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Journal of Chromatography B, 849 (2007) 163–180
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

Analytical methodologies for the detection and structural characterization of phosphorylated proteins☆

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Received 22 May 2006; accepted 28 June 2006
Available online 7 August 2006

Abstract

Phosphorylation of proteins is a frequent post-translational modification affecting a great number of fundamental cellular functions in living organisms. Because of its key role in many biological processes, much effort has been spent over the time on the development of analytical methodologies for characterizing phosphoproteins. In the past decade, mass spectrometry-based techniques have emerged as a viable alternative to more traditional methods of phosphorylation analysis, providing accurate information for a purified protein on the number of the occurring phosphate groups and their exact localization on the polypeptide sequence. This review summarizes the analytical methodologies currently available for the analysis of protein phosphorylation, emphasizing novel mass spectrometry (MS) technologies and dedicated biochemical procedures that have been recently introduced in this field. A formidable armamentarium is now available for selective enrichment, exaustive structural characterization and quantitative determination of the modification degree for phosphopeptides/phosphoproteins. These methodologies are now successfully applied to the global analysis of cellular proteome repertoire according a holistic approach, allowing the quantitative study of phosphoproteomes on a dynamic time-course basis. The enormous complexity of the protein phosphorylation pattern inside the cell and its dynamic modification will grant important challenges to future scientists, contributing significantly to deeper insights into cellular processes and cell regulation.

Keywords: Phosphorylation; Proteomics; Electrophoresis; Mass spectrometry

Contents

☆ "This paper is part of a special volume entitled "Analytical Tools for Proteomics", guest edited by Erich Heftmann."

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doi:10.1016/j.jchromb.2006.06.033

1. Introduction

Protein phosphorylation is one of the most abundant post-translational modifications and a key biological process regulating a great number of essential biochemical reactions in living organisms. This reversible reaction may affect 3D structure and function of proteins, e.g. substrate recognition and enzymatic activity [1,2] and, modulating protein localization, may control complex formation and degradation. Accordingly, it has major influence on various fundamental cellular functions including metabolic maintenance, gene expression, cell division, signal transduction, cytoskeletal regulation and apoptosis [3,4].

Although examples of protein *N*-phosphorylation on His/Lys residues, *S*-phosphorylation on Cys and acyl-phosphorylation on Glu/Asp residues have been reported in literature for different organisms, *O*-phosphorylation of Ser, Thr and Tyr residues is the most frequent reaction [5]. This process has an essential role in eukaryotic cells life [1–4], as demonstrated by the occurrence of 510 protein kinases and 100 phosphoprotein phosphatases in the human genome (2% of the total coding sequences) [6]. In fact, it has been estimated that 40% of all proteins in a eukaryotic cell are phosphorylated at any one time, and there are more than 100,000 predicted phosphorylation sites in mammalian proteomes [7]. This evaluation well parallels with the exponential number of experimentally verified phosphorylated sites daily annotated in dedicated databases. For instance, the Phospho.ELM database (phosphor.elm.eu.org) currently lists 5315 ascertained *O*-phosphorylation sites for 1805 proteins derived from eukaryotes, the human protein reference database (www.hprd.org) contains 3652 reported phosphorylation sites on 1240 human proteins, and PhosphoSite (www.phosphosite.org) reports 6084 non-redundant *O*-phosphorylation sites on 2430 human and mouse proteins. However, a careful analysis of all database entries, together with an experimental quantitation of the phosphoamino acid content, evidences a value of 1800/200/1 for pSer/pThr/pTyr ratio in vertebrate cells, demonstrating that Ser and Thr residues undergo phosphorylation more often than Tyr. These data contrast with the comparable number of kinases and phosphatases having Ser/Thr specificity with respect to Tyr ones [6] and suggest a higher gain for Tyr phosphorylation-based signalling processes.

Because of its key role in many biological processes, much effort has been spent on the development of methods for characterizing protein *O*-phosphorylation. Traditional methodologies involve incorporation of ^{32}P into proteins by procedures based on using of radioactive ATP [8–10]. Radioactive proteins are detected during subsequent fractionation (e.g. different types of liquid chromatography or two-dimensional gel electrophoresis), identified and analyzed for their phosphoamino acid content [10]. Phosphorylation site(s) are determined by protein proteolytic digestion, electrophoretic/chromatographic separation of radiolabeled peptides, and further analysis by Edman sequencing [11,12]. However, these techniques are tedious, require massive quantities of phosphorylated proteins and involve the use of significant amounts of radioactivity. Although full characterization of phosphoproteins remains an analytical challenge, in the past decade mass spectrometry (MS) has emerged as a viable alternative to more traditional methods of phosphorylation analysis [13–15]. In fact, MS can easily provide accurate information on the molecular mass of an intact phosphorylated polypeptide; these data, together with calculation of the theoretical mass of the unmodified species and/or treatment with phosphatase, allow determination of the average number of attached phosphate groups [16–19]. A detailed analysis of the sites and stoichiometry of phosphorylation is generally based on further MS and MS/MS analysis of peptide fragments generated by digestion of the phosphoprotein by site-specific proteases [16–19]. Moreover, depending on their high sensitivity, MS techniques are fully compatible with the poor protein amounts recoverable by electrophoretic/chromatographic separations of biological fluid/cell lysate samples.

In general, a comprehensive analysis of proteins phosphorylation (phosphoproteomics) involves identification of all phosphoproteins, localization on polypeptide sequence of the amino acids that are phosphorylated and quantitative evaluation of their relative phosphorylation degree. Accordingly, phosphoproteome analysis is a huge and challenging task. In fact, the stoichiometry of protein phosphorylation within the cell may be relatively low [20]; only a small fraction of the available intracellular pool of a protein is phosphorylated at any given time. Especially regarding signalling pathways phosphorylation rates, only 1–2% of the entire protein amount is present in a phosphorylated form [21]. Another experimental difficulty arises from protein phosphorylation heterogeneity; most phosphoproteins undergo modification on more than one amino acid, generating different phosphorylated forms and complex phosphorylation patterns. In this case, not all the residues are phosphorylated in a similar quantitative fashion. The first two issues emphasize the importance of the analytical techniques to be used for studying protein phosphorylation, which should present a broad dynamic range of detection, ensuring a quick localization of modification sites as well as a quantitative evaluation of their phosphorylation extent. Moreover, since the cooperative activity of kinases and phosphatases is highly dynamic and intensely

regulated, different phosphorylation cycles may take place on a very short timescale. This observation introduces the need for optimized reproducible procedures to "freeze" protein phosphorylation status during sample preparation and purification steps, and the development of holistic approaches for the study of phosphoproteins on a time-corse basis. The latter issue is related to the general problems encountered during global analysis of proteome repertoires, where thousands of proteins occurring in a wide dynamic range within cell may be selectively accumulated for a specific class of molecules by dedicated enrichment procedures and analysed. On this basis, the decipherment of the phosphoproteome is a huge and challenging task with regard to the dynamics and different kinds of phosphorylation generating a variety of phosphoproteins that are not accessible to a distinct analysis method altogether [16–19]. This review summarizes the analytical methodologies currently available for the analysis of protein *O*-phosphorylation, emphasizing novel approaches and techniques that have been recently introduced in this field.

2. Sample preparation

To preserve protein phosphorylation status in the course of the entire analytical process, a fundamental part of the phosphoprotein analysis is the use of suitable sample preparation conditions during each purification steps. In fact, different phosphatase and kinase activities present in lysed cells/biological fluids can generate phosphorylation artefacts, determining occurrence of non-natural hyper- or hypo-phosphorylated species. Accordingly, particular attention must be paid with regard to lysis conditions, buffers and protease inhibitors used. To limit enzymatic activities, working at low temperatures is recommended. Undesired protein dephosphorylation by all different phosphatases present in the sample can be suppressed by integrating buffers with specific inhibitors [22], which must be chosen as exhaustive cocktails to block all phosphatase activities.

In addition, since phosphorylated amino acids present different reactivities in various buffers (e.g. pSer and pThr residues are subjected to β-elimination in alkaline conditions, whereas pTyr is relatively more stable), the analysis scheme and methodologies should be decided in advance, choosing experimental conditions suitable for the specific type of phosphorylation investigated. In this regard, although the analyzed phosphorylation type is often known, a preliminary investigation on the post-translational modification nature (i.e. *O*-, *N*-, *S*- and acyl-phosphorylation) may be useful to avoid unpleasant specific over-estimations. Since the four known phosphorylations are labile to different solvents or pH-states (e.g., *N*-phosphates are labile to acidic conditions while *O*-phosphates are stable at low pH-values), the type of the phosphorylated residues may be identified by incubation with various solvents [23].

3. Detection of phosphoproteins

Occurrence of phosphorylated species within complex protein mixtures is usually revealed following specific sample fractionation by liquid chromatography and/or two-dimensional gel electrophoresis (2DE) procedures. The most sensitive methodologies for *O*-phosphoprotein detection are based on the incorporation of ^{32}P into phosphoproteins and subsequent Cerenkov-radiation measurement of each separated fraction (chromatography) or radioimmunoblotting (electrophoresis) [24,25]. A major advantage of this approach is that pSer, pThr and pTyr are simultaneously detected and phosphorylation measurement is achieved quantitatively. In general, introduction of ^{32}P groups can be done either in vivo (by addition of radioactive ATP to the investigated type of living cells) or in vitro (by incubating the protein sample with radioactive ATP and a specific kinase/kinases pool). Major drawbacks of using living organisms are related to stress-modulated artificial phosphorylation phenomena generated following introduction of radioactivity within cells, or are emphasized in the case of constitutively phosphorylated proteins with low phosphate turnover rates, which may escape detection as result of the small amount of radioactivity incorporated. A specific phosphorylation can also occur during in vitro experiments because of the very high reagent concentrations and reaction conditions. After an appropriate incubation time, sample generated either by in vivo or in vitro procedures can be subjected to downstream analysis steps.

