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INVITED REVIEW

Tools for phospho- and glycoproteomics of plasma membranes

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Abstract Analysis of plasma membrane proteins and their posttranslational modifications is considered as important for identification of disease markers and targets for drug treatment. Due to their insolubility in water, studying of plasma membrane proteins using mass spectrometry has been difficult for a long time. Recent technological developments in sample preparation together with important improvements in mass spectrometric analysis have facilitated analysis of these proteins and their posttranslational modifications. Now, large scale proteomic analyses allow identification of thousands of membrane proteins from minute amounts of sample. Optimized protocols for affinity enrichment of phosphorylated and glycosylated peptides have set new dimensions in the depth of characterization of these posttranslational modifications of plasma membrane proteins. Here, I summarize recent advances in proteomic technology for the characterization of the cell surface proteins and their modifications. In the focus are approaches allowing large scale mapping rather than analytical methods suitable for studying individual proteins or non-complex mixtures.

Keywords Plasma membrane protein · Mass spectrometry · Glycosylation · Phosphorylation

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Introduction

Biological membranes are distinctively ordered arrays of lipids interacting with proteins, and carbohydrates covalently bound to the lipids and/or the proteins. Proteins interact with lipids in a variety of ways. Integral membrane proteins (IMP) are embedded, to various extends, in the lipid bilayer, whereas other proteins associate with membranes via interactions with polar moieties of the lipids and IMPs. An additional group comprises proteins that strongly associate with membranes via covalently bound lipid "anchors". The protein content in the membranes is variable. Dependent on the cell type and specific functions proteins constitute 25–75% of the membrane mass.

Cells use different types of membranes for organization of subcellular organelles and plasma membrane (PM), which constitutes the cell surface and separate cytoplasm from the extracellular environment. PM not only encloses the cell, but also maintains the essential differences between the cytoplasm and the cell's surroundings. It is also responsible for exclusion of toxic ions and molecules from the cell, the accumulation of cell nutrients, and energy transduction. Moreover, PM is involved in cell motility, cell division, signal transduction, and interactions with molecules and other cells. Although the basic organization of the PM is provided by the lipids, the specific cellular functions are carried out by the proteins.

Despite significant technological advances, identification and characterization of PM proteins remains a challenging task in biology. Since the majority of the proteins is low abundant, in classical biochemical approaches an over-expression in heterologous systems and purification of individual proteins is usually required. Furthermore, these methods are limited to analysis of single proteins or their

non-complex mixtures. In contrast, proteomic technology offers a direct way for characterization of proteins without any extensive purification. In particular, precision mass spectrometry based proteomics enables identification and relative quantitation of thousands of cellular proteins in large-scale experiments (Aebersold and Mann 2003; Cravatt et al. 2007; Mann et al. 2001; Mann and Kelleher 2008). Importantly, proteomics has the advantage to characterize proteins and their posttranslational modifications in their in vivo status without any need for site labeling with radioisotopes or mutational substitution of residues of interest.

Plasma membrane proteins belong to extensively modified proteins. As the consequence of their bipartite extra/intra cellular localization, PM proteins are glycosylated on their extracellular domains and phosphorylated on their cytoplasmic domains. In this article, advances in the identification and characterization of these modifications are summarized. Since the use of appropriate methods for studying membrane proteins is the prerequisite for characterization of posttranslational modifications (PTMs) of PM proteins, this article also summarizes methods that have been developed for analysis of membrane proteins.

In advance, I would like to apologize to any author whose work contributing to PM proteomics was overlooked or was beyond the scope of this article. I would like to refer readers, particularly interested in analysis of integral membrane proteins, to an exhaustive review by Speers and Wu (Speers and Wu 2007).

Enrichment of plasma membrane proteins

Plasma membrane constitutes only few percent of the total membranes in the cells. In proteomic analyses of total lysates, PM proteins represent 10–20% of all identified proteins (Table 1). Dependent on the instrumentation, this can allow detection of 100–200 PM proteins in a single-run experiment (Nagaraj et al. 2008). Since the expected number of the PM proteins is higher about one order of magnitude, more information on these proteins can be obtained either by extensive fractionation and extended measuring times or by biochemical enrichment of PM. Alternatively, the content of the PM proteins in sample can be increased by affinity isolation of glycosylated or chemically tagged proteins.

