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# ANALYSIS OF PROTEIN PHOSPHORYLATION BY MASS SPECTROMETRY

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***Abstract:*** Characterization of protein modification by phosphorylation is one of the major tasks that need to be accomplished in the post-genomic era. Phosphorylation is a key reversible modification occurring mainly on serine, threonine and tyrosine residues that can regulate enzymatic activity, subcellular localization, complex formation and degradation of proteins. Phosphorylation, the process by which a phosphate group is attached to a pre-existing protein, is an evolutionarily and metabolically cheap way to change the protein's surface and properties. Analysis of these modifications presents formidable challenges but their determination generates indispensable insight into biological function. The combination of function- or structure-based purification of modified 'subproteomes', such as phosphorylated proteins or modified membrane proteins, with mass spectrometry is proving particularly successful. To map modification sites in molecular detail, novel mass spectrometric peptide sequencing and analysis technologies hold tremendous potential. Mass spectrometry based methods are the methods of choice for the identification of phosphorylation sites; however biochemical pre-fractionation and enrichment protocols will be needed to produce suitable samples in the case of low-stoichiometry phosphorylation. In this review, we will examine and catalogue how proteomics techniques can be used to answer specific questions related to protein phosphorylation. Hence, we will discuss the different mass spectrometric methods for enrichment of phospho-proteins and -peptides, and then the various technologies used for their identification and quantitation.

***Keywords***: Protein phosphorylation; Mass spectrometry.

## 1. Introduction

Phosphorylation, the process by which a phosphate group is attached to a pre-existing protein, has been known to be a significant regulatory mechanism in many organisms controlling a wide variety of biological functions (Huberd *et al.*, 1993; Manning *et al.*, 2002; Koch *et al*., 1991; Manning *et al.*, 2002). The reaction is catalyzed by a set of enzymes called kinases that form one of the largest protein families known. As the reaction is readily reversed by another group of enzymes called phosphatases, phosphorylation is a pivotal regulatory mechanism that plays critical roles in the regulation of many metabolic pathways and cellular processes, including cell cycle, growth or differentiation (Hunter *et al.*, 1995). In fact, nearly 2% (>2000 genes) of the human genome encodes for protein kinases, with an estimated 30% of all proteins thought to exist in phosphorylated forms (Manning *et al.*, 2002).

The determination of phosphorylation sites is the basis for a deeper understanding of cellular regulation and will allow conclusions to be made about the enzymes involved in specific regulatory pathways. Because aberrant phosphorylation events are known to occur in many diseases, including various types of cancer, this holds huge promise for the definition of new drug targets. Phosphorylation, from the history of its discovery to methodological advances and biological aspects, is covered by a number of excellent and exhaustive reviews (Hunter *et al.*, 1995, Cohen *et al.*, 2002; Reinders *et al.*, 2005).

Despite phosphorylation being acknowledged as a crucial modification involved in many cellular

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events, determining the sites of phosphorylation on proteins is not a routine task. Traditional methods for the analysis of phosphoproteins include radioactive labeling of the protein with $^{32}P$ to monitor phosphorylation, and Edman degradation chemistry on phosphopeptides to localize the site of phosphorylation (Yan *et al.*, 1998). Although some success has been reported with these methods, these techniques have the disadvantages of being relatively time consuming and laborious and requires large amounts of purified protein. In addition, Edman degradation does not work on proteins/peptides with blocked N-termini (i.e., N-terminal acetylation). Recently, mass spectrometry based methods have emerged as powerful and preferred tools for the analysis of post-translational modifications including phosphorylation due to higher sensitivity, selectively, and speed compared to most biochemical techniques (McLachlin *et al.*, 2001; Annan *et al.*, 2001; Mann *et al.*, 2002). Mass spectrometry approaches for the analysis of phosphorylation sites have mostly relied on using an instrument capable of performing tandem mass spectrometry (MS/MS) experiments to determine the sequence and location of phosphorylation sites on peptides. In this review we will discuss about different mass spectrometric methods and also we will address the difficulties that arise during the analysis of low abundant phosphopeptides present in a complex mixture.