Phosphoproteins can also be selectively visualized on electrophoretic gels either using phosphoprotein specific stains [26–28] or by western blotting techniques [29], without problems associated to handling radioactive reagents. In addition to a reduced sensitivity with respect to ^{32}P-based procedures, both approaches are hampered by additional blotting steps [26,29]. Recent introduction of small-molecule organic fluorophore Pro-Q Diamond dye facilitated sensitive detection of phosphoproteins directly into electrophoretic gels [27,30]. This non-covalent reagent, specific for all types of *O*-phosphorylation or just for Tyr-phosphorylation (depending on the respective staining protocols), also showed a good compatibility to subsequent staining by conventional dyes and MS analysis. Fig. 1 illustrates the application of Pro-Q Diamond dye in selective detection of phosphoproteins from a Jurkat cell lysate [27].

Femtomolar amounts of phosphoproteins can also be routinely detected by western blotting techniques using phosphospecific antibodies [29]. Although ideal reagents should be able to selectively recognize phosphorylation or specific phosphoamino acids in a non-sequence-specific fashion, method specificity and sensitivity are significantly affected in reality by antibody nature [31]. Thus, effective pTyr-specific antibodies with a poor cross-reactivity toward Tyr or pSer/pThr residues [32–34] have been counterbalanced with western blotting reagents directed toward pSer/pThr having a limited selectivity, being highly affected by the phosphorylation consensus sequence [35]. Nevertheless, different examples of proteome phosphorylation studies using these approaches have been reported [25,36]. In principle, this strategy can be applied to the study of any phosphorylation pathway in vivo, although its applicability is limited by dilution of proteins into different 2DE spots because of differing phosphorylation states, the occurrence of unrelated proteins co-migrating with the protein of interest leading to false identification conclusions,

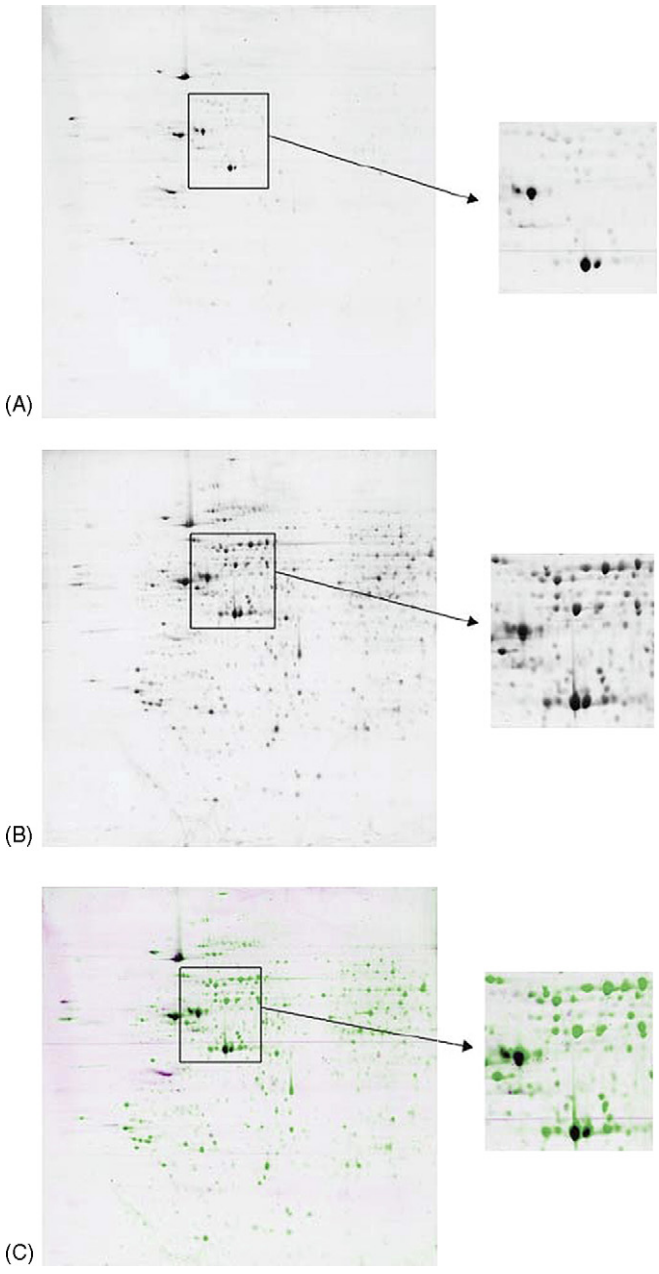

Fig. 1. Serial dichromatic detection of phosphorylated and unphosphorylated proteins from Jurkat cells (150 μg) using Pro-Q Diamond dye and SYPRO Ruby protein gel stain [27]. (Panel A) Gel stained with Pro-Q Diamond dye and imaged using a 532 nm SHG laser and 580 nm long pass filter. The position of heat shock protein 90 is highlighted. (Panel B) Same gel post-stained with SYPRO Ruby reagent and subsequently imaged with a 473 nm SHG lase and 580 nm long pass filter. (Panel C) Differential display map highlighting phosphorylated proteins (magenta) and total proteins (green). Cropped regions of the gels are shown to the right. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

and the reliability of the non-sequence-specific antibodies used to detect pSer/pThr. Thus, global approaches for the recognition of pTyr/pSer/pThr-containing proteins in 2DE are generally accomplished by a combination of various methodologies [29,35].

Since *O*-phosphates are relatively stable in acid conditions, *O*-phosphoproteins can also be detected by revealing *O*-phosphoamino acids in protein hydrolysates. Resulting pSer, pThr and pTyr are routinely separated by 2D thin layer chromatography procedures [37] and detected using the ninhydrin, western blotting (only for pTyr), and/or autoradiography (in the case of ^{32}P-labeled proteins). Phosphorylated amino acids can also be separated by strong anion exchange chromatography after derivatization with a fluorenylmethoxycarbonyl- or dabsyl-moiety [38,39]. Unfortunately, chromatographic procedures usually require picomolar amounts of phosphoamino acids and thus cannot compete with sensitivity/structural information nowadays achieved by MS.

4. Enrichment of phosphoproteins

As mentioned above, only a fraction of the total proteins in a proteome is phosphorylated at any given time. Thus, different methods for the selective enrichment of phosphoproteins from complex protein mixtures have been developed, before their subsequent chromatographic/electrophoretic fractionation [16–19]. A survey of the different strategies for phosphoproteins enrichment is shown in Fig. 2; these procedures, coupled to various analytical methodologies for detection and structural analysis, allow exhaustive identification and characterization of phosphorylated species.

Antibodies are routinely used to immunoprecipitate specific proteins. There are several commercially available antibodies that tightly bind to pTyr residues in a generic fashion, which have been used to effectively immunoprecipitate and enrich pTyr-containing proteins from whole cellular extracts [40,41]. Although there is a wide agreement about the limited availability of anti-pSer/pThr antibodies for selective enrichment of proteins having phosphorylated Ser or Thr residues [16,17], a recent application has been reported in literature to this purpose [42]. In parallel, examples of selective enrichment in phosphoproteins by specific anti-pTyr/pSer/pThr antibodies immobilized on agarose columns have been also described [43,44].

Another commonly used strategy for selective enrichment of phosphoproteins is immobilized metal affinity chromatography (IMAC) [45,46]. Phosphoproteins are bound to the stationary phase by electrostatic interactions of the phosphate moiety with positively charged metal-ions (Fe^{3+}, Ga^{3+}, Al^{3+} or Zr^{4+}), which are immobilized on the column material via iminodiacetic acid, nitriloacetic acid or Tris-(carboxymethyl)-ethylendiamine linkers [47]. Different columns are commercially available although some are not described for nature details. Non-phosphorylated species can be washed away and the phosphoproteins may be eluted by salt and/or pH-gradients. An IMAC (Fe^{3+})-based technique allowing recovery up to 90% of phosphoproteins and compatible with 2DE has been described [48]. On the other hand, a novel methodology termed metal oxide affinity chromatography (MOAC) of enriching phosphoproteins, based on the affinity of the phosphate group for $A1(OH)_3$, has been recently introduced [49]. Authors claimed that this method is more selective, more cost-effective and easily applicable to optimization than commercial IMAC-based phosphoprotein-enrichment kits, but practical experiences are very limited up to now.

