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Phosphoproteome Analysis

Roberto Raggiaschi,[1,2] Stefano Gotta,[1] and Georg C. Terstappen[1]

Protein phosphorylation is directly or indirectly involved in all important cellular events. The understanding of its regulatory role requires the discovery of the proteins involved in these processes and how, where and when protein phosphorylation takes place. Investigation of the phosphoproteome of a cell is becoming feasible today although it still represents a very difficult task especially if quantitative comparisons have to be made. Several different experimental strategies can be employed to explore phosphoproteomes and this review will cover the most important ones such as incorporation of radiolabeled phosphate into proteins, application of specific antibodies against phosphorylated residues and direct staining of phosphorylated proteins in polyacrylamide gels. Moreover, methods to enrich phosphorylated proteins such as affinity chromatography (IMAC) and immunoprecipitation as well as mass spectrometry for identification of phosphorylated peptides and phosphorylation sites are also described.

KEY WORDS: Phosphorylation; phosphoprotein detection strategies; phosphoprotein enrichment methods; mass spectrometry analysis.

ABBREVIATIONS: IMAC: immobilized metal affinity chromatography; 1D: one dimensional; 2D: two dimensional; ECL: enhanced chemiluminescence; pY: phosphotyrosine; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; MP: multiplex proteomics; DIGE: difference gel electrophoresis; SILAC: stable isotope labeling with amino acids in cell culture; CID: collision-induced dissociation; amu: atomic mass unit; ESI: electrospray ionization; λ PPase: lambda phosphatase.

PHOSPHORYLATION AND ITS IMPORTANCE FOR BIOLOGICAL PROCESSES

Post translational modifications of proteins are considered to be one of the major determinants regarding the complexity of higher organisms [1]. At least more than 200 different types of post translational modifications are known [2, 3] of which only a few are reversible and important for the regulation of biological processes. The most studied modification is protein phosphorylation which was described for the first time by Edwin Krebs and Edmond Fisher in 1955 as being indispensable for the conversion of glycogen phosphorylase from an inactive to an active form [4]. Since then the interest in protein phosphorylation and its role in regulating protein functions has been steadily increasing. Nowadays it is well known that all processes

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regulated by protein phosphorylation are reversibly controlled by the combined action of two different classes of enzymes: protein kinases which catalyze the transfer of phosphoryl groups from a high-energy compound to a nucleophilic acceptor on an aminoacid side-chain of proteins and protein phosphatases which catalyze a water-driven hydrolysis of phosphoester bonds.

The importance and key role of protein phosphorylation and dephosphorylation is also supported by the high number of protein kinases and protein phosphatases present in the human genome, where they constitute about 2% of all genes [5–7].

Many cellular processes such as metabolism, transcription, translation, cell cycle progression, cytoskeletal rearrangement, cell movement, apoptosis and differentiation are controlled by phosphorylation of proteins directly involved in them. Moreover, phosphorylation plays a key role in information transfer inside the cell governing signal transduction pathways, in intercellular communication during development, in activation of the immune system and in the functioning of the nervous system. In combination with its reversibility, phosphorylation is a very frequently occurring post translational modification in eukaryotic cells and target proteins belong to different classes comprising structural proteins, molecular chaperones, enzymes and transcription factors. In fact, it is estimated that 30% of all cellular proteins are phosphorylated, often at multiple sites, and each phosphorylation event might have a distinct effect on protein function. Defects or alterations of protein kinases and phosphatases might result in severe damage of cells, which might lead to pathological conditions such as cancer and neurodegeneration [8, 9]. The most common type of phosphorylation is O-phosphorylation which occurs on serine, threonine and tyrosine aminoacids with a ratio of about 1000/100/1 [10]. Other types of phosphorylation occurring on histidine, arginine, lysine, aspartic acid, glutamic acid and cysteine residues are also known [11] but much less studied since such modifications are chemically labile and thus very special techniques are necessary for their investigation.

Analysis of the whole complement of phosphorylated proteins in a cell, the so-called phosphoproteome, was an attractive study subject since the discovery of phosphorylation as a key regulatory mechanism of cell life. However, only today, with the improvement of knowledge and technologies it has also become a realistic goal.

