Exploring the Phosphoproteome with Mass Spectrometry

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Abstract: Protein phosphorylation is a reversible post-translational modification crucial in the control of numerous regulatory pathways. Understanding the highly interconnected nature of such networks requires new broader-scale analysis techniques. This report summarizes recent advances in the use of mass spectrometry to assess phosphorylation events in ever more complex systems.

INTRODUCTION

Perhaps the most intriguing finding to arise from the Human Genome Project is the relatively modest increase in the number of genes that humans possess compared to other lower eukaryotic organisms [1]. This clearly suggests that the functional evolution of proteins results more from combinatorial diversification of regulatory networks than from a proportional increase in gene number [2]. Among the more than 200 protein modifications described to date, only a limited subset have been demonstrated to be reversible, and thus of potential importance in actively regulating biological responses. Of these, protein phosphorylation is by far the most studied. Although generally exerting its regulatory function by altering the three dimensional shape and thus the function of a target protein, phosphorylation has also been implicated in such diverse processes as subcellular localization, complex formation, regulation of transcription factors, and the modulation of protein lifetime [3]. Additionally, multisite phosphorylation can enable several such effects to operate simultaneously in the same protein [4, 5].

The phosphorylation state of a particular protein at any given time is exquisitely controlled by the actions of two families of enzymes. Protein kinases affect the addition of a phosphate group primarily to serine, threonine, and tyrosine residues in eukaryotes, while protein phosphatases catalyze the removal of a phosphate group from these residues. The modification of histidine, arginine, and lysine residues has also been reported. It is estimated that approximately 1000 unique kinases and 500 phosphatases are encoded by the human genome, and that nearly one-third of all proteins are phosphorylated at any given time.

The ultimate goal of phosphoproteomics is nothing less than the complete characterization of this highly sophisticated regulatory network. At its basest level, this involves the identification of all phosphoproteins as well as the specific site(s) of their modification. Additionally, both the stoichiometry as well as the temporal organization of individual phosphorylation events with respect to other such modifications need to be determined. Traditionally, such studies have involved the incorporation of ^{32}P into proteins through the use of radiolabeled ATP, and the subsequent isolation and characterization of the resulting radioactive species after fractionation by such techniques as two-dimensional (2D) gel electrophoresis or peptide mapping as well as HPLC (7). These methodologies remain extremely valuable when used to investigate very specific issues, such as the ability of an *in vitro* kinase assay to faithfully reproduce its naturally occurring phosphorylation pattern [6] or the identification of *in vivo* substrates of an individual kinase using a novel chemical genomics approach [7]. However, these techniques are relatively laborious, and thus often ill-suited to characterize the myriad of changes that occur in these highly dynamic and interconnected regulatory pathways.

Over the past decade, mass spectrometry (MS) has become an increasingly powerful tool for the characterization of biomacromolecules, and several excellent reviews have recently highlighted the use of MS in protein phosphorylation analysis [8-10]. In this report, we outline recent advances in both sample preparation as well as MS for the detection and quantification of protein phosphorylation. Additionally, the potential use of such methodologies to investigate other related areas such as the determination of enzyme activity or the selectivity of small molecule inhibitors will also briefly be discussed.

THE NEED FOR ENRICHMENT METHODS

Despite the recent rapid advancements in MS-based protein characterization techniques, it must be noted that the comprehensive analysis of a protein's phosphorylation profile is still a very challenging undertaking. Most regulatory proteins are expressed at only low copy numbers per cell, necessitating their enrichment with respect to other more abundant species. Additionally, the stoichiometry of phosphorylation is generally relatively low, while multisite phosphorylation can lead to several heterogeneous forms of the same protein. Even in those cases where relatively homogeneous samples of a phosphorylated protein can be obtained, further challenges remain. For example, the mass spectrometric responses of phosphopeptides have been shown to be significantly suppressed in the presence of non-phosphorylated peptides, making the detection of the phosphorylated species more difficult. Such issues were clearly illustrated by a study presented at the ABRF 2003 annual meeting, in which only three groups out of 106 that originally requested samples were able to correctly identify the two sites of phosphorylation in a relatively simple test

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mixture. In light of these issues, numerous methodologies have been investigated to enrich phosphorylated peptides and/or proteins with respect to unphosphorylated species.

Phosphospecific Antibodies

In the absence of radioactive labeling, the presence of a series of spots on a 2D polyacrylamide gel with similar molecular weights but different isoelectric points may indicate differential phosphorylation. However, the limited loading capacities of 2D gels [11] combined with the relatively low abundances of most regulatory proteins makes this a relatively inefficient method for putatively identifying potentially interesting phosphorylated species. Therefore, complex protein samples are often first enriched by immunoprecipitation with commercially available antibodies that bind to phosphotyrosine (pY) in a generic fashion [12]. Although there have been only limited reports of effective protein immunoprecipitation/ enrichment using antibodies against phosphoserine (pS) and phosphothreonine (pT) [13], these species have successfully been used in Western-blot analyses [14].

