The application of mass spectrometry to membrane proteomics

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Membrane proteins perform some of the most important functions in the cell, including the regulation of cell signaling through surface receptors, cell–cell interactions, and the intracellular compartmentalization of organelles. Recent developments in proteomic strategies have focused on the inclusion of membrane proteins in high-throughput analyses. While slow and steady progress continues to be made in gel-based technologies, significant advances have been reported in non-gel shotgun methods using liquid chromatography coupled to mass spectrometry (LC/MS). These latter strategies facilitate the identification of large numbers of membrane proteins and modifications, and have the potential to provide insights into protein topology and orientation in membranes.

Membranes play a critical role in cellular structure by providing a physical barrier between the cell and its environment and the various subcellular compartments within eukaryotic cells. Although the basic structure and function of biological membranes is provided by the lipid bilayer, membrane-spanning proteins confer unique compartment-specific functions and communication between separated environments. The plasma membrane regulates the exchange of information between the cell and its environment through signaling mechanisms and the mediation of the transport of ions and solutes; intracellular membranes regulate the different environments within organelles and the cytosol. Each type of cellular membrane has characteristic functional properties. Accordingly, the total number and types of proteins and lipids in a given membrane are highly variable. Integral membrane proteins are amphipathic—composed of regions that are hydrophobic and regions that are hydrophilic. However, the very amphiphilic nature that allows them to be localized in the membrane also makes them notoriously difficult to study.

Although proteomics technologies have made rapid progress in the analysis of soluble proteins in recent years, membrane proteins have lagged behind and are typically under-represented in datasets. Thus, whereas it has been estimated that 20–30% of the human genome encodes membrane proteins[1,2], the representation of membrane proteins reported in existing analyses is much lower.

The membrane proteome can be defined as the entire complement of membrane proteins present in a cell under a specific condition and at a specific time. Interest in defining membrane proteomes stems not only from the role of membrane proteins in fundamental biological processes, such as cell signaling (for example, G-protein coupled receptors (GPCRs)), cell–cell interactions (integrins and adhesion proteins), the intracellular compartmentalization of organelles (kinase-anchoring proteins), ion and solute transport (potassium channels), and energy generation (bacteriorhodopsin, ATP synthase), but also from the possibility of profiling cell surface membrane proteins in specific cell types or pathogens for research on vaccines and biomedical therapeutics.

The importance of membrane proteins in drug discovery cannot be overemphasized—currently they account for ~70% of all known pharmaceutical drug targets (a recent article reports that 25% of all current experimental and marketed drugs target class 1 and class 2 GPCRs alone[3]). There is also growing interest in the use of therapeutic monoclonal antibodies to target cell surface proteins uniquely expressed on diseased cells or tissues.

In this review, we focus on mass spectrometry methodologies for identifying membrane proteins and their post-translational modifications. We first summarize gel-based approaches (the classical proteomic approach) and then describe progress with 'shotgun' methods in which complex protein mixtures are proteolytically digested before separation and analysis by LC/MS. In addition, we discuss the capacity of current LC/MS approaches to quantify relative differences between membrane samples as well as their potential to facilitate characterization of membrane protein topology. For the sake of brevity, we do not cover advances in computational approaches or yeast two-hybrid technology. These can complement the information from LC/MS approaches by providing information on interaction networks among membrane proteins (for further information, see refs. 4–6).

Gel-based methods

Traditionally, proteomic analyses of complex protein samples involve the resolution of proteins using two-dimensional gel electrophoresis followed by the identification of resolved proteins by mass spectrometry[7,8]. The limitations of this approach for membrane proteins are well documented[8]. The major obstacle remains solubility for two reasons: first, many hydrophobic proteins are not solublilized in the non-detergent isoelectric focusing sample buffer; and second, solubilized proteins are prone to precipitation at their isoelectric point. Limited dynamic range of detection is also an issue because membrane proteins are typically lower in abundance when compared with soluble proteins. However, it is now clear that subcellular fractionation and directed biochemical enrichments can overcome many of these abundance issues.

