Protein chemistry and mass spectrometry in biochemical research

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Contents

List of abbreviations

2D	two-dimensional
APC	antigen presenting cell
ATZ	anilinothiazolinone
BCR	B cell receptor
CD	cluster of differenciation
CID	collisionally-induced dissociation
Da	dalton
DAP	death -associated protein
DCSIGN	dentritic cell specific ICAM3 grabbing nonintegrin
DIM	detergent -insoluble membrane
DRM	detergent-resistent membrane
DSG	disuccinimidyl glutarate
DSS	disuccinimidyl suberate
ECD	electron capture dissociation
EDC	1-Ethyl-3-(3'-dimethylaminopropyl) carbodiimide
ESI	electrospray ionization
ER	endoplasmatic reticulum
FAB	fast atom bombardment
FTICR	Fourier transform ion cyclotron resonance
FTMS	Fourier transform mass spectrometer
Gal	galactose
GaINAc	N-acetylgalactosamine
GEM	glycosphingolipid-enriched membrane
GIcIslAc	N-acetylglucosamine
GM	ganglioside
GMO	genetically modified organism
GPI	glycosylphosphatidylinositol
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
ICAM	intercellular adhesion molecule

ICR	ion -cyclotron resonance
IEF	isoelectric focusing
IPG	immobilized pH gradient
ITAM	tyrosine -based activation motif
ITIM	tyrosine -based inhibition motif
KIR	killer cell immunoglobulin-like receptor
LAT	the linker for activation of T cells
Lck	tyrosine kinase
LFA	lymphocyte function -associated antigen
m/z	mass -to -charge ratio
MALDI	matrix -assisted laser desorption/ionization
Man	mannose
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MPa	megapascal
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NK	natural killer
PKC	protein kinase C
PLRP	polymer based reverse phase
PMF	peptide mass fingerprinting
PTH	phenylthiohydantoin
PVDF	polyvinylidene difluoride
Ras	protein kinase
RNK	rat natural killer
RP	reverse -phase
SMAC	supramolecular activation cluster
TCR	T cell receptor
TOF	time -of -flight
UV	ultraviolet
ZAP	tyrosine kinase

Abbreviations of amino acids in three and one letter code are used according to IUPAC

I. THE IMMUNE SYSTEM

1.1 Introduction to lymphocytes and their receptors

Lymphocytes are small white blood cells that bear the major responsibility for carrying out the activities of the immune system. The two major classes of lymphocytes are B cells, which grow to maturity independent of the thymus, and T cells, which are processed in the thymus. Both B cells and T cells recognize specific antigens. B cells produce substances which are called antibodies to body's fluids or humors. These antibodies interact with circulating antigens such as bacteria, viruses or toxic molecules. On the other hand, T cell attack their targets directly. They are able to destroy body cells which are infected by viruses or malignantly transformed.

Although both type of lymphocytes look similarly under the microscope, on their surfaces they bear quit different set of molecules. There are not only disparities among T and B cells, but also among subsets of these cells. Every mature T cell, for instance, carries a marker known as CD3. Most helper T cells carry a CD4 marker, a molecule that recognizes MHC class II antigens. A molecule known as CD8, which recognizes MI -IC class I antigens, is found on many suppressor/cytotoxic T cells. Different T cells have different classes of antigen receptors -either alpha/beta or gamma/delta.

1.2 B cell receptors (BCRs)

B lymphocytes recognize antigens via their antigen -specific receptors, the BCRs. Signals caused by the recognition of soluble molecules, cytokines or adhesion molecules (direct contact between cells), induce the maturation of B lymphocytes. The BCR and antibodies belong to immunoglobulins. The receptor is composed of two heavy and two light domains with variable regions on the N -termini of their polypeptide chains. BCR, the transmembrane immunoglobulin, is expressed on the cell surface. Formation of a complex between BCR and other transmembrane molecules CD79a and CD7913 is responsible for the full activation of B lymphocyte.

1.3 T cell receptors (TCRs)

The T cell receptor is a heterodimer composed either of alpha and beta or gamma and delta polypeptide chains. Amino acid sequencing analysis shows a surprising similarity to the domain structure of the immunoglobulins. Each chain has a variable region domain and a constant region domain. Similarly to antibodies, the variable region of the TCR is created by gene rearrangement and selection of minigenes in a random order to create diversity of antigen recognition [Claverie *et al.,* 1989]. The alpha and beta chains are associated with a group of five proteins called CD3. The transmembrane region of the TCR is composed of positively charged amino acids. It is thought that this allows the TCR to associate with coreceptors (like CD3) that have negatively charged transmembrane regions. These coreceptors are vital for the propagation of the signal from the TCR into the cell [Allison *et al.,*2001]. The antigens that TCRs bind are small peptide fragments, or epitopes, displayed by MI -IC molecules on the surface of cells. Cytotoxic T lymphocyte TCRs recognize epitopes displayed by MHC class I molecules on the surface of almost every cell in the body, so it can distinguish between "self' -antigens and foreign antigens (viral -infected cells), as well as being sensitive to the amount of self -antigen presented (increased number of self -antigens in malignant cells). Helper T cell and inflammatory T cell TCRs recognize epitopes displayed by MHC class II molecules on the surface of antigen -presenting immune cells, such as macrophages, that engulf foreign particles and microorganisms, dendritic cells that present antigen to T cells, and B cells that produce antibodies. The binding of the epitope to the TCR involves T cell surface coreceptors CD8 on cytotoxic T lymphocytes and CD4 on helper and inflammatory T cells. The CD8 and CD4 surface glycoproteins recognize MHC class I and II molecules, respectively. The binding of a TCR to an epitope can result in a signal that is sent to the nucleus to induce a response [Janeway & Travers, 1994].

1.4 Signal transduction through the TCR

Signaling through TCRs start with aggregation of T -cell receptors themselves when the formation of peptide/MHC complexes occurs on the surface of a target cell. For the signaling, the presence of the coreceptor molecules CD4 and CD8 is required. This results in aggregation of the receptor associated tyrosine kinases, which leads to activation of growth factors or hormones. Phosphorylation of substrates by receptor associated tyrosine kinases like CD3 and chain (bind and activate cytosolic kinase ZAP -70) plays also an important role in T -cell receptor mediated signaling. It was observed that T -cell surface molecule CD 45 (leukocyte common antigen) is also involved, due to its tyrosine -specific phosphatase activity, in signal transduction. CD45 is a transmembrane molecule associated directly with TCR. Cells that lack CD45 are defective in signaling through TCR.

The activation of receptor associated tyrosine kinases leads to tyrosine phosphorylation of enzyme phospholipase C. This enzyme breaks down phosphatidylinositol to diacylglycerol and inositol, which raise the intracellular calcium ion concentration. Intracellular calcium ions and activated protein kinase C are both responsible for cellular response that requires new gene expression.

1.5 The role of lipid rafts in T -cell signaling

During the signalization, most of the signaling proteins and receptors are localised in specific areas of plasma membrane. This areas are chemically and physically different from the surrounding space. Formation of domains composed of glycosphingolipids, cholesterol and other lipids was observed. This rigid units are also called lipid rafts or membrane microdomains. In literature we can find another synonyms for lipid rafts such as DIM (detergent -insoluble membranes), DRMs (detergent-resistent membranes) or GEMs (glycosphingolipid-enriched membranes) [Brown & London, 1998]. For example, T cell antigen receptor molecule and other proteins involved in signaling such as p56 Lck, LAT and protein kinase C are recruited to lipid rafts. The disturbance of lipid rafts by methyl-13-cyclodextrin, an agent that extracts cellular cholesterol, leads to abrogation of signaling but also in hyperactivation of the Ras -controlled signaling pathway [Scheel *et al.*, 1999]. Due to

the ability to separate different proteins and lipids in the plasma membrane, lipid rafts are involved in several important cellular processes such as signal transduction, membrane fusion, or protein trafficking [Chamberlain *et al.* 2001].

1.6 The composition of lipid rafts

The major biochemical characteristic of lipid rafts is their insolubility in non-ionic detergents such as Triton X 100, Brij 58 or NP -40 in the cold. The high amount of cholesterol and other lipids allows the isolation of lipid rafts in sucrose density gradients. Proteins which interact with lipid rafts could be found on inner or outer leaflets of plasma membrane. These proteins are usually modified by the addition of saturated lipid groups such as glycosylphosphatidylinositol anchor (GPI) or palmitate S-acyl groups (acylation).

The lipid raft structure is held together by hydrophobic interactions between saturated fatty acid residues (sphingomyelin, GM3) and intercalating cholesterol molecules. Another important lipid is cereatnide, which plays essensial role in aggregation of lipid rafts [Hofejgi, 2005].

1.7 Formation of an immunological synapse

During the adhesion between T cell and antigen presenting cell (APC), formation of TCR-MHC complex was observed as discussed previously. But not only signaling molecules are involved in this process. Adhesion molecules, such as LFA-1 (integrins) or CD2-CD58 and DCSIGN-ICAM-3 (both non-intergrins) overcome the barrier of cell -cell contact posed by the negatively charged glycocalyx of T cell and APC [Springer, 1990, Shaw & Dustin, 1997]. The distance between cells is then 50 — 100nm and cannot be spanned by the TCR and MHC-peptide complex, which interacts at 15nm [Garcia *et al.*, 1996]. The adhesion molecules such as CD2 and CD58 bring the solution of this problem by creating thousands of transients, low affinity interactions [Shaw & Dustin, 1997].

Actin cytoskeleton of T -cell is involved in rearragement of TCR and adhesion molecules during the interaction with MHC-peptide complexes present at the surface of

antigen -presenting cells. T -cell stops moving and generates a central area rich in integrins banked by the bulk of TCR [Grakoui *et al.,* 1999]. After several minutes, TCR molecules are transported to the central area of the cell contact, the intergrins and other adhesion molecules are forced into a surrounding ring. This structure is stable for several hours and is required for full activation of T -cell [Monks *et al.,* 19981. The structure of an immunological synapse is schematically shown in figure 1.