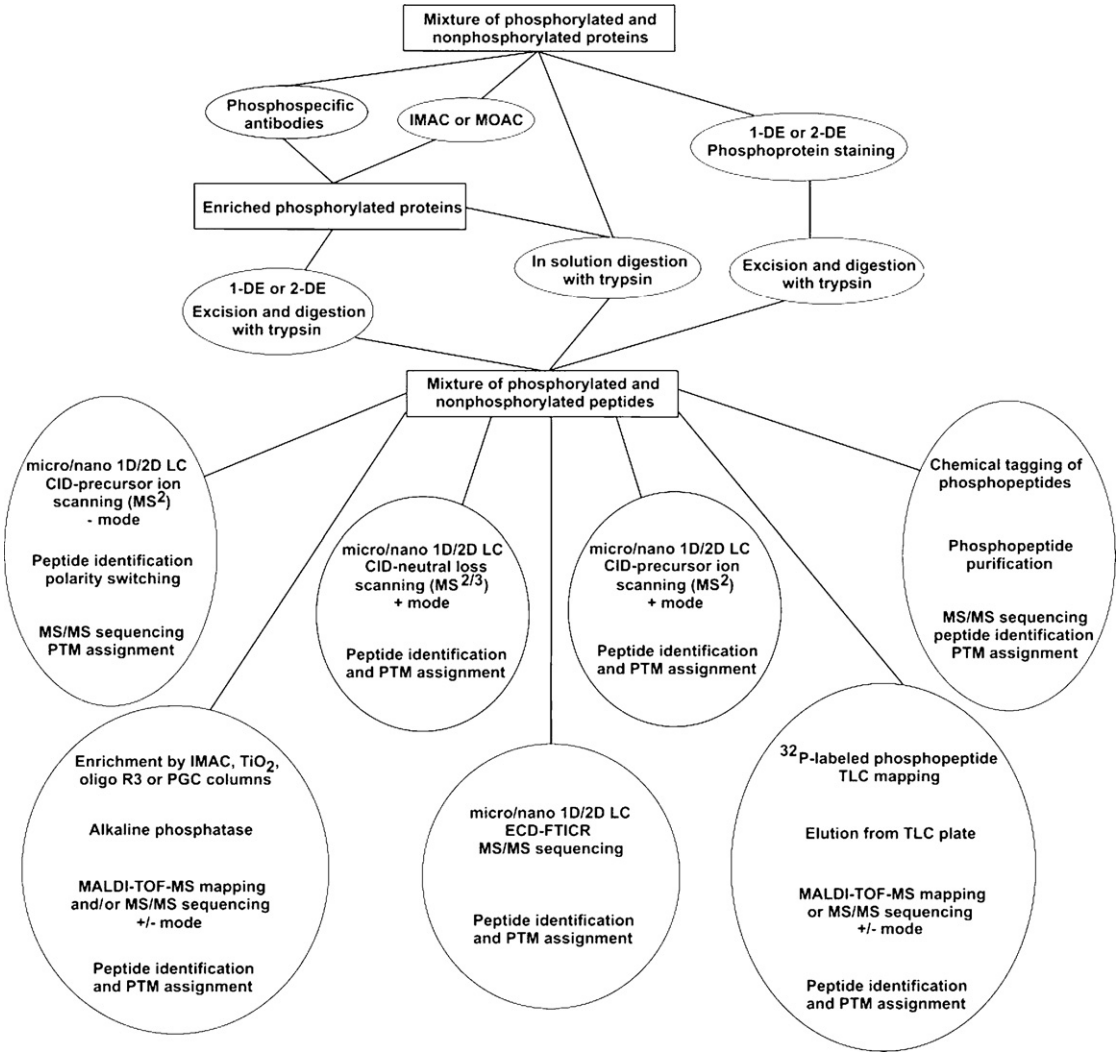

Fig. 2. Different techniques for the enrichment and the analysis of phosphoproteins based on MS procedures. PTM, post-translational modification.

5. Enrichment of phosphopeptides

After the purification/resolution of a specific phosphoprotein by LC or 2DE procedures, a detailed MS characterization of its phosphorylation status is generally accomplished. In fact, depending on its high sensitivity and the peculiar possibility to associate unique mass values to specific modifications, MS has become an incomparable technique for the analysis of protein post-translational modifications [13].

In general, MS analysis of the phosphopeptides generated after protein proteolytic digestion has emerged as a fundamental step for characterization of phosphoproteins. In fact, "bottom-up" MS approaches, dealing with peptides derived from protein digests, are still finding a wider diffusion than recent "top-down" techniques, referring to direct analysis of whole phosphoproteins [50]. In the former cases, every phosphorylated component of the protein digest should be detected to obtain complete information. Unluckily, MS analysis of a peptide digest rarely provides the entire protein sequence coverage. Moreover, phosphorylation is often sub-stoichiometric, such that the phosphopeptides are present in lower abundance than other non-modified peptides. Finally, phosphopeptides are negatively charged whereas MS analysis is generally performed in the positive mode. Accordingly, the mass spectrometric response of a phosphopeptide may be suppressed relative to its non-phosphorylated counterpart, and this suppression tends to be enhanced in the presence of other peptides. This observation has been routinely verified in the course of a large number of matrix assisted laser desorption ionization (MALDI)-based MS studies [16–19], where phosphopeptides were not revealed or occurred as associated to poor intense signals. In contrast, the argument that phosphopeptides analysis is problematic as result of (a) increased hydrophilicity with concomitant loss during loading onto reversed-phase columns, (b) selective suppression of the ionization of phosphopeptides in the presence of unmodified peptides [16–19], has not been proved as correct when using electrospray (ESI)-based instruments and H_3PO_4 as LC solvent additive [51]. In this case, the authors showed that the analysis of phosphopeptides in complex peptide mixtures is hampered mainly by the large excess (in number and relative amount) of non-modified peptides with respect of the phosphopeptides present therein. Accordingly, analysis of phos-

phopeptides using MALDI- and ESI-based MS approaches is greatly facilitated when the number of non-phosphorylated peptides has been reduced to a minimum (i.e. the phosphopeptides have been enriched). This consideration is particularly evident for the recent approaches that have been described to globally characterize proteome phosphorylation status by directly analyzing the complex phosphopeptide mixture generated following proteolytic digestion of a crude cellular extract, without any previous enrichment, purification/resolution of the phosphoproteins. These methodologies have been developed to directly identify modified proteins and assign sites of phosphorylation of low-abundant proteins when limiting amounts of the proteins are available [16–19]. Thus, several strategies have been introduced and widely used to selectively enrich protein digests for phosphorylated peptides [7] (Fig. 2). Although some examples have been reported in literature [52], immunoprecipitation of phosphopeptides by specific antibodies is not an experimental approach widely used [53] and other methods have to be applied.

Phosphopeptides have been enriched by chromatographic adsorption on a polymer-based reverse-phase perfusion resin (oligo R3), originally designed for oligonucleotides purification [54,55]. Alternatively, porous graphitic carbon columns have been used to this purpose [56]. The application of miniaturized columns for the enrichment of phosphopeptides corresponded to the most frequent use of IMAC technology. In fact, IMAC micro-columns, mainly loaded with $Fe^{3+}$and Ga^{3+} ions [57,58], have been successfully used in off-line and on-line applications for the detection of phosphopeptides using MS [58–62]. A major drawback is the specificity of this procedure, which results variable because of the affinity of the resin for acidic groups (Asp and Glu) and to electron donors (e.g. His) [59]. The latter problem can be overcome by prior esterification of the side chain carboxylate [63] using HCl-saturated, dried methanol [64]. However, reaction conditions have to be chosen carefully to avoid both incomplete reaction and cross-reactivity with other residues, which may increase sample complexity and interfere with subsequent MS analysis. In addition, multiply phosphorylated peptides are more enriched and the recovery of phosphopeptides appears to be largely dependent on the type of metal ion, column material and the elution procedure used. A comparison of different commercially available IMAC resins and protocols has been recently published [65]; with further refinement, authors claimed that this technique may offer the best hope for large-scale phosphorylation analysis. A valid alternative to IMAC resins for decreasing the co-purification of acidic peptides came from the recent introduction of $A1(OH)_3$ [49], ZrO_2 [66] and TiO_2-based [67] stationary phases. In the latter two cases, only organic phosphates have been verified to bind specifically to the column under acidic conditions, allowing removal of all non-phosphorylated peptides. Elution of the phosphopeptides is done at an alkaline pH. Depending on MS technique chosen for further structural characterization, different sample loading protocols based on use of 2,5-dihydroxy-benzoic acid (for MALDI analysis) or phosphoric acid (for LC-ESI analysis) have been developed. Whatever their nature, the use of these resins for phosphopeptide-enriching is an essential step before MS analysis, ensuring low background levels of unphosphorylated species.

Further approaches use chemical tagging of the phosphate groups as alternative methods to enrich phosphopeptides from complex peptide mixtures. A survey of the different chemical reactions is shown in Fig. 3. The first strategy makes use of β-elimination reaction, occurring when pSer and pThr residues are exposed to alkaline conditions, and subsequent Michael-type addition of nucleophiles [12,68,69]. Oda et al. [68] and Goshe et al. [69] used ethanedithiol as a nucleophile, which introduces a new reactive thiol group serving as a linker for attachment of a biotinylated affinity tag, via a maleinimide-group. Tagged peptides are isolated by subsequent affinity chromatography on avidin resin. Care must be taken to properly block the SH groups of Cys residues before derivatizations. To this purpose, performic acid oxidation [68] is preferred over alkylation [69] because alkylated Cys/Met residues may also undergo β-elimination [70]. Other phosphopeptide enrichment strategies such as IMAC may be coupled successfully to derivatization methods [71]. Major drawbacks of this tagging methodology are: (i) it is not applicable to Tyr phosphorylation, (ii) yields from the β-elimination reaction tend to be substoichiometric, (iii) *O*-linked sugar moieties may also undergo β-elimination, generating intermediates affording false phosphorylation assignment, (iv) poor solubility of the thiol compound in water. In general, since all reactions are performed in a single tube, losses resulting from multiple purification steps are minimized and this procedure is easier to perform than the method described below. Different technical improvements to the original approach, either still based on biotin-containing tags or on direct affinity purification of the free SH groups by other methods, have been reported to reduce above mentioned problems [72–76]. A modification of this strategy, based on the direct use of a 6-(mercapto-acetylamino)-hexanoic acid functionalized resin as Michael reaction donor, has been recently reported [77]. In this case, derivatization and binding to the stationary phase are performed in a single reaction. After an acid-mediated release step, phosphopeptides are labeled with a 6-(2-mercapto-acetylamine)-hexanoic amide tag at phosphorylation sites and characterized by MS procedures.

