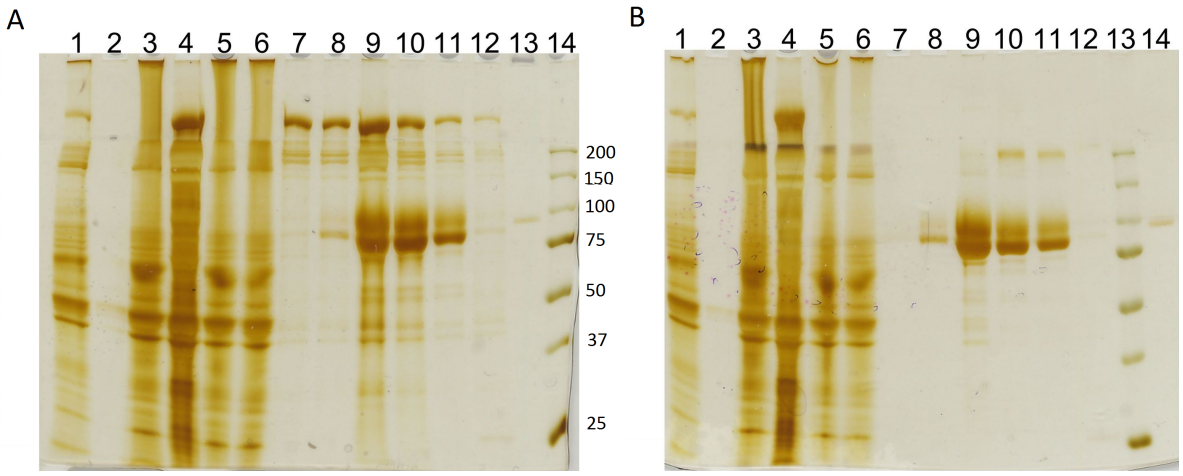

Figure 10: Analysis of fractions from AviEXSTL2 purification via Streptavidin mutein in two buffers. All the samples were heated at 95°C for 10 minutes in sample buffer, separated by SDS-PAGE and visualised by silver staining. **Panel A**: AviEXSTL2 purification in buffer A (137mM NaCl, 2.7mM KCl, 12.7mM phosphate, pH 7.2); 1: medium (12 µl); 2: medium after concentration (1.2 µl); 3: flow through after concentration (12 µl); 4: pellet (12µl); 5: load (1.2 µl); 6: flow through (1.2 µl); 7: wash 3 (12 µl); 8: elution 1 (12 µl); 9: elution 2 (12 µl); 10: elution 3 (12 µl); 11: elution 4 (12 µl); 12: resin (12 µl); 13: recombinant GCPII (3 ng); 14: All blue marker (Bio-rad, 3 µl). **Panel B:** AviEXSTL2 purification in buffer B (150mM NaCl and 50mM phosphate, pH 7.4); 1: medium (12 µl); 2: medium after concentration (1.2 µl); 3: flow through after concentration (12 µl); 4: pellet (12µl); 5: load (1.2 µl); 6: flow through (1.2 µl); 7: wash 3 (12 µl); 8: elution 1 (12 µl); 9: elution 2 (12 µl); 10: elution 3 (12 µl); 11: elution 4 (12 µl); 12: resin (12 µl); 13: recombinant GCPII (3 ng); 14: All blue marker (3 µl).

The yield of purification was 2.35 mg/1 l of medium for the sample in buffer A and 0.81 mg/1 l of medium for the sample in buffer B with estimated purity from SDS gel of around 80 %. Purified proteins were analysed by thermofluor. 1 µg of recombinant GCPII in phosphate buffer B was used as a standard. All the samples yielded a negative peak typical for folded proteins, therefore both proteins were folded. The minimum for both samples of AviEXSTL2 was around 49°C, as can be seen in figure 11, page 50.

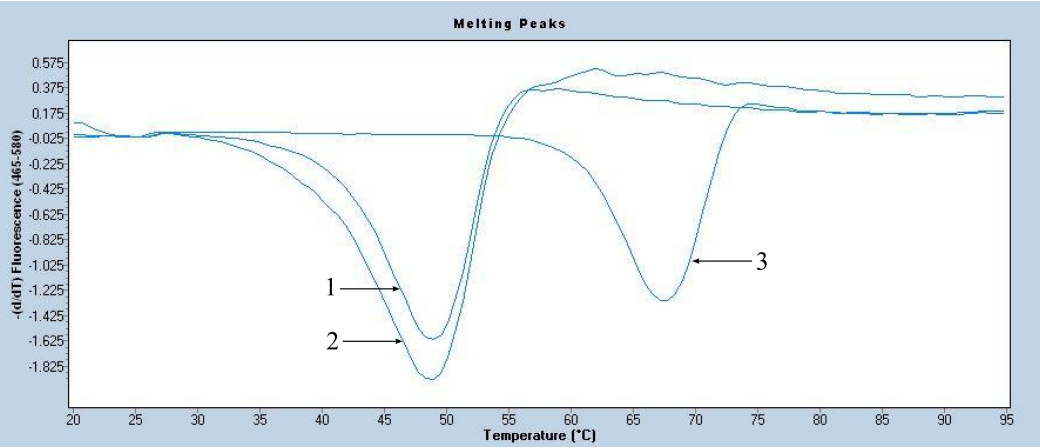

Figure 11: Thermofluor experiment with samples purified in buffer A (137mM NaCl, 2.7mM KCl, 12.7mM phosphate, pH 7.2) and buffer B (150mM NaCl and 50mM phosphate, pH 7.4); 1: AviEXSTL2 in buffer A; 2: AviEXSTL2 in buffer B; 3: recombinant GCPII.

When optimising the purification of AviEXSTL2, several buffers were tried. Tris buffer routinely used for recombinant GCPII purification resulted in partly unfolded protein with high initial fluorescence in thermofluor analysis (data not shown). Another routinely used buffer, phosphate buffer A, resulted in elution of protein with contamination of high molecular weight as can be seen in figure 10, page 49. Slow freezing of AviEXSTL2 at -20°C leads to unfolded structure of the protein observed as linear curve instead of a negative peak from thermofluor (data not shown). When frozen fast in liquid nitrogen followed by thawing and immediate structure analysis using thermofluor, the protein remained folded (data not shown). To investigate which buffer is the most suitable for AviEXSTL2 purification and storage, a screen of buffers was performed.

6.1 Stability of proteins in different buffers

Because the position of negative peaks of AviEXSTL2 in previous thermofluor experiments differed and because the sample in Tris buffer had a high initial

fluorescence, a buffer screen on thermofluor was performed. 14 buffers at various pH values were tried (see chapter 5.1.6). There were two sets of each buffer, one without salt, the other one with ionic strenght of 200mM salt (NaCl). The result is summarised in the table 1.

buffer	pH	Tm in water solution	Tm in solution with 200mM NaCl
Sodium acetate	4.5	48.4 x	56.8
Sodium citrate	4.7	55.1	58.0
Sodium acetate	5.0	52.1	59.3
Potassium phosphate	5.0	58.0	59.8
Sodium phosphate	5.5	62.5	62.1
Sodium citrate	5.5	56.1 x	58.7
MES	5.8	56.0	60.6
Potassium phosphate	6.0	62.0	62.8
MES	6.2	57.6	60.6
Sodium phosphate	6.5	59.1	59.4
MES	6.5	59.6	60.9
Potassium phosphate	7.0	56.9	57.8
HEPES	7.0	59.7	61.5
Sodium phosphate	**7.5**	**65.1**	52.4
Ammonium acetate	7.3	68.4 x	59.5
Tris	7.5	58.2	58.0
Imidazole	8.0	60.9 x	57.7
HEPES	8.0	57.8	56.7
Tris	8.0	55.5	55.0
Bicin	8.0	48.4	50.4
Tris	8.5	54.6	53.5
Bicin	9.0	-	41.0 x
dH_2O	-	56.2	57.4

Table 1: Buffer screen for AviEXSTL2; 100 mM buffers were tried at various pH and both in water solution and in 200mM NaCl. The cross stands for high initial fluorescence. The most suitable bufer is in bold. Tm = melting temperature.

Protein in some buffers gave a signal with high initial fluorescence, such as sodium acetate, probably due to the presence of partly or completely unfolded protein. The highest melting temperature with normal initial fluorescence was measured in the sample with phosphate buffer at pH 7.5, therefore the protein

is the most stable in it and this buffer was used for further experiments.

6.2 Gel filtration of AviEXSTL2

Next, we decided to purify the AviEXSTL2 obtained by affinity chromatography using gel fitration. First, a column calibration was performed (see chapter 5.2). For this purpose, a mixture of standard proteins of molecular weights ranging from 6,500 to 440,000 was used. A column for size exclusion chromatography was transferred to a PBS buffer. Two sets of calibration samples were run on the column. The graph in the panel A, figure 12, page 53 shows that standard proteins were separated according to their molecular weights, all the peaks were clearly separated without any irregularities. The column could be thus used for sample analysis.