Subcellular fractionation

Methods for subcellular fractionation of cells and tissues were already developed a half century ago. These protocols involve gentle homogenization of the biological material that preserves integrity of some organelles, and lead to formation of vesicles from disrupted ones. In sequential centrifugation steps, the homogenate is separated in fractions differing in size of the organelles and the vesicles. A final purification of PM can be accomplished by a density gradient centrifugation of the fractions in sucrose gradients or other density media. Although subcellular fractionation allows several-fold enrichment of PM, this approach usually requires large amounts of starting material, and therefore it is almost limited to studies on cultured cells and tissues which are available in large amounts. Since in many applications the sample amount is not a limiting factor, the subcellular fractionation techniques are frequently used for enrichment of PM proteins in proteomic analyses. They allow isolation of fractions consisting of 40–50% PM proteins (Lund et al. 2009; Olsen et al. 2004; Zhang et al. 2005).

Table 1 Percentage of integral and plasma membrane proteins identified by LC–MS/MS in total lysates of human cells and mouse tissues

GO term	Integral to membrane (%)	Plasma membrane (%)
HeLa cells	16.9	11.4
MCF7 cells	17.0	9.8
Brain	20.5	20.0
Heart	13.4	11.7
Lung	13.9	14.3
Spleen	10.7	10.8
Thymus	10.8	10.1
Liver	20.2	12.7
FFPE mouse liver	20.1	13.2

FFPE formalin fixed and paraffin embedded tissue

Whole SDS-lysates were processed according to the FASP (filter aided sample preparation) procedure. Data from Ostasiewicz et al. (2010), Wisniewski et al. (2009b), and unpublished results

Phase partitioning

The partitioning of organelles or solubilized proteins have been frequently used in the past. In aqueous-polymer two-phase systems using dextran and polyethylene glycol (PEG), cellular membranes accumulate in the upper PEG-phase. To increase the content of PM, in a second step, the PEG phase has to be re-extracted with a lectin–dextran conjugate. In proteomics, this approach was successfully applied for purification of PM from the rat brain (Schindler et al. 2006). In that study, about 500 proteins were identified and 42% out of them were PM proteins. In another work, the two phase partitioning was used in combination with a sucrose density gradient purification of plasma membranes (Cao et al. 2006). This work resulted in

identification of about 400 proteins out of which 67% were integral membrane proteins.

Cell surface coating

Chaney and Jacobson (1983) developed a method for coating of intact cultured cells with colloidal silica. This method offers a straightforward way for isolation of the PM fraction by centrifugation. Rahbar and Fenselau (2004, 2005) applied this technique for mapping of PM proteins of cancer cell lines obtaining fractions containing about 40% of the PM proteins. Analysis of these fractions led to the identification of 366 and 540 proteins in two separate studies. Even though, the colloidal silica coating was initially considered to be used for PM isolation from cultured cells, Schnitzler et al. used this technique for isolation of PM from the rat lung (Durr et al. 2004; Oh et al. 2004). In a recent report of this group, application of the coating technique combined with four different mass spectrometry-based approaches resulted in characterization of 1,833 proteins, including more than 500 integral membrane proteins in the PM preparations from endothelial cells (Li et al. 2009).

Isolation and analysis of crude membrane fractions

Preparation of total membrane fractions from animal tissue or clinical samples by depletion of soluble proteins from tissue homogenates allows identification of considerable numbers of PM proteins. Instead of gentle homogenization of tissue, we have proposed to use a high speed blender for disintegrating subcellular structures (Nielsen et al. 2005). Subsequent extraction with high salt, sodium carbonate and urea results in fractions containing up to 75% membrane proteins. Analysis of such fractions prepared from different tissues revealed that about 20% of their content were PM proteins (Nagaraj et al. 2008). Recently, a combination of this method with an uncomplicated pipette-tip-SAX fractionation has been applied to analysis of the mouse hippocampus. In this work, 1,604 IMPs and 906 PM proteins were identified (Wisniewski et al. 2009a).

Surface biotinylation

Biotinylation of surface proteins for PM enrichment from cultured cells was tested by several groups (Nunomura et al. 2005; Sostaric et al. 2006; Zhao et al. 2004). This strategy involves chemical coupling of amine- or thiol-reactive biotinylation reagents to proteins located on the cell surface. After solubilization of the membranes and the protein digestion, biotinylated peptides are affinity purified on resins with immobilized avidin. Similar to the PM coating strategy, the surface biotinylation approach is more suitable for analysis of cells in suspension rather than for tissues. In addition, possible contaminations by cytoplasmic and nuclear proteins originating from broken cells can seriously affect the results of the analysis.