## 2. Mass Spectrometric Methods of Analysis of Phosphoproteins

### *Use of a Hybrid Triple-quadrupole/linear-ion-trap mass Spectrometer*

The mass spectrometer needs to be coupled on-line with nanoflow reverse phase-C18 HPLC. An additional pump capable of generating nanoliter flow rates from a 1 ml syringe is also required for the analysis. The LC-MS/MS analysis of phosphopeptides consists of 2 steps: A) detection of phosphorylated peptides by a selective scan function, i.e., a precursor ion scan for *m/z* 79 ($PO_3^{3-}$) in negative mode, where the first quadrupole Q1 scans the *m/z* range of possible intact phosphopeptide precursors. The second quadrupole Q2 serves as a collision cell where precursors are fragmented by collisionally induced dissociation and Q3 is set to continually monitor production of the marker fragment *m/z* 79 ($PO_3^{3-}$) and B) MS/MS analysis of the phosphopeptide sequence in positive mode. On hybrid triple quadrupole/linear ion trap mass spectrometers these two steps can be carried out in a single integrated experiment (Hager *et al.*, 2002; Le Blanc *et al.*, 2003; Williamson *et al.*, 2006).

A neutral loss scan is performed on a triple quadrupole instrument by scanning the ionized peptides or proteins through the first quadrupole (Q1). The ions are then fragmented in the second quadrupole (Q2) through low-energy collisions with a collision gas in the collision cell. The third quadrupole is then scanned with a fixed offset to Q1, specific to the modification and the charge state of the precursor peptide, transmitting the precursor ion minus the modification, i.e., the product ion of the neutral loss, to the detector (Schlosser *et al.*, 2001). This neutral loss analysis identifies the presence of a modification as well as the associated precursor ion. A precursor-ion scanning method that can be performed in the positive mode has recently been developed for the specific detection of phosphotyrosine-containing peptides (Steen *et al.*, 2001; Steen *et al.*, 2001). This method is based on the ability to selectively detect the immonium ions of phosphotyrosine residues that have an m/z value of 216.043. Immonium ions are generated by double cleavage of the peptide backbone. Highly sensitive MRM experiments are used to trigger dependent acquisition of product ion scans (MS/MS) where triple quadrupole instruments are capable of performing MRM experiments (Bruins *et al.*, 1987). This involves using the first quadrupole in resolving mode (RF and DC voltages applied) so that a specific *m/z* is allowed to pass. These ions are accelerated into the collision cell (a quadrupole used in RF mode only) where they collide with gas molecules and fragment (CID). The third quadrupole is also operated in resolving mode so that it passes only one of the fragment ions from the target compound. When combined with chromatography, this makes the mass spectrometer a highly specific mass detector for the target molecule. It is highly unlikely that isobaric compounds that may co-elute with the

target compound will also have an identical fragment mass. This makes MRM methods ideal for quantifying compounds in a complex biological matrix. These MRM transitions are used to trigger dependent linear ion trap scans: enhanced resolution and enhanced product ion scans. An EPI scan is similar to a traditional product ion scan on a triple quadrupole instrument, in that the fragments are generated by accelerating ions into a collision cell. The difference, however, is that an EPI scan uses the linear ion trap to trap the resulting fragments and perform a mass scan (Hager *et al.*, 2003). If such an instrument is not available though, the two parts can also be carried out independently on separate equipments.

Special consideration should be given to the choice of solvents and organic modifiers that are used for the reverse-phase separation and LC-MS/MS analysis. For a single experiment, analysis conditions are needed that allow for both negative and positive mode electrospray ionization. A weak acidic modifier has been shown to enable electrospray analysis of phosphopeptides in both polarities at comparable sensitivity. From a chromatographic point of view, trifluoroacetic acid is the organic modifier of choice as it provides the best separation because of its strong ion-pairing properties. As TFA in practice is severely detrimental to negative-mode electrospray ionisation, formic acid instead is usually chosen as the organic modifier. Another challenge implied with negative-mode electrospray ionization is the possibility of high-voltage corona discharge, or "arching," leading to corrosion of the sprayer needle and irreproducible ionisation conditions. The post-column addition of isopropanol as a "make-up solvent" via a T-piece reduces the voltage necessary to achieve ionisation in negative mode, thus significantly reducing the danger of corona discharge. Isopropanol can be premixed with organic modifier and acetonitrile to achieve constant modifier concentration and reduce the otherwise high back-pressure of the more viscous isopropanol.