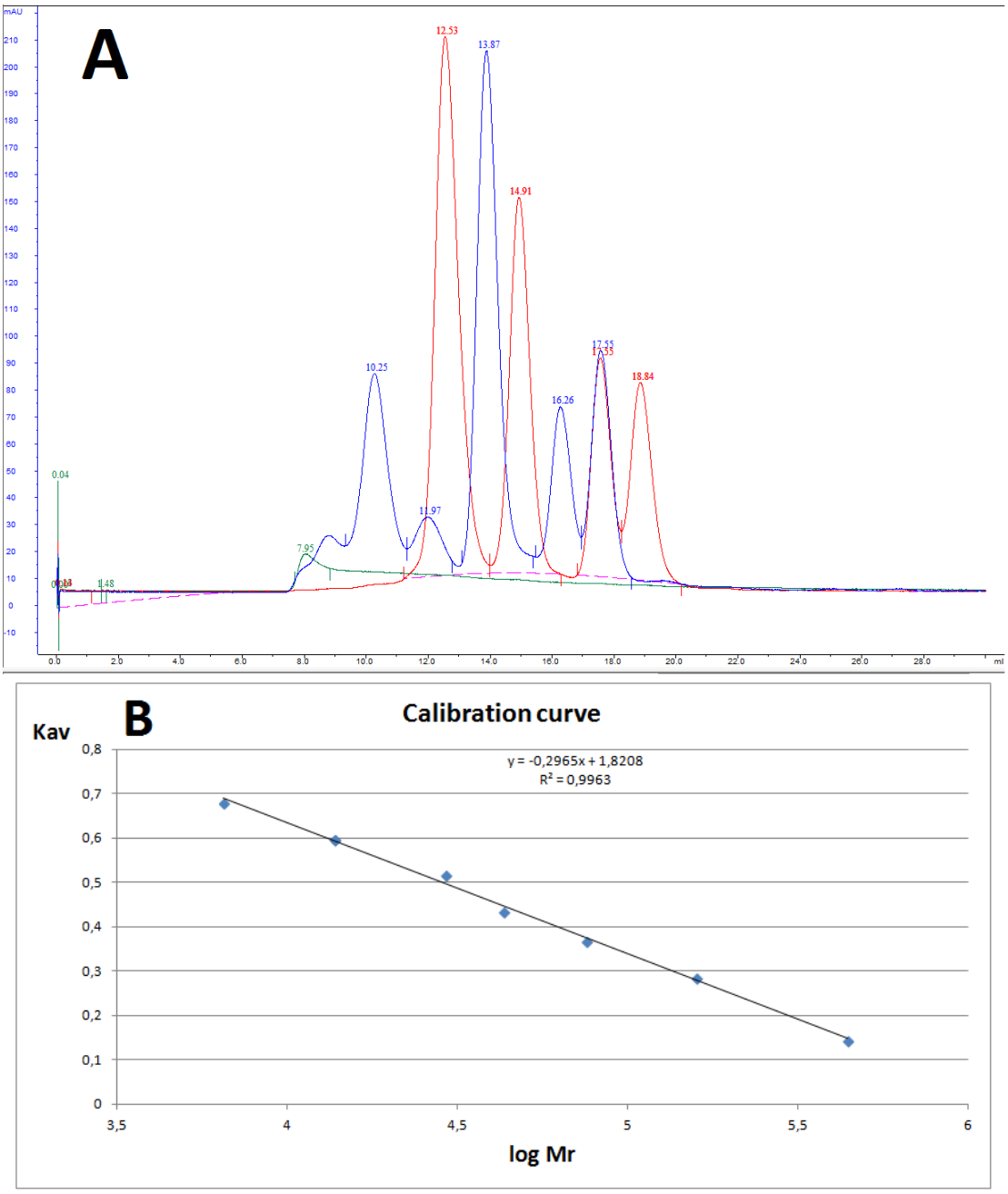

Figure 12: Calibration of the column; **Panel A:** absorbance in mAU (milli absorbance units) is plotted against elution volume. First set of calibration samples is in blue, second is in red. The results of these two measurements are overlapped. Peaks from the left to the right - blue: ferritin (10.25 ml), conalbumine (13.87 ml), carbonic anhydrase (16.26 ml), ribonuclease A (17.55 ml); red: aldolase (12.53 ml), ovalbumine (14.91 ml), ribonuclease A (17.55 ml), aprotinine (18.84 ml). **Panel B:** Calibration curve showing the dependence of Kav on log Mr. The R^2 factor is 0.9963.

A calibration curve was prepared by plotting partition coefficient Kav (see chapter 5.2) against log Mr (figure 12, page 53). Samples of purified AviEXSTL2 in buffer A and buffer B were purified by gel filtration (figure 13 on page 54). While gel filtration of the sample in buffer A resulted in two peaks, one at elution volume of 8 ml, second at elution volume from 9.5 to 12.5 ml, gel filtration of the sample in buffer B resulted in only one peak at elution volume from 9.5 to 12.5 ml corresponding to the second peak of the previous sample. This asymmetrical peak, both in buffer A and in buffer B, had a side peak on the left side originating from the heterogeneity (doubleband) which was also visible on polyacrylamide gels (figure 14, page 55).

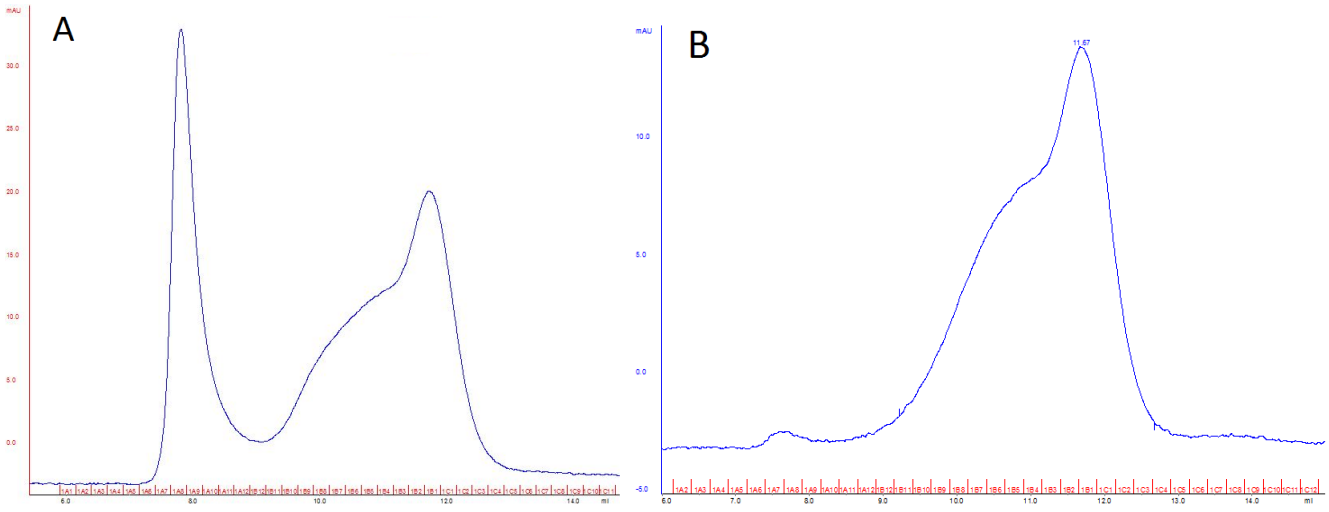

Figure 13: Gel filtration of AviEXSTL2; **Panel A:** sample in buffer A, **Panel B**: sample in buffer B.

The fractions collected from gel filtration were analysed by SDS-PAGE and visualised by silver staining (figure 14, page 55). SDS gel confirms the heterogeneity observed in the gel filtration experiment.

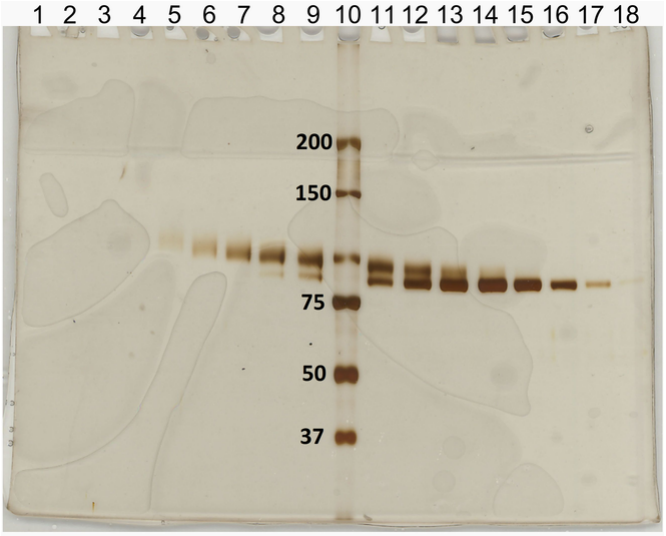

Figure 14: Analysis of selected fractions from gel filtration of AviEXSTL2 in buffer B. Fractions from the smallest elution volume to the biggest. The volume of each fraction loaded was 15 µl. The samples were heated at 95°C in sample buffer for 10 minutes and separated by SDS-PAGE and visualised by Silver staining. Elution volumes of fractions from gel filtration are in the brackets. 1: B12 (3 ml); 2: B11 (3..25 ml); 3: B10 (3.5 ml); 4: B9 (3.75 ml); 5: B8 4 ml); 6: B7 (4.25 ml); 7: B6 (4.5 ml); 8: B5 (4.75 ml); 9: B4 (5 ml); 10: All blue marker (1 µl); 11: B3 (5.25 ml); 12: B2 (5.5 ml); 13: B1 (5.75 ml); 14: C1 (25 ml); 15: C2 (6.25 ml); 16: C3 (6.5 ml); 17: C4 (6.75 ml); 18: C5 (7 ml).

7 AviEXSTL2 LC-MS analysis and N-terminal sequencing

Fractions B8 (separated upper band, elution volume 4 ml) and C4 (separated lower band, elution volume 6.75 ml) from the gel filtration were separated by SDS-PAGE, the gel was then stained by Silver blue dye (see chapter 5.5) and the bands were analysed by LC-MS by in house service. Interestingly, no diference between the upper and lower band was observed since both samples were identified as AviEXSTL2.

Identical gel was blotted and analysed by N-terminal sequencing analysis (see chapter 5.7). The analysis showed that the N-terminus of proteins from both bands, upper and lower, are the same and therefore the heterogeneity seen as a double band is not caused by processing at the N-terminus.