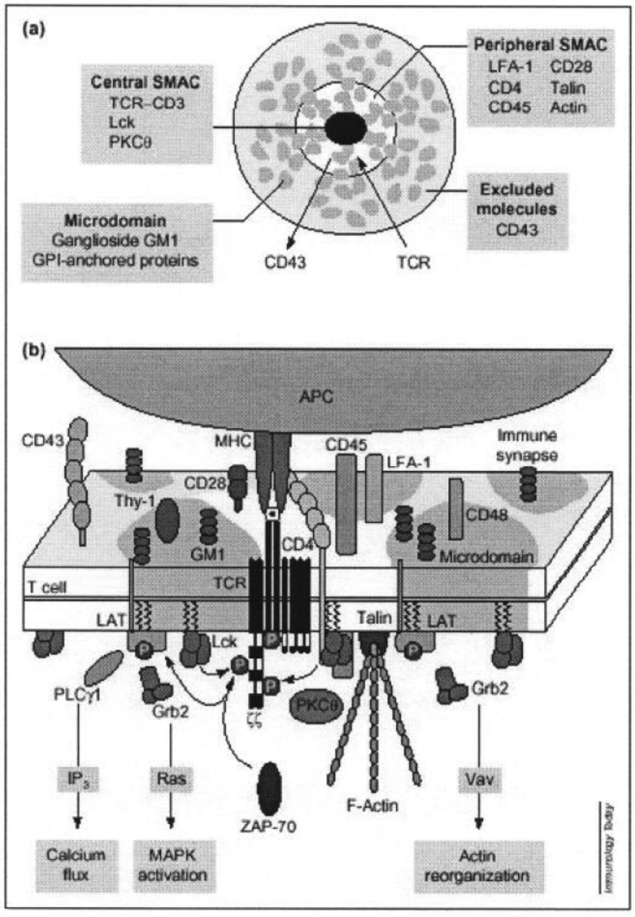

Fig. I *a) Top view of immunological synapse (T-cell-APC). During the contact of the cells, signalling proteins Lck, Fyn and PKC accumulate in the central supramolecular activation cluster (SMAC). Other molecules (LFA-I, CD4, CD45 and talin) involving in actin polymerization and crosslinking accumulate into peripheral SMAC. The third group of proteins (CD43 and other adhesion molecules) is excluded from the central part of the synapse and plays an important role in adhesion processes.*
b) *Side view of immunological synapse (T-cell-APC).*
TCR recognition of peptide-MHC complex. Microdomain Lck and CD4 are both responsible for T -cell signaling. Recruitment of ZAP -70 to CD3(ITAMs and its activation by Lck/Fyn kinases leads in phosphorylation of several intracellular molecules. Phosphorylation of LAT results in coupling the TCR signals leading in calcium fluxes, MAPK activation and actin cytoskeleton reorganization Illangumaran, 20001

1.8 The role of NK cell receptors

NK cell are a very important subset of lymphocytes. They represent 10-20% of peripheral blood lymphocytes. Several typical molecules such as CD56 and CD16 are observed on their surface. NK cells are distributed in peripheral blood, bone marrow, spleen and liver. During early phase of inflammatory processes they migrate to lymph nodes or lymphatics and so represent an important defence mechanism against various intracellular pathogens [Moretta *et al.,* 2002]. It is known that NK cells can directly lyse some tumor and virally infected cells. The functions of these NK cells are critical in the host defense against cancer or viruses and in transplantation. The regulation of balance between inhibitory and stimulatory receptors on the cell surface plays an important role in NK cell mediated cytotoxicity. Nearly all cells of the body express endogenous MHC class I molecules. Many of the inhibitory receptors expressed by NK cells recognize their MI -IC class I and provide protection for cells that express normal amounts of class I on their cell surface ("missing self hypothesis"). The NK cells recognize the molecules on target cells via members of distinct inhibitory receptors ("negative signaling"). The lack of MHC class I molecule on transformed or infected cells causes the activation of stimulatory receptors. The contact between these cells and NK cells leads to initiation of cytotoxicity and cytokine production. The inhibitory receptors are characterized by immunoreceptor tyrosine -based inhibition motif (ITIM) in their cytoplasmatic domain. The typical example of inhibitory receptors is the killer cell immunoglobulin-like receptor (KIR), which belongs to immunoglobulin superfamily receptors [Lanier L., 2001]. KIRs with two immunoglobulin domains (KIR2D) recognize groups of HLA-C allotypes rather than the complex MHC class I - peptide.

C -type lectins like molecules are the second group of MHC class I inhibitory receptors. To these molecules belong Ly-49 homodimer and NKG2D/CD94 heterodimer. All three molecules are membres of natural killer complex on mouse chromosome 6 and human chromosome 12 [Colonna *et al.,* 2000].

Activating receptors are small transmembrane adaptor proteins that contain immunoreceptor tyrosine -based activation motifs (ITAMs). ITAMs are presenting in proteins such as CD3y, CD3.5, CD3E, chain, Iga, Igf3, FceRIy and DAP12. NK cells express three of the ITAM-bearing transmembrane adaptor proteins: chain, FceRIy and DAP12. Another

two activating NK cell receptors were identified on the NK cell surface: NKR-P1 and CD69. Inhibition and activation processes of NK cells are schematically shown in figure 2.

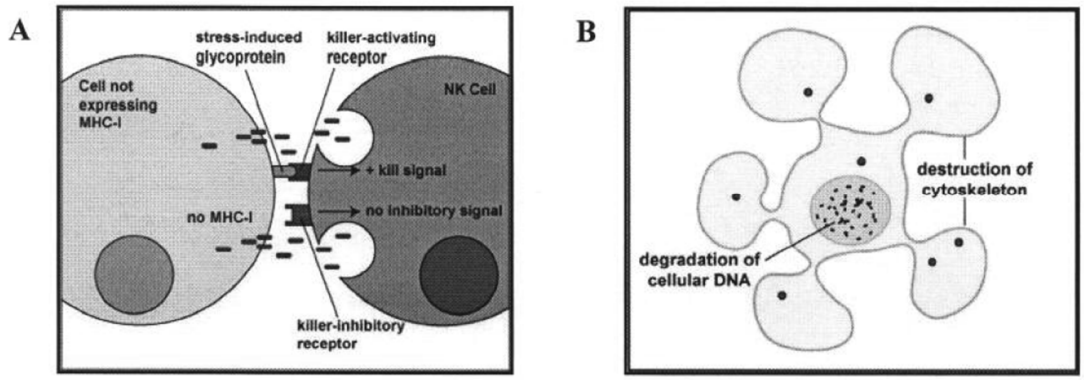

Fig. 2 A) *Viruses and malignant transformation can sometimes interfere with the ability of the infected cell or tumor cell to express MHC class I molecules. Without the signal from the killer -inhibitory receptor, the kill signal from the killer -activating signal is not overridden and the NK cell releases pore -forming proteins called performs, proteolytic enzymes called granzymes, and chemokines.*

B) *Granzymes pass through the pores and activate the enzymes that lead to apoptosis of the infected cell by means of destruction of its structural cytoskeleton proteins and by chromosomal degradation. As a result, the cell breaks into fragments that are subsequently removed by phagocytes. Perform= can also sometimes result in cell lysis.*

1.9 NKR-P1 and CD69 — two important activating NK cell receptors

The NKR-P1 belongs to type II transmembrane glycoproteins of the C -type lectin superfamily. It is also located in the NK gene complex on mouse chromosome 6, rat chromosome 4 and human chromosome 12p12 -p13. There are structural differences between isoforms of NKR-P1 in their cytoplasmatic domains. It was observed that all rodent NKR-P1 proteins express the CXCP motif (also found in CD4 and CD8, where X is any amino acid). This motif interacts with phosphorylated p56 Lck molecule. On the other hand, human NKR-P1 lacks this motif. Ryan *et al.* have shown that loss of NKR-P1 in rat RNK-16 NK leukemia cell line results in the inability to kill mouse tumor cell targets [Ryan *et al.*, 1995]. It was shown that the presence of calcium was needed for the correct folding of this protein *in vitro.* Futherrnore, under physiological pH calcium could not be removed by chelating agents from

the protein, but under both low and high pH, calcium is released, whereas in classic C -type lectins, the binding of calcium remain stable in the alkaline enviroment.

CD69 is a 60kDa disulfide -linked homodimer consisting of two subunits. It is type II transmembrane glycoprotein widely expressed on hematopoietic cell such as neutrophiles, monocytes, T cells, B cells and NK cells. It is related to C -type lectins. The transmembrane domain is responsible for cell signaling and cellular expression. The neck region is important for dimerisation of the protein and the globular domain mediates the binding of monoclonal antibodies and physiological ligands [Sancho *et al.*, 2000]. The presence of calcium in CD69 results in structural changes, which lead to formation of high -affinity carbohydrate -binding site [PavliZek*etal.*, 2003].