Making membrane protein analyzable

The key step in the bottom-up proteomics is digestion of proteins. Most of the cytosolic, nuclear or other soluble proteins can efficiently be digested in buffers that are compatible with endoproteinases and do not interfere with the downstream mass spectrometric protein analysis. In contrast, IMPs that are embedded in the lipid membrane are only partially accessible to the enzymes and have to be released from the membrane for an efficient digestion. For this purpose, detergents enabling transfer of IMPs into detergent complexes are almost indispensable reagents. Unfortunately, detergents are highly susceptible for ionization, and therefore cannot accompany proteins in mass spectrometric analysis. To circumvent these analytical problems, different methods for generation of peptides from membrane proteins have been developed.

Digestion of methanol solubilized membranes

Since the biological membranes can be partially solubilized with organic solvents, several groups have developed 'in-solution' digestion protocols for analysis of membrane proteins. Blonder et al. developed a method that uses buffered 60% methanol for membrane solubilization and for the tryptic digestion (Blonder et al. 2002). This method was applied to variety of samples and allowed identification of considerable numbers of proteins of which 40–50% were IMPs (Blonder et al. 2004). In a more complex variation of this method, a two step digestion with trypsin and chymotrypsin was used for analysis of bacterial membranes allowing identification of 326 integral membrane proteins (Fischer et al. 2006). The critical parameter of this approach is the organic solvent that strongly reduces protease activities, and therefore its concentration has to be thoroughly selected.

Surface 'shaving'

A direct digestion of the soluble parts of the IMP in the suspension of enriched membranes offers another way for identification of membrane proteins without the use of detergents. Two different approaches employing either Proteinase K (Wu et al. 2003) or LysC and trypsin (Olsen et al. 2004) for release of the peptides were developed. Digestion of brain homogenate with Proteinase K followed by a multidimensional fractionation resulted in mapping of

about 1,600 proteins out of which 28% were IMPs. The two step LysC and trypsin-digestion based approaches were used in several studies to analyze crude (Le Bihan et al. 2006) and enriched plasma membranes (Nielsen et al. 2005; Olsen et al. 2004, 2007) of the mouse brain. In these studies, about 40% of the identified proteins were IMPs.

'In-gel' digestion

The commonly used SDS PAGE technique offers an efficient way to fractionate detergent-solubilized proteins, and found a great appreciation in proteomics. In this technique, the in-gel trapped proteins can directly be digested in the gel, because detergents are removed already during the gel fixation (Shevchenko et al. 1996).

A variation of the 'in-gel' digestion method is the basis for the 'Tube Digestion' approach for analysis of the membrane proteins in which proteins are solubilized in detergents, and directly incorporated into a polyacrylamide gel matrix without electrophoresis (Lu and Zhu 2005). Detergents are subsequently removed from the gel matrix, whereas proteins remain trapped in the gel. Although 'in-gel' digestion technique is very popular, its major disadvantage is moderate peptide-yields when compared to 'in-solution' digests (Katayama et al. 2004; Shevchenko et al. 2006). In addition, difficulties in scaling-up are an important limitation of the 'in-gel' approach making it less suitable for studying PTMs. In addition, the 'in-gel' digestion is practically limited to trypsin, because other highly specific endoproteinases such as LysC, GluC, ArgC, and AspN are less efficient likely due to their inability to penetrate polyacrylamide gels.

Acid labile detergents

Difficulties with the removal of traditional detergents led to development of acid labile detergents that can be decomposed after protein solubilization and digestion. The applicability of the so called "mass spectrometry compatible" detergents RapiGest and PPS Silent has been tested by several groups. Indeed, these reagents do not interfere with the mass spectrometric analysis, but they result in less PM protein identifications when compared, for example, to methanol-based extraction procedure (Ye et al. 2009). Large-scale analyses of brain proteome that employed the mass spectrometry compatible surfactants did not show obvious advantages of using these expensive detergents (Chen et al. 2007, 2008).

