Protein chemistry and mass spectrometry in biochemical research

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THE IMMUNE SYSTEM

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 MHC 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.
The role of lipid rafts in T-cell signaling

During the signalization, most of the signaling proteins and receptors are localized in specific areas of plasma membrane. This area is 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-resistant 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<sub>Lck</sub>, LAT and protein kinase C are recruited to lipid rafts. The disturbance of lipid rafts by methyl-β-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].

The major biochemical characteristic of lipid rafts is their insolvency 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 ceramide, which plays essential role in aggregation of lipid rafts [Hořejší, 2005].

ENZYMES

Introduction to the structure of β-N-acetylgalactosaminidase

β-N-acetylgalactosaminidase is one of the most abundant enzymes found in many species from bacteria to human. The enzyme is involved in several 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 to the ability of effective transglycosylation of β-GlcNAc and β-GalNAc, the research interest was focused on the use of the enzyme for chemoenzymatic synthesis of biological important oligosaccharides [Kfen et al., 1994, Rajnochová et al., 1997, Weigneronová et al., 2003, Krist et al., 2001].
β-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 *Streptomycetes plicatus* [Mark et al., 2001, Williams et al., 2002]. β-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 β-N-acetylhexosaminidases form dimeric molecules in human composed of two subunits α and β. The dimeric form of the molecule is very important for the activity of the enzyme. Fungal and yeast β-N-acetylhexosaminidases tend to form homodimers. The posttranslational modifications such as N-glycosylation [Jones & Kosman, 1980] or O-glycosylation [Plíhal 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, O-glycosylation seem to be necessary for full enzymatic activity. The fungal β-N-acetylhexosaminidase from *Aspergillus oryzae* is composed of signal peptide, propeptide, zincin-like and catalyical domain (Figure 1). The N-glycosylated oligosaccharide structures containing up to eleven mannoses were found in the C-terminal part of the catalytic domain [Plíhal et al., 2004].

The propeptide of β-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 the *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 O-glycosylation of the C-terminal part of the propeptide. Other biochemical studies determined the structures of O-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. 1: Fungal β-N-acetylhexosaminidase is composed of four parts: signal peptide, propeptide, zincin-like domain and catalytic domain. Propeptide is on its C-termini O-glycosylated, catalytic domain is N-glycosylated.

METHODS

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.

Mass spectrometry

Mass spectrometry is a powerful tool for analysis of proteins or peptides prepared by proteomical approaches including two dimensional chromatography, RP-HPLC or biochemical processes. The major advantages of this technique are 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.

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 methods 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 identify a 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 specific avidity antibodies. Therefore we developed unbiased strategy for determination of the GEMs proteome.
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 application 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 convenient 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 organelles such as mitochondria and Golgi apparatus. 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 detection 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 especially 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 develop 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 protocol 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 chaotrophic 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 spectrometer.

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 integrare membrane proteins (CD9) and peripheral proteins (galactin 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 receptors. 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 expression strategies to obtain soluble proteins. Futhermore, these proteins represent a problem for many recombinant expression 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 expression system in which the segment coding for the entire extracellular portion of NK cell receptors is expressed by placing the corresponding gene fragment just after the initiation methionin of the pET bacterial expression vectors. The proteins are produced into inclusion bodies from which they need to be solubilised in denaturing 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 expression construct in which the membrane-proximal
dimerisation cysteine residue has been omitted, 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 $^{15}$NH$_4$Cl as a single nitrogen source. The homogenously isotopically labeled protein was then used for $^1$H-$^{15}$N 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 crystallography: 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 [Bezouška 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
existence of a single binding site for calcium per carbohydrate recognition domain having Kd of approximately 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 IC50 as low as 10^{-10} M making it the ligand nearly as efficient as the natural triantenary nonasaccharide NTRIA (IC50=10^{-11} M).

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 success has been achieved with the human CD69 for which preparations stable both upon heating and long term storage have been obtained.

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 β-N-acetylhexosaminidase gene revealed the occurrence 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 KKSKKR 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 β-N-acetylhexosaminidase. Because this enzyme is secreted 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 β-N-acetylhexosaminidase examined by SDS-PAGE under reducing and nonreducing conditions was identical precluding the occurrence of intermolecular bridges. The structure of three intramolecular bridges
that connect Cys\textsuperscript{290}-Cys\textsuperscript{351}, Cys\textsuperscript{448}-Cys\textsuperscript{483}, and Cys\textsuperscript{583}-Cys\textsuperscript{590} 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 \(\beta\)-N-acetylhexosaminidases are expressed as dimers. This feature also holds for our enzyme as revealed by gel filtration experiment. This conclusion was further 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. \(O\)-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)\textsubscript{n} attached to serine and threonine residues in the C terminal part of the propeptide (S\textsuperscript{83}, S\textsuperscript{84}, T\textsuperscript{78}, T\textsuperscript{80}). The functional significance of \(O\)-glycosylation is in mediating the proper interaction of the propeptide with its corresponding catalytic unit. Evidence for this role has been obtained from reconstitution experiments in which the enzymatic activity of the catalytic unit of \(\beta\)-N-acetylhexosaminidase from \textit{Aspergillus oryzae} was reconstituted either with the homologous propeptide, or by \(O\)-glycosylated propeptide from a closely related fungus, or by nonglycosylated propeptide expressed in bacteria.

Our results related to \(\beta\)-N-acetylhexosaminidase were published in the journal \textit{Biochemical Society Transactions} and are the subjects of two prepared publications. Currently we are aiming at confirmation of our findings using protein crystallography. Moreover, we are trying to set up and appropriate expression system for molecular mutagenesis. Because of the critical role for glycosylation we seek the homogenous expression system in which the expression plasmids will be transfected into filamentous fungi in which the expression of the wild type enzyme will be prevented through genetic deletion.
REFERENCES

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