II. ENZYMES

2.1 Enzymes

Enzymes are highly efficient biocatalysts specific for a particular chemical reaction involving the synthesis, degradation, or alteration of a compound. Microbial enzymes have largely replaced the traditional plant and animal enzymes used in industry; hydrolytic enzymes are most commonly used in biotechnology. A variety of microbial enzymes are used in baking, brewing and textile industries, animal feed, pulp and paper industry, fruit juice, meat and fish processing, detergents, dairy, and leather industries. Because of enzyme's very specific catalytic properties, only small quantities of them are required to perform the desired conversion, and product yields are often higher than those obtained with chemical routes. Enzymes are derived from natural resources, such as fungi and bacteria, and then are genetically engineered or "molecularly evolved" in the laboratory to impart specific new properties. The new genetic material is inserted into the nucleus of a recombinant host, such as the fungus *Aspergillus* or the bacteria *Bacillus* (both used in food -component production) that is easy to scale up via fermentation. The reason for using recombinant hosts is to ensure product safety (some isolated, wildtype hosts may produce toxins or be pathogenic), higher purity, fewer unwanted byproducts and a favorable economic process. Today, 95% of all industrial enzymes are made by manufacturing genetically modified organisms (GMO) in contained factories. The criteria that industrial enzymes must meet should be reflected in the library of microorganisms that are screened. For example, enzymes for detergent use must show optimal performance at high pH values and temperatures of 20-50°C. On the other hand, feed enzymes work at neutral pH values and 37°C (i.e., the ambient temperature of the gut). Moreover, feed enzymes must also show extreme stability at high temperatures, as they are co -formulated with the feed, and at low pH values, since they have to survive passage through the stomach. Secreted enzymes, which are produced extracellularly by bacteria, archaea and fungi, have the highest probability to live up to the diverse and often extreme conditions. In contrast to intracellular enzymes, which are often stabilized in the cytoplasm, secreted enzymes have been optimized to work in environments by natural selection, as they have evolved over thousands of years. Examples of microbial sources for secreted enzymes

are alkaline lakes with very high pH values, an acid spring with low pH values, a geothermal area with temperatures above the boiling point of water, and polar regions [Vorgias & Antranikian, 2004].

2.2 Introduction to the structure of p-N-acetylhexosaminidase

P-N-acetylhexosaminidase is one of the most abundand enzymes found in several species from bacteria to human. The enzyme is involved in many important biological processes and the dysfunction of the enzyme leads to different diseases. The most common dysfunctions of the enzyme are related to lysosomal storage disorders known as Tay -Sachs [Sachs, 1887, Tay, 1881] and Sandhoff disease [Sandhoff *et al.,* 1968]. Due the ability of effective transglycosylation of p-G1cNAc and p-GaINAc, the research interest was focused on the use of the enzyme for chemoenzymatic synthesis of biological important oligosaccharides [Ken *et al.,* 1994, Rajnochova *et al.,* 1997, Weignerova *et al.,* 2003, Krist *et al.,* 20011.

0-N-acetylhexosaminidase (EC 3.2.1.52) belongs to exoglycosidases and its hydrolase activity leads to hydrolysis of terminal N-acetyl-D-galactosamine or N-acetyl-D-glucosamine. This enzyme is also involved in degradation of chitin into its monosaccharides. In fungus, the enzyme is used for the formation of septa, germ tubes and fruit bodies [Gooday *et al.* 1992, Bulawa, 1993, Cheng *et al.,* 2000].

The first structure of the enzyme was described for the bacterial enzymes from *Serratia marcescens* [Tews *et al.,* 1996, Prag *et al.,* 2000] and *Streptomyces plicatus* [Mark *et al.,* 2001, Williams *et al.,* 2002]. 13-N-acetylhexosaminidase is a member of family 20 of glycosyl hydrolases. The tertiary structure of the enzyme is composed of a TIM barrel motif, in the enzyme catalytic centre. It was found that 13-N-acetylhexosaminidases form dimeric molecules in human composed of two subunits a and p. The dimeric form of the molecule is very important for the activity of the enzyme. Fungal and yeast f3-N-acetylhexosaminidases tend to form homodimers. The postranslational modifications such as N-glycosylation [Jones & Kosman, 1980] or 0-glycosylation [Plihal *et al.,* 2004] has been observed in these enzymes. The unique role of these glycosylations was shown in deglycosylation experiments. N-glycosylation increased the stability and solubility of the enzyme, 0-glycosylation seem to

be necessary for full enzymatic activity. The fungal ii-N-acetylhexosaminidase from *Aspergillus cuyzae* is composed of signal peptide, propeptide, zincin-like and catalytical domain (Figure 3). The N-glycosylated oligosaccharide structures containing up to eleven mannoses were found in the C -terminal part of the catalytic domain [Plihal *et al.*, 2004].

The propeptide of 13-N-acetylhexosaminidase is a very important part of the enzyme. Propeptide is a 10kDa large peptide and its molecular weight was evaluated by mass spectrometry, which corresponds to the theoretical molecular weight determined from the sequence of *hexA* gene. It was observed that under the denaturating conditions, the propeptide is cleaved from the catalytical domain. These results show the noncovalent association between propeptide and catalytical domain of the enzyme. Moreover, detail mass spectrometry analysis reveal the 0-glycosylation of the C -terminal part of the propeptide. Other biochemical studies determined the structures of 0-glycans composed of up to four mannoses terminated with galactose.

The noncovalent association of propeptide is required for dimerization of the catalytic subunits, for stabilization and serves as an intramolecular regulator by keeping it in the biologically active conformation.

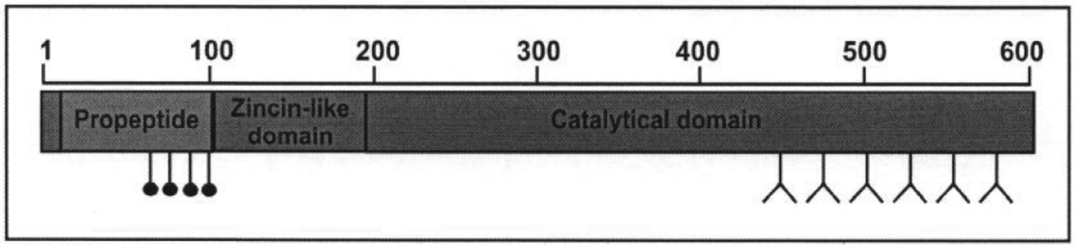

Fig. *3: Fungal /J-N-acetylhexosaminidase is composed offour parts: signal peptide, propeptide, domain and catalytical domain. Propeptide is on its C -termini 0-glycosylated, catalytical domain is N-glycosylated.*

III. METHODS

3.1 Introduction to proteomics

A new term proteomics is used in today's biological and biochemical research for the large scale identification and characterization of proteins, their posttranslational modifications such as phosphorylation or glycosylation, for evaluation of protein -protein interactions and quantification. The term proteome is a linguistic equivalent to the concept of genome that describes all proteins and their modifications in the lifetime of the cell and can be viewed as a tool for determining gene functions. Several techniques and their combination are used for the proteomic analysis. Protein separation by two-dimensional electrophoresis (2D), reverse -phase -high performance liquid chromatography (RP-HPLC), mass spectrometry (MS) and amino -terminal sequence analysis using the Edman degradation belongs to the most common techniques in this respect.

3.1.2 Two-dimensional gel electrophoresis (2D)

This powerfull technique is commonly used for the separation of complex protein mixtures such as whole cell lysates. Isoelectric focusing (IEF) in the first dimension separates proteins on the basis of their charge. Separation by molecular weights of the proteins is then the second step. Due to such orthogonal separations of proteins, 2D is more efficient that the classical one-dimensional electrophoresis and also allows to separate proteins on the basis of their postranslational modifications.

Several new methods were developed over the years to improve the reproducibility and sensitivity of 2D separations. Development of immobilized pH gradients **(IPG)** gel by Angelika Gorg and colleagues in 1985 [Gorg *et al.,* 1985] brought revolution in isoelectric focusing of proteins. The other advantage of IPG is the possibility to select the length and pH range and so increase the resolution. Visualization of proteins includes several staining techniques that can be used in analytical or preparative gels.

3.1.3 Reversed -phase chromatography (RP-HPLC)

The basis of RP-HPLC is the separation of proteins according to the hydrophobic interactions that occurs between the amido acid side chains on the proteins and the hydrophobic surface of the chromatographic medium. RP-HPLC has been widely used for the separation, purification and analysis of molecules such as proteins or peptides.

On the other hand, the separations involving organic chemicals and low pH in solvents could lead to the loss of biological activity, denaturation and unfolding of proteins. The use of appropriate chromatographic medium and optimal set up of separation conditions is very important for succesful separation.

The most widely used chromatographic medium is based on silica. This medium is used for protein or peptide separations due to its mechanically strenght and high separation efficiencies. Silica -based materials could differ in the pore size of the matrices. The particle size is in range 5-10µm, containing C 4, C8 or C18 alkyl chains. For more hydrophobic samples C4 and C8 alkyl chains are usually used, for more hyfrophilic samples C18 alkyl chain is used.

Mobile phase is composed of an organic solvent, aqueous component and ion -pairing reagent. Organic solvent is used to decrease the polarity of aqueous mobile phase, causing the elution of proteins from the column. The ion -pairing agent is used to increase the selectivity.

3.1.4 Amino -terminal sequencing of polypeptides using Edman degradation

The stepwise degradation of peptides was first applied by Abderhalden and Brockmann in 1930 [Abderhalden & Brockmann, 1930]. Phenylisocyanate was used as amino group coupling reagent for the production of an intermediate that rearranged under acidic conditions, cleaving the derivatized terminal amino acid from parent peptide. Pehr Victor Edman [Edman, 1949] changed the coupling agent to phenylisothiocyanate that yields a more readily cyclized intermediate. This, together with the cleavage under anhydrous conditions, contributed to an efficient removal of the derivatized amino acid without the destruction of the remaining peptide. One amino acid is identified by one cycle of Edman chemistry and one cycle of RP-HPLC to analyze the PTH amino acid. **All** steps in degradation processes could be done automatically using an automated sequencer. Edman

and Begg [Edman & Begg, 1967] constructed a prototype of protein sequenator in 1967. Automation of the degradation steps and separations of PTH amino acids in high performance instrument, allows to identify up to 40 - 50 N -termini amino acid residues.

The Edman degradation procedure is divided into three steps: coupling, cleavage and conversion. In coupling reaction, the phenylisothiocyanate chemically modifies the free amino -terminal a -amino group of a polypeptide to form a phenylthiocarbamyl. In the cleavage reaction, the phenylthiocarbamyl amino residue is cleaved from polypeptide chain with anhydrous acid. An unstable anilinothiazolinone (ATZ) amino acid is formed. The polypeptide chain has „new" a -amino group and another cycle of coupling and cleavage can be repeated. In the third step, conversion, the unstable ATZ amino acid is converted to the more stable phenylthiohydantoin (PTH) amino acid derivate under acidic condition. The degradation cycle is schown in figure 4. Using HPLC, each PTH-amino acid is identified by the evution time of the peak it produces on chromatogram.