PHOSPHOPROTEOME

The development and optimization of protocols for the enrichment of phosphorylated proteins or peptides is essential to investigate and elucidate the phosphorylation events occurring in a cell at a given time as well as to identify phosphorylation sites in proteins. Because of the complexity of protein phosphorylation patterns, a comprehensive analysis can only be achieved by employing several different experimental strategies all aiming at selective enrichment of phosphorylated proteins. A proteomics approach has the advantage that, instead of studying changes of the phosphorylation of a single protein, all phosphoproteins in a cell are investigated at the same time. However, experimental analysis of phosphorylated proteins is not an easy task for several reasons : phosphorylation is generally a sub-stoichiometric reaction indicating that only a small fraction of a

given protein is phosphorylated; the possibility that a protein can be phosphorylated on different sites implies the existence of phosphoproteins in different forms and thus it is necessary to identify the phosphorylation sites in order to distinguish between the phosphorylated forms; the often low abundance of phosphoproteins (especially those involved in regulatory processes such as signal transduction) which require a phosphoprotein enrichment strategy; the existence of phosphatase enzymes which can dephosphorylate aminoacid residues unless special precautions are taken; the dynamic nature of protein phosphorylation which results in constant modifications of the phosphoproteome of a cell [12, 13].

PHOSPHORYLATION DETECTION STRATEGIES

While the identification of phosphorylated proteins and of phosphorylation sites was greatly improved by the introduction of mass spectrometry, the detection of phosphorylated proteins of a cell still relies on the application of optimized ''classical'' methods.

Isotopic Labeling of Phosphoproteins

The use of the inorganic phosphate isotopes ^{32}P or ^{33}P to radiolabel phosphorylated proteins is probably the oldest method used to study protein phosphorylation. It is still largely employed because it is simple and reliable for studying phosphorylation events *in vitro* and in cellular models. Metabolic labeling with radioactive inorganic phosphate followed by cell lysis and separation of labeled proteins by 1D or 2D gel electrophoresis and autoradiography or image acquisition by PhosphorImager systems for visualization of phosphorylated proteins is the most common workflow pursued in proteomics studies when an isotopic labeling strategy is considered. A comparison of the performance of ^{32}P and ^{33}P in labeling proteins (in particular cytoplasmic proteins of MRC−5 cells) has been described [14]. Results showed that ^{33}P labeled proteins produced a more ''neat'' image of the phosphoproteome with a higher degree of resolution even if a longer exposition time was required. Apart from considering environmental and safety aspects of using radioactive isotopes, metabolic labeling has other important disadvantages: it can only be used to investigate phosphoproteins in viable cells since the radioactive isotope has to be taken up from the medium and metabolized, and consequently it is not applicable for proteomics studies of human post-mortem tissue or biopsies; due to the high sensitivity, subsequent identification of detected phosphorylated proteins can be very difficult because of low amounts of proteins; the standard dose employed induces DNA fragmentation, elevates p53 tumor suppressor protein levels and results in cell cycle arrest or apoptosis [15–17].

Western Blotting Employing Phosphospecific Antibodies

Western blotting is a quite old technique [18] which requires the availability of specific antibodies to detected proteins transferred from a 1D or 2D gel [19] to a solid membrane support. The development of antibodies against common protein epitopes allows the identification of proteins sharing the same characteristics such as

phosphorylated proteins. For phosphoproteome analysis phosphoserine, phosphothreonine and phosphotyrosine represent the common epitopes which are recognized by specific antibodies that are routinely employed. Analysis of phosphoproteome using Western blotting techniques is improved by combining high-resolution two-dimensional electrophoresis (which resolves the highest number of protein species) with highly selective anti-phosphoantibodies and the ECL detection system (Fig. 1) [20, 21]. The selectivity and affinity characteristics of the antibodies are of major importance since a large number of "false positive" interactions can be detected, reducing the applicability of such an approach. While excellent anti-phosphotyrosine antibodies are available (PY−20, PY−100 and 4G10 hybridoma clones), better anti-phosphoserine and anti-phosphothreonine antibodies are needed. This is probably also one of the major reasons why tyrosine phosphorylation, which is much less frequent in cells than serine/threonine phosphorylation, is much more studied.