8 Investigation of possible cause of heterogeneity of AviEXSTL2

To investigate whether the heterogeneity is caused by glycosylation, samples of several fractions from the gel filtration experiment were deglycosylated in urea by PNGase F (see chapter 5.4) and compared with their glycosylated forms using Western blot (see chapter 5.6). Samples were loaded on a polyacrylamide gel, separated and transferred to a nitrocellulose membrane by wet transfer. The bands were visualised by Neutravidin-HRP conjugate and West Pico luminiscent substrate (Thermo Fisher Scientific). The result is summarised in figure 15, page 57.

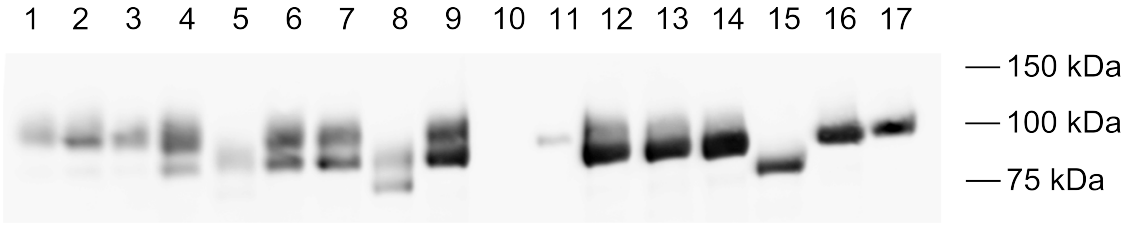

Figure 15: Analysis of glycosylation of heterogeneity of AviEXSTL2 samples from gel filtration. The samples were separated by SDS-PAGE, transferred to a membrane, incubated with Neutravidin-HRP and visualised by West Pico chemiluminiscent substrate. The volume of all samples loaded is 10 µl. The elution volume of the fraction on gel filtration is in the brackets. 1: B8 (form with higher molecular weight, 4 ml); 2: B7 (form with higher molecular weight, 4.25 ml); 3: B6 (form with higher molecular weight, 4.5 ml); 4: B5 (form with both molecular weights, 4.75 ml); 5: B5+6 deglycosylated (form with both molecular weights, 4.75 ml); 6: B4 (form with both molecular weights, 5 ml); 7: B3 (form with both molecular weights, 5.25 ml); 8: B3 deglycosylated (form with both molecular weights, 5.25 ml); 9: B2 (form with both molecular weights, 5.4 ml); 10: All blue marker (2 µl); 11: recombinant GCPII (20 ng); 12: B1 (form with both molecular weights, 5.75 ml); 13: C1 (form with lower molecular weight, 6 ml); 14: C2 (form with lower molecular weight, 6.25 ml); 15: C2 deglycosylated (form with lower molecular weight, 6.25 ml); 16: C3 (form with lower molecular weight, 6.5 ml); 17: C4 (form with lower molecular weight, 6.75 ml).

Both bands in deglycosylated fraction B3 (lane 8) moved towards lower molecular weights as well as deglycosylated samples B5+6 (lane 5) and C2 (lane 15). All the deglycosylated bands moved the same distance, separate upper band, separate lower band and a mixture of both.

A commercially available antibody against the extracellular part of Naaladase L2 was used to confirm the presence of Naaladase L2 by Western blot (see chapter 5.6). The antibody was developed against a peptide from the extracellular part of Naaladase L2. It was tested on both normal and deglycosylated protein. Cytochrome c and recombinant GCPII were used as negative controls. As can be seen in figure 16, page 57, the antibody binds to both normal and deglycosylated AviEXSTL2. The antibody did not recognise homologous recombinant GCPII. However, it detected cytochrome c. The amount of 500 ng of protein was clearly visible, though the intensity of the band containing 250 ng of the protein was very low.

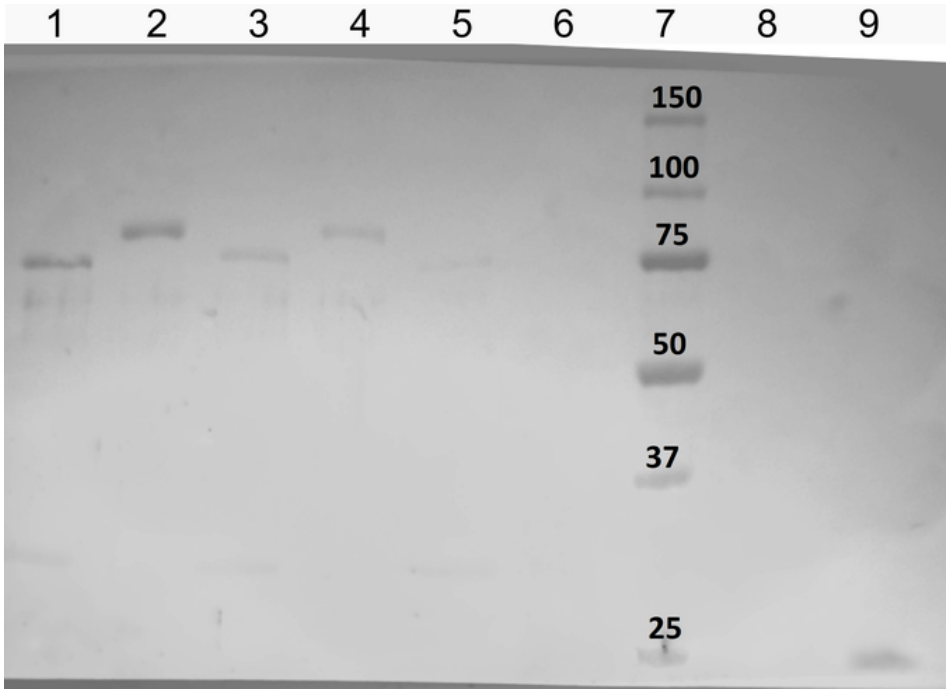

Figure 16: Western blot analysis of purified AviEXSTL2 using α-NaalL2 antibody. The sample was separated by SDS-PAGE, transferred to a membrane and incubated with the α-NaalL2 antibody. Goat anti mouse IgG-HRP was used as a secondary antibody. The visualisation was done by West Femto chemiluminiscent substrate. 1: AviEXSTL2 deglyc. (1 µg); 2: AviEXSTL2 (1 µg); 3: AviEXSTL2 deglyc. (0.5 µg); 4: AviEXSTL2 (0.5 µg); 5: AviEXSTL2 deglyc. (250ng); 6: AviEXSTL2 (250 ng); 7: All blue marker (2 µl); 8: recombinant GCPII (20 ng); 9: Cytochrome c (12.5 µg).

9 Analysis of putative proteolytical activity of AviEXSTL2

AviEXSTL2 was tested for proteolytical activity using PICS (proteomic identification of protease cleavage sites) assay. The experiment was performed by Michal Svoboda at IOCB. Both purified upper and lower bands were studied separately, no activity was observed.

10 Expression of the intracellular part of Naaladase L2

Two different transiently transfected HEK cells were prepared (see chapter 6.2). A2TT cells are transiently transfected cells with the biotinylated intracellular and transmembrane part (amino acids 1 - 154) of Naaladase L2, the expected molecular weight of the protein expressed was around 35 kDa (figure 17, page 59). A2NC are empty cells treated the same way as A2TT cells serving as a negative control. Cells A2BA17TT are transiently transfected cells with only the intracellular biotinylated part of Naaladase L2 (amino acids 1 - 124) with cells A2BA17NC serving as a negative control. The protein molecular weight was expected to be around 22 kDa. Additionally, two different stably transfected cells (395 and 400) were used. These cells express the first 124 amino acids of Naaladase L2, the resulting protein with expected molecular weight around 22 kDa would be also biotinylated. They were prepared previously in our laboratory by colleagues.

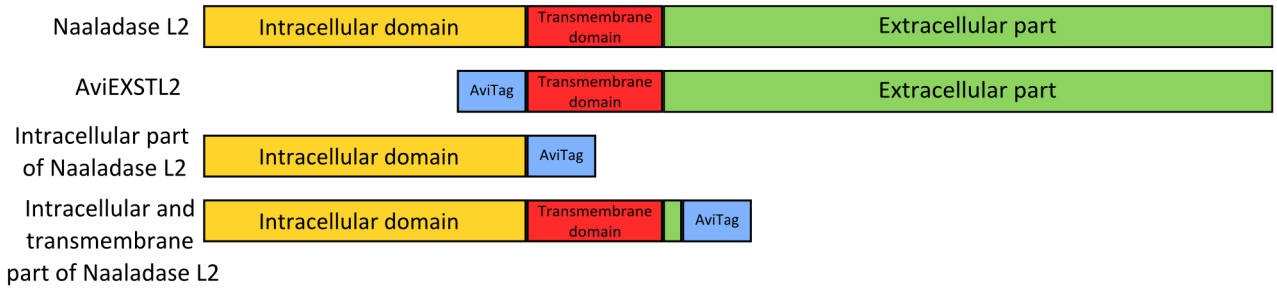

Figure 17: Constructs used for transfection of the cells. At the top is the full-length protein. Second is AviEXSTL2, the extracellular part of Naaladase L2. Third is the intracellular part of Naaladase L2 consisting of the first 124 amino acid. The last is the protein consisting of the intracellular and transmembrane part of Naaladase L2 (the first 154 amino acids).

The cells transfected with the six expression plasmids described above were lysed in RIPA buffer. The concentration of protein was measured in all cell lysates by Bradford protein assay. Sample containing 50 µg of protein of each of the cells were separated on 12% polyacrylamide gel and transferred to a nitrocellulose membrane by wet transfer. The membrane was incubated in Neutravidin-HRP and the bands were visualised by West Femto chemiluminiscent substrate (Thermo Fisher Scientific) (figure 18, page 60).