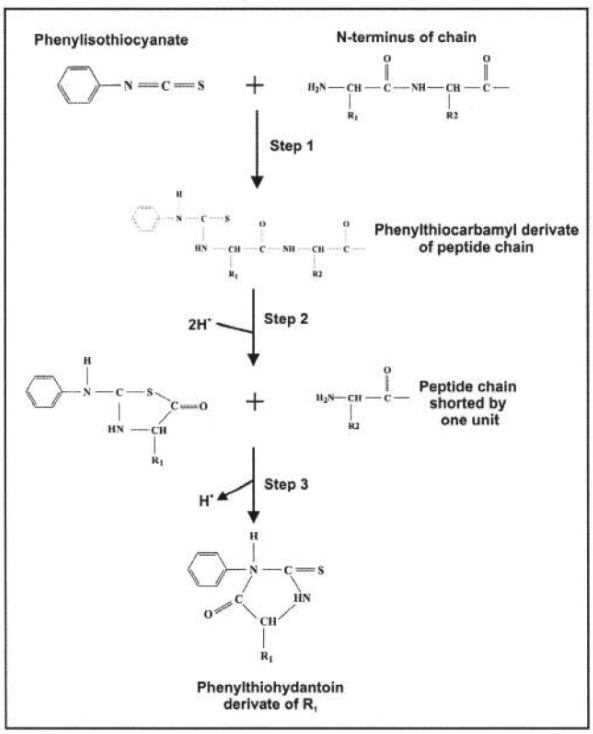

Fig. 4: *The three steps of N -terminal Ednam degradation (step I -coupling, step 2 -cleavage, step 3 -conversion)*

3.1.5 Mass spectrometry

Mass spectrometry is a powerful tool for analysis of proteins or peptides prepared by proteomical approches including two dimensional chromatography, RP-HPLC or biochemical processes. The major advantages of this technique is its sensitivity, speed and accuracy.

Mass spectrometry provides the scientist with two fundamental pieces of information. The first is the mass of a single molecule of interest. The mass of a molecule is obviously represented by the sum of the masses of the atoms being present in the molecule. However, the mass tells us nothing about the internal architecture of the atoms in the molecule. The second type of information is obtained from fragmentation of the molecular ion. Since the fragmentation process reflects chemical structure of the molecule, by looking at the spectrum it is possible to simply deduce the information what the original molecule looks like.

The birth of mass spectrometry falls to the early beginning of the last century. The ability to separate molecules based on different mass and charge was first described in 1912 by J.J. Thompson. In fact, this area of science has started in 1897, when this great physicist had demonstrated the existence of the electron as an electrically charged particle and measured its mass -to -charge *(m/z)* ratio. This is exactly the same measurement made by mass spectrometrists today, but now we measure the *m/z* ratio not of electrons, but of individual charged atoms and molecules. The initial breakthrough application of the new method was the discovery and detailed investigation of the isotopes of the elements. First contribution of mass spectrometry to modern chemistry appeared in the area of petroleum industry, slowly followed by many other applications including environmental analysis, forensic science, and diagnosis of metabolic disorders. All the early applications of mass spectrometry required evaporation of analyzed compound into gas phase before converting its neutral molecules into ions. Thus, only sufficiently stable compounds, able to survive the heating necessary for their evaporation, could be analyzed. This condition excluded the use of mass spectrometry in biological sciences because most of larger molecules of biological importance decompose before evaporation. Although some technical improvements and developments were designed to circumvent this limitation, mass spectrometry was of little importance for protein chemists until 1981. In that year Barber introduced fast atom bombardment (FAB), a soft ionization method gently generating ions from the analyte, and thus pioneered peptide and protein

analysis by mass spectrometry [Barber *et al.*, 1981]. However, the major milestone in the mass spectrometric analysis of biomolecules was the nearly simultaneous invention of two ionization techniques, the electrospray ionization (ESI) developed by John Fenn [Fenn *et al.*, 1989] and matrix-assisted laser desorption/ionization (MALDI) introduced by Tanaka, Hillenkamp and Karas [Tanaka *et al.*, Hillenkamp *et al.*, 1986]. These methods solved the difficult problem of generating ions from large, nonvolatile compounds such as peptides and proteins without significant analyte fragmentation.

Efficient conversion of neutral peptide and protein molecules from liquid or solid state into the gas phase macromolecular ions without their degradation is one of the greatest breakthroughs in the analytical chemistry and physics of the last century. After ionization the ions are separated and detected by a variety of ways using ion motion in electromagnetic fields and specific properties of charged particles. To distinguish between different ions, they are separated in the ion transfer system (analyzer) or directly at the detector according their m/z values. Signal intensity of an ion at given *m/z* represents their abundance in the sample. Mass spectrum, the graphical outcome of mass spectrometric analysis, shows then intensities of the ions plotted against corresponding *m/z* values.

Mass spectrometer consists of four major parts: ion source, analyzer, detector and data processing system.

Ion source device makes ions from an electroneutral analyte and transfers the species to a gas phase. There are several types of ion source divices. For the analysis of biopolymers, such as proteins, oligosaccharides and nucleic acids, the only suitable techniques are so called soft ionization techniques — ESI (electrospray ionization) and MALDI (matrix-assisted laser desorption ionization). ESI, for example, allows to identify non-covalent complexes without their disruption.

MALDI creates ions by excitation of a sample that is mixed with matrix component with a laser, figure 5B. The laser beam strikes the mixture of protein or peptide with the crystalline matrix, which has the absorption wavelenght close to the laser wavelenght, and so excites it into the gas phase. The single charged ions entry then into the analyzer. MALDI is usually used for determination of complex mixtures of analytes and is most often used with TOF (time-of-flight) or ICR (ion-cyclotron resonance) analyzers. The process of MALDI ionization is schematically shown in figure 5A.

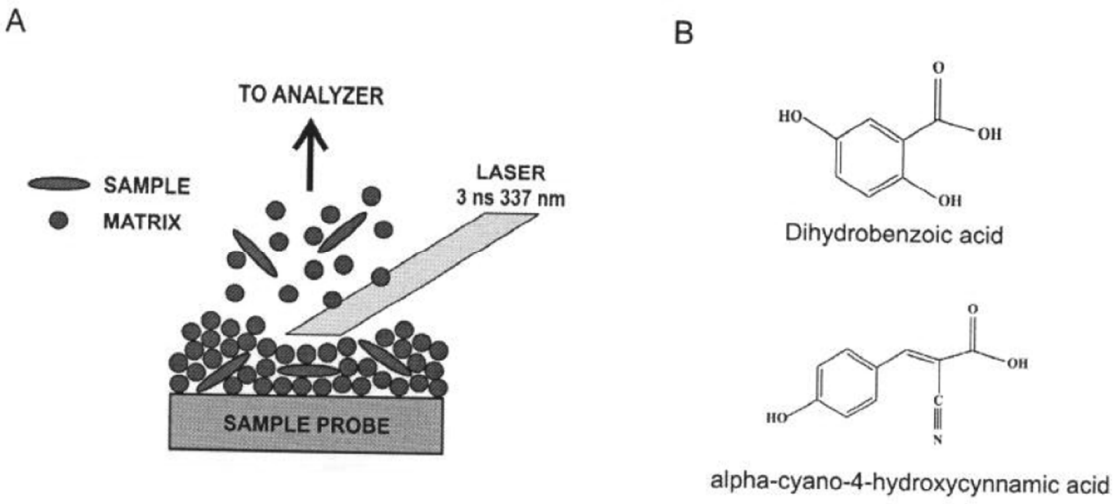

Fig. 5: A) MALDI *ionization process.* ***B)*** *Example of two matrixes most often used in* ***MALDI*** *ionization*

Electrospray ionization (ESI) creates ions by applying a potential to a flowing liquid that contains the analyte and solvent molecules. A spray of microdroplets is created by application of high electric tension through the needle. The solvent, which carry the analyte, is removed by heated capillary before entering the mass spectrometer. ESI produces multiply charged ions that require mass spectral deconvolution. Micro/nano-HPLC system is usually connected with ESI ionization. The created ions enter to ion -trap, quadrupole or ICR **onalyzators. Very complex mixtures** of analytes could be analyzed by ESI-MS, but the interpretation of spectra is difficult due to the increase of molecular weights of molecules and their number.

Time of flight (TOF) analyzer is suitable for analysis of intact masses of peptides and proteins. For the characterization of the structure of biomolecules or identification of proteins via microsequencing strategy, the tandem mass spectrometer represents the desired instrumentation. The tandem MS then can be done either in space or in time. For MS/MS in time the best example is the ion trap. Here the ions are isolated, fragmented and selected fragment ion can be isolated and fragmented again and this process can be repeated many times (up to MS 1°). For fragmentation in space, two analyzers are usually combined in one instrument. The first serves as the precursor ion selector and the second performs the MS/MS

experiment. An alternative for those who do not have tandem mass spectrometer is the use of controlled in -source fragmentation.

3.1.6 Protein identification and characterization

Nowadays, the two major peptide and protein characterization strategies based on mass spectrometry are employed: top -down and bottom -up. In the top -down approach we analyze the protein of interest without submitting it to chemical or enzymatic cleavage. Tandem mass spectrometry on the intact protein offers wholesome view on the protein molecule including identification of post -translational modifications. The development of the top -down methodology has been possible mostly due to high resolution Fourier transform ion cyclotron resonance mass spectrometry and its advantageous combination with electrospray ionization. The bottom -up approach starts with chemical or proteolytic cleavage of a protein being to be characterized. The obtained mixture of small peptide fragments can be analyzed by any type of mass analyzer typically in combination with ESI or MALDI technique.