A comparison of the specificity and reliability of commercially available anti-phosphoserine and anti-phosphothreonine antibodies was performed by Grønborg *et al.* [22], who used the best antibodies to perform a large scale differential analysis of phosphorylated proteins in cells treated with calyculin A (a protein phosphatase inhibitor) that led to the identification of several phosphorylation sites and phosphorylated peptides which were not even predicted by dedicated prediction software [22].

Anti-phosphotyrosine and -phosphoserine antibodies have been used to analyze signal transduction pathways [23, 24] in quiescent NIH 3T3 cells stimulated with platelet-derived growth factor (PDGF) which resulted in detection of 260 tyrosine phosphorylated and 300 serine phosphorylated proteins. Recently, a new clone for the detection of phosphotyrosine (PY-KD1) has been generated in order to evaluate a prototype microarry platform for tracking temporal and positional pY changes across the cellular proteome [25].

Although Western blotting allows the detection of very low abundant phosphoproteins, (especially when combined with ECL detection) this method is not very suitable for quantitative analysis due to the variability of the amount of proteins transferred to the membrane.

Direct Staining of Phosphoproteins

The easiest way to analyze the phosphoproteome of a cell, tissue or organism, is to employ reagents designed to selectively detect phosphoproteins directly in SDS-PAGE gels. Since 1970 several methods have been described [26, 27] to stain phosphoproteins directly in gels but the low specificity and sensitivity prevented those methods from being routinely applied. In fact, in some cases such dyes did not specifically discriminate between phosphorylated and unphosphorylated proteins whereas in other cases only phosphoserine and phosphothreonine were detected. Recently, a novel fluorescent-based dye system has been introduced (Pro-Q Diamond^{TM}) [28, 29]. This dye selectively stains phosphorylated proteins employing a very simple experimental protocol. In order to obtain good results two steps are critical: fixing after electrophoretic separation in order to thoroughly remove SDS from the gel to reduce background and destaining because Pro-Q Diamond^{TM} is a reversible dye (i.e. short de-staining times will result in a high background while long