### *Use of MALDI TOF TOF Mass Spectrometer for Phosphopeptide Analysis*

Sample is usually dissolved in 50% ACN in water containing 0.1% TFA and mixed with alpha cyano 4-hydroxycinnamic acid (5mg/ml) in 1:1 (v/v) ratio. MALDI analysis of phosphopeptide is generally carried out in the reflector mode where in-gel trypsin digested sample analyses are carried out using a mass range from 700 to 4000 m/z. The threshold criteria for MS/MS triggering are generally set as follows: mass range, 750–4000 Da; minimum signal-to-noise ratio, 15; precursors/spot, 50. CID is generally performed at a collision energy of 1 keV. Phosphopeptides may be identified simply by examination of the loss of 98 or 80 Da from the precursor mass in the MS/MS spectrum. Data can be analyzed using a search engine like Mascot (Matrix Science) against a list of protein sequences downloaded from the National Center for Biotechnology Information (*www.ncbi.nlm.nih.gov*). Searches allow up to two missed cleavages for a given enzyme and MS and MS/MS tolerances of 0.4 Da. Variable modifications include carbamimidomethylation of cysteine, deamidation of asparagine and glutamine, oxidation of methionine, phosphorylation of serine, threonine, and tyrosine. It is sometimes possible to differentiate between serine or threonine and tyrosine phosphorylation on phosphopeptides using MALDI-TOF. In the positive ion mode, the tendency for serine or threonine phosphopeptides to show a predominant neutral loss of 98 Da (owing to $H_3PO_4$ loss) as compared with a loss of 80 Da (owing to $HPO_3$ loss) can be used to differentiate them from tyrosine phosphopeptides, which generally show only a loss of 80 Da (Annan *et al.*, 1996).

### *Use of Ion trap for Phosphopeptide Analysis*

Tandem mass spectrometry of phosphopeptide ions, using CAD, often produces product ions dominated by the neutral loss of phosphoric acid. A method, termed Pseudo $MS^n$, for phosphopeptide ion dissociation in quadrupole ion trap mass spectrometers utilizes induction of collisional activation of product ions - those resulting from neutral loss (es) of phosphoric acid following activation of the precursor ion. Thus, the principal neutral loss product ions are converted into a variety of structurally informative species. Since product ions from both the original precursor activation and all subsequent neutral loss product activations are

simultaneously stored, the method generates a "composite" spectrum containing fragments derived from multiple precursors.

### *Use of QqTof for Phosphopeptide Identification*

Using high-resolution instruments, such as those operating on the QqTof principle (with a Q2-pulsing function), the immonium ion of tyrosine can be easily distinguished from other peptide fragment ions. Phosphopeptides that are barely detected in the original MS scan can be easily identified using this approach. Once the phosphotyrosine-containing peptides are located, they can be sequenced in the product ion MS/MS mode without any need for switching polarity of the ion source. This scanning method is sensitive and tyrosine phosphorylation sites from subpicomole amounts of gel-separated proteins have been successfully identified. Unfortunately, owing to the lability of phosphoserine and phosphothreonine residues, this method cannot be applied for identification of phosphorylation events involving these amino acids. In the MS/MS spectrum, a spacing of 69 Da (owing to dehydroalanine) or 83 Da (owing to dehydroaminobutyric acid) indicates the exact location of phosphorylated serine and threonine residues, respectively. The drawbacks of this method are the incidence of false-positive signals as well as the fact that the charge state of the phosphopeptide has to be known in advance. It is also possible to use QqTof mass spectrometers for such experiments in an automated data-dependent acquisition mode.