In the bottom -up methodology, proteins are traditionally identified by peptide mass fingerprinting (PMF) or peptide microsequencing. PMF approach uses simple measurement of peptide masses [Pappin *etal.,* 1993], while peptide microsequencing requires tandem mass spectrometry [Hunt *et al.,* 1986]. PMF is based on the fact that the determination of precise masses of peptides derived from a protein by sequence -specific proteolysis (typically using trypsin) may serve as an effective means of protein identification. Proteins of different amino acid sequences produce after proteolysis sets of peptides, the masses of which constitute mass fingerprints unique for a specific protein. Therefore, if a sequence database is searched using selected peptide masses; the protein of interest is easily identified. However, if the sequence of protein to be identified or a sequence of a homologous protein is missing in the database, the PMF method fails. In that case, one has to turn to the alternative strategy of protein identification using peptide microsequencing. Here, protein is identified on the basis of a partial amino acid sequence which is deduced from a MS/MS spectrum obtained by tandem mass spectrometry. Fragment ion spectra are generated by the process of collisionally-induced dissociation (CID), in which the peptide ion to be analyzed is isolated and fragmented in a collision cell. MS/MS experiment can be performed either in time on single mass spectrometer represented by an ion trap, or in space on an instrument equipped with two

analyzers. The first one preselects the precursor ion for the subsequent MS/MS analysis and the second one detects its fragment ions.

Protein identification would be impossible without software tools which correlate mass spectrometric data with sequence databases. There is a variety of searching programs for peptide mass fingerprinting and all of them are accessible *via* Internet. The first one called MOWSE was developed in 1993 and has been already replaced by more sophisticated algorithms like ProFound [Zhang & Chait, 2000] and MASCOT [Perkins *et al.,* 1999]. The major relevant programs for interpretation of MS/MS data of peptides are Sequest [Eng *et al.,* 1994] and MASCOT. Both algorithms first create a list of sequences having the same mass as the fragmented peptide. The fragment ions expected for each candidate sequence are then compared to the observed MS/MS spectrum, in order to determine which candidate sequence fits to the experimental data. This approach could be easily automated and adapted to rapid identification of proteins as well as their post -translational modifications. Taking advantage of mass spectrometry in combination with proper software tools obviously represents a highly efficient means for identification and characterization of peptides and proteins.

Mass spectrometry is considered as an indispensable tool for the structure analysis of a broad spectrum of biomolecules such as peptides, proteins, nucleic acids, and oligosaccharides. In the field of peptide and protein characterization, mass spectrometry is employed for accurate determination of molecular weight, protein identification based either on peptide mass fingerprinting or on amino acid sequencing, for identification and localization of post -translational modifications [Aebersold & Goodlett, 2003] as well as for absolute and relative quantitation of protein expression [Gygi *et al.,* 1999]. The method is also suited for analysis of protein mixtures and identification of components of protein complexes. Because of the relative softness of electrospray ionization, mass spectrometry also enables detection of intact non -covalent complexes which are transferred into gas -phase without any degradation. To exploit effectively the high potential of mass spectrometry, it is often useful to combine it with wet chemistry reactions and classical biochemical methods or, if analyzing complex peptide or protein mixtures, with separation techniques like liquid chromatography or electrophoresis. For example, tryptic digestion performed in a mixture of normal and isotopic ($H_2{}^{18}O$) water [Schnolzer *et al.,* 1996] or sulfonation of the N -terminal group of a peptide [Keough *et al.,* 2002] facilitate the sequence readout in MS/MS spectra of peptides. Another trick utilizes esterification which modifies each carboxy group within the

peptide sequence [Hunt *et al.,* 1986]. Thus, the simple comparison of peptide mass before and after derivatization reveals number of amino acid residues having COOH functions present in the original peptide. The combination of mass spectrometry with multidimensional techniques enhances the dynamic range of the method, pushes the detection limit down and allows detection of minor components present in the analyzed mixture. All these efforts and tricks have made mass spectrometry an even more powerful technique allowing smart solving of biological problems.

3.1.7 The use of chemical cross -linking

Protein cross -linking is in today's research widely used for different biological and biochemical studies such as three dimensional structures of proteins, studies of enzyme-substrate complexes, and molecular associations in cell membranes. Cross -linkers are also useful for solid -phase immobilization of proteins, hapten-carrier conjugation, antibody-enzyme conjugation, and immunotoxin and other labeled protein reagent preparation.

Cross -linkers are divided into two main groups: heterofunctional and homofunctional. Heterofunctional agents containing different functional groups connected by a flexible spacer arm and are able to attack reactive groups on proteins (i.e., carboxy group, primary amino group on lysine or N -terminus). On the other hand, homoftmctional cross -linkers containing the same functional groups and also attack the same reactive groups on proteins or peptides. A few cross -linking reagents (i.e., trifunctional cross -linkers) have three reactive groups to make trimeric complexes.

Most protein cross -linking reactions are side chain reactions and are nucleophilic, resulting in a portion of the end of the cross -linker being displaced in the reaction (the leaving group). Many factors must be considered to obtain optimal cross -linking for a particular application. Factors that affect protein folding (e.g., pH, salt, additives and temperature) may alter conjugation results. Other factors such as protein concentration, cross -linker concentration, number of reactive functional groups on the surface of a protein, cross -linker spacer arm length, and conjugation buffer composition must also be considered [Hermanson, 1996]. The most common cross -linkers are: primary amine reactive agents (N-Hydroxysuccinimide esters) that react with primary amines (lysine and amino termini),

sulfhydryl reactive (maleimides, haloacetyls and pyridyl disulfides are common thiol-reactive groups), carboxyl reactive (EDC, carboxylate groups can be coupled to primary amines at pH 4.0-7.0). EDC is a zero -length cross -linker that reacts with carboxyls and activates them to couple to primary amines, forming amide bonds. Photoreactive cross -linkers are also used for determination of protein -protein interaction *in vivo* by exposure of a photoreactive group to short wavelength UV light.

3.1.8 Evaluation of the cysteine status

Disulfide linkages are one of most important structural elements in proteins and peptides. They have an irreplaceable role in establishing and maintaining the structural fold of a protein.

The traditional approach to determine the cysteine status is based on comparative **RP-HPLC** of peptides that were digested with specific proteases to obtain peptides containing disulfide bond. The retention times of nondisulfide-containing peptides will be the same in both nonreducing and reducing peptide maps, whereas under reducing conditions, disulfide bridges will be broken with the resultant loss of bridged peptides in the reduced peptide map and concomitant appearance of their reduced peptide components. Disulfide containing peptides could be identified by tandem mass spectrometry or Edman degradation. For succesfull determination of the cysteine status, it is important to match large set of peptides by multistep enzymatic or chemical digestions.

The development of new instrumentation techniques (Fourier transform (FT) MS) allows to identify cystine peptides more easily. FTMS instrument is schematically shown in figure 6. Electron capture dissociation (ECD) [Zubarev *et al.,* 1998] is based on the dissociative recombination of multiply protonated polypeptide molecules with low -energy electrons. Polypeptide polycations initialy capture an electron in a high orbit and following neutralization leads to excitation of radical species that rapidly undergoes bond cleavage. Disulfide bonds and some other bonds dissociate with higher rates. [Zubarev *et al.,* 1999] ECD has only been used in Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS).

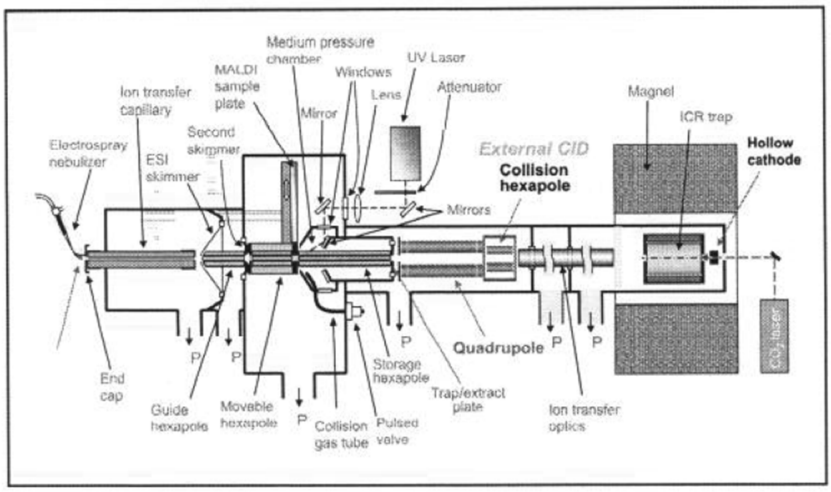

Fig. 6: *The schema of Fourier transform ion cyclotron resonance mass spectrometer*

3.1.9 Isolation of plasma membrane microdomains

The lymphocyte receptors have an irreplaceable role in the whole immunity system. They recognise complicated structures on other cells and are able to initiate reaction leading to cell answer. A lot of studies was done to characterize the structure or function of receptors from different cell types. In our laboratory, we have focused on the identification of protein associated with membrane microdomains, which are also involved in signal transduction, and could be present in recognition processes such as formation of the immunological synapse. There are several method that could be used for studying the proteins associated with lipid rafts, i.e. immunostaining methods, light and confocal microscopy etc. We decided to use mass spectrometry, which allows us to identified large number of proteins in one experiment. Unstimulated Jurkat T cell line served as a model cell line, due to its well elaborated and nonexpensive cultivation.