Most refinements are directed at sample preparation and involve improved solubilization of membrane fractions with either organic solvents[9,10] or nonionic[11]/zwitterionic[12] detergents before gel analysis

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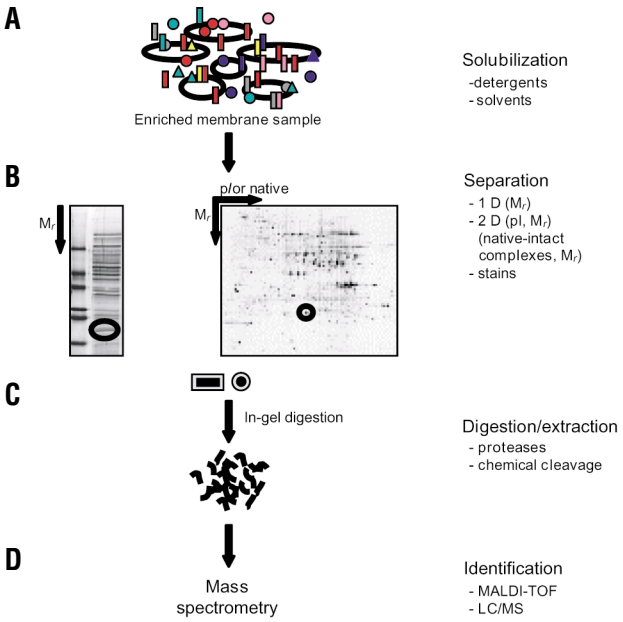

Figure 1. Gel-based analysis of membrane proteins. Four general steps are shown: (A) solubilization; (B) separation; (C) digestion/extraction; and (D) identification.

(Fig. 1A). Other strategies are targeted at the first dimension (the isoelectric focusing step) (Fig. 1B). As membrane proteins become increasingly insoluble as they near their isoelectric point (pI), the simplest solution is to eliminate that step.

In fact, many investigators have returned to the ageless workhorse of the one-dimensional gel[9,13,14] and coupled it with mass spectrometry for protein identification. The limitation of this approach is the increased protein complexity in each one-dimensional gel band. This problem can easily be overcome either by the use of liquid chromatography to resolve the extracted peptides or by increased mass accuracy of the mass detection. Alternatively, isoelectric focusing can be replaced with a different separation strategy. Brookes *et al.*[15] use two-dimensional blue native gel electrophoresis, in which membrane protein complexes remain intact during the first dimension, which is performed under native conditions, and are subsequently resolved during the denaturing sodium dodecyl sulfate (SDS) second dimension. This analysis is unique because it facilitates the functional analysis of membrane protein complexes on two-dimensional gels[15]. Devreese *et al.*[16] have used a similar approach to identify and profile mitochondrial proteins.

Once the proteins are resolved on gels (one-dimensional or two-dimensional), proteins are visualized using various stains (Fig. 1B). Progress in this technology has been directed at issues of sensitivity, reproducibility, and quantification. These developments affect all proteins (soluble and membrane)[17,18] and, therefore, will not be discussed further here. Once the protein bands or spots have been selected, they are digested into peptides, which are then extracted from the gel for subsequent identification by mass spectrometry (Fig. 1C). A limitation of the in-gel digestion approaches for membrane proteins is the size and hydrophobicity of the fragments generated by trypsin, the typical protease used. High-sequence coverage is difficult to obtain because the membrane-spanning segments are either not readily accessible to proteolytic enzymes or lack the specific proteolytic cleavage sites. These difficulties can sometimes be overcome with the use of a combination of proteases and chemical cleavage methods. Van Montfort *et al.*[19,20] coupled trypsin digestions with subsequent cyanogen bromide cleavage. When compared with individual cleavage methods, the sequence coverage of the hydrophobic-membrane domain doubled, whereas coverage of the soluble domain remained the same.

Gel-based approaches provide a useful analytical tool for the generation of global protein expression profiles of complex protein samples. While they have limitations, they do enable quantitative comparisons of thousands of proteins simultaneously. Biochemical enrichments using organic solvents and subsequent analysis on one-dimensional gels or modified two-dimensional gels (excluding isoelectric focusing) provide a targeted solution to optimize the inclusion of membrane proteins in these analyses. Unfortunately, no one strategy provides a global solution for all membrane proteins, and conditions are typically optimized for each membrane-enriched sample.