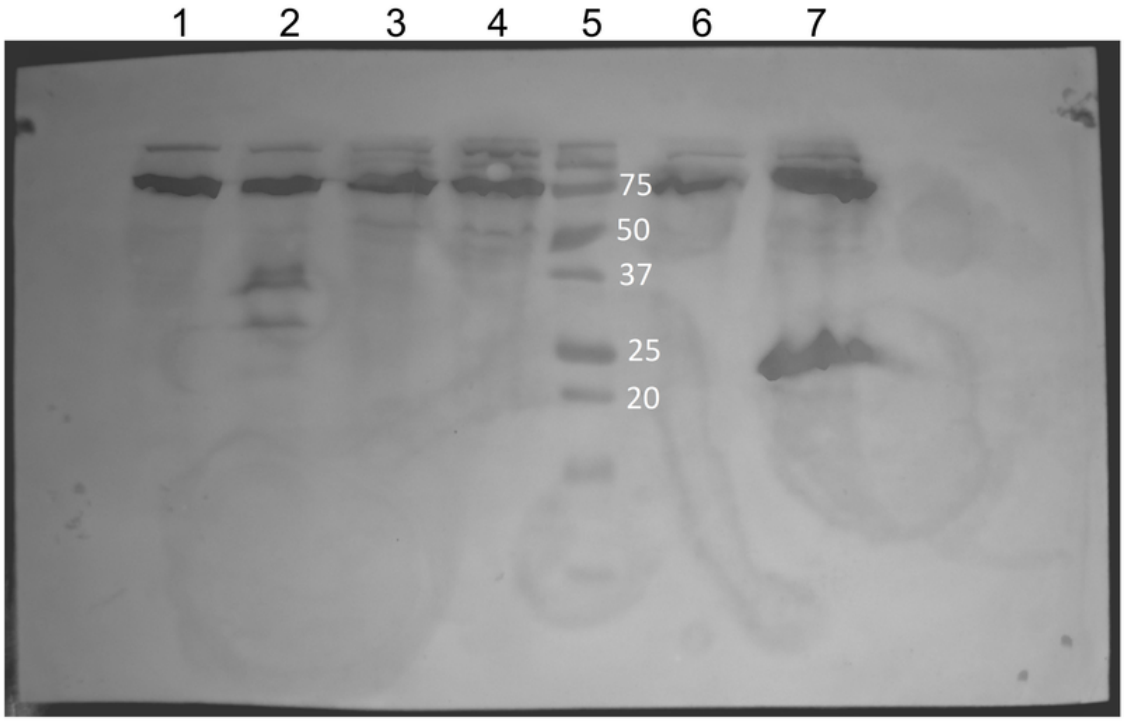

Figure 18: Analysis of the expression of intracellular part of Naaladase L2 in transfected cells. Cell lysates were loaded on a polyacrylamide gel, separated, transferred to a membrane, incubated with Neutravidin-HRP and visualised by West Femto chemiluminiscent substrate. 1: A2NC (50 µg); 2: A2TT (50 µg); 3: 395 (50 µg); 4: 400 (50 µg); 5: All blue marker (2 µl); 6: A2BA17NC (50 µg); 7: A2BA17TT (50 µg).

A clearly visible band in lane 7 shows that A2BA17TT cells expressed the expected protein of molecular weight around 22 kDa, the cells 395 and 400 (lanes 3 and 4) did not show any protein expression. The cells A2TT (lane 2) expressed some protein resulting in a weak band of molecular weight around 35 kDa, therefore no further experiment was performed with lysates from these cells. All the negative controls were without bands of appropriate molecular weights. All the following experiments were performed with lysates from A2BA17TT and A2BA17NC cells.

To determine the approximate amount of protein expressed, several dilutions of lysate were separated on 18% polyacrylamide gel, transferred to nitrocellulose membrane, incubated with Neutravidin-HRP and visualised by

West femto luminiscent substrate (Thermo Fisher Scientific). As a calibration curve a set of various dilutions of pure AviEXST protein was used (data not shown). The amount of the intracellular part of Naaladase L2 (molecular weight around 22 kDa) was determined by comparison of the bands of recombinant GCPII and bands of the lysate of the intracellular part of Naaladase L2. The band of intracellular part of Naaladase L2 of 1 µg of total protein was almost as intensive as the band of 1 ng of recombinant GCPII. Therefore the amount of the intracellular part of Naaladase L2 in lysate is almost 1 ng/µg of total protein. The total amount of the desired protein was thus approximately 300 ng (data not shown).

10.1 Analysis of the pellets from the lysates

The pellets from the lysation of cells were examined to determine whether some insoluble protein remained in this fraction. The pellets were resuspended in sample buffer and loaded on a 18% polyacrylamide gel, transferred to a membrane, incubated with Neutravidin-HRP and visualised by West Femto chemiluminiscent substrate (Thermo Fisher Scientific). There was no signal of the molecular weight of either around 22 kDa or 35 kDa in the samples (data not shown), suggesting that no insoluble recombinant protein was expressed in these cells.

11 Pull-down of the intracellular part of Naaladase L2 from the cell lysate

To investigate the putative binding partner of Naaladase L2, the intracellular part of Naaladase L2 was pulled-down from the cell lysate using Streptavidin agarose (see chapter 5.8.2) and the elution was analysed by MS (see chapter

5.5). Two parallel experiments were performed, one with the cells containing the protein (TT) and one with negative control (NC). The elution was done by boiling the resin in 40 µl of sample buffer for SDS-PAGE. All the fractions were loaded on a polyacrylamide gel in the amount of 10 µl, transferred to a nitrocellulose membrane, incubated with Neutravidin-HRP and visualised by West Femto chemiluminiscent substrate (Thermo Fisher Scientific). Despite a small amount of protein remained bound to the resin (figure 19, page 62, lane 1), most of it was successfully purified (lane 6). All samples of negative control remained clear, without any visible bands.

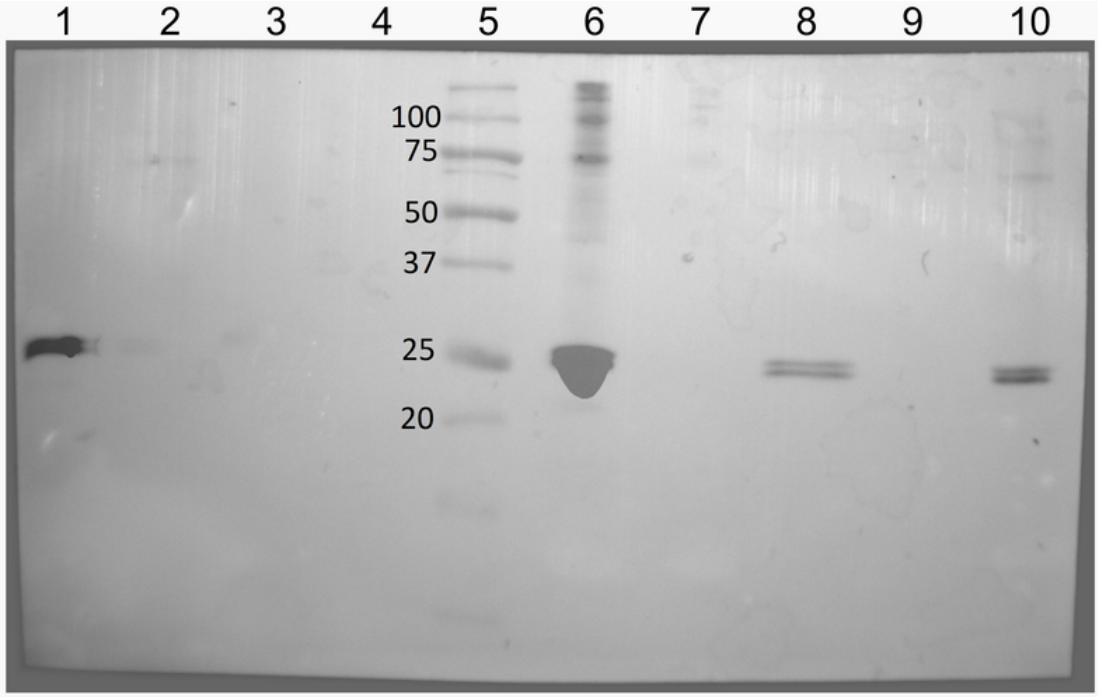

Figure 19: Purification of the intracellular part of Naaladase L2 from the cell lysate via Streptavidin agarose. All the fractions from purification were separated by SDS-PAGE, transferred using wet transfer and visualised. A strong band in lane 6 shows a successful purification. TT stands for positive sample, NC for negative control. 1: resin TT (10 µl); 2: wash 1 NC (10 µl); 3: wash 1 TT (10 µl); 4: elution NC (5 µl); 5: All blue marker (2 µl); 6: elution TT (5 µl); 7: wash 3 NC (10 µl); 8: wash 3 TT (10 µl); 9: FT NC (10 µl); 10: FT TT (10 µl).

35 µl of the elution sample were loaded on a polyacrylamide gel and stained by Silver blue. The whole lane containing pulled-down proteins from TT cells

was analysed by MS by in house service (see chapter 5.5). The same was done with the lane containing the negative control sample.

The lysate with the intracellular part of Naaladase L2 was applied on Streptavidin agarose followed by addition of prostate tissue lysate. The elution was done by 40 µl of sample buffer for SDS-PAGE. Selected fractions were loaded on a polyacrylamide gel, transferred to a nitrocellulose membrane, incubated with Neutravidin-HRP and visualised by West Femto chemiluminiscent substrate (Thermo Fisher Scientific).