Using an alternative chemistry, Aebersold and co-workers developed a second strategy generally applicable to peptides containing pTyr, pSer and pTher residues [78]. Phosphopeptides were bound to a thiol-containing compound (1-amino-2-thioethane), via phosphoamidate-bonds, and subsequently immobilized by covalent attachment to glass beads containing immobilized iodoacetyl groups (Fig. 3), washed, and released by treatment with acid. The phosphate moieties are not removed from the respective residues, so native phosphopeptides are recovered after elution. Major drawback of this method is the need of quantitative protection reactions (i) of the NH_2 moiety of the peptides (by *tert*-butyloxycarbonyl chemistry) and (ii) of the carboxylate moiety (by amidation), to prevent intra- and inter-molecular condensations. Accordingly, this approach requires several chemical reactions and purification steps before MS analysis, which could lead to substantial losses. Thus, it requires large amounts of sample to be analyzed and only abun-

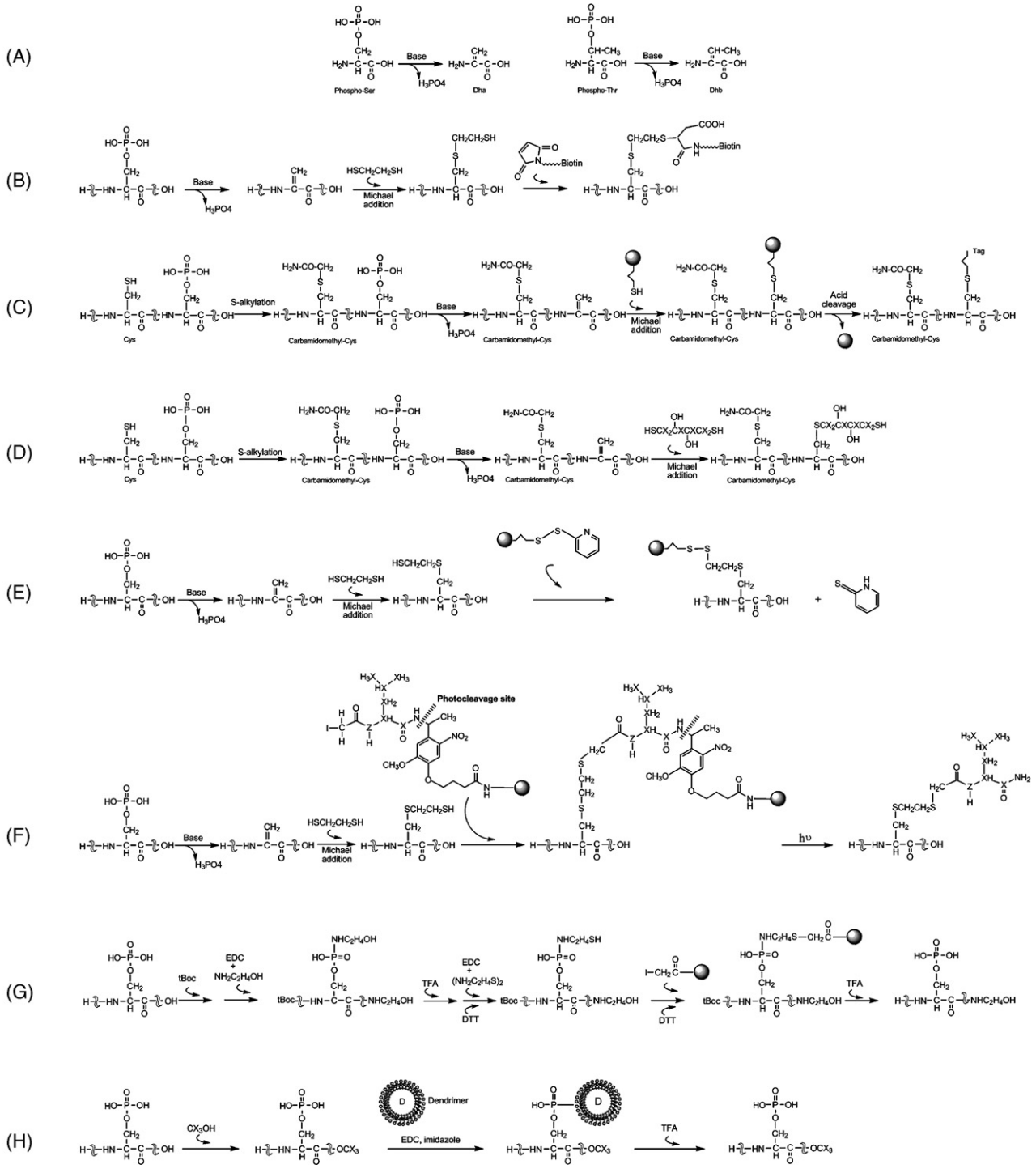

Fig. 3. Various strategies for the enrichment of phosphopeptides based on different chemical modification reactions. (Panel A) Base-catalyzed β-elimination of pSer and pThr yielding Dha and Dhb, respectively. Enrichment approaches by chemical tagging of the phosphate group based on a β-elimination/Michael addition chemistry modified according to Oda et al. [68] (panel B), Tseng et al. [77] (panel C), Vosseller et al. [72] (panel D), McLachlin and Chait [75] (panel E), Qian et al. [185] (panel F). Enrichment approaches by chemical tagging of the phosphate group based on a carbodiimide condensation chemistry modified according to Zhou et al. [78] (panel G) and Tao et al. [79] (panel H). Depending on the possibility to perform quantitative measurements, X corresponds to H or D (panel D and H); similarly X and Z correspond to ^{12}C or ^{13}C and ^{14}N or ^{15}N, respectively (Panel F).

dant proteins are trapped. The same authors recently reported an improved strategy based on a single-step covalent conjugation of the phosphopeptides to a dendrimeric soluble polymeric support, catalyzed by carbodiimide and imidazole [79]. Also in this case, preliminary protection of the peptide carboxylate moieties (by esterification) was necessary to prevent undesired side-reactions. Modified phosphopeptides were released from the dendrimer via acid hydrolysis and analyzed by different MS techniques. This procedure allowed a quantitative measurement of different phosphorylation states (see below).

All phosphopeptide tagging strategies described above have been thoroughly improved during the last years, decreasing detection limits down to the low femtomolar range. In general, all approaches need a careful monitoring of the experimental conditions to ensure reaction completeness and prevent undesired reactions, which may result in increased sample complexity, unexpected changes in peptide masses and possible loss of certain phosphopeptides. Nevertheless, these methods are promising and could be coupled to other fractionation steps to improve the overall recovery of low-abundance proteins.

6. Chromatographic separation of phosphopeptides

Application of separative techniques to a peptide mixture generated from protein digestion is an obvious method to reduce the sample complexity. Thus, LC or CE have been used for resolution of phosphopeptides from protein digests [80,81], reducing problems associated to their further positive ion mode MS characterization, mainly related to the concomitant large excess of non-modified peptides (for MALDI and ESI), and weak ionization/suppression phenomena (for MALDI). In general, CE applications found a limited use as result of a non-simple connection set-up to the mass spectrometer and the necessity to load small sample volumes [82].

A very simple fractionation method dedicated to MALDI–TOF-MS analysis has been also described, which uses step elution of peptides from reversed-phase beads [55]. The peptides are eluted in 3–5 fractions, which are less complex than the entire mixture. Problems related to phosphopeptides loss during the fractionation procedure were limited by the use of phosphoric acid as eluant acidifier, but have to be taken into consideration.

Depending on the amounts of phosphopeptides recoverable from spot digests (1–1000 fmol), capillary (operating at 1–10 μl/min) [83] or nano (operating at 100–200 nl/min) [84] C_{18} material-packed columns have been used for LC separation of complex peptide mixtures containing phosphopeptides, ensuring a good reproducibility and convenient automation [85]. Both techniques allow a general elution of a peptide generally in a peak lasting 10–40 s; thus, different hundreds of peptides may be separated and analyzed. Eluted fractions are directly eluted into an ESI-based tandem mass spectrometer (more frequent) or spotted onto a MALDI target for further off-line MS characterization (less frequent). By using a microscale precolumn, a 10-port valve and an additional pump, large volumes can be loaded (up to 100 μL) [86]; after a valve switching, the concentrated and desalted peptides are eluted onto the separation column at nanoscale flow rates. To avoid specific loss of phosphopeptides, the use of inert tubing, efficiently capped silica-based columns and the addition of 0.1–1.0% of H_3PO_4 to the solvents is recommended [87,88]; in general, this additive reduces the phosphopeptides hydrophilicity, avoiding losses associated to uneffective interaction with the stationary phase.

Recently, 2D chromatography has been also used to resolve phosphopeptides from complex mixtures [89–94]. At first, the protein digest is separated on a strong cation exchange column and collected as separated fractions; then, each fraction is resolved on a C_{18} nano-LC column directly connected to a mass spectrometer. In the data-dependent acquisition mode, the instrument can be set to automatically fragment and collect MS/MS data on any number of peaks observed in the MS spectrum based on their intensity, *m/z* value or charge state. Different tandem mass spectrometric techniques have been used to this purpose, ensuring a general sensitivity of 10–500 fmol for detection of phosphopeptides in the MS/MS mode.

7. Recognition of phosphopeptides

7.1. MALDI-mass mapping and phosphatase treatment

Once the nature of a protein has been identified, its phosphorylation status can be ascertained by revealing the occurrence of phoshopeptides within its proteolytic digest. Peptide mass mapping experiments have been widely used to this purpose. In fact, a direct mass measurement for all peptides within the digest, together its comparison with the theoretically expected values, highlighted phosphopeptides as those molecular species having a molecular mass shifted by multiples of +80 Da (HPO_3 = 80 Da). These experiments are generally performed by direct MALDI–TOF-MS analysis of protein digests [13,95]. However, exhaustive sequence coverage of phosphoproteins by MALDI–TOF-MS mapping procedures is not straightforward as result of the partial results obtainable in a single experiment, and the already mentioned sub-stoichiometric abundance, weak ionization and suppression phenomena during positive ion mode analysis of phosphopeptides. Thus, MALDI–TOF-MS analysis of protein digests is generally accomplished after phosphopeptides enrichment on IMAC columns. On the other hand, suppression effects in MALDI spectrum have been partially reduced by the addition of phosphoric acid, and ammonium salts to conventional and 2′,4′,6′-trihydroxy-acetophenone based MALDI-matrices [96–98]. Similarly, MALDI–TOF-MS analysis in the negative ion mode has been successfully used to improve relative signal intensity of phosphopeptides within protein digests [99,100]. A modification of this approach based on the comparative analysis of methyl esterification products in negative and positive ion mode has been recently proposed [101].