Shotgun chromatography/mass spectrometry methods

Shotgun methods provide a powerful alternative to gels. Proteins are first digested with proteases into a more complex peptide mixture that is then analyzed directly by LC/MS. Subsequently, protein identifications are determined by database searching software. This general approach is rapid and readily automated, but requires significant computing resources for data analysis. As with gel-based methods, the solubility of membrane proteins is also the major challenge for non-gel shotgun approaches.

Membrane solubilization strategies. Three methods have recently been deployed to analyze enriched membrane fractions and address the solubility issue by using detergents[21], organic solvents[22,23], and organic acids[24] compatible with subsequent proteolytic digestion/chemical cleavage, and separation and analysis by LC/MS (Fig. 2).

Our group[24] provided the first large-scale proteomic analysis that included a substantial proportion of membrane proteins (Fig. 2A). In their approach, an enriched yeast membrane fraction is solubilized with 90% formic acid in the presence of cyanogen bromide. The concentrated organic acid provides the solubilization agent, and cyanogen bromide, functional under acidic conditions, allows many embedded membrane proteins to be cleaved. These large fragments are subsequently further digested with a serial digestion using endoproteinase LysC and trypsin to produce smaller peptide fragments more amenable to analysis by tandem mass spectrometry. Multidimensional protein identification technology (MudPIT) allows the separation of the resulting complex mixture of peptides with two-dimensional high-performance liquid chromatography separation (by charge and hydrophobicity) coupled in-line with tandem mass spectrometry. In this study, 131 integral membrane proteins were identified, with three or more predicted transmembrane domains from the 1,484 total yeast proteins identified. The method is robust and broadly applicable to various complex membrane samples. However, this approach is limited to the qualitative identification of proteins within the mixture.

Another method developed by Han *et al.*[21] uses a detergent to solubilize the membrane proteins (Fig. 2B). A membrane-enriched microsomal fraction is solubilized by boiling in 0.5% SDS and, following isotope-coded affinity tag (ICAT) labeling, is diluted to reduce the concentration of SDS. At low concentrations, SDS is compatible with tryptic digestion. The resultant peptide mixture is fractionated by three steps of chromatography to both enrich for tagged peptides and to resolve them for analysis by mass spectrometry. In this study, Han *et al.*[21] identified 491 proteins, a proportion of which were *bona fide* integral membrane proteins. The advantages of this technique are the enrichment of less abundant proteins and the quantification of differences between samples using ICAT reagents. It is difficult to optimize the efficiency of labeling using these reagents, however, and peptides lacking cysteine are not enriched and need to be analyzed separately[25].