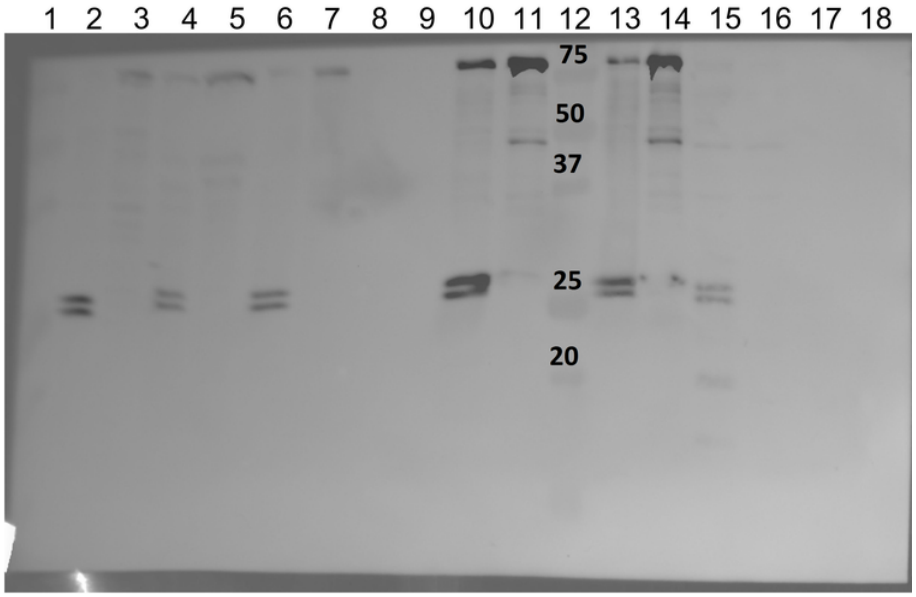

Figure 20: Pull-down experiment using prostate tissue lysate. The intracellular part of Naaladase L2 was immobilised on Streptavidin agarose and a prostate tissue lysate was applied. The elution was done by heating at 95°C in 40 µl of sample buffer. All the fractions were separated on a polyacrylamide gel, transferred to a nitrocellulose membrane and visualised by Neutravidin-HRP and West Femto chemiluminiscent substrate (Thermo Fisher Scientific). 1:All blue marker (1 µl); 2: load TT (0.5 µl); 3: load NC (0.5 µl); 4: FT TT (10 µl); 5: FT NC (10 µl); 6: wash 1 TT (10 µl); 7: wash 1 NC (10 µl); 8: wash 3 TT (10 µl); 9: wash 3 NC (10 µl); 10: elution TT (5 µl); 11: elution NC (5 µl); 12: All blue marker (2 µl); 13: resin TT (10 µl); 14: resin NC (10 µl); 15: FT2 TT (10 µl); 16: FT2 NC (10 µl); 17: wash 4 TT (10 µl); 18: wash 4 NC (10 µl).

Some protein remained bound to the resin as can be seen in lane 13 in figure 20. The elution sample loaded resulted in an intensive band, while in nega-

tive control there was no such band. Some non-specificically bound proteins of high molecular weights are visible in elution and resin fractions, higher intensity was in negative controls. Elution fractions (lanes 10 and 11, figure 20, page 63) were loaded on a polyacrylamide gel and stained by Silver blue and analysed the same way by in house service as in previous experiment.

The database of recognised proteins gained from mass spectrometry for each experiment was processed in two ways. First, only the proteins present in the positive sample but not in the negative sample were selected. Second, the proteins were sorted by the number of unused peptides, that is by number of unique peptide evidence related to a given gene detected. The ones with the highest difference between positive sample and negative control were selected. Resulting four lists of proteins (two from each experiment), each with several thousands hits, were searched for proteins present in both pull-down experiments. Nine peptides came out as a result: CCR4-NOT transcription complex, subunit 1, centrosomal protein 135kDa, dephospho-CoA kinase domain-containing protein, myomegalin, protein regulator of cytokinesis 1, Schlafen family member 11, triple functional domain protein, trimethyllysine dioxygenase, mitochondrial and AP-2 complex subunit alpha-2. These proteins were then analysed by the DAVID online annotation tool [88] suggesting role in cytoskelet forming and organisation and in binding nucleotide phosphates such as ATP.

Part V

Discussion

Naaladase L2 is a transmembrane protein encoded by the NaaladL2 gene. Its mRNA levels correlate with Gleason score used in prostate cancer stage evaluation. It was suggested as a potential marker of this disease since its overexpression leads to elevated cell migration and invasion. Naaladase L2 is not yet well characterised and its function remains unclear.

We set to express and purify the recombinant extracellular part of Naaladase L2. Since it is a homologue of well-studied GCPII, first step was to test Naaladase L2 for the GCPII proteolytic activity. Then we started investigating the intracellular part for potential interaction partners.

First, the extracellular part of Naaladase L2, AviEXSTL2, was expressed and purified via Streptavidin mutein resin and its folding was analysed. It was observed that protein frozen slowly and stored at -20°C which was then thawed did not possess folded structure. On the other hand, protein stored at 4°C which was analysed immediately after purification was folded. This observation is in contrast to results obtained when using recombinant extracellular part of GCPII suggesting that AviEXSTL2 protein cannot undergo slow freezing while keeping its native structure. When frozen in liquid nitrogen, the protein remains folded. Also, melting temperature of recombinant GCPII is higher than the one of AviEXSTL2. The difference is caused by different amino acid composition and protein structure. Recombinant GCPII belongs among the proteins with above-average high melting temperatures.

AviEXSTL2 behaves differently than recombinant GCPII as was described above, it was thus possible that the buffer for recombinant GCPII might not

be the most suitable for AviEXSTL2.

To investigate which buffer should be used for AviEXSTL2 storage and handling, a buffer screen was performed using a wide variety of buffers of different pH values. The most suitable buffer was phosphate buffer because the melting temperature of AviEXSTL2 in this buffer was the highest. Some measurements resulted in highly located beginnings of curves (see table 1, page 51). The reason for this probably is that in these buffers the protein precipitates and unfolds even at room temperature resulting in binding dye to their revealed hydrophobic cores. Another explanation would be that the protein was not fully folded even before the experiment, however, most curves had a clear minimum and were not elevated from the very beginning. It can be concluded that some buffers support the protein folded structure, such as phosphate buffer that was selected as the most suitable one for further experiments.

Therefore, two phosphate buffers were selected for another affinity purification of AviEXSTL2 followed by gel filtration. In contrast to phosphate buffer B (150mM phosphate, 450 mM NaCl, pH 7.4) where only one peak (corresponding to AviEXSTL2) was visible, sample in phosphate buffer A (137mM NaCl, 2.7mM KCl, 12.7mM phosphate, pH 7.2) resulted in two peaks. The additional peak was probably caused by non-specific impurity which was also visible on polyacrylamide gels from purification at 100 to 250 kDa. The peak of AviEXSTL2 (see figure 13, panel A, page 54) consisted of the main peak and a sidepeak at the left side. Analysis of fractions from gel filtration revealed that the main peak and its side peak correspond to the upper and lower band respectively in elution fractions in figure 10 on page 49.

To investigate the heterogeneity, fractions from gel filtration were separated by SDS-PAGE and the upper band and lower band were analysed by MS and

N-terminal sequencing. However, no difference between the proteins forming the two bands was found. This suggests that purified AviEXSTL2 consists of two parts of slightly different molecular weights. To determine, whether the heterogeneity is caused by glycosylation, sample of the upper band, lower band and a mixture of both bands was deglycosylated and analysed by Western blot (see figure 15 on page 57). Glycosylated, non-treated, proteins were used as a control. The peaks in the sample containing both bands shifted, solitary upper band and lower band shifted the same distance as well. Both upper and lower bands are therefore glycosylated and glycosylation itself cannot account for the observed difference.

Since the protein is not well-studied, only one monoclonal antibody against Naaladase L2 is available. It was used for confirmation of the identity of the protein purified (see figure 16 on page 57). This antibody can be used for further experiments since it distinguishes between recombinant GCPII and AviEXSTL2, though its sensitivity is quite low. The amount of 250 ng of protein was barely visible. The antibody also bound to cytochrome c used as a negative control. For that reason, a cross-reactivity might occur and further characterisation of the antibody is necessary.

To determine if AviEXSTL2 possesses the same proteolytic activity as recombinant GCPII, that is cleavage of NAAG [9, 10], or a similar one, a PICS (proteomic identification of protease cleavage sites) experiment was performed on each upper and lower band separately. No activity was detected on either one of the samples. The role of Naaladase L2 is therefore different from GCPII or GCPIII.

Further experiments were done with the intracellular part of Naaladase L2. Since Naaladase L2 possesses a relatively large intracellular part and it is a transmembrane protein, its function could lay in interacting with other

proteins on the intracellular side as is common among transmembrane receptors. For this reason, two constructs of intracellular part were produced by transient transfection of HEK cells, one with the transmembrane and intracellular part, the other with only the intracellular part. Only the second mentioned was expressed in sufficient amount and was used for further experiments. Additionally, two stable clones expressing the same protein that were made previously in our lab were not expressed, probably due to freezing and thawing (see figure 18, page 60). No protein remained in the pellet produced during the cell lysation, hence the protein consisting of the intracellular part of Naaladase L2 was well soluble and cytosolic (data not shown).

The intracellular part of Naaladase L2 was purified via Streptavidin agarose and analysed by MS (performed by in house service). As well as the extracellular part even the intracellular consists of two bands as can be seen in figure 19 on page 62. The investigation of the cause of this heterogeneity was also postponed for when the function of Naaladase L2 is known.

Pull-down experiments followed by MS analysis revealed a possible role of Naaladase L2 in cytoskeletal organisation and forming. Data from both experiments, that is pull-down and pull-down using a prostate tissue lysate, were processed the same way. Since the possibility of false positivity is high, an intersection of the data gathered from the two experiments was done to lower the amount of false hits. Nine proteins were found to be possible interacting partners of Naaladase L2 (CCR4-NOT, CEP135, dephospho-CoA kinase domain-containing protein, phosphodiesterase 4D interacting protein, protein regulator of cytokinesis 1, Schlafen family member 11, triple functional domain, trimethyllysine dioxygenase (mitochondrial) and AP2A2.