Since the presence of isobaric peptides in the sample can complicate MALDI–TOF-MS recognition of phosphopeptides within peptide digests, MS analysis has been used in combination with phosphatase treatment to specifically identify phosphopeptides [59,102]. In this method, phosphopeptides are characterized as typical species presenting the unique mass shift owing to loss of phosphate (80 Da or multiples) after treatment with phosphatase. As an example, the MALDI–TOF-MS spectrum of human JNK-interacting protein 1 digests captured by the IMAC(Ga^{3+}) column before and after treatment with alkaline phosphatase is shown (Fig. 4). This widely applied strategy has been quantitatively evaluated [95], and the authors concluded that few pmoles are necessary for a reliable characterization of protein phosphorylation. This amount is the result of the need to obtain high coverage of the protein in the MS

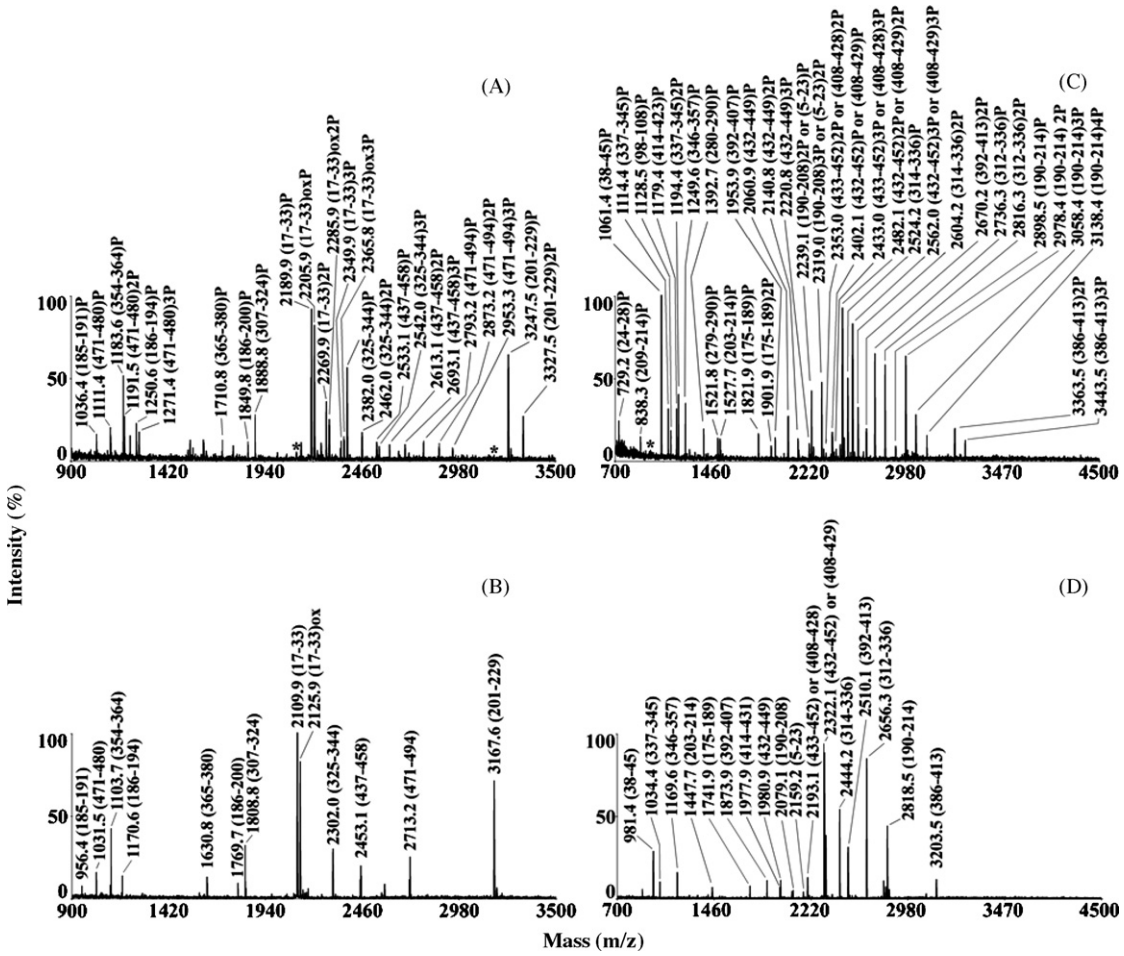

Fig. 4. MALDI–TOF-MS spectrum of human JNK-interacting protein 1 phosphopeptides captured by the IMAC(Ga^{3+}) column before and after treatment with alkaline phosphatase [123]. The spectrum of tryptic digest before and after treatment with alkaline phosphatase is shown in panels A and B, respectively. The spectrum of endoprotease AspN digest before and after treatment with alkaline phosphatase is shown in panels C and D, respectively. Metastable peaks formed by the loss of HPO_3^{2-} from phosphopeptides are marked with an asterisk. Assignment of signals present in panels A and C to specific phosphopeptides was performed on the basis of the measured mass, protease specificity, presence of Ser, Thr and Tyr within peptide sequence and signals observed in panels B and D.

analysis. To obtain as much information from a given sample as possible, phosphatase has been applied directly on the MALDI target after the initial mass map has been acquired [95,103,104].

Direct detection of phosphopeptides by MALDI–TOF-MS in the positive ion mode can also be achieved on the basis of their tendency to give metastable decomposition and/or CID, losing meta-phosphoric ($-HPO_3$) and phosphoric acid ($-H_3PO_4$) under mass spectrometric conditions [105,106]. Accordingly, fragment ions are not distinguishable from their parents during linear mode measurements, but are detected as low-resolution signals present at a lower apparent mass in spectra collected in reflector mode [105]. In general, peptides bearing pSer and pThr are differentiated from those containing pTyr because the former present 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), whereas the latter generally show only a loss of 80 Da [105,107]. Major drawbacks of this approach are again related to the decreased ionization of phosphopeptides in complex mixtures due to ionization suppression effects. Different chemical derivatization approaches based on β-elimination/Michael addition chemistry have been developed to limit problems related to weak ionization/suppression phenomena for phosphopeptides during positive ion mode MALDI-mapping analysis of protein digests. The use of different thiols has been reported to convert phosphate moiety into uncharged/positively charged functionalities, thus generating easily ionisable peptide derivatives [73,108] and/or non-natural phosphospecific proteolytic sites [109]. In particular, derivatization with a mixture of two alkanethiols made phosphopeptide-specific derivatives readily distinguishable due to their signal intensity and characteristic ion pair signature [73]. On the other hand, Shokat and co-workers used cysteamine to convert pSer and pThr into aminoethylcysteine and β-methylaminoethylcysteine, respectively; these residues are selectively recognized by Lys-specific proteases, allowing direct mapping sites of phosphorylation by specific MALDI–TOF-MS detection of newly generated peptides following derivatization [109].

7.2. Stepped skimmer potential

Application of a high potential to the skimmer region of an ESI device determines the loss of phosphate groups from

phosphopeptides [110]. Thus, an increase of the skimmer potential during the low part of a negative ion mass scan makes phosphopeptides selectively recognizable on triple quadrupole instruments, by the specific occurrence of signals at m/z −63 (PO^-) and −79 (PO_3^-). As the scan continues to higher masses, the skimmer potential can be lowered and the ion polarity switched to positive ion mode [111]. This modification during the second part of the scan allows determination of the phosphopeptide masses at highest sensitivity (hundreds fmol level).

7.3. Precursor ion scanning

During tandem mass spectrometry experiments, collision-induced dissociation (CID) of modified peptides produces not only sequence-specific fragments but sometimes characteristic fragment ions that are specific for a defined type of modification, also known as 'reporter ions'. Accordingly, phosphopeptides have been identified in precursor-ion scanning (PIS) of protein digests because of their peculiar loss of phosphate (PO_3^-) ($m/z = -79$) under basic conditions [112–114]. ESI-triple quadrupole mass spectrometers operating in negative ion mode are generally used for this purpose. In this method, the first quadrupole is scanned over the full mass range of the instrument, the second quadrupole is used for CID and the third quadrupole is set up to selectively pass only $m/z = -79$ ions. Detection of PO_3^- identifies the corresponding precursor phosphopeptide ion by its m/z value. Using nano-ESI, subpicomolar amounts of phosphopeptides have been selectively detected in protein digests [55,113–116]. To allow further phosphopeptide MS/MS characterization (implying a change in polarity and rebuffering of the sample) and improved detection of low abundance phosphopeptides in protein digests, a multidimensional approach has been developed using a LC separation in acid solvents [117]. Part of the column eluant is split off, electrosprayed into the mass spectrometer and subjected to CID in the high-pressure region close to the skimmer. Monitoring for the presence of phosphate-derived anions at $m/z = -63$ and −79 is used to detect phosphopeptides, without scanning the mass analyzers. The remainder eluant is collected as fractions. Thus, each phosphopeptides-containing fraction is in part redissolved in a basic solvent and analyzed in negative ion mode for their molecular mass using the precursor ion ($m/z = -79$) scan method, and in part solved in an acid solvent and subjected to positive ion mode MS/MS analysis to identify phosphorylation site (see below). Despite a picomolar amount sensitivity, the method is a powerful tool because of its high selectivity and its applicability for pSer, pThr and pTyr residues. An innovative methodology based on the analysis of phosphate-specific high-mass fragment ions generated by the peculiar loss of phosphate (PO_3^-) under basic conditions and selective recognition of their associated signals at $m/z = [M\text{-}n\text{H-}79]^{(n-1)-}$ has been recently proposed [118]. These variable ions are formed with high efficiency at moderate collision offset values as the counterparts of the established phosphopeptide marker ion found at $m/z = -79$ and allow sensitive detection of pSer/pThr/pTyr-containing peptides due to the low background level in MS/MS spectra. A precursor-ion scanning method performed in the positive mode has been also proposed for the specific detection of pTyr-containing peptides [119,120]. This method is based on the ability to fragment peptides and selectively detect the immonium ions of pTyr residues (with $m/z = 216.043$) from other peptide fragment ions such as b-ions AsnThr or GlySer (both $m/z = 216.098$ Da) and y2-ion-NH_3 AspVa1 ($m/z = 216.069$ Da), etc. This approach has been made possible using high-resolution mass spectrometers, such as those operating on the q-TOF principle having a Q2-pulsing function [121]. Once the pTyr-containing peptides are detected in the original MS scan, they can be sequenced in the product ion MS/MS mode without changing polarity of the ion source. A subpicomolar amount sensitivity for detection of tyrosine phosphorylation sites from protein digests has been reached.