## 3. Detection of the Site of Phosphorylation

### *Use of CID*

Different phosphorylation sites could be confidently assigned by high quality MS/MS spectra, even when the peptide contained multiple possible sites. The MS/MS data obtained for the peptide containing phosphate groups attached at different sites can give some clue in predicting the site of phosphorylation. In this, the observed b (b-$H_3PO_4$) and y (y-H3$PO_4$) ion series allow for assignment of both the peptide sequence and the site of phosphorylation. A situation can be assumed where the b-series fragments from $b_n$ down to a $b_{n-m}$ ion all show the ability to lose $H_3PO_4$ (-98 amu), while the $b_{n-m-1}$ ion is present only in its nonphosphorylated state, indicating that it is the n-m-1th residue from the N-terminus that is phosphorylated. In addition, the mass difference between the $b_{n-m}$ and $b_{n-m-1}$ ion is consistent with the mass of a phosphothreonine residue. Hybrid Qtrap instrument can produce high spectral quality and completeness of the collision cell fragmentation pattern, diminishing the need for additional $MS^3$ experiments to determine the sequence of the peptide and the site of phosphorylation on most phosphopeptides.

### *Use of ETD*

The precise determination of the location of phosphorylation is crucial to the understanding of the regulation mechanisms. Mass spectrometry analysis combining database search has become the choice of many proteomics laboratories. However, these MS-based techniques are not without limitations and may produce false positive identifications due to insufficient amount of peptide sequence MS/MS information. In some cases loss of phosphate groups occurred preferentially in CID and could not provide information on the phosphorylation sites. McLafferty et. al., introduced an MS-based technique called electron capture dissociation in 1998 which provided a way to obtain MS/MS spectra complementary to CID data. Recently, a variation of the ECD technique has been developed and it has been shown to minimize false positives when using CID and database search for identification. The novel technique has been referred to as ETD, which can provide similar results to the ECD process. An additional observation for the ETD technique is that the ion-molecule collision process is much gentler than the CID process and the phosphate remains attached to the amino acid during the collision process, which allows the specific location of the phosphate to be observed. Analyte cations are first accumulated in the ion trap and the selected precursor is isolated. The negative ions namely fluoranthene are then pulsed into the ion trap. This Fluoranthene anion radical is generated and used as the reactant for ETD. Electron transfer and fragmentation through ion-ion interaction occur in the ion trap and the fragments are scanned. However, ETD is better suited for highly charged

species like triple or higher charge states since higher the charge states, greater will be the internal energy of the molecule of same mass and less external energy is required to break the molecule to its fragments. Doubly charged peptides are more difficult to fragment using ETD technique and in such case CID seems to be more effective.

## 4. Quantitative Phosphoproteome Analysis

Although defining the precise sites of phosphorylation yields important information that can be related to the biological function of phosphoproteins, the quantitative evaluation of the extent of phosphorylation at a given site or in relation to other phosphorylation sites within the same protein, is critical for the interpretation in terms of biological significance. This is why quantitation of phosphorylation is particularly important. A given protein might be in more than one signaling pathway with different stimuli inducing overlapping patterns of phosphorylation. That means a given site might not be phosphorylated at all, phosphorylated in a minority of molecules or, in an extreme case, on all the molecules of that protein. When a population of molecules from unsynchronized cells is analyzed, this situation corresponds to detection of unphosphorylated, weakly phosphorylated or highly phosphorylated peptides containing the residue. Similarly, the ratio of phosphorylation of a protein on multiple residues might be crucial for its function. Mass spectrometric approaches to quantitative phosphorylation generally use stable isotope dilution whereby two samples are differentially labeled with mass-encoded tags such that the samples can be mixed and analyzed simultaneously.