Phase transfer surfactants' approach

Ishihama et al. (Masuda et al. 2008) evaluated about 30 additives in terms of the enhancement of protease activity and solubilization of membrane proteins. They showed that deoxycholate which is effective in membrane solubilization, and does not inhibit trypsin can be quantitatively extracted (transferred) from the digest with ethyl acetate. The phase transfer surfactant (PTS) protocol was applied to an analysis of membrane enriched fraction from HeLa cells. The digest was analyzed by two-dimensional LC–MS/MS. In 12-LC–MS/MS runs, 1,450 proteins including 764 membrane proteins were identified. Direct comparison showed that the PTS protocol is more efficient than the RapiGest (see above) based one (Masuda et al. 2008). In another PTS based study, 545 membrane proteins were identified in an *E. coli* lysate (Masuda et al. 2009).

Filter aided sample preparation

Detergents can be depleted from protein extracts using organic solvents such as ethanol (Lu et al. 2009). Unfortunately, removal of the detergent often results in protein pellets that are not soluble in aqueous solutions, and even in the presence of strong denaturants such as 8 M urea, a portion of hydrophobic proteins remains in the pellet. Alternatively, detergents can be dissociated and removed from proteins on desalting gel filtration columns equilibrated with 8 M urea (Nagaraj et al. 2008). Since column desalting leads to sample dilution, the method is not useful for small amounts of sample. To circumvent this technical problem, we have developed the filter aided sample preparation (FASP) method (Wisniewski et al. 2009b). In FASP, a sample solubilized in SDS is retained and concentrated into microliter volumes in the disposable protein ultrafiltration unit. The unit then acts as a 'proteomic reactor' for detergent removal, buffer exchange, chemical modification, and protein digestion. Importantly, depletion of detergent is accompanied with elution of lipids. During peptide elution, the filter retains high molecular substances such as undigested protein and DNA. Advantages of the FASP method for analysis of membrane proteomes were demonstrated by analysis of the mouse hippocampus (Wisniewski et al. 2009a) and mouse N-glycoproteome (Zielinska et al. 2010). These studies provided the largest reported PM proteome for a single MS experiment reported to date. The FASP protocol allows digestion of proteins under conditions that are optimal for the enzymes, and therefore it is compatible with all commonly used endoproteinases (Wisniewski et al. 2009b). Importantly, applicability of FASP to wide range of sample amounts, ranging from one hundred HeLa cells (Wisniewski and Mann 2009) to 200 mg of mouse brain tissue (Wisniewski et al. 2010) was demonstrated.

Mapping of glycosylation sites

Glycosylation is an abundant covalent modification occurring on more than half of the proteins in biological systems (Van den Steen et al. 1998). Typically, the protein glycosylation is categorized as either N- or O-linked (Spiro 2002). The N-glycosylation, which is by far the most common cell surface modification, occurs at asparaginyl residues within the consensus sequence N-!P–S|T, where !P is any amino acid except for proline. The O-glycosylation happens on serine and threonine residues. N-galactosamine (GalNAc) is usually the first sugar attached. Unlike to the N-glycosylation, for the O-glycosylation no consensus sequence defining the site was identified. An important variation of the O-glycosylation is the intracellular O-GlcNAc (Wells et al. 2001).

The extracellular surface of cells is densely covered with carbohydrate moieties attached to PM proteins. Glycosylation confers proteins with specific function on the cell surface including cell–cell communication, receptor–ligand interactions, immune response, apoptosis, and pathogenesis of many diseases (Varki et al. 2009; Woods et al. 1994). Changes in glycosylation accompany diseases, including immune deficiencies, neurodegeneration, and cancer (Dennis et al. 2009; Lowe and Marth 2003).

Proteomic mapping of glycosylation typically starts with enrichment of proteins or peptides carrying glycans. Following deglycosylation or chemical substitution of the glycan, the glycosylation sites are identified by the tandem mass spectrometry.

Glycan enrichment

Enrichment of proteins or peptides carrying glycan moieties can be achieved by one of the following methods: an affinity capture, a chemical coupling of the sugar residues to solid supports, or by chromatographic approaches.

The lectin affinity capturing is the most commonly used technique that relies on binding sugar moieties to immobilized lectins and subsequent washing-out unbound molecules. Final elution of the glycoproteins and glycopeptides is carried out using competing sugars or chaotropic reagents.