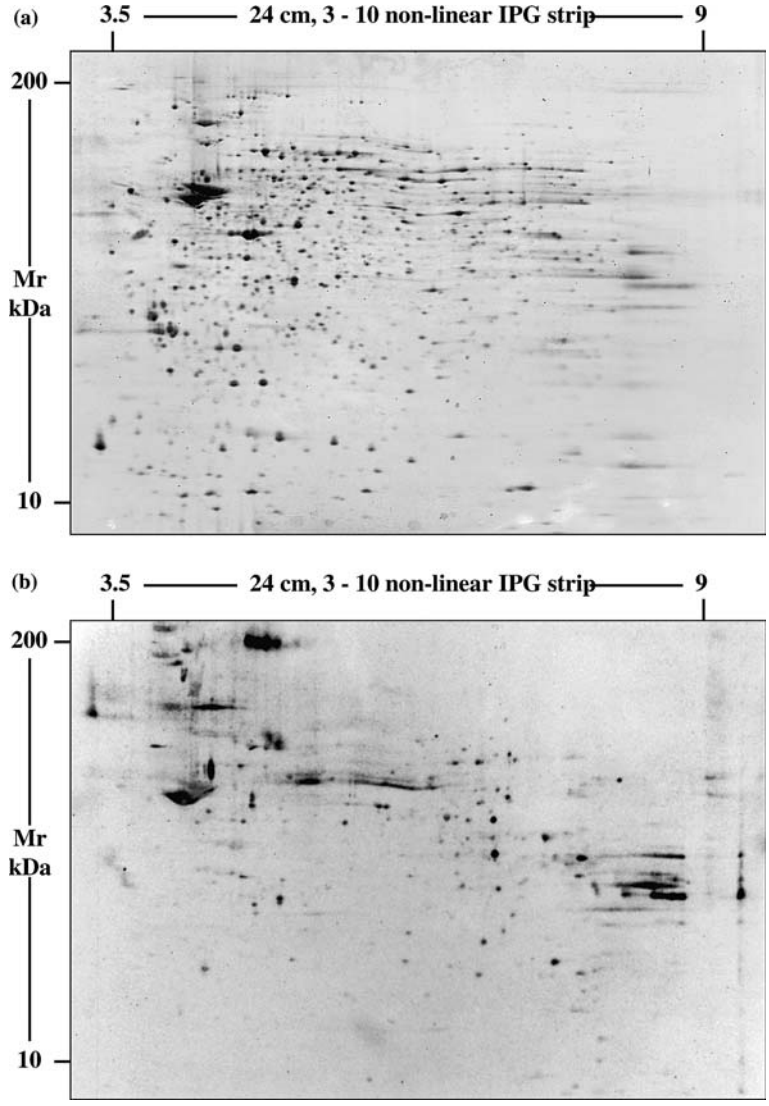

Fig. 1. Comparison of a 2D gel image of rat cortical neuron proteins stained with SYPRO Ruby (a) to detect total proteins and a 2D Western blot (b) of the same sample showing phosphotyrosine proteins detected using anti-phosphotyrosine antibodies and the ECL system.

de-staining times will decrease the number of detected phosphoproteins). The sensitivity of staining depends on the number of phosphorylated residues present in the protein. About 16 ng for pepsin (1 phosphorylated residue) and 2 ng for α casein (8 phosphorylated residues) was the detection limit. Although this sensitivity is quite good, it is not sufficient for comprehensive analysis of the phosphoproteome. Pro-Q Diamond^{TM} which is compatible with mass spectrometry analysis allows also *Multiplex Proteomics* (MP) i.e. detecting phosphorylated proteins and total proteins

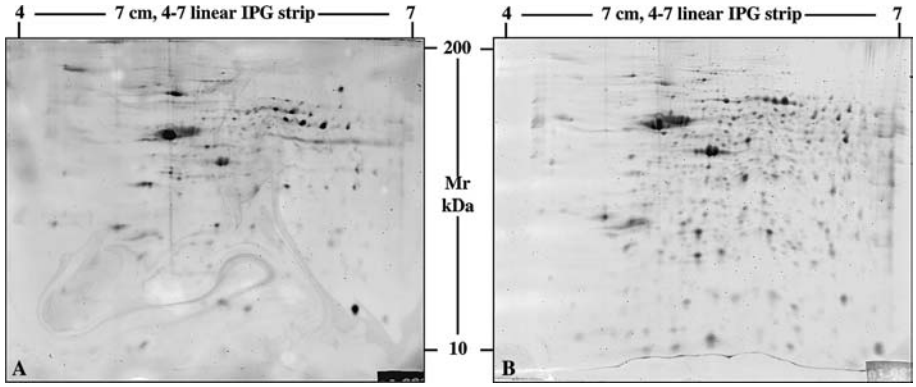

Fig. 2. Direct staining with ProQ-Diamond and its use for Multiplex Proteomics. Rat cortical neurons were analysed by 2D gel electrophoresis and stained with ProQ-Diamond (A) to detect phosphorylated proteins followed by Sypro Ruby staining of the same gel (B) to detect total proteins. It is evident that some spots are more intense when stained with ProQ-Diamond demonstrating a high level of phosphorylation.

(with the SYPRO Ruby dye) on the same gel (Fig. 2). Application of MP to 2D gel electrophoresis experiments allows distinguishing a slightly phosphorylated, high abundant protein from a highly phosphorylated, low abundant protein by comparing the results of the two different colorations. A similar kind of MP was already in use at the beginning of the 1990s using a dual labeling protocol with ^{32}P and ^{35}S to detect phosphorylated and total proteins in the same sample [30].