Each phosphopeptide thus appears as two peaks in the mass spectrum, and the relative abundances of the peaks reflect the amount of the phosphopetide in each sample. This can be accomplished by metabolic labeling of proteins in cell culture (Oda *et al.*, 1999; Ibarrrola *et al.*, 2003) or subsequent chemical labeling of functional groups such as peptide N termini or C termini (Liu *et al.*, 2002; Yao *et al.*, 2001). *In vitro* labeling (Gygi *et al.*, 1999) is used to quantitate proteins by labeling cysteine residue by a method called isotope-coded affinity tagging. A variation to this strategy is to introduce a biotin tag into phosphoserine and phosphothreonine residues by β-elimination and Michael addition reaction (Goshe *et al.*, 2002). Other methods take advantage of this reaction for attaching different tags for a quantitative analysis of phosphorylation without any enrichment (Goshe *et al.*, 2002; Weckwerth *et al.*, 2000; Adamczyk *et al.*, 2002). Several other methods that use chemical moieties to make peptides heavier have also been developed (Munchbach *et al.*, 2002; Zhang *et al.*, 2001; Cagney *et al.*, 2002). Other methods use the treatment with phosphatase in conjunction with isotopic labeling. In this approach, the quantitative phosphorylation in individual samples is measured by dividing a sample in two, labeling each with the light/heavy forms of a mass tag and treating one sample with phosphatase before recombining the fractions (Zhang *et al.*, 2002; Hegeman *et al.*, 2004). An *in vivo* labeling has been used to label yeast proteins by growing them in 15N-labeled media that labels all proteins without any further manipulation and thus to quantitate the extent of phosphorylation (Oda *et al.*, 1999). An alternative approach is to label proteins *in vivo*, designated stable isotope labeling by amino acid in cell culture, which uses amino acid containing a stable isotope, has been developed (Ong *et al.*, 2002).

## 5. Detecting Changes in Site Specific Phosphorylation

A targeted approach using MRM, which is a very unique technique, is necessary to specifically determine the relative changes in phosphorylation sample set. A computer program, namely Multiple Reaction Monitoring initiated detection and sequencing, was developed to simplify the process of developing an MRM method based on the primary amino acid sequence. The program performs a theoretical enzymatic digest of the protein, selects peptides containing a specified type of phosphorylation (S/T or Y), and calculates the Q1 and Q3 *m/z* values for various charge states and fragment ions. A set of unique MRM transitions can be carefully designed to include the neutral loss and characteristic y-$H_3PO_4$ and b-$H_3PO_4$

fragments unique to the position of the phosphoserine to target each possible phosphopeptide and any potential enzymatic miscleavages due to positioning of phosphorylation groups. The overlay of MRM transitions localized to the eluted peak profile and the simultaneous acquisition of a full scan MS/MS spectrum correctly identifies the phosphopeptide and validates the specificity of the MRM assay. Therefore using this workflow strategy, samples of different states can be analyzed for relative quantitation.

## 6. Difficulties Encountered during Phosphoproteome Analysis

Despite the advances in mass spectrometric identification of phosphopeptides, several difficulties still remain. Thus, mass spectrometry technique suffers from the often low stoichiometry of phosphorylation leading to low signal intensities that tend to disappear into the background. Second, the signals in the mass spectrometer arising out of phosphopeptide have also been observed to be lower than its non-phosphorylated version. Lastly, the MS/MS properties of phosphopeptides undergoing low energy CAD fragmentation are, in most cases, challenging to interpret. Several approaches have been reported to deal with the stoichiometry challenge (Reinders *et al.*, 2005):

- chemical replacement of the phosphate group by other functionalities that enhance ionization efficiency and MS/MS fragmentation behavior, e.g., by elimination and subsequent Michael addition. Because of incomplete reaction and necessity of purification of the product this approach usually requires an increased amount of peptide.
- affinity enrichment of phosphorylated species, e.g., by IMAC on $Fe^{3+}$ or $Ga^{3+}$ matrices. $ZrO_2$ or $TiO_2$ have also been successfully used for this purpose. However, the enrichment is rarely specific and acidic peptides are likely to be enriched as well.
- alternatively, peptides phosphorylated on tyrosine can be purified using anti-P-Tyr-antibodies. Antibodies to detect P-Ser and P-Thr with high specificity are not available even though these form the bulk of cellular phosphorylation sites.

The phosphorylation-specific analytical method would have to introduce some sort of filter that will allow systematic screening for phosphorylated compounds.