7.4. Neutral loss scanning

Also in this case CID allows selective detection of pSer/pThr-containing peptides as result of gas-phase β-elimination reaction determining a neutral loss of phosphoric acid (−98 Da) or a dephosphorylation (−80 Da) [122]. pSer/pThr-containing peptides are recognized in tandem mass spectrometry experiments by searching for doubly, triply and quadruply charged peptide ions showing an apparent loss of $m/z = 49$, 32.6 and 24.5, with respect to parent ions; this approach is known as neutral loss scanning (NLS). pTyr-containing peptides are generally more resistant to this loss. Method drawbacks are the incidence of false-positive signals in automatic visualization of peptides affected by neutral loss events as well as the dominant occurrence of phosphate-loss specific fragment ion in the MS/MS spectra [94]. The latter problem determinates a limited production of polypeptide backbone fragmentation ions, otherwise useful for modification site assignment. Similarly to PIS, NLS-driven tandem mass spectrometry experiments are performed in automated data-dependent acquisition mode on instruments with sufficient resolution and fast scanning rate, i.e. triple quadruple [122], ion trap [94,123] and q-TOF mass spectrometers [93,124].

7.5. ^{31}P detection

Element mass spectrometry can be used to monitor specific occurrence of ^{31}P into peptide samples by inductively coupled plasma (ICP) ionization-based instruments [125]. Although this method necessitates the use of a dedicated instrumentation (with a 2500–3000 resolution) and determination of analyte molecular mass in a separate measurement, it allows detection of phosphopeptides at subpicomolar amounts. Moreover, being not affected by suppression effects or varying ionization responses, absolute quantitation can be obtained. Later on, it was also demonstrated that the average phosphate content of proteins can be determined with ICP-MS by measuring the $^{31}P/^{32}S$ ratio [126]. Very recently, the same authors proposed a combination of μLC-ICP-MS and imaging laser ablation-ICP-MS techniques for the quantitative estimation of the phosphorylation degree of a cellular proteome [127]

8. Identification of phosphorylation sites

After phosphopeptide identification, assignment of the phosphorylation site(s) has to be performed. Sometimes, the peptide has only one Ser, Thr or Tyr residue, making obvious identification of the modified amino acid(s); in contrast, in most cases fragmentation of the peptide and characterization of the fragments is necessary. This is the case of some hyperphosphorylated proteins, whose phosphorylation sites elucidation sometimes needs the examination of phosphopeptides generated from different protein digests and exhaustive identification of all sites of phosphorylation remains a most challenging analytical task.

8.1. Collision-induced dissociation

CID is the most common MS method of identifying sites of phosphorylation. Both MALDI- and ESI-sources may be used, but generally modification assignment is achieved more easily by using doubly or triply charged parent ions produced in an ESI source. Generated fragment ions are measured in triple quadrupole- [55,117], ion trap- [94,123], hybrid q-TOF [93,128] and Fourier transform ion cyclotron resonance (FTICR)- [129,130] mass analyzers. Since negative-polarity MS/MS spectra are mostly of poor quality, phosphopeptide fragmentation experiments are usually performed in positive ion mode. To avoid problems associated to polarity switch during the experiment, determining decreased scanning rates and increased failing instrument susceptibility, phosphopeptide detection approaches using ESI-negative ion mode measurements (some PIS methods) are combined to off-line positive ion mode MS/MS experiments. In contrast, phosphopeptide detection by ESI-positive ion mode (some PIS and all NLS methods) and fragmentation experiments are directly performed simultaneously. In the latter case, a LC device is generally coupled on-line to the ESI-based mass spectrometer for an improvement of the phosphopeptides resolution from complex proteolytic mixtures (see above) and related probability of effective phosphopeptide CID analysis. In fact, MS analysis is usually carried out automatically by repeated MS scanning of the chromatographic peaks, and subsequent multiple selection of the most intense ions from a given scan for CID-generating fragmentation data for many peptides (including phosphopeptides) in the sample. In such an experiment, the achievement of good fragmentation data for phosphopeptides may be hindered by the presence of more co-eluting peptides (limiting the time available for recording good MS/MS spectra of each species) and their occurrence as sub-stoichiometric species (limiting the relative parent ion MS signal intensity) [51]. Accordingly, PIS or NLS-driven automated CID acquisition procedures (see above) are routinely used during shot-gun analysis of phoshopeptide-containing mixtures [93,94,129,130]. Moreover, the availability of analyzers with high mass accuracy (i.e. FTICR) is now allowing the use of accurately determined molecular masses to easily identify phosphopeptides. In this sense, different internal calibration procedures have been developed to improve the mass accuracy in the course of shot-gun LC-based phosphoproteomic analysis [129,130].

Additional methods for phosphorylation assignment are based on preventive detection of phosphopeptides (usually by MALDI-TOF-mass mapping) and further on-line LC–ESI-MS/MS analysis of ascertained phosphopeptides for obtaining sequence information [90]. In this approach, the mass spectrometer is programmed to specifically fragment phosphopeptide species having a defined *m/z* value, although a weak ion response, ignoring all other peptides. This approach has been widely applied to the characterization of a number of phosphorylation sites [90,129,131].

As mentioned above, loss of HPO_3 or H_3PO_4 is the favored fragmentation event in positive ion mode, dominating over the production of polypeptide backbone fragmentation ions, useful for sequence determination and modification site assignment. However, even in this case, useful fragmentation information can be obtained by carefully analysing weaker backbone fragment ions. A large number of positive examples have been reported in literature [93,94,123,128–131]. In addition, dedicated softwares have been developed to predict precursor and neutral loss fragment ion *m/z* values useful for multiple reaction monitoring experiments [132]. This prediction triggered data dependent product ion scans on a hybrid quadrupole linear ion trap instrument. Anyway, to prevent loss of informative data, MS^3 experiments have been recently used for phosphorylation site assignment of pSer/pThr-containing phosphoproteins [94,133]. The fragment ion generated by neutral loss in MS^2-mode is used for further fragmentation. The resulting MS^3-spectrum provides significantly more structural information than the MS^2-spectrum, with a spacing of 69 Da (owing to dehydroalanine, Dha) or 83 Da (owing to dehydroaminobutyric acid, Dhb) indicative of the exact location of pSer and pThr residues, respectively [94]. As an example, Fig. 5 shows the MS^2 and MS^3 analysis of the tryptic phosphopeptide from 14-3-3 protein of *Giardia duodenalis* [134]. In general, the experiment may be performed in data-dependent mode, with NLS triggering automatic MS^3 acquisition [94,135]. The reduced abundance of fragment ions in MS^3 experiments observed in a conventional ion trap mass spectrometer has been balanced by the greater ion capacity of the newer linear ion trap devices [136]. Both the MS^2- and the MS^3-spectrum are checked for consistency, combined in a "composite" spectrum and subjected to database search by a dedicated algorithm [137].

Depending on the liability of the phosphate moiety in CID experiments, various authors have reported different phosphorylation site identification procedures by converting the phosphate moiety into different easily ionizable functionalities, according to the β-elimination/Michael addition chemistry described above, and further CID analysis [72,73,75]. Various reagents have been used to this purpose, sometimes facilitating concomitant phosphopeptides enrichment and subsequent MS analysis [68–76]. Fig. 3 shows some of the reagents used. In this case, resulting peptide derivatives have been widely characterized by shot-gun approaches using CID, yielding more complete sequence information. Derivatization with dimethylaminoethanethiol or thioethylpyridine has been used to generate modified peptides easily detectable by PIS experiments searching for 2-dimethylaminoethanesulfoxide (*m/z* = 122.06) [138]

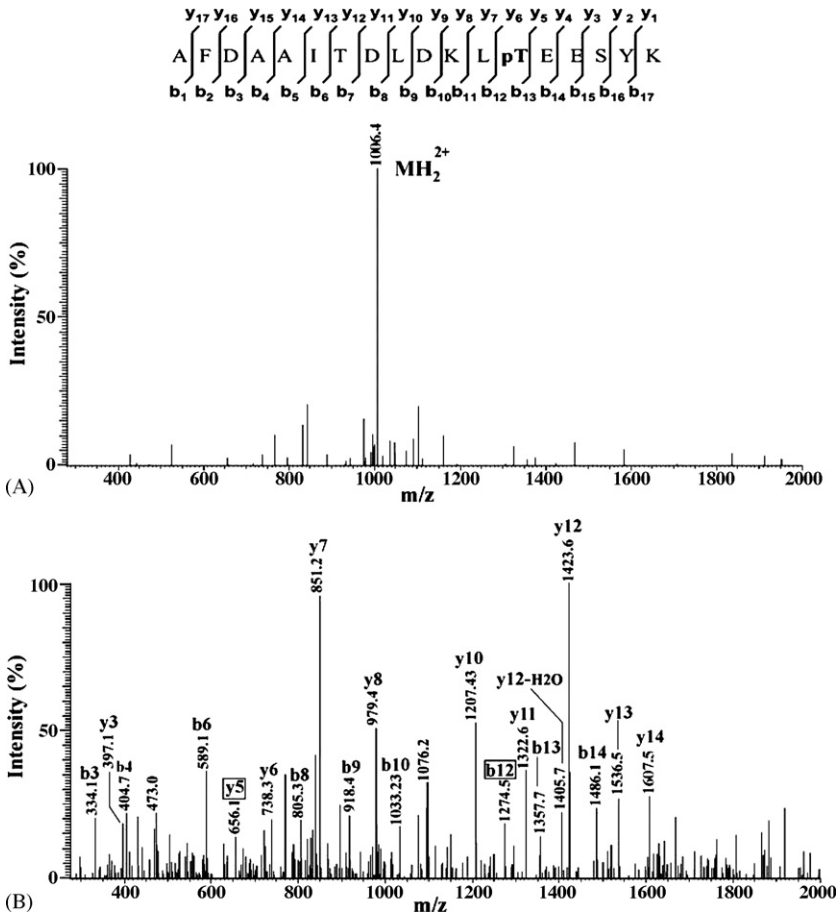

Fig. 5. Phosphorylation of 14-3-3 protein from *Giardia duodenalis* [134]. (Panel A) MS^2 of the double charged ion at *m/z* 1054.6 corresponding to phosphorylated peptide (202–219). The sequence of the tryptic peptide is reported in the box. Signal at *m/z* 1006.4 derives from collision-induced loss of phosphoric acid from parent ion. (Panel B) MS^3 spectrum of signal at *m/z* 1006.4; *y* and *b* fragment ions refers to Dhb containing sequence. Boxed signals most clearly identify Thr214 as the phosphorylated residue.

and 2-ethylene-pyridine ion (*m/z* = 106.14) [139], respectively, obtainable by low energy CID.