Detection of Phosphoproteins Employing Protein Phosphatases

Phosphorylation of proteins leads to a change of the net charge of proteins and thus the migration behavior during 2D gel electrophoresis. Accordingly, the charge variation occurring after phosphatase treatment can be exploited to discriminate phosphorylated from unphosphorylated proteins (Fig. 3) [31]. Phosphatase treated and untreated samples are analyzed by 2D electrophoresis and the resulting 2D maps compared in order to detect differences in migration corresponding to phosphorylated proteins. This experimental strategy capitalizes on the specific enzymatic activity of λ-phosphatase (λPPase) on phosphoserine, phosphothreonine, phosphotyrosine and phosphohistidine residues, combined with the high resolution power of 2D gel electrophoresis and has led to the identification of some novel phosphoproteins in cultured rat fibroblasts [31]. An improvement of this experimental strategy was recently achieved by employing DIGE technology (see Westermeier; this issue) for the detection of variations in protein migration after λPPase treatment [32]. Since DIGE eliminates gel-to-gel variability thus allowing separation of two proteomes on the same gel, detection of changes in protein patterns is greatly facilitated (Fig. 4). A detailed analysis of gel images of proteins form rat cortical neurons allowed the identification of novel phosphorylated proteins [32].

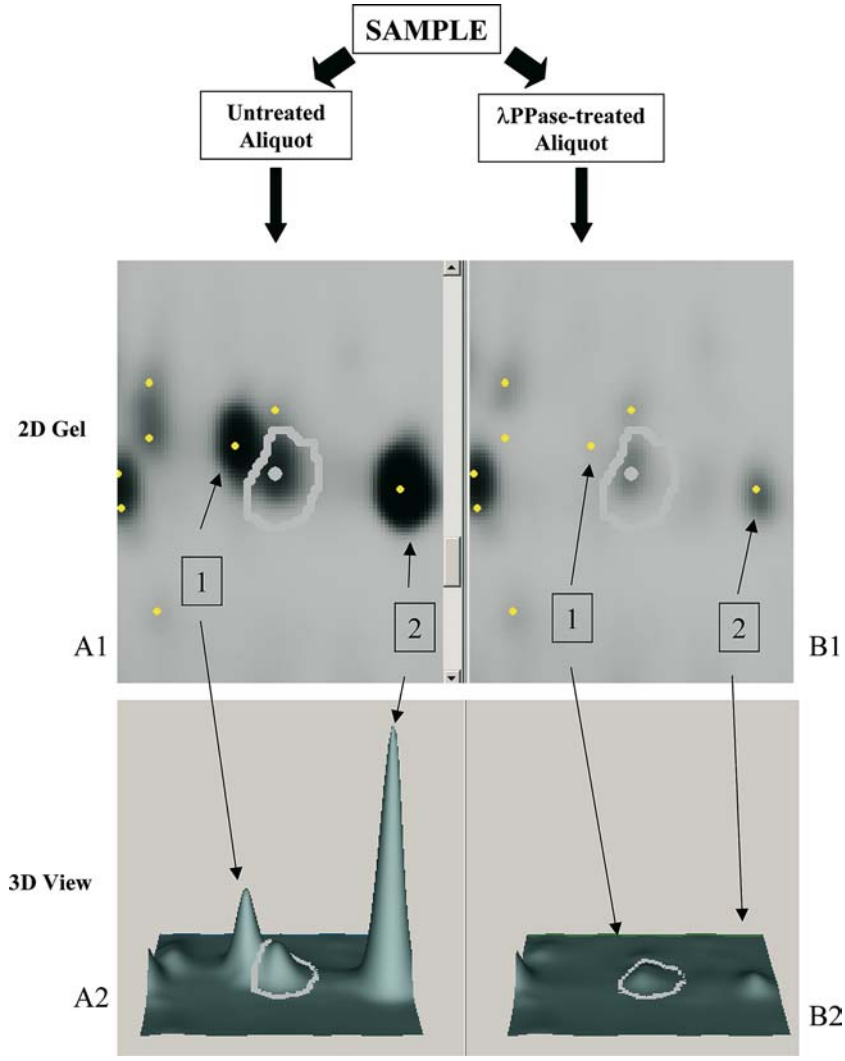

Fig. 3. Detection of phosphoproteins employing λ-phosphatase (λPPase). The starting sample is divided in two aliquots, one of which is treated with λ-PPase. Subsequently, both samples are analysed by 2D gel electrophoresis. Phosphorylated proteins are identified by comparison of the 2D protein patterns exploiting the different migration 'behaviour' due to loss of phosphate groups as can be seen by comparing spots 1 and 2 in parts A1 and B1 of this figure which represent enlarged views of a 2D gel. Comparing these spots in a 3D view (see A2 and B2), differences in spot volume are even more evident.