## 7. Precautions Required during Sample Preparation

Preparation of a biological sample should include phosphates and protease inhibitors in the cell lysis buffer to minimize de-phosphorylation and degradation of the phosphoprotein, respectively. Usually, 2 mM sodium vanadate or 50 µM calyculin and protease inhibitor cocktail (2.5 µg/mL leupeptin, 2.5 µg/mL pepstatin, 2.5 µg/mL aprotinin, 2.5 µg/mL antipain, and 2.5 µg/mL chymostatin) are added to the cell lysis buffer before processing the protein samples.

## 8. Phosphoproteome Enrichment

A major obstacle in the study of phosphorylated proteins is that they comprise only a small fraction of the total protein in a cellular lysate. Thus, many phosphoproteins cannot be identified in a cell extract. As a consequence, a number of techniques have been developed to partially purify or to preferentially enrich phosphopeptides from a complex mixture namely IMAC, specific chemical derivatization and immunoprecipitation.

### *Immobilized Metal Affinity Chromatography*

The use of (miniaturized) IMAC columns was developed for the enrichment of phosphopeptides and exploits the high affinity of a phosphate group to cations such as $Zn^{2+}$, $Fe^{3+}$, and $Ga^{3+}$ (Andersson *et al.*, 1986; Posewitz *et al.*, 1999). IMAC has been successfully used either in off-line or on-line formats for the detection of phosphopeptides using MS (Posewitz *et al.*, 1999; Nevile *et al.*, 1997; Stendballe *et al.*, 2001; Trinidad *et al.*, 2006). Although this approach is useful, the main disadvantage is the co-elution of peptides having high content of acidic amino acids, histidine or cysteine (Chicz *et al.*, 1990). In addition, multiple phosphorylated peptides are also enriched and the recovery of

phosphopeptides appears to be largely dependent on the type of metal ion, column material and the elution procedure used. Esterification of acidic residues before IMAC enrichment has permitted a much higher specificity (Ficarro et al. 2002) by eliminating the non-specific binding and enabling the determination of a large number of phosphorylation sites in proteins from whole-cell lysates. Moreover, this approach requires that all phosphoproteins in a cell lysate be analyzed to detect those that undergo changes.

Recently Larsen and colleagues reported the use of titanium dioxide ($TiO_2$) as a potent chelator for phosphopeptides which can be used upstream of mass spectrometry sequencing (Larsen et al., 2005). Authors reported a highly selective phosphopeptide enrichment method using $TiO_2$ columns. Peptide samples are combined with dihydroxybenzoic acid to enhance specific binding of phosphorylated peptides eliminating competition from non-phosphorylated peptides. In comparison with IMAC, this procedure proved superior in terms of specificity and sensitivity of phosphopeptide binding (Larsen et al., 2005). In addition, the $TiO_2$ purification was fast and can be used in combination with HPLC coupled to either MALDI-MS/MS or ESI-MS/MS.

### *Specific Chemical Derivatization*

Another way to isolate phosphorylated proteins or peptides is to take advantage of the unique chemistry of phosphoamino acids in peptides. Till date, two methods have been reported that use chemical modification of the phosphate moiety as a strategy to enrich phosphopeptides from complex mixtures. The first method uses a β-elimination reaction that occurs when phosphoserine and phosphothreonine residues are exposed to strong alkaline conditions (Oda *et al.*, 2001; Meyer *et al.*, 1993). The resulting dehydroalanine or dehydroaminobutyric acid residues can be detected, after chemical modification with EtSH, using tandem mass spectrometry (MS/MS) (Resing *et al.*, 1993; Jaffe *et al.*, 1998). The same strategy can be used to attach biotinylated moieties to purify phosphoproteins or peptides. Thus, EtSH is used as a nucleophile, which provides a new reactive thiol group serving as a linker for attachment of a biotinylated affinity tag (Oda *et al.*, 2001). However, an undesired side effect involving side chains on cysteine and methionine residues can still occur. To overcome this problem the sample is first treated with performic acid, leading to oxidation of these residues, thereby inactivating them. The second method is an alternate strategy to isolate phosphotyrosine-containing peptides in addition to those containing phosphoserine and phosphothreonine residues (Zhou *et al.*, 2001). The main feature of this method is that a transient carbodiimide (ethyl carbodiimide) catalyzes the addition of cystamine to phosphate moieties, which then allows purification of phosphopeptides on glass beads containing immobilized iodoacetyl groups. Elution of phosphopeptides is performed by cleavage of phosphoroamidate bonds by TFA. The major disadvantage of these two methods is that the current chemistries require significant amounts of protein or peptide for identification by MS to be successful. In addition, the specificity of these methods has not been confirmed yet. Several proteins were isolated using these methods that are not known phosphoproteins. Nevertheless, these approaches are promising and could be coupled to other fractionation steps to improve the overall recovery of low-abundance proteins.