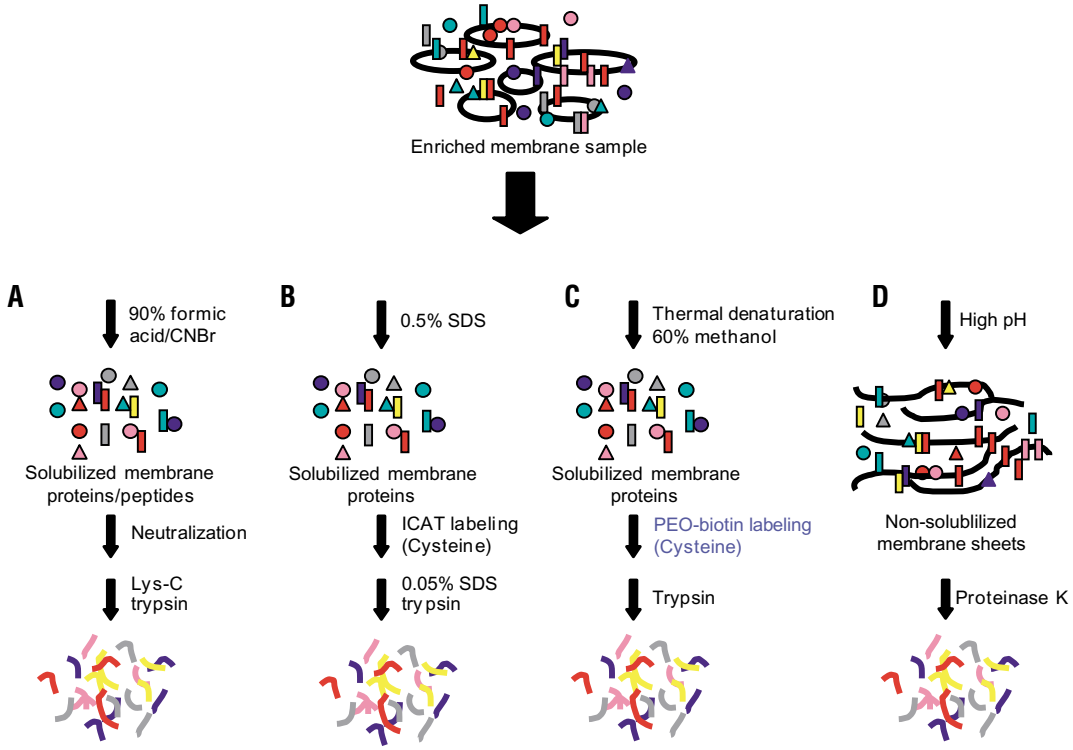

Figure 2. Shotgun proteomic analysis of membrane proteins. Comparison of the four most current methods used to prepare membrane proteins for analysis. (A) Formic acid/CNBr solubilization followed by neutralization and further digestion with Lys-C and trypsin. (B) SDS detergent solubilization followed by ICAT labeling and digestion with trypsin/0.05% SDS. (C) Organic solvent solubilization, followed by ICAT labeling (optional, to allow quantification between samples) and digestion with trypsin. (D) Membrane fractionation at high pH and digestion with proteinase K.

Membrane proteins can also be solubilized using organic solvents, as shown by Blonder *et al.*[22] (Fig. 2C). Using an enriched membrane sample, the proteins are thermally denatured and sonicated in 60% organic solvent (methanol) in the presence of trypsin. The resultant peptide mixture is then analyzed by LC/MS. When this approach was applied to *Deinococcus radiodurans,* 15% of the 503 proteins identified were predicted membrane proteins. More recently, this method has been coupled with ICAT-like biotinylating reagents to allow the enrichment of low-abundance membrane proteins and quantitative comparisons[23] (Fig. 2C). Like the Han *et al.*[21] approach, the advantages of using this technique are the capabilities to enrich for less abundant proteins and to quantify differences between samples.

All three of these methods are effective and optimize the identifications of membrane proteins. Furthermore, both the SDS[21] and organic solvent[22,23] approaches allow relative quantitative comparisons of enriched cysteine-containing peptides to be obtained from different samples. However, they are still far from being comprehensive. In each case, the membranes are solubilized, and protein topology is lost. Also, although identification of covalent modifications is possible, it is certainly not probable. Finally, because specific proteases are used, sequence coverage on the proteins identified is limited (see "Protease specificity and sequence coverage").

Membrane fractionation by high pH. In contrast to the membrane solubilization approaches, we have developed a strategy to analyze membrane proteins from nonsolubilized membrane samples[26] (Fig. 2D). Sealed membrane vesicles are agitated at high pH to produce intact membrane 'sheets'[27]. Proteinase K, a robust nonspecific protease, is used to cleave exposed soluble domains on the integral membrane proteins. A major advantage of this approach is the tolerance of sample diversity and complexity. When we employed this approach in the analysis of a crude rat brain homogenate, 1,610 proteins were identified, 28.2% of which were predicted membrane proteins[26].

Post-translational modifications

Though a major objective of proteomics is the systematic identification of all proteins expressed in a cell or tissue, comprehensive insight into protein function also requires the analysis of covalent modifications (and particularly of functionally critical modifications, such as phosphorylation and glycosylation). Over 200 different protein modifications have been described, and yet they are absent from the proteomic literature (for a review, see ref. 28 and p. 255). Two-dimensional gel electrophoresis provides an indication of the number of modified forms of specific proteins and allows comparisons between samples. However, these analyses target individual proteins for which the identification of the modification typically requires additional molecular tools and biochemical approaches.

LC/MS approaches provide an alternative strategy with a greatly enhanced level of sensitivity for detecting mass changes due to covalent modifications. Much like a two-dimensional gel electrophoresis experiment, measurement of the intact molecular weights of proteins provides a means of detecting the different functional forms of a protein, such as covalent modifications and truncations. However, as with all methods using molecular weight of a protein to measure post-translational modification, the use of careful controls will be critical to avoid possible artifacts and false assignments.