It has been also reported that side-chain fragmentation of phosphopeptides in CID may be humpered by gas-phase derivatization using various borate or borane derivatives prior to the fragmentation [140]. In CID, the $[M + B\text{-}2H]^+$-ion is generally observed, which undergoes preferentially backbone fragmentation. The effect is believed to occur upon binding of the boron to the phosphate group and nearby nucleophilic sites.

The availability of instruments in which a MALDI source has been coupled to tandem mass spectrometers, such as quadrupole-TOF (MALDI–q-TOF), TOF–TOF (MALDI–TOF–TOF) or ion trap (MALDI-ion trap), is now allowing an increased efficiency and sample throughput because identification of phosphopeptides and assignment of the modification sites based on MS/MS sequencing can be performed on a single sample spot [119,141–146]. Some of the drawbacks of MALDI (i.e. limited sequence coverage without previous digest fractionation and weak ionization/suppression phenomena for phosphopeptides) are not overcome by the use of these instruments and enrichment/derivatization of phosphopeptides would still be of great value to increase relative ion intensity of phosphopeptide precursor ions (see above). In general, a significant loss of HPO_3 or the elements of H_3PO_4 from phosphopeptides has been reported in positive ion mode, resulting in a characteristic pair or series of peaks in the spectrum. The dephosphorylated ion formed by this prompt loss was observed to produce easily interpretable CID fragmentation patterns, where formerly pSer and pThr residues were identified by the presence of Dha and Dhb residues, respectively [146].

A precursor ion discovery method has been developed on q-TOF mass spectrometers for studying protein phosphorylation. Phosphorylated peptides are automatically discovered and identified in a way similar to that of the use of precursor ion or neutral loss scanning, but without the need to scan the quadrupole mass filter [147]. Similarly, an identification method for the identification of femtomolar amounts of phosphopeptides by MALDI–q-TOF MS in positive and negative ion modes, after methyl esterification, has been recently reported [101]. This method has been successfully applied to the study of fibroblast growth factor receptor phosphorylation.

Phosphopeptides have also been detected and assigned for modification sites in the course of vacuum or atmospheric pressure MALDI–ion trap-MS experiments [145,148]. In addition to pSer and pThr residues, unexpected elimination of the elements of H_3PO_4 from pTyr-containing phosphopeptides was

also observed under atomospheric pressure ionization conditions [149]. Based on vacuum MALDI–IT-MS/MS analysis data, a strategy termed hypothesis-driven multiple-stage mass spectrometry (HMS-MS) was developed for femtomolar detection and assignment of phosphopeptides derived from phosphoprotein digests. In this strategy, all of the potential sites of phosphorylation in a given protein were postulated as phosphorylated. Using this assumption, all the *m*/*z* values of all the theoretically possible singly charged phosphopeptide ions were calculated. The presence of pSer or pThr in measured peptides was verified on the basis of the occurrence of phosphate loss in MS^2 experiments. Subsequent MS^3 analysis of the $(M + H\text{-}98)^+$ peaks allowed to confirm or reject the hypotheses of phosphopeptide occurrence [150]. Efficient assignment of phosphorylation sites has been recently obtained also by MALDI–TOF–TOF-MS analysis [151,152]. Also in this case, HMS-MS was used to correctly assign phoshorylation sites in phosphopeptides immobilized on IMAC beads, previously identified by phosphatase treatment [104].

8.2. Post-source decay

Phosphate loss is the dominant metastable and CID fragmentation pathway during MALDI–TOF-MS analysis of phosphopeptides. Despite of the interpretation of these spectra can be challenging, direct assignment of phosphorylation sites has been possible in some cases on the basis of the low intensity signals associated to polypeptide backbone fragmentation observed in the course of post-source decay (PSD) experiments [105,153–155]. When the phosphorylation occurs within target sequences of Pro-directed kinases (Ser-Pro and Thr-Pro motifs), cleavage of the intervening amide bond is highly preferred, making characterization much easier [156].

8.3. Electron capture dissociation

Electron capture dissociation (ECD) is a relatively new technique for dissociation of proteins and peptides into fragments, and involves irradiation of ESI-produced multiply charged ions with a stream of sub-thermal electrons in a FTICR mass spectrometer [157,158]. On the basis of the interpretation of the *c*- and *z*-fragment ions generated, it has emerged as a powerful technology for the sequencing of proteins and peptides [159]. Recently, it has also been successfully applied to the assignment of phosphorylated residues in peptides [160–162]. In fact, contrarily to CID and PSD, no loss of phosphoric acid, phosphate or water from the parent phosphopeptide or the fragments is observed during ECD-based sequencing. The phosphorylated amino acid side chain remains intact and the peptide backbone is subjected to an effective fragmentation [160]; this phenomenon makes the identification of the phosphorylation site(s) simpler than in CID experiments. Owing to FTICR superior resolution and mass accuracy, large peptides and proteins not amenable by other techniques can be studied [163–165]; this makes phosphorylation status analysis an experiment directly performed, without any need for additional enzymatic digestion [161,166]. The most significant limitation to the use of ECD-based approaches is the availability of expensive instrumentation and well-experienced personnel. On the basis of the promising characteristics of this technique, a database independent search algorithm for the detection-phosphorylated peptides from data sets originated from nano-LC–ECD-FTICR-MS experiments has been developed [167].

An alternative method, known as electron transfer dissociation (ETD), has been recently developed using a modified linear IT-system and yields fragmentation patterns similar to ECD [168]. Thereby, electrons are transferred to the protein/peptide ions from anions generated by a chemical ionization source with methane buffer gas. This technique has been applied to a limited number of proteins [169].

9. Quantitative phosphoproteomics

Phosphorylation on multiple Ser, Thr and Tyr residues may be an important modulator of a phosphoprotein function and its modification degree may be indicative of the portion of active enzymatic species. Since a phosphoprotein may be involved in more signalling pathways, the quantitative phosphorylation status of a specific residue can be variable as result of the different stimuli inducing partially overlapping patterns of phosphorylation. Thus, the understanding of the dynamics of the quantitative modification on specific residues may be suggestive of important cellular processes as well as of molecular mechanisms associated to cell regulation. In addition to procedures based on metabolic and phosphorylation site labeling with ^{32}P, different MS-based approaches have been developed for accurate quantitative determination of phosphorylation status of phosphoproteins, to be eventually used in comparative investigations.

A very early quantitation method used LC-separation of the phosphopeptide from its unmodified counterpart (as identified by MS), quantitative amino acid analysis, and integration of the two chromatographic peptide peaks [170]. For sensitivity reasons, this method was initially adapted to ESI-MS measurements [171] but considering the different ionization tendencies of both phosphopeptide and peptide species, it provided very rough estimations of phosphorylation stoichiometry. In contrast, a direct quantitative measurement of protein/peptide phosphorylation status can be obtained by ICP-MS [125,172]. As mentioned in chapter 7.5, ^{31}P signal intensity is directly proportional to the molar P content of the sample. Unfortunately, the sample is pyrolyzed by ICP-ionization and no information on phosphorylation site can be gained. To obtain quantitative and structural data, the peptide digest has to be divided in two aliquots for ICP-MS and ESI- or MALDI–MS/MS-techniques, respectively. On the other hand, absolute quantitation of a phosphopeptide was achieved by analyzing in parallel the sample with the heavy isotope labeled synthetic phosphopeptide [173]. However, this method is restricted to samples with a known structure and limited by the economic costs of isotopically labeled phosphopeptides to be synthesized.

Two strategies have been recently introduced for the simultaneous assignment of phosphorylation sites and relative quantitative measurement of phosphorylation degree between multiples samples. Both are based on the MS measurement of

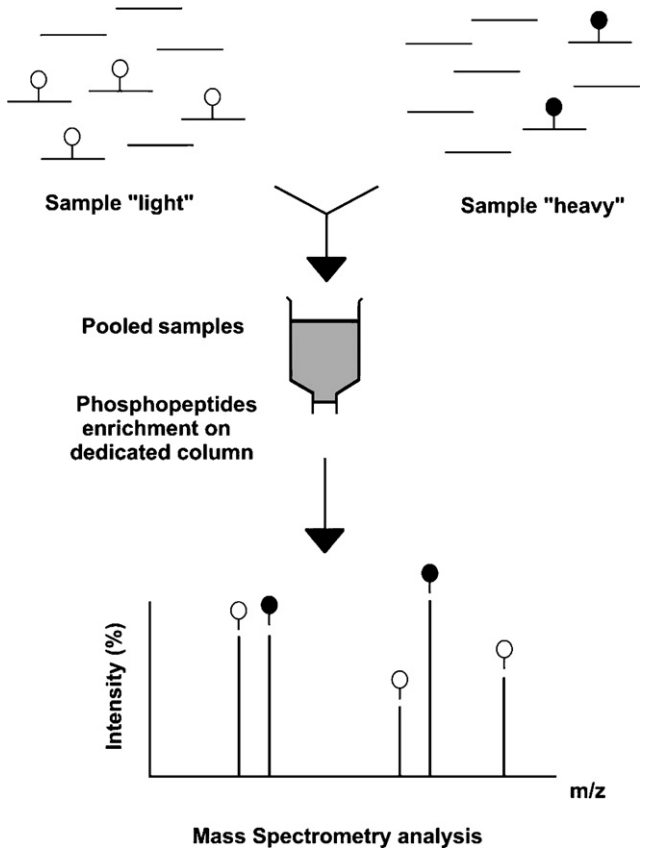

Fig. 6. Quantitative phosphorylation analysis based on phosphopeptide enrichment and MS experiments. Labeling of the samples may be done either in vivo or in vitro. Samples are mixed and loaded on a dedicated column for phosphopeptides enrichment. The resulting fraction is analyzed by MS; specific signals are integrated and used for relative quantitation.