The general approach for GEMs isolation was based on the fact that GEMs are resistant to lysis with non-ionic detergent while the surrounding plasma membrane is dissolved at the same time. The detergent treated whole cell lysates were separated by density gradient ultracentrifugation in which the soluble molecules were pelleted and the GEMs were targeted to the low density fractions. The flotation of GEMs is caused by the high content of cholesterol and other lipids. Separated GEMs were then characterized using immunochemical techniques such as blotting and/or immunoprecipitation but several reports were also

published where the GEMs protein composition was identified by mass spectrometry [Foster 2003, von Haller *et al.,* 2003]. In order to increase the effectiveness of GEM separation we enriched the plasma membranes prior to the ultracentrifugation. We used gentle homogenization procedure in which the plasma membrane is recovered in the form of sheets. Simple stepwise centrifugation procedure in which we removed nuclei, microsomes and the cytosolic content allowed to increase the sample load in the subsequent ultracentrifugation step. In the majority of published studies the detergent used for GEMs isolation was Triton X-100. However, the composition of GEMs may vary considerably depending on the extraction conditions and, therefore, we employed very mild detergent Brij 58 known to preserve weakly associated proteins, more stringent detergent NP -40 (an equivalent of Triton X-100 widely used for GEMs isolation), and non -detergent conditions introduced to eliminate non-specific associations [Prinetti *et al.,* 1999]. Because of the increasing evidence for heterogeneity of microdomains and occurrence of heavy GEMs associated with cytoskeleton we decided to follow protein profiles individually in all fractions [Pike, 2003]. The mapping of GEMs by classical immunochemical approaches is difficult or nearly impossible as it is dependent on the existence of high avidity specific antibodies. Therefore we developed unbiased strategy for determination of the GEMs proteome. Scheme of methods used for identification of proteins associated with lipid rafts is shown in figure 7.

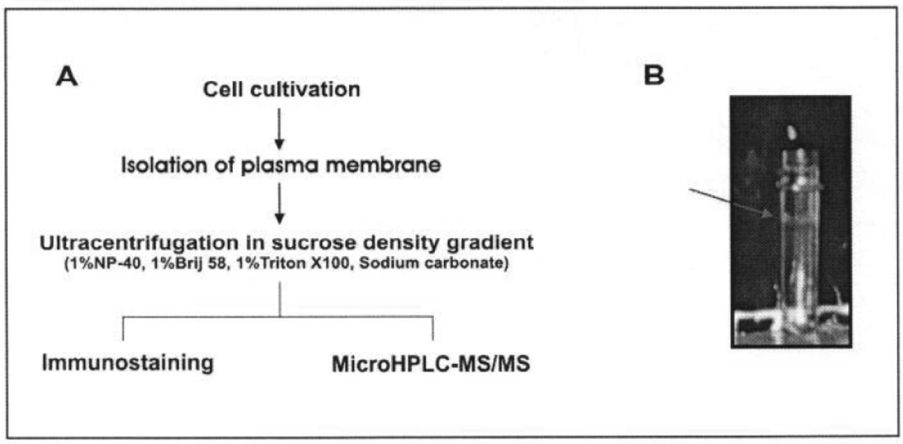

Fig. 7: A) Schema used for isolation of proteins associated with plasma membrane microdomains with different extraction conditions. Detection of proteins was done by both biochemical technique (western blot and immunostaining with monoclonal antibodies) and analytical technique (microRP-HPLC "on line" connected with mass spectrometer, switching between MS and MS/MS mode).
***B)** Photography of a tube after ultracentrifugation in sucrose density gradient. During ulracentrifugation, membrane microdomains floting w the fractions with lower concentration of sucrose, due to the high content of lipids. The arrow shows an "opalescent" ring, a marker of the succesful isolation, containing membrane microdomains.*

3.2 Evaluation of methods

3.2.1 Liguid chromatography

The chromatographical methods are very common in biochemical research. The separations are based on the size (gel filtration), charge (ionex chromatography), hydrophilic character (hydrophilic chromatography) and isoelectric point (chromatofocusation). In my work, I usually used the separation under the high pressure to gain better resolution of protein or peptide mixtures. The shorter separation time of this technique allows analysing of unstable molecules. On the other hand, separation under the high pressure can leads to denaturation of proteins and to the loss of the activity in some enzymes (P-N-acetylhexosaminidase).

The development of new separation materials gives a lot of possibilities to choose the most appropriate separation medium according to the chemical character of analytes. For separation of proteins or peptides, we usually used separation materials based on the organic gels, such as Q-Sepharose or S-Sepharose, silica gels (reversed phase separation) or polymer material. The separations occurred under the room temperature, pressure between 2 and 11 MPa and the analytes were monitored by UV detector in a wavelenght range of 214 — 280 nm.

Polystyrene/divinylbenzene macroporous spherical particals (PLRP-S column) were used as a column medium for separation of proteins and peptides. The advantage of this material is in its high chemical and physical stability across the complete pH range and its long lifetime. Moreover, only little nonspecific interactions of analytes with this material were observed.

3.2.2 Mass spectrometry and Edman degradation

Mass spectrometry together with automated Edman degradation are in today's biochemical research the two most often tools used for detection and characterization of proteins and peptides. Both techniques usually required preseparation of proteins or peptides

by electrophoresis or liquid chromatography. Electrotransfer of separated proteins from gel to PVDF membrane is used in the case of Edman degradation. We used the method of peptide mass finger printing for the identification of proteins from one or two dimensional gels. Both ionization techniques, MALDI and ESI, were used for more detail characterization. The combination of microRP-HPLC and tandem mass spectrometry were the most efficient tools for the identification of complex protein mixtures. On the other hand, MALDI ionization was most often used for the identification of proteins separated on 2D gels, due to its higher sensitivity, little or no fragmentation, fast sample preparation, higher tolerant to detergents and salts. The development of an ion cyclotron resonance mass spectrometer together with sophisticated programs for spectra evaluation, allows the identification of molecules with higher sensitivity and accuracy. We have used FTICR instrument for the evaluation of the cysteine status of several proteins and for identification of peptides, which were modified by different cross -linking agents. These studies can be used for the better protein homology modelling of three dimensional structures.

Despite no progress in development of Edman sequencer, this technique is very useful for the identification of N -termini sequence of proteins or peptides, which can not be identified by other techniques such as mass spectrometry. The modern software allows not only qualitative but also quantitative evaluation of each PTH amino acid. One of the limitations of the Edman sequencer is the sample wash out from the reaction vessel. This problem is solved by introduction of the polymeric quartenary ammonium salt Polybrene [Klapper *et al.,* 1978], which anchored proteins or peptides and so allowing sequencing of hydrophobic molecules.

3.2.3 Isolation of plasma membrane microdomains

Characterization of proteins which are involved in signal transduction or other plasma membrane processes, is in today's immunology one of the most studied direction. A lot of studies were done by classical immunological approaches such immunostaining. In this work, we have focused on non -classical approach using mass spectrometry. For the succesful analysis of proteins associated with lipid rafts by mass spectrometry, it was necessary to gain a guit large amount of material. Due to high prices of cultivation medium and problems with

the growth of more interesting NK cells, we have decided to start the analysis with Jurkat T cell line. For the isolation of lipid rafts, we have used special technigues for plasma membrane homogenization. Due to local increase of temperature by homogenization with ultrasound, the cells were passed through the syringe. All the separation steps were done in cold and with high amount of the inhibitors of proteases. Due to differences in the lipid composition of membrane microdomains, we have used four different extraction conditions. One of the biggest advantages of mass spectrometry is the possibility to identify proteins, which can not be expected to be involved in lipid rafts. The limitation of the immunostaining technique is the amount of known monoclonal antibodies against proteins associated with lipid rafts. On the other hand, immunostaning is more sensitive than mass spectrometry and is sometimes used as a prescreaning method for the detection of the markers of lipid rafts. Using mass spectrometry we were able to identify not only typical markers of lipid rafts such as Lck, flottilin or Ras proteins but also cytoskeletal proteins, adhesion molecules or proteins, which were not yet identified in lipid rafts.

3.2.4 Characterization of protein structure by mass spectrometry

Several common techniques are used for the structure characterization of proteins such as NMR spectroscopy, Raman vibrational spectroscopy or X — ray crystallography. Sometimes it could be doubtful whether the structure based on X-ray data corresponds to the real native state of the protein and so we employed the combination of chemical cross -linking and Fourier transform -ion cyclotron resonance MS to get as close as possible to physiological conditions. We have used different cross -linking agents such as DSS (disuccinimidyl suberate), DSG (disuccinimidyl glutarate) and EDC (1-Ethy1-3-(3-dimethylaminopropyl) carbodiimide). Chemical crosslinking can yield low -resolution structure information on the distance constraints within a molecule. Mass spectrometry is used due to its high sensitivity allowing rapid analysis of complex mixtures obtained from enzymatic digest of cross -linking reaction mixtures. We have used cross -linking methods for characterization of the dimeric form of the enzyme (3-N-acetylhexosaminidase and for the structure characterization of rat CD69 molecule. The aim of the study is to disclose the influence of calcium ion on the fine structure of CD69 and its carbohydrate binding site. We have obtained the recombinant

soluble form of rat CD69 after recombinant expression in *E. coli* and *in vitro* refolding. Since the protein modeling and docking studies revealed changes in the carbohydrate binding site upon incorporation of calcium [Pavlidek *et al.*, 2003] we prepared the protein with and without calcium. We have succesfully optimalized the reaction conditions to form mainly internally cross -linked protein avoiding dimer formation. Using FT -MS instrumentation we were able to identify the modified peptides. The detailed characterization of differences between protein with and without calcium is under progress.

We have used two approaches to determine the cysteine status of 13-N-acetylhexosaminidase. First, the methods based on comparative HPLC separation of enzymatic digests of treated and nontreated proteins with reducing agents such as dithiotreitol or 0-mercaptoethanol. Collected fractions were analysed by MALDI-TOF mass spectrometer. The disadvantage of this method is the lower sequence coverage of some proteins. The second approach was based on the analysis of enzymatic digested peptides from a native protein by Fourier transform -ion cyclotron resonance mass spectrometer, which is "online" connected with microHLPC system. Due to the high sensitivity and resolution of the FT -MS instrument, we are able to identify more peptides from the protein and so increase the sequence coverage. The advantages of this method are the speed and simplicity of sample preparation and automated data interpretation by programs. On the other hand the price of FTICR mass spectrometer is still very high.