To purify the glycan carrying proteins or peptides, various lectins can be used. Concanavalin A (ConA), which binds to mannose and wheat germ agglutinin (WGA), which binds to sialic acid and to N-acetylglucosamine are the most commonly used ones. The lectins can either be used separately to isolate specific glycan moieties (Bunkenborg et al. 2004; Ghosh et al. 2004; Kaji et al. 2003; Xiong et al. 2003) or as a mixture. For example, to capture all three classes of N-glycosylated peptides, a multi-lectin enrichment was employed (Yang et al. 2006). ConA, WGA and the *Ricinus communis* agglutinin (RCA, capturing galactose modified at the 3-0 position, as well as terminal galactose) were selected for an in-depth mapping of N-glycosylation sites in mouse tissues (Zielinska et al. 2010). Another lectin, Jancalin was useful for enrichment of a core-1 *O*-GalNAcα type carbohydrate-carrying glycopeptides from bovine serum (Darula and Medzihradszky 2009).

The efficiency of the lectin based enrichments depends on the composition of samples and the properties of a used affinity resin. To improve the enrichment quality, double lectin affinity purification on the protein and on the peptide level was applied for the analysis of human plasma proteins (Bunkenborg et al. 2004). Recently, we have introduced an alternative to column chromatography approach using ultrafiltration units instead of affinity columns for lectin-capturing of glycosylated peptides (N-glyco-FASP) (Zielinska et al. 2010). In the N-glyco-FASP method, lectins do not need to be coupled to a solid support because they are retained by a filter with a nominal cut-off of 30 kDa, and therefore any lectin or mixture of lectins can be employed. In this approach, it is important that peptides applied to the filter are free of the enzyme used for protein digestion. This can be easily achieved using FASP protocol. Using N-glyco-FASP, we observed 50-100-fold enrichment in glycosylated peptides. This high purification degree presumably reflects both high purity of the peptide mixtures prepared by the FASP method and the "homogenic", in solution, separation system free of resin particles. Also high yields are typical for the N-glyco-FASP method, because it allows on routine basis mapping of 1,000–2,000 N-glycosylation sites in a single LC–MS/MS run from as low as 100–200 μg of total protein.

The hydrazide chemistry has been developed for the selective conjugation and the analysis of the N-linked glycopeptides (Zhang et al. 2003). This method bases on the covalent attachment of glycoproteins via their carbohydrate moieties to a solid support, and a subsequent release of glycopeptides with PNGaseF. An alternative glycan-capturing method using the hydrazide chemistry for immobilization of glycopeptides providing higher yields, and specificity was reported by Sun et al. 2007). This approach enabled identification of 286 peptides with the N-!P–S|T consensus sequence from microsomal fraction of an ovarian cell line.

The hydrazide chemistry is also the basis for the cell surface capturing (CSC) technology (Gundry et al. 2009; Wollscheid et al. 2009). This method promises selective identification of plasma membrane proteins from native or cultured cells. The CSC protocol involves covalent tagging of the carbohydrate containing proteins with biocytin hydrazide, digestion of RapiGest solubilized proteins, and affinity isolation of the tagged peptides on streptavidin-beads.

After the PNGaseF treatment, the released peptides are analyzed by LC–MS/MS. So far, application of CSC resulted only in the identification of moderate numbers of the identified N-glycosylation sites. For example, CSC enabled mapping of 235 N-glycosylation sites in C2C12 myoblast cells (Gundry et al. 2009).

Approaches to maximize the numbers of N-glycosylation sites identified by complementary hydrazide and lectin based experiments did not provide expected improvement. For example, using such approach, a relatively low number of 132 N-linked glycosylation sites could be identified in HeLa cell lysates (McDonald et al. 2009). In addition to the lectin and the hydrazide based enrichment methods, a few other techniques were reported. These include reaction with boronic acid (Sparbier et al. 2005), and a chromatographic enrichment of the glycopeptides by HILIC (Hagglund et al. 2004) or the gel filtration (Alvarez-Manilla et al. 2006). Potential of these methods remains to be evaluated.