Several groups have attempted direct analysis of intact membrane proteins and identification of their covalent modifications. In this approach, the protein mixtures are first solubilized and then chromatographically resolved. Intact membrane proteins of up to 61 kDa[29–32] have been analyzed directly by LC/MS.

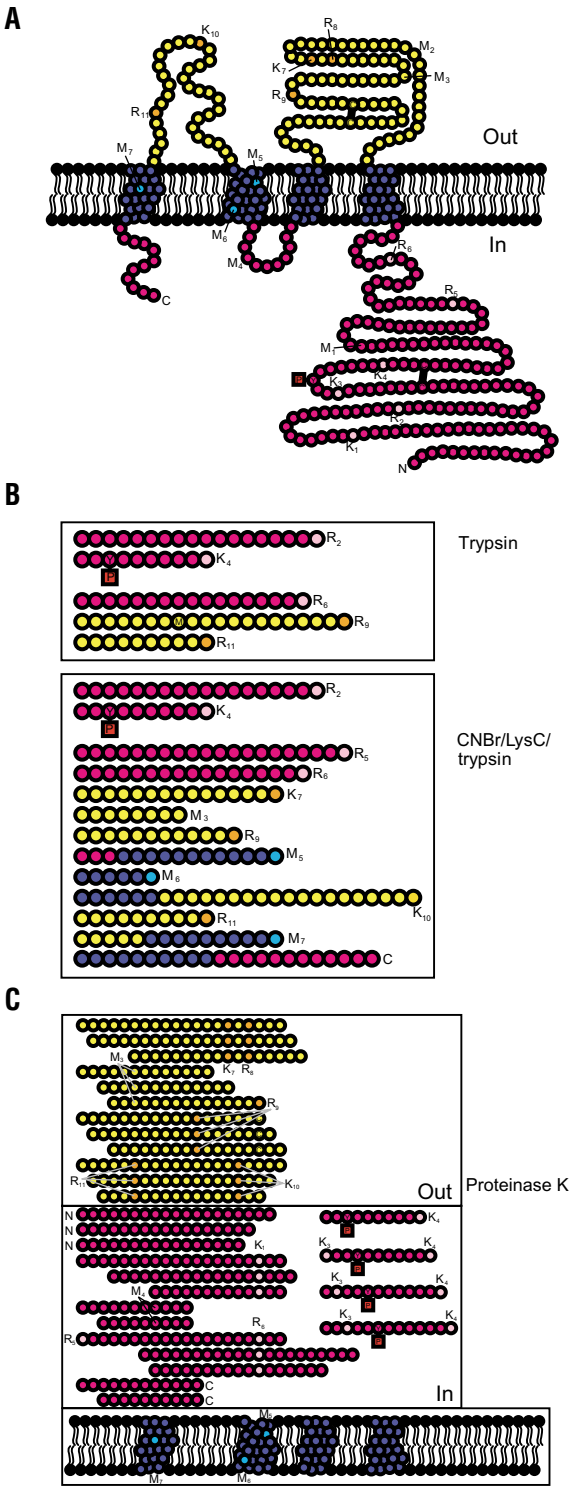

Figure 3. Shotgun proteomic analysis of a hypothetical membrane protein. (A) Hypothetical membrane protein is depicted with four transmembrane domains, soluble domains on both sides of the lipid bilayer, and one phosphorylation site. Amino acid residues are depicted with colored circles (domains inside the membrane compartment, pink/purple; domains outside the membrane compartment, yellow/orange; and transmembrane domains, blue/teal). Arginine (R), lysine (K), and methionine (M) residues are labeled and numbered from amino terminus (N) to carboxy terminus (C). Cysteine residues (C) and one phosphorylation site (P) are also labeled. (B) Digestion of hypothetical membrane protein with trypsin and CNBr/trypsin. All expected peptides with lengths of 25 amino acids or less are shown for each digestion. (C) Digestion of hypothetical membrane protein with Proteinase K. A small representation of possible peptides generated are shown.