CCR4-NOT transcription factor subunit 1 plays an important role in mRNA deadenylation by acting as a scaffold protein for other subunits [33]. CEP135

is a centrosomal protein involved in the cell cycle. Its activity enables forming of the centriole where it also acts as a scaffold for a different protein, CEP250 [89, 90]. Dephospho-CoA kinase domain-containing protein is involved in coenzyme A biosynthesis [33]. Phosphodiesterase 4D interacting protein or myomegalin is a protein whose funcion is not yet well known [33]. Protein regulator of cytokinesis 1 is involved in cytokinesis as its name implies. It is a microtubule associated protein necessary to maintaining the spindle midzone which is a subcellular structure segregating chromosomes between cells in cell division [91]. Schlafen family member 11 might be involved in cell cycle arrest and induction of apoptosis caused by exogenously induced DNA damage [92]. Triple functional domain, also called trio rho guanine nucelotide exchange factor is a protein of several functions. It interacts with proteins regulating actin cytoskeleton and proteins from the Ras superfamily involved in regulation and timing of cell division [93, 94]. Trimethyllysine dioxygenase, mitochondrial is an enzyme converting trimethyllysine to hydroxytrimethyllysine in matrix of mitochondrion. Naaladase L2 is transmembrane protein, therefore it is improbable that these two proteins interact in vivo [95]. AP2A2 (adaptor protein complex 2, alpha subunit 2) is a protein enabling protein transport from the membrane to the cytosol. It helps clathrin-dependent endocytosis and acts as a scaffolding platform for endocytic accessory proteins [96].

Many of the proteins mentioned above play a crucial role in cytoskelet organisation and cytoskeletal changes in cell division. This finding is in agreement with the observed fact that prostate cancer tissue with overexpressed Naaladase L2 tends to be more invasive [35]. Further experiments have to be performed to confirm and evaluate interacting partners from this preliminary list.

Part VI

Conclusion

Extracellular part of Naaladase L2 (AviEXSTL2) was prepared and purified using affinity chromatography and gel filtration. The purification yielded heterogenous product that could be separated by SDS-PAGE.

The recombinant extracellular part of Naaladase L2 showed no proteolytic activity.

Possible cause for AviEXSTL2 heterogeneity on SDS-PAGE was investigated and remains unclear.

Intracellular part of Naaladase L2 was prepared and used for a pull down experiment. Nine proteins were found to be associated with this protein suggesting a role in cytoskeletal changes and cell division.

References

[1] D. S. O'Keefe, S. L. Su, D. J. Bacich, Y. Horiguchi, Y. Luo, C. T. Powell, D. Zandvliet, P. J. Russell, P. L. Molloy, N. J. Nowak, T. B. Shows, C. Mullins, R. A. Vonder Haar, W. R. Fair, and W. D. Heston, "Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene.," *Biochimica et biophysica acta*, vol. 1443, no. 1-2, pp. 113–127, 1998.

[2] U. V. Berger, R. E. Carter, M. McKee, and J. T. Coyle, "N-acetylated alpha-linked acidic dipeptidase is expressed by non-myelinating Schwann cells in the peripheral nervous system.," *Journal of Neurocytology*, vol. 24, no. 2, pp. 99–109, 1995.

[3] J. S. Horoszewicz, E. Kawinski, and G. P. Murphy, "Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients.," *Anticancer Research*, vol. 7, no. 5B, pp. 927–935, 1987.

[4] E. H. Holmes, T. G. Greene, W. T. Tino, A. L. Boynton, H. C. Aldape, S. L. Misrock, and G. P. Murphy, "Analysis of glycosylation of prostate-specific membrane antigen derived from LNCaP cells, prostatic carcinoma tumors, and serum from prostate cancer patients," *The Prostate*, vol. 29, no. 7, pp. 25–29, 1996.

[5] N. Schülke, O. a. Varlamova, G. P. Donovan, D. Ma, J. P. Gardner, D. M. Morrissey, R. R. Arrigale, C. Zhan, A. J. Chodera, K. G. Surowitz, P. J. Maddon, W. D. W. Heston, and W. C. Olson, "The homodimer of prostate-specific membrane antigen is a functional target for cancer therapy.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 22, pp. 12590–12595, 2003.

[6] R. Israeli, C. Powell, W. Fair, and W. Heston, "Molecular cloning of a complementary DNA encoding a prostate-specific membrane antigen," *Cancer Research*, vol. 53, no. 2, pp. 227–230, 1993.

[7] N. D. Rawlings and A. J. Barrett, "Structure of membrane glutamate carboxypeptidase," *Biochimica et Biophysica Acta - Protein Structure and Molecular Enzymology*, vol. 1339, no. 2, pp. 247–252, 1997.

[8] N. Rawlings, M. Waller, A. Barrett, and A. Bateman, "Merops: the database of proteolytic enzymes, their substrates and inhibitors," *Nucleic Acids Research*, vol. 42, pp. D503–D509, 2014.

[9] P. Sacha, J. Zamecnik, C. Barinka, K. Hlouchova, A. Vicha, P. Mlcochova, I. Hilgert, T. Eckschlager, and J. Konvalinka, "Expression of glutamate carboxypeptidase II in human brain.," *Neuroscience*, vol. 144, no. 4, pp. 1361–1372, 2007.

[10] M. B. Robinson, R. D. Blakely, R. Couto, and J. T. Coyle, "Hydrolysis of the brain dipeptide N-acetyl-L-aspartyl-L-glutamate. Identification and characterization of a novel N-acetylated alpha-linked acidic dipeptidase activity from rat brain.," *The Journal of Biological Chemistry*, vol. 262, no. 30, pp. 14498–14506, 1987.

[11] J. Pinto, B. Suffoletto, T. Berzin, and C. Qiao, "Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells.," *Clinical Cancer Research*, vol. 2, no. 9, pp. 1445–1451, 1996.

[12] R. Carter, "Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 2, pp. 749–753, 1996.

[13] J. R. Mesters, C. Barinka, W. Li, T. Tsukamoto, P. Majer, B. S. Slusher, J. Konvalinka, and R. Hilgenfeld, "Structure of glutamate carboxypeptidase II, a drug target in neuronal damage and prostate cancer.," *The EMBO Journal*, vol. 25, no. 6, pp. 1375–1384, 2006.

[14] M. I. Davis, M. J. Bennett, L. M. Thomas, and P. J. Bjorkman, "Crystal structure of prostate-specific membrane antigen, a tumor marker and peptidase.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 17, pp. 5981–5986, 2005.

[15] R. Israeli, C. Powell, J. Corr, W. Fair, and W. Heston, "Expression of the prostate-specific membrane antigen," *Cancer Research*, vol. 54, no. 7, pp. 1807–1811, 1994.

[16] A. C. Cunha, B. Weigle, A. Kiessling, M. Bachmann, and E. P. Rieber, "Tissue-specificity of prostate specific antigens: comparative analysis of transcript levels in prostate and non-prostatic tissues.," *Cancer Letters*, vol. 236, no. 2, pp. 229–238, 2006.

[17] Y. Kinoshita, K. Kuratsukuri, S. Landas, K. Imaida, P. M. Rovito, C. Y. Wang, and G. P. Haas, "Expression of prostate-specific membrane antigen in normal and malignant human tissues.," *World Journal of Surgery*, vol. 30, no. 4, pp. 628–636, 2006.

[18] P. Mhawech-Fauceglia, S. Zhang, L. Terracciano, G. Sauter, A. Chadhuri, F. R. Herrmann, and R. Penetrante, "Prostate-specific membrane antigen (PSMA) protein expression in normal and neoplastic tissues and its sensitivity and specificity in prostate adenocarcinoma: an immunohistochemical study using mutiple tumour tissue microarray technique.," *Histopathology*, vol. 50, no. 4, pp. 472–483, 2007.

[19] A. Baccala, L. Sercia, J. Li, W. Heston, and M. Zhou, "Expression of prostate-specific membrane antigen in tumor-associated neovasculature of renal neoplasms.," *Urology*, vol. 70, no. 2, pp. 385–390, 2007.

[20] D. Mahadevan and J. W. Saldanha, "The extracellular regions of PSMA and the transferrin receptor contain an aminopeptidase domain: implications for drug design.," *Protein science: a publication of the Protein Society*, vol. 8, no. 11, pp. 2546–2549, 1999.

[21] A. K. Rajasekaran, G. Anilkumar, and J. J. Christiansen, "Is prostate-specific membrane antigen a multifunctional protein?," *American Journal of Physiology. Cell physiology*, vol. 288, no. 5, pp. C975–981, 2005.

[22] G. P. Murphy, P. B. Snow, J. Brandt, A. Elgamal, and M. K. Brawer, "Evaluation of prostate cancer patients receiving multiple staging tests, including ProstaScint scintiscans.," *The Prostate*, vol. 42, no. 2, pp. 145–149, 2000.

[23] D. S. O'Keefe, D. J. Bacich, and W. D. W. Heston, "Comparative analysis of prostate-specific membrane antigen (PSMA) versus a prostate-specific membrane antigen-like gene.," *The Prostate*, vol. 58, no. 2, pp. 200–210, 2004.

[24] C. Barinka, P. Mlcochová, P. Sácha, I. Hilgert, P. Majer, B. S. Slusher, V. Horejsí, and J. Konvalinka, "Amino acids at the N- and C-termini of human glutamate carboxypeptidase II are required for enzymatic activity and proper folding.," *European Journal of Biochemistry / FEBS*, vol. 271, no. 13, pp. 2782–2790, 2004.