chemically similar peptides showing identical ionization tendencies but different stable-isotope content [174]. The "light" and the "heavy" form of the same polypeptide, corresponding to the different states to be quantitatively compared, are generated by exclusive introduction of light and heavy isotope forms of the label, mixed together, eventually digested and subjected to MS comparison of relative peptide ion intensities (Fig. 6) [175]. This general principle has been extended to phosphoproteomics, making stable-isotope labeling as the method of choice for relative quantitative measurements of phosphorylation abundance [7]. In this case, quantitative differences in phosphorylation between the two-pooled samples are reflected by a change in the intensity ratios of the "light" and the "heavy" form of phosphorylated peptides as well as their unlabeled counterparts. Finally, sequencing of the light form by MS/MS is required to assign phosphorylated residues within the peptide sequence. The most crucial point within these methods is the choice of the incorporated isotope. Since deuterium can modify peptide retention times [176], ^{15}N- and ^{13}C-isotopes have been used to an increasing extent during LC-based MS quantitations [177].

The first strategy is based on in vivo labeling of proteins by the occurrence of ^{15}N-isotopes [175] or ^{13}C/^{15}N-labeled amino acids (SILAC) [178] within the growth medium. This approach is limited to situations where cells may be grown on labeled media and does not involve intrinsic enrichment for phosphorylated species. Accordingly, it has to be coupled to phosphopeptide-enrichment methods to avoid suppression of signals from phosphopeptides and achieve higher throughput [135,179]. In general, the most frequently used amino acids for stable-isotope labeling are Arg and Lys. In fact, Ser, Thr and Tyr have been excluded from candidates for isotopic labeling because of their conversion during their catabolism to other labeled amino acids, leading to erroneous results. This approach has been successfully used in quantitative phosphorylation profiling of HeLa cells [179], ERK/p90 ribosomal S6 kinase-signaling cassette and its targets [180] and yeast pheromone-signalling pathway [135] under different conditions.

In the second strategy, labels are introduced into the peptides/proteins by in vitro methods, using mass tags [69,181,182]. Reagents containing stable isotopes can either be attached to ubiquitous peptide functional groups [63,183,184], or replace the phosphate-moiety itself using the β-elimination/Michael addition chemistry [182]. Thus, the "light" and the "heavy" form of the reagent are independently reacted with digests deriving from the two different states to be compared. The two samples are mixed and subjected to MS analysis. Again, the ratio of the peak intensities of the normal and labeled phosphopeptides gives the stoichiometry of phosphorylation. As an example, Weckwerth et al. replaced the phosphate group of phosphopeptides by β-elimination, followed by Michael addition of ethanethiol or its fully deuterated version [182], during quantitative comparison of pSer/pThr-containing proteins in two separate phosphorylation states. No enrichment was performed in this case. An improved version of this methodology involved the use of normal and deuterated dithiothreitol as reagents, followed by enrichment of modified phosphopeptides by thiol chromatography [72]. Similarly, conversion of the phosphate moiety to isotopically labeled residues has also been combined with affinity tagging of peptides and proteins for selective enrichment [40] as in the case of the phosphoprotein isotope-coded affinity tag-(PhIAT) [69,185] or phosphoprotein isotope-coded solid-phase tag (PhIST)-techniques [185]. This approach has been used for quantitative phosphorylation profiling of MCF-7 human breast cancer cells [185].

If the modification is directed on ubiquitous groups, selective enrichment of phosphopeptide derivatives by IMAC procedures is necessary to remove unphosphorylated peptides humpering MS analysis and to achieve higher throughput. IMAC(Fe^{3+}) microcolumns have been used in the case of phosphopeptide quantitation using amine-reactive isobaric tagging reagent (iTRAQ). This reagent allowed ESI and MALDI tandem mass spectrometry-based comparison between four different phosphorylation states on Ser and Tyr residues for proteins isolated by gel electrophoresis at low- to sub-picomole amounts [152,186]. On the other hand, normal and deuterated propionic anhydride was used to label peptide digests generated for SDS-PAGE resolved phosphoproteins from two separated conditions [187]. Mixed digests were submitted to HMS-MS method and quantitation was obtained by relative intensity measurement of the two isotopic forms generated by the dominant loss of H_3PO_4 during MS/MS experiments. This approach was successfully applied to

MALDI–q-TOF and MALDI-ion trap MS/MS analysis of mouse brain extracts from animals treated with a psychostimulant drug. The use of carboxylate methylesterification and a dendrimeric trapping procedure has been adopted for the quantitative determination of pTyr-containing peptides in T-cell phosphoproteins subjected to pervanadate treatment [79].

A series of isotope-free MS methods for the relative and absolute quantitation of protein phosphorylation have also been proposed, which are based on LC–ESI-MS techniques [188–190]. Ruse et al. described a procedure that uses selected ion monitoring to determine the chromatographic peak areas of specific peptides from the digest of the protein of interest [188]. The extent of phosphorylation was determined from the ratio of the phosphopeptide peak area to the peak area of an unmodified reference peptide that acts as internal standard, correcting for variations in protein amounts and peptide recovery in the digest preparation procedure. Analysis of unphosphorylated peptide counterpart is performed in parallel. A similar methodology was used for quantification of gel-separated proteins and their phosphorylation sites using unlabeled internal standards [189]. In this approach, integrated chromatographic peak areas of phosphopeptide analytes from proteins under study were normalized to those of a non-isotopically labeled internal standard protein spiked into the excised gel samples just prior to in-gel digestion. This method of peak area measurement with an internal standard was used to investigate the effects of pervanadate on protein phosphorylation in the WEHI-231 B lymphoma cell line. Finally, a robust stable isotope-free MS method for relative and absolute quantitation of phosphorylation stoichiometries has been proposed by Kirschner and co-workers [190]. This procedure monitors the unmodified proteolytic peptides derived from the protein of interest and identifies peptides that are suitable for normalization purposes. Changes in phosphorylation stoichiometry are measured by monitoring the changes in the normalized ion currents of the phosphopeptide(s) of interest. Absolute phosphorylation stoichiometry are measured by monitoring the ion currents of a phosphopeptide and its unmodified cognate as the signal intensity changes of both peptide species are correlated [190].

10. Conclusions and future perspectives

A large variety of powerful analytical devices and MS methodologies for detecting and structurally characterizing phosphoproteins is now available. It is obvious that there is no a single method that supersedes all others for analysis. Important parameters in deciding the most appropriate approach are the quantity of the protein available, the nature of phosphorylated residue(s) (Ser, Thr and/or Tyr), the degree of purification of the phosphoprotein under investigation and finally if a global analysis is desired. Regardless of the method used, enrichment of phosphorylated protein or peptide improves the likelihood of success. Despite this formidable armamentarium, complete characterization of the phosphorylation state of a protein isolated in small quantities from a biological sample still remains far from routine. This is particular evident for hyperphosphorylated proteins whose structural characterization still remains a challenging task.

In this review, we reported on analytical devices and MS-based techniques/strategies that have been recently introduced to qualitatively/quantitatively characterize *O*-modification sites in phosphoproteins. Since MS is certainly the technique of choice for phosphopeptides characterization, we focused on methods not involving the use of ^{32}P, although MS-based procedures can certainly be combined to classical methods of phosphoprotein analysis. On the basis of the reported advances in sample preparation techniques as well as instrumentations, identification and localization of phosphorylation sites in proteins is more accessible today. These improvements as well as the above mentioned approaches for global analysis are now allowing prototype phosphoproteomic studies on collections of proteins sampled at an organelle-wide or cell-wide level. Recent examples include the phosphoproteome profiling of whole-cell yeast lysate [25], yeast pheromone signalling pathways [134], rice [191], developing mouse brain [92], mouse liver [192], HeLa cell nucleus [94], human post-synaptic density preparations [93], human skin fibroblasts [193], human colon adenocarcinoma cells [194], human hepatocytes [195], human cardiomyocites [196], human mitotic spindle [124] and others. All these data are now updating phosphorylation databases of eukaryotic proteins as well as constituting the starting point for iterative approaches directed to the identification of novel phosphorylation motifs from large-scale data sets [197]. Certainly, phosphoproteomics will remain a field of increasing importance in the next future. The enormous complexity of the protein phosphorylation pattern inside the cell will grant important challenges to future scientists. Better strategies/methodologies for the enrichment of phosphopeptides/phosphoproteins and for quantitative evaluation of phosphorylation changes in phosphoproteomes will help researchers in studies contributing significantly to deeper insights into cellular processes and cell regulation.

11. Nomenclature

CID	collision-induced dissociation
2DE	two-dimensional gel electrophoresis
Dha	dehydroalanine
Dhb	dehydrobutyrine
ECD	electron chemical dissociation
ESI	electrospray
ETD	electron transfer dissociation
FTICR	Fourier transform ion cyclotron resonance
HMS-MS	hypothesis-driven multiple-stage mass spectrometry
ICP	inductively coupled plasma
IMAC	immobilized metal affinity chromatography
IT	ion trap
LC	liquid chromatography
MALDI	matrix assisted laser desorption ionization
MS	mass spectrometry
MOAC	metal oxide affinity chromatography
NLS	neutral loss scanning
pSer	phosphoserine
pThr	phosphothreonine
pTyr	phosphotyrosine

PIS precursor-ion scanning
PSD post-source decay
q-TOF quadrupole time of flight
SDS-PAGE sodium dodecyl sulphate poly-acrylamide gel electrophoresis
SILAC stable isotope labeling with amino acids in culture
TOF time of flight

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