The phosphatase-based method allows an easy identification of phosphorylated proteins expressed in a sample but it is less suitable for the quantification of variations of protein phosphorylation patterns comparing two different samples. The complexity of the analysis (a minimum of four gels is necessary to study two different conditions) and the variability of the efficacy of enzymatic action are the main reasons for this.

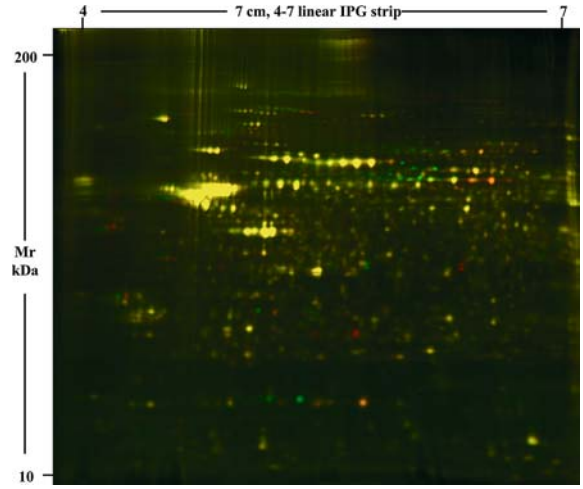

Fig. 4. Detection of phosphoproteins employing λ-phosphatase (λPPase) combined with DIGE technology. As can be seen from the photograph of a 2D DIGE gel, phosphorylated proteins are easily detected since colours reveal the origin of spots. Green spots which are only present in the untreated sample, correspond to phosphorylated proteins whereas red spots represent the unphosphorylated forms of proteins. Yellow spots represent proteins in both samples which are not affected by λ-PPase treatment. Please refer also to figure 3.

ENRICHMENT OF PHOSPHOPROTEINS

Even if the phosphoproteome can be analyzed in total protein lysates as described above, enrichment strategies are essential to elucidate and identify very low expressed phosphoproteins such as many signaling molecules and receptors. Immobilized metal affinity chromatography (IMAC) and immunoprecipitation are the most widely used methods in this regard.

Phosphopeptide and Phosphoprotein Enrichment Using Immobilized Metal Affinity Chromatography (IMAC)

The capacity of some metal ions such as Fe^{3+} to react with phosphoaminoacid is the basis for affinity purification methods routinely applied to reduce sample complexity in order to analyze low-abundance proteins. Already in 1957 [33] it was revealed that phosphoserine has a binding constant above 10^{13} for Fe^{3+} but only in 1975 immobilized metal affinity chromatography was introduced. Since then, IMAC was mainly used for the separation and purification of histidine-tagged recombinant proteins. Its use for the isolation of phosphopeptides after proteolysis prior to MS analysis was introduced in 1997 [34]. In the first publication describing a large scale phosphoproteome analysis using IMAC enrichment from whole cell lysates, more than 1000 phosphopeptides were detected and about 383 phosphorylation sites determined on 216 sequenced peptides in *Saccharomyces cerevisiae* [35]. Binding of unphosphorylated peptides containing high numbers of acidic residues such as

glutamic and aspartic acid (which is the major limitation of this method) was prevented by using methyl esterification of acidic residues prior to IMAC enrichment. At present, this method is almost exclusively used for enrichment of phosphopeptides since its application with phosphoproteins has further disadvantages such as the high dilution of samples and the high salt concentration of the fractions eluted from IMAC columns which need further treatment prior to subsequent analysis by gel electrophoretic techniques [36].