Whitelegge and colleagues[30,31] used organic solvents to maintain the solubility of membrane proteins followed by LC/MS analysis. Cadene and Chait[32] used nonionic and zwitterionic detergents followed by analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. Drawbacks of these approaches are the relatively large sample requirements[31] and limitations on the complexity of the sample[31,32]. Additionally, measurement of the intact molecular weight of a protein does not ensure the accurate identification of the protein.

New approaches for 'top-down proteomics' of intact proteins use methods to fragment intact proteins (for example, Fourier-transform mass spectrometers (FT-MS) in conjunction with electron capture dissociation). Meng *et al.*[33] have successfully coupled this strategy with the use of an acid-labile surfactant to analyze intact proteins. The method has yet to be applied to integral membrane proteins, but holds promise if membrane proteins can be maintained in solution during the analysis.

Covalent modifications have also been studied using shotgun LC/MS methods. However, the majority of the reported modifications are on soluble proteins from purified samples and simple mixtures. Several recent approaches have targeted the identification of covalent modifications from relatively complex mixtures and focus on protein phosphorylations using chemical derivatization[34–36] or affinity strategies for phosphopeptide enrichment[37].

Our group[38] has developed an alternative approach that is broadly applicable to the identification of multiple covalent modifications using multiple proteases (including nonspecific proteases) to increase the number of peptides covering a given modification. Although successful for the analysis of soluble proteins with a range of complexities, these methods have, however, had limited success when applied to membrane samples.

With this problem in mind, we have[26] devised a method for analyzing membrane proteins from nonsolubilized membrane sheets that may provide a general method of identifying covalent modifications on membrane proteins. Using the general approach developed previously in our lab to identify covalent modifications of simple mixtures of soluble proteins[38], the selection of Proteinase K increases the production of overlapping peptides covering a given modification on membrane proteins[26]. In a single series of measurements, the largest number of predicted membrane proteins (454) and four different types of modifications (79 including phosphorylation) were reported from a total of 1,610 identifications from a rat brain homogenate. The 79 modifications were identified on 51 total proteins, of which 24 were predicted membrane proteins. Even though the method was developed for the analysis of membrane proteins, this method is applicable to protein samples (soluble and/or membrane) of a large range of complexities, from protein complexes to enriched membrane fractions to whole cell lysates.

Protein topology in membranes

Another benefit resulting from the analysis of membrane proteins in the context of membrane sheets is the unique opportunity to map the soluble regions of predicted transmembrane proteins[26]. In this approach, Proteinase K is used to cleave accessible soluble domains of membrane proteins for subsequent analysis by MudPIT to identify the peptides. When this strategy is combined with protease protection strategies, protein topology can be determined (as shown for two membrane proteins from a rat liver Golgi preparation in ref. 26).

Using a hypothetical membrane protein as a model, it is clear that current shotgun approaches (Fig. 2) suffer from several limitations. As shown in Figure 3A, the depicted hypothetical protein is composed of multiple domains: three soluble domains located

Protease specificity and sequence coverage

Many proteomic approaches involve the digestion of intact proteins followed by the analysis of the resulting peptide fragments. Traditionally, specific proteases (e.g. trypsin) have been used almost exclusively. However, non-specific enzymes offer the unique advantage of creating overlapping peptides throughout the protein sequence. These overlapping peptides increase both the protein sequence coverage and the peptide mixture complexity. Specific enzymes usually produce a peptide mixture with well defined complexity and peptides with predicted cleavage specificity. This cleavage specificity can be used by database search algorithms to facilitate the identification of the peptide sequence. In contrast, the peptide mixtures resulting from non-specific enzymes (e.g. Proteinase K) result in samples of variable complexity that can complicate the computational analysis of the peptide spectra. Nevertheless, if the LC/MS system is capable of handling the complexity of the non-specifically cleaved peptide mixture and database search algorithms are used that are capable of identifying peptides without cleavage specificity, the improved sequence coverage attained can provide a unique system for the comprehensive analysis of proteins.

inside the membrane-bound compartment (pink/purple); four hydrophobic transmembrane domains (blue/teal); and two soluble hydrophilic loops located outside the membrane-bound compartment (yellow/orange).