Site detection of N-glycosylation

The deglycosylation with the PNGaseF is commonly used for the release of N-linked glycans, and allows an identification of a glycosylation site as deaminated asparagine. Since deamidation also happens spontaneously in proteins, it would be difficult to discriminate between the enzymatic and spontaneous deamidation. For this reason, tagging of the deglycosylation sites appears to be important. The PNGaseF deglycosylation in ^{18}O-water is an elegant and a relatively simple way to introduce an isotope tag (Kuster and Mann 1999). By this procedure, each deglycosylated site can be easily identified by a mass shift of 3 Da in respect to its amidated counterpart. This approach was used for identification of the N-glycosylation sites by Kaji et al. (2003). Their protocol called 'Isotope coded glycosylation site-specific tagging' (IGOT) was successfully applied to analysis of *Caenorhabditis elegans* (Kaji et al. 2007) providing the first large scale map of N-glycosylation sites. In total, 1,465 N-glycosylation sites on 829 proteins were mapped. The ^{18}O-tagging of the deglycosylated sites also was incorporated into the N-Glyco-FASP approach (Zielinska et al. 2010). This study mapped 6,367 unique N-glycosylation sites on 2,352 proteins in four mouse tissues and blood plasma. A total of 31% of the N-linked glycoproteome were in the 'plasma membrane' Gene Ontology (GO) category and 25% in 'extracellular region' one. Taking into account non-exclusive localization in GO, 52% of the N-glycoproteome is located at the outside or beyond the plasma membrane (832 of 1,594 N-glycoproteins with GO annotation).

Beside these topological constraints, the vast majority (96.5%) of N-glycosylated asparagines match the stringent glycosylation consensus sequence N-!P-[S|T]. Sequence motif analysis reveals additional constraints on the known sequence recognition pattern. Both in mouse (Zielinska et al. 2010) and in *C. elegans* (Kaji et al. 2007), proline were found as under represented on the third position relative to the asparagines. Threonine occurs more often than serine on the second position, in contrast to non-glycosylated sites that match the motif by chance. The mouse N-glycoproteomic study also showed that other motifs such as N–X–C, N–X–V and N–G are overrepresented among the sites that do not match with the canonical sequence.

Site detection of O-glycosylation

In contrast to the N-glycosylation, mapping of the O-linked glycosylation is much more difficult because of the lack of an efficient deglycosylation enzyme comparable to the PNGaseF. For this reason, detection of the O-glycosites frequently relies on chemical derivatization of the sites (Durham and Regnier 2006; Hanisch et al. 2001; Hanisch et al. 2009; Li et al. 2003). While applying chemical methods, it is important to realize that they suffer from a variety of drawbacks. Harsh conditions used in these methods can affect polypeptide backbone and some amino acid moieties and can result in artifacts (Hanisch et al. 2009).

Recent approaches allowing direct localization of the O-glycosites using alternative to the CID fragmentation methods might provide novel solutions in analysis of this type of glycosylation (Darula and Medzihradszky 2009; Seipert et al. 2009). Darula and Medzihradsky (2009) using the ETD-fragmentation technique mapped 26 glycosylation sites in serum proteins. It is noteworthy that no other single study to date has yielded so much information about *O*-linked glycosylation sites. Since the methods for analysis of the O-glycosylation still are at a development stage, there are no proteomic studies on this PTM on the PM proteins.

Cytoplasmic domains of plasma membrane proteins are extensively phosphorylated

Owing the technical difficulties in studying membrane proteins, analyses of phosphorylation on the PM proteins were limited to single proteins. These approaches require overexpression and/or extensive purification of a protein of interest. Although they can provide detailed maps of phosphorylation sites, they cannot provide biological system-wide insights.

Protein phosphorylation is the most frequently studied posttranslational modification using proteomic techniques. Since digests of total lysates of eukaryotic cells contain only about 1% of phosphorylated peptides, identification of phosphorylation sites requires specific phosphopeptide enrichment. This is usually achieved by TiO_2 (Larsen et al. 2005) or metal affinity chromatography (IMAC) (Nuhse et al. 2003; Stensballe et al. 2001). Identification of less abundant phosphotyrosine sites can be facilitated by immunoaffinity capture using anti-phosphotyrosine antibodies (Ballif et al. 2008).

During the recent years, many proteomic studies focusing on mapping of phosphorylation sites and their dynamics appeared. Interestingly, these analyses provide information on relatively high numbers of PM proteins. For example, the study of the HeLa cell phosphoproteome revealed that 8.7% out of 6,600 sites were located on PM proteins (Olsen et al. 2006). Large scale study on *Caenorhabditis elegans* resulted in the identification of more than 600 (9.5% of total) phosphorylation sites located on PM proteins (Zielinska et al. 2010).

Alternative to the analysis of phosphoproteomes using total lysates, enriched PM protein fractions can be used for focused identification of phosphorylation sites. For example, Thingholm et al. (2008) used a two step enrichment method. First, plasma membranes were purified for stem cells and then tryptic phosphopeptides were captured on TiO_2 beads. This study allowed identification of 703 phosphorylation sites on 376 proteins. Despite extensive subcellular fractionation, only 30–40% out of these proteins originated from plasma membrane. In another study, Tan et al. (2008) using enriched PM fractions identified 217 unique phosphorylation sites corresponding to 158 phosphoproteins from the mouse liver.