[25] M. N. Pangalos, J. M. Neefs, M. Somers, P. Verhasselt, M. Bekkers, L. van der Helm, E. Fraiponts, D. Ashton, and R. D. Gordon, "Isolation and expression of novel human glutamate carboxypeptidases with N-acetylated alpha-linked acidic dipeptidase and dipeptidyl peptidase IV activity.," *The Journal of biological chemistry*, vol. 274, no. 13, pp. 8470–8483, 1999.

[26] T. Bzdega, T. Turi, B. Wroblewska, D. She, H. S. Chung, H. Kim, and J. H. Neale, "Molecular Cloning of a Peptidase Against N-Acetylaspartylglutamate from a Rat

Hippocampal cDNA Library," *Journal of Neurochemistry*, vol. 69, no. 6, pp. 2270–2277, 2002.

[27] K. Hlouchova, V. Navratil, J. Tykvart, P. Sacha, and J. Konvalinka, "GCPII Variants, Paralogs and Orthologs," *Current Medicinal Chemistry*, vol. 19, no. 9, pp. 1316–1322, 2012.

[28] K. Hlouchova, C. Barinka, V. Klusak, P. Sacha, P. Mlcochova, P. Majer, L. Rulisek, and J. Konvalinka, "Biochemical characterization of human glutamate carboxypeptidase III.," *Journal of Neurochemistry*, vol. 101, no. 3, pp. 682–696, 2007.

[29] D. Bacich and E. Ramadan, "Deletion of the glutamate carboxypeptidase II gene in mice reveals a second enzyme activity that hydrolyzes N-acetylaspartylglutamate," *Journal of Neurochemistry*, vol. 83, no. 1, pp. 20–29, 2002.

[30] F. Collard, D. Vertommen, S. Constantinescu, L. Buts, and E. Van Schaftingen, "Molecular identification of β-citrylglutamate hydrolase as glutamate carboxypeptidase 3.," *The Journal of Biological Chemistry*, vol. 286, no. 44, pp. 38220–38230, 2011.

[31] T. Bzdega, S. L. Crowe, E. R. Ramadan, K. H. Sciarretta, R. T. Olszewski, O. A. Ojeifo, V. A. Rafalski, B. Wroblewska, and J. H. Neale, "The cloning and characterization of a second brain enzyme with NAAG peptidase activity.," *Journal of Neurochemistry*, vol. 89, no. 3, pp. 627–635, 2004.

[32] J. Tykvart, C. Barinka, M. Svoboda, V. Navratil, R. Soucek, M. Hubalek, M. Hradilek, P. Sacha, J. Lubkowski, and J. Konvalinka, "Structural and Biochemical Characterization of a Novel Aminopeptidase from Human Intestine.," *The Journal of Biological Chemistry*, vol. 290, no. 18, pp. 11321–11336, 2015.

[33] Uniprot, "/www.uniprot.org/blast/uniprot/B2015012173JLQKU320?fil=taxonomy:9604," accessed 21.01.2015.

[34] E. T. Tonkin, M. Smith, P. Eichhorn, S. Jones, B. Imamwerdi, S. Lindsay, M. Jackson, T.-J. Wang, M. Ireland, J. Burn, I. D. Krantz, P. Carr, and T. Strachan, "A giant novel gene undergoing extensive alternative splicing is severed by a Cornelia de Lange-associated translocation breakpoint at 3q26.3.," *Human Genetics*, vol. 115, no. 2, pp. 139–148, 2004.

[35] H. C. Whitaker, L. L. Shiong, J. D. Kay, H. Grönberg, a. Y. Warren, A. Seipel, F. Wiklund, B. Thomas, P. Wiklund, J. L. Miller, S. Menon, A. Ramos-Montoya, S. L. Vowler, C. Massie, L. Egevad, and D. E. Neal, "N-acetyl-L-aspartyl-L-glutamate peptidase-like 2 is overexpressed in cancer and promotes a pro-migratory and pro-metastatic phenotype.," *Oncogene*, vol. 33, no. 45, pp. 5274–5287, 2014.

[36] Y. Hao, X. Liu, X. Lu, X. Yang, L. Wang, S. Chen, H. Li, J. Li, J. Cao, J. Chen, Y. Li, L. Zhao, Y. Shi, C. Shen, W. Yan, J. He, J. Huang, and D. Gu, "Genome-wide association study in Han Chinese identifies three novel loci for human height.," *Human Genetics*, vol. 132, no. 6, pp. 681–689, 2013.

[37] D. Burgner, S. Davila, W. B. Breunis, S. B. Ng, Y. Li, C. Bonnard, L. Ling, V. J. Wright, A. Thalamuthu, M. Odam, C. Shimizu, J. C. Burns, M. Levin, T. W. Kuijpers, and M. L. Hibberd, "A genome-wide association study identifies novel and functionally related susceptibility Loci for Kawasaki disease.," *PLoS Genetics*, vol. 5, no. 1, p. 1000319, 2009.

[38] K. Borg, P. Stankiewicz, E. Bocian, A. Kruczek, E. Obersztyn, J. R. Lupski, and T. Mazurczak, "Molecular analysis of a constitutional complex genome rearrangement with 11 breakpoints involving chromosomes 3, 11, 12, and 21 and a approximately 0.5-Mb submicroscopic deletion in a patient with mild mental retardation.," *Human Genetics*, vol. 118, no. 2, pp. 267–275, 2005.

[39] A. Millson, D. Lagrave, M. J. H. Willis, L. R. Rowe, E. Lyon, and S. T. South, "Chromosomal loss of 3q26.3-3q26.32, involving a partial neuroligin 1 deletion, identified by genomic microarray in a child with microcephaly, seizure disorder, and severe intellectual disability.," *American Journal of Medical Genetics*, vol. 158A, no. 1, pp. 159–165, 2012.

[40] C. D. Chen, D. S. Welsbie, C. Tran, S. H. Baek, R. Chen, R. Vessella, M. G. Rosenfeld, and C. L. Sawyers, "Molecular determinants of resistance to antiandrogen therapy.," *Nature Medicine*, vol. 10, no. 1, pp. 33–39, 2004.

[41] Z. Wang, Y. Hao, and a. W. Lowe, "The Adenocarcinoma-Associated Antigen, AGR2, Promotes Tumor Growth, Cell Migration, and Cellular Transformation," *Cancer Research*, vol. 68, no. 2, pp. 492–497, 2008.

[42] P. Dean, W. Scouten, P. Elvig, and J. Wienfordner, "Affinity Chromatography - Bioselective Adsorption on Inert Matrices," *FEBS Letters*, vol. 138, no. 1, pp. 151–152, 1982.

[43] Imgarcade, "Chromatography Protein Purification, http://imgarcade.com/1/affinity-chromatography-protein-purification/, accessed 15.03.2015."

[44] G. Patchornik, "Purification of His-tagged proteins with [desthiobiotin-BSA-EDTA] conjugates exhibiting resistance to EDTA.," *Bioconjugate chemistry*, vol. 19, no. 3, pp. 673–679, 2008.

[45] K. Terpe, "Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems.," *Applied Microbiology and Biotechnology*, vol. 60, no. 5, pp. 523–533, 2003.

[46] T. P. Hopp, K. S. Prickett, V. L. Price, R. T. Libby, C. J. March, D. Pat Cerretti, D. L. Urdal, and P. J. Conlon, "A Short Polypeptide Marker Sequence Useful for Recombinant Protein Identification and Purification," *Bio/Technology*, vol. 6, no. 10, pp. 1204–1210, 1988.

[47] S. J. Duellman, N. E. Thompson, and R. R. Burgess, "An epitope tag derived from human transcription factor IIB that reacts with a polyol-responsive monoclonal antibody.," *Protein Expression and Purification*, vol. 35, no. 1, pp. 147–55, 2004.

[48] R. Stofko-Hahn, D. Carr, and J. Scott, "A single step purification for recombinant proteins Characterization of a microtubule associated protein (MAP 2) fragment which associates with the type II," *FEBS Letters*, vol. 302, no. 3, pp. 1–4, 1992.

[49] D. Smith and K. Johnson, "Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase," *Gene*, vol. 67, no. 1, pp. 31–40, 1988.

[50] O. Puig, F. Caspary, G. Rigaut, B. Rutz, E. Bouveret, E. Bragado-Nilsson, M. Wilm, and B. Séraphin, "The tandem affinity purification (TAP) method: a general procedure of protein complex purification.," *Methods*, vol. 24, no. 3, pp. 218–229, 2001.

[51] W. Dougherty, S. Cary, and T. Parks, "Molecular genetic analysis of a plant virus polyprotein cleavage site: a model," *Virology*, vol. 364, no. 1989, pp. 356–364, 1989.

[52] G. Rigaut, A. Shevchenko, B. Rutz, and M. Wilm, "A generic protein purification method for protein complex characterization and proteome exploration," *Nature Biotechnology*, vol. 17, no. 10, pp. 1030–1032, 1999.

[53] L. Huber, "Is proteomics heading in the wrong direction?," *Nature Reviews Molecular Cell Biology*, vol. 4, no. 1, pp. 74–80, 2003.

[54] N. J. Totowa, "Protein-Protein Interactions," *Methods in Molecular Biology*, vol. 26, no. 1, pp. 167–174, 2004.

[55] D. G. Edmondson and S. Y. Roth, *Identification of protein interactions by far Western analysis.*, vol. Chapter 20. 2001.

[56] Life Technologies, "Far Western Blot, Life Technologies, https://www.lifetechnologies.com/order/catalog/product/23500, accessed 26.04.2015."

[57] S. Fields and O.-k. Song, "A novel genetic system to detect protein-protein interactions," *Nature*, vol. 340, no. 6230, pp. 245–246, 1989.