### *Immunoprecipitation*

Antibodies are routinely used to immunoprecipitate specific proteins. Consequently, phospho-specific antibodies can be used to selectively immunoprecipitate phosphorylated proteins depending on the specificity of the antibody. As for Western blot (see above) antiphosphotyrosine antibodies are the most reliably and widely used in order to enrich tyrosine-phosphorylated proteins from complex mixtures. These antibodies can be used to immunoprecipitate, and therefore to enrich, tyrosine phosphorylated proteins from complex mixtures of proteins such as cell lysates. Although these antibodies have been relatively effective at enriching and identifying low-abundance tyrosine phosphorylated proteins, it has been showed that the existing immunopurification protocols for phospho-tyrosine (pY) containing peptides have a poor selectivity (Zhang *et al.*, 2006). Recently, a method using a pY antibody

for pY peptide purification from an enzymatically digested protein extract combined with LC-MS/MS was applied to large scale pY analysis in cancer cells (Hager *et al.*, 2003). Currently, there are no antibodies that are suitable for enriching proteins that are phosphorylated on serine or threonine residues, and thus these proteins must be enriched using the alternative methods described above.

## 9. Summary

Protein phosphorylation is widely prevalent posttranslational modification that is very critical for many cellular processes like cell differentiation, cell growth and migration. However, detection of phosphorylated proteins is extremely challenging mainly due to their low abundance and stoichiometry. The phosphoproteome consists of the entire complement of phosphorylated proteins in cells, which is mapped or analysed not only for the identification of phosphorylation sites, but also for the quantitation of phosphorylation events in signal transduction pathways in a time dependent manner. The analysis of the phosphoproteome relies on techniques such as radioactive labeling, mass spectrometry and Edman-sequencing, usually coupled to upstream enrichment steps, which are used to increase the amount of phosphorylated species in the monitoring step. Current techniques for the analysis of the phosphoproteome have been reviewed in this paper. Many advances have been made on the enrichment and detection of phosphoproteins, but these processes are still not straightforward for several reasons: the stoichiometry of phosphorylation is usually very low, the phosphorylated sites on proteins vary, signalling molecules are present at low abundance within cells, minor phosphorylation sites might be difficult to identify due to a very limited dynamic range of most analytical techniques to study phosphorylation, and precautions need to be taken to inhibit phosphatase activity during preparation and purification steps of cell lysates. The enrichment in phosphoprotein or phosphopeptide content, prior to the respective analysis, circumvents some of the challenges presented. Moreover, these approaches help to understand the intricate cellular networks and regulation of pathways, as well as identifying new proteins involved in these processes that might reveal potential therapeutic strategies.

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## *Abbreviations*

MS, mass spectrometry; CID, collision induced dissociation; HPLC, high performance liquid chromatography; LC, liquid chromatography; MRM, multiple reaction monitoring; EPI, enhanced product ion; TFA, trifluoroacetic; CAD, collision-activated dissociation; MALDI TOF, matrix assisted laser desorption ionization time of flight; ETD, electron transfer dissociation**;** ECD, electron capture dissociation; ICAT, isotope-coded affinity tagging; SILAC, stable isotope labeling by amino acid in cell culture; IMAC, immobilized metal affinity chromatography; ESI, electrospray ionization; EtSH, ethanethiol.

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