Recently, taking advantage of the FASP method for sample preparation, we have performed an in depth analysis of phosphorylation sites in mouse brain (Wisniewski et al. 2010). This study allowed mapping of 12,035 phosphorylation sites on 4,579 brain proteins. Gene Ontology annotation reveals that 23% of identified sites are located on plasma membrane proteins, including a large number of ion channels and transporter proteins. An important outcome of this study was the finding that 95% of phosphorylation sites on PM proteins were localized to predicted cytosolic domains and 5% to predicted extracellular locations. Almost all of the latter represent sites on proteins for which little experimental evidence exists, and therefore it seems that the predicted extracellular locations are in fact cytosolic. In another study, Nuhse et al. (2004) also have demonstrated that high number of predicted cytosolic domains was incorrectly assigned by prediction programs. Current bioinformatic tools for analysis of transmembrane protein topology rely on prediction of transmembrane segments (TMs). Since structure of the transmembrane helices is not highly stringent in terms of their amino acid composition, length, and geometry (deviating from the rod to loop structure), the prediction of some TMs is difficult.

Several previous proteomic studies also emphasized that phosphoproteomics data can be used for empirical evidence of the topology of transmembrane proteins (Nuhse et al. 2004; Wu and Yates 2003). For independent verification of membrane topology, N-glycosylation data can be used because N-glycosylation sites on PM proteins are exclusively located in the extracellular space. Large scale proteomic studies providing experimental constraints, defining cytoplasmic and extracellular domains by phosphorylation and glycosylation sites, respectively, will improve topology prediction algorithms in the future.

Integral plasma membrane proteins often associate with each other or with cytoplasmic proteins in specific and functional complexes. For example, high voltage activated calcium channels (Ca_v) are composed of the pore forming 1α subunit (Dolphin 2009), membrane anchored extracellular $\alpha 2\delta$ subunit (Davies et al. 2010; Klugbauer et al. 2003) and the cytoplasmic β-subunit (Dolphin 2003). Large scale phosphoproteomic studies can provide unique insights in posttranslational modification within such complexes. In total, our analysis revealed 94 phosphorylation sites of the Ca_v-channels and their auxiliary subunits (Wisniewski et al. 2010). Modifications on the 1α subunit were found in the N and C-terminal polypeptides and the inter-repeat loops I/II and III/IV (Fig. 1). From our phosphorylation data, we were able to map up to 13 phosphorylation sites ($Ca_v2.1$) to the C-terminal polypeptides and up to 11 to the II/III linker of $Ca_v2.2$. Phosphorylation sites on β subunit are located in the sequences flanking the SH3 and GK domains, and could modulate binding mediated by them (Fig. 1). Interestingly, these sites conform to Calmodulin kinase II and PKA motifs, matching with the known involvement of these kinases in regulation of the calcium channel (Abiria and Colbran 2010; Gerhardstein et al. 1999). Most of the phosphorylation sites on 1α and β subunits match consensus motifs of many of different kinases. It is likely that these kinases are also involved in regulation of the calcium channels. We did not find any phosphorylation sites on the $\alpha 2\delta$ subunit that is an extracellular, membrane anchored (Davies et al. 2010) and extensively glycosylated protein (Zielinska et al. 2010).

Outlook: perspectives and limitations

Proteomic analysis of plasma membrane proteins has been considered for many years as difficult. This opinion became common because 2D-PAGE methods were

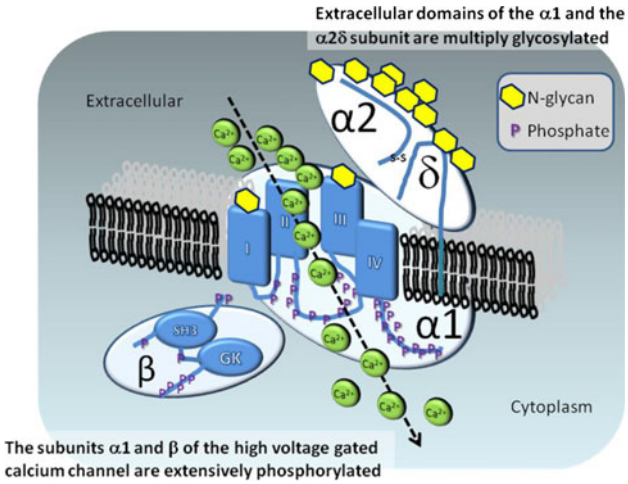

Fig. 1 Subunits 1α and β of the high voltage gated calcium channel are extensively phosphorylated, whereas extracellular loops of the 1α and the α1/δ subunits are N-glycosylated. Data from Wisniewski et al. (2010), Zielinska et al. (2010)