IV. RESULTS

See the attach file

V. DISCUSSION OF THE RESULTS

Until the most recent period individual components of membrane microdomains have been identified using immunochemical and biochemical techniques. However, the rapid progress in the development of various proteomic techniques allowed its aplication for the analysis of the protein profiles of membrane microdomains. Since even the sensitive immunochemical and mass spectrometry techniques require at least 10 8 homogenous cells, cell lines are often used as a conveniount source of the starting material. For the analysis of membrane microdomains of T cells, the T cell line Jurkat is often used because it is easily cultured under standard conditions. There are, however, several experimentant strategies for the proteomic analysis of lipid rafts of these cells. The initial proteomic work in this field has been published by von Haller *et al.,* 2003. These authors used unfractionated whole Jurkat cells from which the rafts were extracted using Triton X100. The subsequent proteomic analysis of the whole cell material was technically sound but suffered from the presence of a number of proteins that had no relation to plasma membrane surface proteins. These contaminations were caused mainly by proteins extracted from membrane microdomains occurring in intracellular organels such as mitochondria and Golgi aparatus. Moreover, the proteins identified by these authors were provided in the form of list of protein hits without any suggestions about their functional importance.

In order to overcome the problems of the above study, we have introduced several methodical improvements. First, highly purified fraction of plasma membranes was isolated from the starting material in order to concentrate the relevant proteins, and separate them from the contaminants. Moreover, we used a combination of several extraction procedures involving the use of mild detergent Brij 58 as well as the standard (more stringent) detergents Triton X-100 and NP -40. Also, in order to remove the nonspecifically associated proteins we employed alkaline extraction in sodium carbonate [Prinetti *et al.,* 1999]. Lipid rafts extracted under all of these conditions were subsequently separated by ultracentrifugation in sucrose density gradients. Two different approaches were used for the analysis of sucrose fractions. First, we took a limited subset of proteins with a known lipid raft distribution status for which monoclonal antibodies were available. Namely, we used protein tyrosine kinase *Lck,* flotillin and CD59 as examples of protein associated with lipid rafts. Immunochemical detETCection

of *Lck* allowed us to evaluate the distribution of raft proteins under various experimental conditions. Using Brij 58 and sodium carbonate, lipid rafts were shown to be distributed mostly in fractions three to five. On the other hand, in the presence of the stringent detergents *Lck* was mostly distributed in fractions five to seven. The use of several extraction conditions is important expecially for tracing those proteins that display equilibrium between the raft and nonraft fraction.

Having evaluated the occurrence of typical raft proteins in individual fractions we were able to use .our second experimental approach, namely micro HPLC-MS/MS. However, for the success of this experiment it was necessary to develope efficient methods to recover proteins from solutions containing sucrose, salts and detergents, and to prepare tryptic peptides suitable for MS analysis. We used a multistep protein precipitation protocal based on combination of trichloracetic acid and acetone precipitation and extraction with organic solvents. This protocol turned to provide high protein recovery but the resulting protein precipitate was obtained in the form difficult for tryptic digestion. We were able to overcome this problem using trypsin digestion in the presence of chaotropic reagents such as urea and ethylmorpholine acetate. Last but not least, the success in protein identification is dependent on the details of mass spectrometry setup. In our hands we obtained optimal results with the use of fused silica capillary columns filled with a microporous Magic C18 matrix. This column was connected to a microflow HPLC system and the outlet of the column was directly connected with an ion trap mass spetrometer. The LC ^{QDECA} (ThermoElectron, CA) was set to acquire a full MS scan between 350 and 1800 m/z followed by full MS/MS scans of the top four ions from the preceding full MS scan. Activation time for collision -induced dissociation was 30 ms and the relative collision energy was set to 42%. Dynamic exclusion was enabled with two repeat counts, repeat duration of 30 s and 3 mm exclusion duration window. Spectra were searched with the SEQUEST Im software against the database created by extracting rat, mouse, swine and human entries from the non -redundant database downloaded from the NCBI ftp site [Eng *et al.,* 1994]. For spectra from a multiply charged peptide, an independent search was performed on both the 2+ and 3+ mass of the parent ion. The search parameters were as follows: no enzyme specificity; mass errors 2 Da for precursor and 0.8 Da for fragment ions; possible modifications: +16 Da for Met and Trp and +57 Da for Cys. SEQUEST results were automatically processed with the DTASelect and Contrast software [Tabb *et al.,* 2002] using the following criteria: XCorr values were 1.6 for singly

charged, 1.8 and 2.7 for doubly and triply charged peptides, respectively; lowest ACn was set to 0.05; maximum Sp ranking was 450, minimum sequence length was 5 amino acids, and maximum sequence length was 30 amino acids. This filtering is not stringent enough to automatically remove all false -positive hits but it retains true hits albeit with lower scores. Such cases include cleavages at proline and formation of doubly and triply charged ions with neutral loss.

All hits were finally validated manually. The criteria taken into account were: continuity of b- or y -ion series, good signal-to-noise ratio, y -ions corresponding to proline should be intense, unassigned intense fragments should correspond either to loss of one of two amino acids from one of the end of the peptides or to doubly and triply charged ions with the neutral loss. Another features taken into consideration were specific cleavage at aspartic acid as well as losses of water (form S, T, D, E), ammonia (from N, Q, K, R), and of CH3SOH from the oxidized methionine.

For identifications based on the single peptide (this includes also cases where 2+ and 3+ of the same mass, oxidized Met or in -source fragments were found) only the fully tryptic peptides with continuous series of b- or y -ions and with higher scores (over 2.6 and 3.5 for doubly and triply charged pepitdes, respectively) were retained. Single peptide identifications based on the MS/MS from a singly charged peptide were excluded.

Using this improved methodology, we could identify several molecules of interest for the biology of Jurkat cells. Proteins that we identified are involved in cellular signaling, cellular adhesion and association with the cytoskeleton. The signaling molecules identified by us include *Lck,* G proteins, LAR kinase and Ras. Cytoskeletal proteins such as actin, tubulin and myosine are well documented components forming a scaffold for protein kinases and participating in the formation of immunological synapse. Both integrale membrane proteins (CD9) and peripheral proteins (galectin 9) were identified among the cell adhesion molecules. CD45 was shown to have a dynamic association with lipid rafts that depends on the cellular activation status. S-100 calgranulin B, protein known to be an intracellular calcium binding protein and marker of T cell lymphoproliferative disorders, was shown by us for the first time to be the component of lipid rafts.

Our results have been presented in the form of a symposium lecture at the annual meeting of the Biochemical Society "BioScience 2004" in Glasgow in July 2004. Moreover, they have also been published in the journal *Biochemical Society Transactions.* The

methodology developed for the analysis of proteins associated with lipid rafts in Jurkat has been employed in several other studies including the analysis of lipid rafts in plasma membranes of rat NK cells [Man *et al.*, 2005]. Our next study of membrane microdomains by mass spectrometry will be done on NK cells isolated from human peripheral blood.

The understanding of structure and function of C type lectin receptors of NK cells is a major area of interest of our laboratory. In order to be able to study the structure and binding properties of these receptors, sufficient amount of soluble and stable proteins have to be produced. This task is particularly difficult in the case of lectin like NK cell recpetors. The preparation of homogenous populations of NK cells is extremely difficult and precludes the isolation of these molecules from natural sources. Moreover, NK cell receptors are transmembrane proteins which require optimising the expresion strategies to obtain soluble proteins. Futhermore, these proteins represent a problem for many recombinant expresion systems mainly because of the high cysteine contents and the need to close correctly several disulfide bridges, both intramolecular and intermolecular. As a result of many years of extensive research in our laboratory and a number of trials and errors we are gradually finding the solutions for the above problems. We use bacterial expresion system in which the segment coding for the entire extracellular portion of NK cell receptors is expresed by placing the corresponding gene fragment just after the initiation methionin of the pET bacterial expresion vectors. The proteins are produced into inclusion bodies from which they need to be solubilised in denaturating solutions and subsequently refolded *in vitro.*

Our interest in CD69 is motivated by an important role that this antigen plays in the activation of NK cells. Since many of the *in vivo* studies with experimental tumor treatment were performed on rats we were interested in the investigation of the structure and binding properties of rat CD69. cDNA for this protein was obtained by RT-PCR using mRNA isolated from rat NK leukemic cell line RNK16. Using a bacterial expresion construct in which the membrane -proximal dimerisation cysteine residue has been omited, we were able to produce a stable pure monomeric rat CD69 suitable for ligand identification studies. Before performing the ligand identification experiments, we have to verify the structure and stability of rat CD69. In addition to classical biochemical techniques (SDS-PAGE under reducing and nonreducing conditions, Western blot using specific antibodies and MALDI

peptide mapping), several very modern techniques have been also employed. For instance FTICR-MS allowed us to verify the intactness of our protein preparation as well as the correct number of disulfide bonds revealed on mass spectra of the reduce protein. Moreover, the protein was also expressed on minimal M9 medium containing I5NRI C1 as a single nitrogen source. The homogenously isotopically labeled protein was then used for 1H - 15N correlation NMR experiments. NMR spectra obtained after 24 hours using 0,28 mM solution of rat CD69 displayed a nice dispersion of signals expected for a well refolded and homogenous protein. This conclusion was also supported by protein crystalography: nice crystals were grown in a hanging drop containing 0,35 mM solution of the protein 24 hours after the addition of the precipitant.

The stable and well refolded rat CD69 was then used for ligand binding studies. Because of the controversy connected with the binding of calcium and carbohydrates to CD69 [Bezougka *et al.*, 1995, Childs *et al.*, 1999], several experiments had to be performed. First we evaluated the binding of calcium. Equilibrium dialysis experiments revealed the existance of a single binding site for calcium per carbohydrate recognition domain having Kd of aproximately 70 µM. In accordance with our previous findings using human monomeric CD69, three binding sites for GlcNAc have been found per one ligand binding domain. The affinity for GlcNAc is quit high with Kd as low as 5,2 mM. Futhermore the binding affinities of some comlex oligosaccharides were even higher. Thus the tribranched tetrasaccharide N346N, when tested in plate inhibition experiments had IC D as low as 10'°M making it the ligand nearly as efficient as the natural triantenary nonasaccharide NTRIA (IC 50=10-11M).