Phosphoprotein Enrichment by Immunoprecipitation

Phospho-specific antibodies can be used to selectively immunoprecipitate phosphorylated proteins depending on the specificity of the antibody. As for Western blotting (see above) anti-phosphotyrosine antibodies are the most reliably and widely used in order to enrich tyrosine-phosphorylated proteins from complex mixtures. After immunoprecitation the phosphotyrosine enriched sample can be analyzed with different analytical methods such as 1D and 2D gel electrophoresis [37, 38]. Also in this case, variations of protein phosphorylation levels are very difficult to characterize unless a combination of particular protein labeling (SILAC) with stable isotopes (^{13}C and ^{15}N) is used [39]. This strategy allowed a quantitative and temporal investigation of tyrosine phosphorylation events of proteins involved in signaling pathways after stimulation with epidermal growth factor [38]. For further experimental details regarding immunoprecipitation refer also to Monti *et al.* (this issue).

DETECTION OF PHOSPHORYLATION BY MS

All hitherto described methods aim to detect and/or enrich the phosphoproteome. Unambiguous proof of phosphoproteins can only be obtained by detection of phosphate group(s) bound to the protein and/or precise determination of their phosphorylation site(s) which is usually accomplished by mass spectrometry.

MS Analysis of Phosphopeptides

Several instrumental techniques have been used to specifically identify phosphopeptides in complex peptide mixtures. The ''stepping voltage'' method [40] combines in source collision-induced dissociation (CID) with negative mode electrospray ionization (ESI) to generate diagnostic ions ($H_2PO_4^-$, PO_3^- and PO_2^-) which are monitored in the low m/z range, and a non-fragmenting mass scan in the high m/z range in a single mass spectrum to identify phosphopeptides. In ''neutral loss scan'' method the experimental setup is designed to monitor the loss of a neutral phosphoric acid moiety (98 amu) from phosphoserine and phosphothreonine containing peptides using a tandem mass spectrometer operated in positive ion mode. ''Precursor ion scan'' method can be used in a similar manner to monitor the loss of a PO_3^- group (79 amu) in negative ion ESI to identify phosphopeptides [41] or of the immonium ion produced at 216 m/z by phosphotyrosine in positive ion ESI [42] to identify phosphotyrosinated peptides. Both, neutral loss and precursor ion methods can also be used in data-dependent analyses to trigger in the same experiment CID to fragment the precursor ion of interest (the phosphopeptide) in

order to obtain sequence information. The major disadvantages of these methods for phosphopeptide identification are the associated increase of instrumental duty cycle time and the low sensitivity, particularly in negative ion ESI, which make them less attractive for analysis of highly complex peptide mixtures which result from large scale proteomics experiments, for instance.

All reported methods aiming at instrumentally sorting phosphopeptides out of their unphosphorylated counterparts face the following problems: phosphorylation is often sub-stoichiometric; the phosphopeptides are present in lower abundance than other peptides from the protein they belong to; MS response of a phosphopeptide may be suppressed relative to its unphosphorylated counterpart and this suppression tends to be enhanced in the presence of other unphosphorylated peptides; negative charge interference in detection induced by the phosphate group becomes even worse when multiple phosphate groups exist in the same peptide. For these reasons it is usually necessary to enrich phosphopeptides prior to MS analysis by affinity chromatography, immunoprecipitation or strong anion exchange (SAX) chromatography [43] (see above).

In order to identify the phosphorylation site(s) of a given phosphopeptide without any prior knowledge of its aminoacid sequence, a proper isolation of the phosphopeptide of interest is necessary. Phosphorylation site(s) identification can be achieved by performing a product ion scan in a tandem (in space or in time) mass spectrometer operated in positive ion mode. With this technique precursor ions are selected according to multiple criteria (such as abundance, mass, charge etc.), fragmented and the resulting ion products are scanned to give a fragmentation spectrum from which the peptide sequence can be inferred. Product ion data dependent scan provides both, identification of the phosphopeptides and determination of the modified residues within the sequences, even in very complex phosphopeptide mixtures, and allows the characterization of hundreds of phosphorylation sites in one experiment [43, 44].

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