Using the method of Blonder *et al.*[22] (Fig. 2C), optimal solubilization and digestion of the hypothetical membrane protein would produce five peptides, one of which is a phosphopeptide (Fig. 3B, top). To identify this protein, any one of these five peptides must produce a tandem mass spectrum with a high confidence of identification (a requirement for all methods). To identify the phosphorylation site, the peptide containing the modification must produce a quality tandem mass spectrum with fragment ions encompassing the modification site (also a requirement for all methods). In the best-case scenario, even if tandem mass spectra were acquired for all five peptides, the percent sequence coverage for the protein would be low. Using the enrichment method of Han *et al.*[21] and Goshe *et al.*[23] (Fig. 2B,C), none of these peptides would be detected. The affinity purification step would not select for any of the peptides because there are no cysteines available for labeling; therefore, neither of the approaches would be able to identify the protein. Separate analysis of the nonenriched, cysteine-containing peptides would easily solve this limitation. Additionally, membrane proteins that produce labeled cysteine-containing peptides of a detectable mass can be quantitative when different samples are compared.

Use of our earlier method combining cyanogen bromide, endoproteinase Lys C, and trypsin for digestion (Fig. 2A)[24] generates thirteen peptides, one of which is a phosphopeptide (Fig. 3B). Again, analysis of any one of the thirteen peptides would result in the identification of the protein, but only the analysis of the phosphorylated peptide would identify the modification site. The sequence coverage provided by using this method is much higher and coverage of transmembrane domains is provided by five of the peptides.

In our new approach (Fig. 2D)[26], the nonspecific enzyme results in the production of a large heterogenous group of peptides and phosphopeptides. Figure 3C displays a representative collection of the potential peptides. Again, as with the other methods, analysis of any one of these peptides would result in the identification of the protein and the analysis of any one of the multiple phosphopeptides would result in the identification of the modification site. Advantages of this method are that all soluble protein domains are potentially accessible for digestion and the increased number of peptides covering the modification site increases the number of peptides available for the assignment of the phosphorylation site in the lumenal domain of the protein (Fig. 3C). Importantly, digestion of the exposed soluble domains of the protein (Fig. 3C, OUT, yellow/orange) can be temporally separated from digestion of the protected soluble domains of the protein (Fig. 3C, IN, pink/purple). The remaining membrane and the embedded transmembrane domains (Fig. 3C, bottom) could further be analyzed using the method of Blonder *et al.*[22], potentially leading to the targeted identifications of membrane spanning domains. This applied global protease protection strategy would allow the characterization of membrane protein topology and the relative localization of soluble proteins in a membrane compartment.

Although this method has potential for the analysis of covalent modifications and membrane protein topology, there are some obvious limitations. Currently, the method does not provide a means for relative quantification between samples. Furthermore, the complexity of the peptide mixture generated is dramatic, and adequate resolution at the liquid chromatography step and computing power are required for their analysis. Nevertheless, the ability to globally analyze covalent modifications and topology of membrane proteins clearly 'raises the bar' and allows many new exciting avenues of proteomic analyses.

Future directions

Less than 1% of the proteins of known structure are membrane proteins. The principles underlying the folding of integral membrane proteins and algorithms for the prediction of membrane-spanning domains are less than adequate in their representation of *in vivo* structure of membrane proteins. For example, predictions made for the three-dimensional structure of chloride channels were found to be incorrect when the crystal structure was solved[39]. Global shotgun analyses of soluble domains of membrane proteins will enable development of improved prediction algorithms as well as aid in the selection of potential domains for further functional and structural analyses.

Analytical methods have been undergoing a renaissance in the past few years as they are improved and adapted for the large-scale analysis of membrane proteomes. The remaining challenges in membrane proteomics involve improved methods to solubilize intact proteins for either direct liquid chromatography separation or two-dimensional gels. Improved analysis of intact proteins will allow the identification of protein isoforms resulting from sequence difference, alternate splicing, and modifications. Coupling shotgun methods with top-down mass spectrometry approaches will enable the identification of modification sites, and enhance the potential to generate complete sequence coverage.

Shotgun proteomics strategies have the potential to identify large numbers of membrane proteins and modifications. The shotgun method also promises the tantalizing prospect of providing insights into the topology of membrane proteins. Though substantial work is required to validate the technology, localizing the membrane-spanning regions of proteins and the polarity (orientation) of their integration into the membrane will help with structural analysis and the identification of receptor-binding and catalytic domains.

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