Our results have been presented in the form of a symposium lecture at the annual meeting of the Biochemical Society "BioScience 2004" in Glasgow in July 2004. Moreover they have also been published in the journal *Biochemical Society Transactions*. The ongoing studies have been concentrated on the preparation of even more stable protein preparations in the form of noncovalent dimers. Until today, the succsess has been achieved with the human CD69 for which preparations stable both upon heating and long term storage have been obtained.

13-N-acety1hexosaminidase from a filamentous fungus *Aspergillus oryzae* is a secreted enzyme known to be involved in chitin degradation and remodeling of the cell wall of the producing organism. Moreover, this enzyme is also important for biotechnologies, in-particular for the synthesis of unique oligosaccharide sequences. Our laboratory became interested in the molecular characterisation of this enzyme and understanding the role of its glycosylation. Molecular cloning and sequencing of 13-N-acetylhexosaminidase gene revealed the occurence of signal peptide, propeptide, zincin like domain, catalytic domain of glycosyl hydrolase 20 family, and C terminal segment. Most of the secreted enzyme is composed of the dimers of the catalytic subunit associated with one or two molecules of the propeptide. Interestingly, the later molecular form has twice as high specific activity as the former form poiting to the essential role of the propeptide in enzyme activation. The propeptide is processed intracellularly through the dibasic peptidases, localized in the endoplasmatic reticulum, that cleave off the KKSKR sequence from the primary structure of the proenzyme. This processing is essential for acquisition of enzymatic activity.

There are six cysteine residues in the catalytic domain of P-N-acetylhexosaminidase. Because this enzyme is secreated into the extracellular (oxidative) environment, these cysteines would be expected to occur in oxidized form creating either intramolecular or intermolecular disulfide bonds. Molecular size of p-N-acetylhexosaminidase examined by SDS-PAGE under reducing and nonreducing conditions was identical precluding the occurence of intermolecular bridges. The structure of three intramolecular bridges that connect Cys290-Cys ^{351}, $^{cys448\_cys483,}$ and Cys ^{583}-Cys $^{59°}$ was determined by a combination of experimental techniques including differential HPLC separation of cystic peptides under both reducing and nonreducing conditions, and advanced MS technique.

Most P-N-acetylhexosaminidases are expressed as dimers. This feature also holds for our enzyme as revealed by gel filtration experiment. This conclusion was futher supported by enzyme cross -linking using a water soluble carbodiimide.

Both the propeptide and the catalytic domain were predicted to be glycosylated. This prediction was confirmed by experiments in which the propeptide was separated from the catalytic subunit under denaturating conditions (pH 2, acetonitrile), and their glycosylation status was studied. All of the predicted sides for N-glycosylation that occur in the catalytic domain were shown to be used for the attachment of N -linked oligosaccharides of high mannose type. Enzyme deglycosylated by EndoH treatment has catalytic properties identical

to the glycosylated enzyme. However the stability of deglycosylated enzyme in acidic environment was significantly lower. 0-glycosylation of the propeptide was analysed using the C terminal tryptic fragment by a combination of experimental techniques. Quantitative carbohydrate analysis of this propeptide together with MALDI MS, fragmentation MS, and automated Edman degradation all indicate the presence of short linear oligosaccharide sequences Gal -(Man)„ attached to serine and threonine residues in the C terminal part of the propeptide (S83, ^{s84}, T^{78}, T^{90}). The functional significance of 0-glycosylation is in mediating the proper interaction of the propeptide with its correspoding catalytic unit. Evidence for this role has been obtained from reconstitution experiments in which the enzymatic activity of the catalytic unit of 13-N-acetylhexosaminidase from *Aspergillus otyzae* was reconstituted either with the homologous propeptide, or by 0-glycosylated propeptide from a closly related fungus, or by nonglycosylated propeptide expressed in bacteria.

Our results related to P-N-acetylhexosaminidase were published in the journal *Biochemical Society Transactions* and are the subjects of two prepared publications. Currently we are aiming at confirmation of our findings using protein crystalography. Moreover, we are trying to set up and apropriate expresion system for molecular mutagenesis. Because of the critical role for glycosylation we seek the homogenous expresion system in which the expresion plasmits will be transfected into filamentous fungi in which the expresion of the wild type enzyme will be prevented through genetic deletion.

VI. REFERENCES

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VII. LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

7.1 List of publications

Pompach P, Man P. Novak P, Havlidek V. Figerova A and Bezougka K.
Mass spectrometry is a powerful tool for identification of proteins associated with lipid rafts of Jurkat T -cell line: *Biochem Soc Trans.* **2004,** 32, 777.

Pavlieek J, Kavan D, **Pompach P,** Novak P, Lukgan 0, and Bezougka K.
Lymphocyte activation receptors: new structural paradigms in group V of C -type animal lectins: *Biochem Soc Trans.* **2004,** 32, 1124.

Plihal 0, Sklendf J, Kmonidkova J, Man P, **Pompach P,** Havlidek V, Kfen V and Bezougka K.
N-glycosylated catalytic unit meets 0-glycosylated propeptide: complex protein architecture in a fungal hexosaminidase: *Biochem Soc Trans.* **2004,** 32, 764.

Kristova V, Martinkova L, Hugakova L, Kuzma M, Rauvolfova J, Kavan D, **Pompach P.** Bezougka K and Kien V. A chemoenzymatic route to mannosamine derivatives bearing different N-acyl groups: *J Biotechnol.* **2005,** 26; 157.

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The isoforms of rat natural killer cell receptor NKR-P1 display a distinct binding of complex saccharide ligands.: *Collect. Czech. Chem. Commun.* **2004,** 69, 631.

Kaplan 0., Vejvoda V., Plihal 0., **Pompach P.,** Kavan D., Fialova P., Bezougka K., Mackova M., Cantarella M., Jirkil V., Kfen V, and Martinkova L.
Purification and characterization of nitrilase from Aspergillus Niger K10, *in press*

Plihal 0., Sklener J., Hofbauerova K., Novak P., Man P., **Pompach P.,** Ryglava **H.,** Pigvejcova A., Kren V., and Bezougka K
Large propeptides of fungal fi-N-acetylhexosaminidases are novel intramolecular regulators involved in the control of activity, architecture and secretion of the parent enzyme, *Manuscript in preparation*

Rikliger E., Vladimir K. Jr., Hofbauerova K., Baumruk V., Novak P., **Pompach P.,** Man P., Plihal 0., Sklendf J., and Bezougka K.
Structure of the dimeric N-glycosylated form of fungal 13-N-acetylhexosaminidase revealed by homology modeling, vibrational spectroscopy, biochemical and cross -linking studies, *Manuscript in preparation*

7.2 Selected oral presentations

X. Cytosleletalni klub, Vranovska Ves, 2002
Pompach P., Novak P., Kavan D., Man P., Havlieek V. and Bezou§ka K.
Identification of proteins associated with lipid rafts ofJurkat T -cell line by mass spectrometry

Winter Symposium on Structure and Function of Proteins, Nove Hrady, 2003
Pompach P., *Man* **P.,** Ettrich R., Plihal **0.,** Sklenae J., Pigvejcova A., Ken V., HavRek V. and Bezougka K.
Mass spectrometry looks into the structure of fi-N-acetylhexosaminidase

BioScience 2004 from molecules to organism, Glasgow, Skotsko, 2004
Pompach P., Man P., Novak P., Figerova A., Havlidek V. and Bezougka K.
Identification of proteins associated with lipid rafts of Jurkat T -cell line by mass spectrometry

XIX. Biochemical Congress, Olomouc, 2004
Pompach P., Man **P., Sklenat J., Plihal 0.,** Havlieek V., Kten V., and Bezougka K.
The importance of having glycosylated cleavable propeptide: the story of fi-N-acetylhexosaminidase

7.3 Selected poster presentations

HUPO 1st World Congress, Versailles, Francie, 2002
Pompach P., Novak P., Man P., Havli6ek V., Bezougka K.
Identification of Signal Transduction Proteins in Lymphocyte Membrane

5th Igler MS-Tage, Innsbruck, Rakousko, 2003
Bezougka K., Man **P., Pompach P.,** Novak P., Havli6ek V., Hilgenfeld R. , Plihal **0.,** Sklende J., Pigvejcova A., Keen V.
Protein Chemistry and Mass Spectrometry in Structural Analysis of Large Proteins

2. setkani eskYch a slovenskych strukturnich biologii, Nove Hrady, 2003
Man P., Novak P., **Pompach P.,** Ulbrichova D., Havli6ek V., Bezougka K.
Full Characterization of Natural Killer Cell Membrane Microdomains

15th European Immunology Congress - EFIS 2003, Rhodos, Reck°, 2003
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Identification of proteins associated with lipid rafts of Jurkat T -cell line by mass spectrometry

16th International Mass Spectrometry Conference, Edinburgh, Skotsko, 2004
Pompach P., Man P., Plihal 0., Sklenif J., Ettrich R., Bezou§ka K., Havlidek V.
Mass spectrometry looks into the structure of 13-N-acetylhexosaminidase

6. Igler MS Tage, Igls, Rakousko, 2005
Pompach P., Novak P., Kavan D., Man P., HavIRek V. and Bezougka K.
Combination of chemical cross -linking and FT -MS for the structural characterization of rat CD69 molecule

VIth European Symposium of The Protein Society, Barcelona, pan'elsko, 2005
Pompach P., Man P., Novak P., Figerova A., Havli6ek V., Bezougka K.:
Identification of Proteins associated with lipid rafts of fur/cat T -cell Line by Mass Spectrometry

H'UPO 4th Annual World Congress, Mnichov, N6mecko, 2005
Pompach P., Novak P., Kavan **D., Man P., Havli6ek V. and Bezou§ka K.**
Combination of chemical cross -linking and FT -MS for the structural characterization of rat CD69 molecule