ineffective in analysis of hydrophobic membrane proteins. Recently, Jungblut et al. (2010) have shown that weak solubility of the membrane proteins in concentrated urea impede gel-entering of the polypeptides making their separation impossible. As a consequence, only limited effort has been made to study posttranslational modifications of this type of proteins. In contrast, currently having sample preparation methods making analysis of membrane proteins as easy as that of soluble proteins, high resolution and high throughput mass-spectrometry has the power to map thousands of modification sites from minute amount of sample.

At the time when proteomic analysis was a slow and cumbersome technique, reduction of sample complexity before analysis was essential. This was also true for studies on PM proteins, and therefore a large number of different biochemical fractionation techniques for isolation of PMs were tested. Some of these approaches provided convincing results, and therefore will play important role in future studies. Other analyses that cannot implement PM fractionation will use an extensive fractionation on the protein and/or peptide level. For example, PM fractionation methods are not applicable for studying clinical samples as these are usually available as formalin fixed and paraffin embedded (FFPE) material.

Recent studies have shown that posttranslational modifications such as phosphorylation and N-glycosylation are preserved in FFPE material and can be identified after fixation reversal (Ostasiewicz et al. 2010; Tian et al. 2009). We have recently shown that phosphoanalysis of FFPE liver allows identification of thousands of phosphorylation sites out of which 16% were on PM proteins (Ostasiewicz et al. 2010). Lectin-based enrichment of the same lysate allowed identification of 1,500 N-linked glycosylation sites. About one-third of them were mapped to PM proteins. These results clearly demonstrated that posttranslational modifications can be analyzed in large-scale fashion from minute amounts of FFPE sample, and that proteomics has the potential for exploration of archival clinical samples.

Proteomic mapping of PTMs relies on digestion and isolation of peptides carrying modifications. There are obvious limitations in the size and amino acid residue composition of the peptides that can be resolved by mass spectrometer. The peptides should be of a length allowing unequivocal identification of the peptide sequence and the PTM site. In the most common MS set-ups, the peptide can carry only a limited number of positive charges. These constraints exclude identification of many sites. For example, large-scale phosphoproteomic studies typically use trypsin or endoproteinase LysC for protein digestion at R and/or K residues, respectively, and for this reason such analyses are almost unable to map phosphorylation sites of protein kinase C because the modified S or T residue is usually flanked by K and/or R. Digestion with another proteinase can generate a longer peptide with the phosphorylation, but the site-containing peptide may comprise too many positively charged residues to be identified. To resolve such peptides, the MS have to be operated in the opposite charge mode (so called negative mode), that so far has been rarely used in proteomics. Alternatively, identification of highly basic peptides can be facilitated using the ETD/ECD fragmentation.

Low abundance of some proteins and low site occupancy by PTMs are factors limiting identification. Considering the sensitivity of the mass spectrometry instrumentation used in routine analysis of complex mixtures, identification of a protein occurring at 1,000 copies per cell precludes an availability of 1,000,000 cells per experiment (Wisniewski 2008). Already, such amount of sample can in many cases be difficult to procure. Taking into account the partial occupancy of PTM sites, the required quantities of sample can increase to unrealistic sizes. Furthermore, one-dimensional separation in front of the mass spectrometer (LC–MS/MS) is not able to deal with large amounts of sample. Thus, extensive fractionation at protein and/or peptide level appears to be inevitable steps in comprehensive characterization of PTMs.

Plasma membrane proteins belong to abundantly posttranslationally modified proteins, and therefore a single polypeptide can occur in many molecular forms differing in their chemical composition and biophysical properties. To manage the diversity of the proteome, a concept of protein species has been introduced and developed (Jungblut et al. 2008; Schluter et al. 2009). The proposed nomenclature has the potential for precise protein description and classification of PM proteins with their PTMs.

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