[58] A. Brückner, C. Polge, N. Lentze, D. Auerbach, and U. Schlattner, "Yeast two-hybrid, a powerful tool for systems biology.," *International Journal of Molecular Sciences*, vol. 10, no. 6, pp. 2763–2788, 2009.

[59] I. Remy, F. X. Campbell-Valois, and S. W. Michnick, "Detection of protein-protein interactions using a simple survival protein-fragment complementation assay based on the enzyme dihydrofolate reductase.," *Nature Protocols*, vol. 2, no. 9, pp. 2120–2125, 2007.

[60] J. N. Pelletier, I. Remy, and S. W. Michnick, "Protein-Fragment Complementation Assays," *Journal of Biomolecular Techniques*, vol. 95, pp. 12141–12146, 1998.

[61] M. A. Batzer, "Phage Display: A Laboratory Manual. Edited by C. F. Barbas III, D. R. Burton, J. K. Scott, and G. J. Silverman," *Analytical Biochemistry*, vol. 294, no. 2, pp. 194–200, 2001.

[62] G. P. Smith and V. A. Petrenko, "Phage Display.," *Chemical Reviews*, vol. 97, no. 2, pp. 391–410, 1997.

[63] S. S. Sidhu, H. B. Lowman, B. C. Cunningham, and J. A. Wells, "Phage display for selection of novel binding peptides," in *Applications of Chimeric Genes and Hybrid Proteins - Part C: Protein-Protein Interactions and Genomics* (S. D. E. Jeremy Thorner and J. N. Abelson, eds.), vol. 328 of *Methods in Enzymology*, pp. 333 – IN5, Academic Press, 2000.

[64] G. C. Terstappen, C. Schlüpen, R. Raggiaschi, and G. Gaviraghi, "Target deconvolution strategies in drug discovery.," *Nature reviews. Drug discovery*, vol. 6, no. 11, pp. 891–903, 2007.

[65] B. Gray and K. Brown, "Combinatorial peptide libraries: mining for cell-binding peptides," *Chemical Reviews*, vol. 114, no. 2, pp. 1020–1081, 2013.

[66] O. H. Aina, R. Liu, J. L. Sutcliffe, J. Marik, C.-X. Pan, and K. S. Lam, "From combinatorial chemistry to cancer-targeting peptides.," *Molecular Pharmaceutics*, vol. 4, no. 5, pp. 631–651, 2007.

[67] C. C. Liu, A. V. Mack, M.-L. Tsao, J. H. Mills, H. S. Lee, H. Choe, M. Farzan, P. G. Schultz, and V. V. Smider, "Protein evolution with an expanded genetic code.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 46, pp. 17688–17693, 2008.

[68] G. Georgiou, "Analysis of large libraries of protein mutants using flow cytometry," *Advances in Protein Chemistry*, vol. 55, pp. 293–315, 2001.

[69] S. Brown, "Engineered iron oxide-adhesion mutants of the Escherichia coli phage lambda receptor.," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 18, pp. 8651–8655, 1992.

[70] J. Francisco, "Production and fluorescence-activated cell sorting of Escherichia coli expressing a functional antibody fragment on the external surface," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 22, pp. 10444–10448, 1993.

[71] A. Christmann, K. Walter, and A. Wentzel, "The cystine knot of a squash-type protease inhibitor as a structural scaffold for Escherichia coli cell surface display of conformationally constrained peptides," *Protein Engineering Design and Selection*, vol. 12, no. 9, pp. 797–806, 1999.

[72] L. Mattheakis, "An in vitro polysome display system for identifying ligands from very large peptide libraries," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 19, pp. 9022–9026, 1994.

[73] R. Roberts and J. Szostak, "RNA-peptide fusions for the in vitro selection of peptides and proteins," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 23, pp. 12297–12302, 1997.

[74] N. Nemoto, E. Miyamoto-Sato, Y. Husimi, and H. Yanagawa, "In vitro virus: bonding of mRNA bearing puromycin at the 3'-terminal end to the C-terminal end of its encoded protein on the ribosome in vitro," *FEBS Letters*, vol. 414, no. 2, pp. 405–408, 1997.

[75] B. K. Cho, M. C. Kieke, E. T. Boder, K. Wittrup, and D. M. Kranz, "A yeast surface display system for the discovery of ligands that trigger cell activation," *Journal of Immunological Methods*, vol. 220, no. 1-2, pp. 179–188, 1998.

[76] C. Oker-Blom, "Baculovirus display strategies: Emerging tools for eukaryotic libraries and gene delivery," *Briefings in functional genomics*, vol. 2, no. 3, pp. 244–253, 2003.

[77] T. Kodadek, "Protein microarrays: prospects and problems," *Chemistry & Biology*, vol. 8, no. 2, pp. 105–115, 2001.

[78] X. Tang and J. E. Bruce, "Chemical cross-linking for protein-protein interaction studies.," *Methods in Molecular Biology*, vol. 492, pp. 283–293, 2009.

[79] R. Kluger and A. Alagic, "Chemical cross-linking and protein-protein interactions: a review with illustrative protocols," *Bioorganic Chemistry*, vol. 32, no. 6, pp. 451–472, 2004.

[80] C. Schmidt and C. Robinson, "A comparative cross-linking strategy to probe conformational changes in protein complexes," *Nature Protocols*, vol. 9, no. 9, pp. 2224–2236, 2014.

[81] J. Borch and P. Roepstorff, "SPR/MS: recovery from sensorchips for protein identification by MALDI-TOF mass spectrometry.," *Methods in Molecular Biology*, vol. 627, pp. 269–281, 2010.

[82] T. Natsume, H. Nakayama, O. Jansson, T. Isobe, K. Takio, and K. Mikoshiba, "Combination of Biomolecular Interaction Analysis and Mass Spectrometric Amino Acid Sequencing," *Analytical Chemistry*, vol. 72, no. 17, pp. 4193–4198, 2000.

[83] W. Boireau, A. Rouleau, G. Lucchi, and P. Ducoroy, "Revisited BIA-MS combination: Entire "on-a-chip" processing leading to the proteins identification at low femtomole to sub-femtomole levels," *Biosensors and Bioelectronics*, vol. 24, no. 5, pp. 1121–1127, 2009.

[84] R. L. Rich and D. G. Myszka, "Why you should be using more SPR biosensor technology.," *Drug discovery today. Technologies*, vol. 1, no. 3, pp. 301–308, 2004.

[85] P. Englebienne, A. V. Hoonacker, and M. Verhas, "Surface plasmon resonance: principles, methods and applications in biomedical sciences," *Spectroscopy*, vol. 17, no. 2-3, pp. 255–273, 2003.

[86] U. B. Ericsson, B. M. Hallberg, G. T. Detitta, N. Dekker, and P. Nordlund, "Thermofluor-based high-throughput stability optimization of proteins for structural studies.," *Analytical Biochemistry*, vol. 357, no. 2, pp. 289–298, 2006.

[87] Bio Rad, "www.bio-rad.com/webroot/web/pdf/lsr/literature/LIT33.pdf," accessed 26.04.2015.

[88] G. Dennis Jr., B. T. Sherman, D. A. Hosack, and J. Yang, "DAVID: Database for Annotation, Visualization, and Integrated Discovery, http://david.abcc.ncifcrf.gov/, accessed 25. 4. 2015."

[89] K. Kim, S. Lee, J. Chang, and K. Rhee, "A novel function of CEP135 as a platform protein of C-NAP1 for its centriolar localization," *Experimental Cell Research*, vol. 314, no. 20, pp. 3692–3700, 2008.

[90] J. Kleylein-Sohn, J. Westendorf, M. Le Clech, R. Habedanck, Y.-D. Stierhof, and E. a. Nigg, "Plk4-induced centriole biogenesis in human cells.," *Developmental Cell*, vol. 13, no. 2, pp. 190–202, 2007.

[91] C. Mollinari, J.-P. Kleman, W. Jiang, G. Schoehn, T. Hunter, and R. L. Margolis, "PRC1 is a microtubule binding and bundling protein essential to maintain the mitotic spindle midzone.," *The Journal of Cell Biology*, vol. 157, no. 7, pp. 1175–1186, 2002.

[92] G. Zoppoli and M. Regairaz, "Putative DNA/RNA helicase Schlafen-11 (SLFN11) sensitizes cancer cells to DNA-damaging agents," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 37, pp. 15030–15035, 2012.

[93] Q. G. Medley, C. Serra-Pagès, E. Iannotti, K. Seipel, M. Tang, S. P. O'Brien, and M. Streuli, "The trio guanine nucleotide exchange factor is a RhoA target. Binding of RhoA to the trio immunoglobulin-like domain.," *The Journal of Biological Chemistry*, vol. 275, no. 46, pp. 36116–36123, 2000.

[94] J. Bellanger, C. Astier, and C. Sardet, "The Rac1-and RhoG-specific GEF domain of Trio targets filamin to remodel cytoskeletal actin," *Nature Cell Biology*, vol. 2, no. 12, pp. 888–892, 2000.

[95] J. Monfregola, A. Cevenini, A. Terracciano, N. van Vlies, S. Arbucci, R. J. a. Wanders, M. D'Urso, F. M. Vaz, and M. V. Ursini, "Functional analysis of TMLH variants and definition of domains required for catalytic activity and mitochondrial targeting.," *Journal of Cellular Physiology*, vol. 204, no. 3, pp. 839–847, 2005.

[96] N. Masuyama, T. Kuronita, R. Tanaka, T. Muto, Y. Hirota, A. Takigawa, H. Fujita, Y. Aso, J. Amano, and Y. Tanaka, "HM1.24 is internalized from lipid rafts by clathrin-mediated endocytosis through interaction with alpha-adaptin.," *The Journal of Biological Chemistry*, vol. 284, no. 23, pp. 15927–15941, 2009.