Using such methodologies, numerous groups have reported extensive lists of proteins that appear to undergo changes in their phosphorylation profile in response to some stimulus [15-17]. Although there is value in knowing the identities of such proteins, only in a very limited number of cases are the actual site(s) of phosphorylation identified. This discrepancy is due to the fact that while protein identity can readily be assigned based on the detection of a limited subset of possible tryptic peptides, the identification of the actual site(s) of phosphorylation requires the detection of the individually modified peptides. As stated before, this task is further complicated due to the suppressed signal of phosphopeptides in complex mixtures. This suppression can be ameliorated by physically separating the phosphopeptides from other species using chromatographic or electrophoretic methods. However, a potentially more powerful approach would involve the active enrichment of phosphopeptides. Although phosphospecific antibodies have generally performed poorly in this regard, other chromatographic techniques have shown significant promise.

Immobilized Metal Affinity Chromatography (IMAC)

Originally developed by Porath for protein adsorption applications [18], IMAC has more recently been applied to the enrichment of phosphopeptides, and numerous studies involving both off-line and on-line implementations have been reported [19-22]. This technique exploits the affinity of a phosphate moiety for certain metal ions (Fe^{3+} or Ga^{3+}) coordinated by multivalent ligands bound to a solid support. After their enrichment, phosphopeptides can easily be eluted from the resin under basic conditions. Several factors have been reported to exert subtle effects on the overall selectivity of the procedure, including the natures of the metal ion, coordinating ligand, and stationary phase employed, as well as the number of phosphorylations on a given peptide. However, the greatest limitation of the technique has been the non-specific binding of peptides rich in glutamic and aspartic acid residues. Recently, Ficarro *et al.* reported that an initial straightforward methyl esterification of the carboxylate moieties greatly increased the overall selectivity of the process [23].

Although IMAC for phosphopeptide enrichment has traditionally been performed in micro-analytical columns before subsequent analysis, alternative approaches have also been described. For example, several consecutive enzymatic reactions and subsequent matrix-assisted laser desorption/ ionization (MALDI) analysis performed directly on the IMAC resin have been reported [24]. Alternatively, research performed in the authors' laboratory has resulted in the production of MALDI targets that can directly affect on-plate IMAC enrichment (manuscript in preparation). The surfaces of these MALDI target plates are patterned with carboxylate ligands in well defined locations using a plasma etching process, and the modified regions are loaded with Fe^{3+} by incubating the entire plate in a 100 mM $FeCl_3$ solution followed by extensive water washing. Peptide mixtures containing phosphorylated species are loaded onto the targets and directly washed with a solution containing 100 mM NaCl, 25% acetonitrile, and 1% acetic acid. After removal of the salt using an 0.1% acetic acid wash solution, MALDI matrix is added and the sample is directly analyzed. Fig. (**1**) shows the significant enhancement obtained for the signal of a native phosphopeptide from equal levels of a tryptic β-casein digest after on-plate IMAC enrichment.

Chemical Modification Enrichment Strategies

In addition to such targeted chromatography-based methods, several chemical derivatization strategies have also been utilized for the selective enrichment of phosphopeptides and/or proteins. pS and pT-containing peptides can undergo a β-elimination reaction under basic conditions, and the resulting dehydroalanine and β-methyldehydroalanine residues can selectively be reacted with various nucleophiles. Several groups have reported the use of ethanedithiol as a nucleophile, and the subsequent reaction of the free thiol group with different sulfhydryl-containing biotin derivatives, thus enabling the isolation of the labeled peptides using an avidin resin [25, 26]. Oda *et al.* correctly noted the necessity of first blocking the thiol group of cysteine residues by performic acid oxidation rather than classical alkylation methods, as alkylated cysteine species can also undergo the same β-elimination reaction, potentially leading to erroneous results. The same group also recognized that β-*N*-acetylglucosamine-modified serine and threonine residues are even more susceptible to this alkali-induced elimination reaction than their phosphorylated analogs. An excellent report by Wells *et al.* details sample preparation techniques that enable these two important post-translational modifications to be analyzed individually [27].

A chemical devivatization strategy has also been reported for the selected enrichment of pY-containing peptides [28]. After methyl esterification of the carboxylate moieties, all phosphorylated peptides are captured onto an imidazole-functionalized resin *via* a carbodiimide-mediated coupling reaction. Subsequent treatment with acid selectively releases pY-containing peptides from the resin in their original form, while other phosphorylated species remain bound to the solid support.

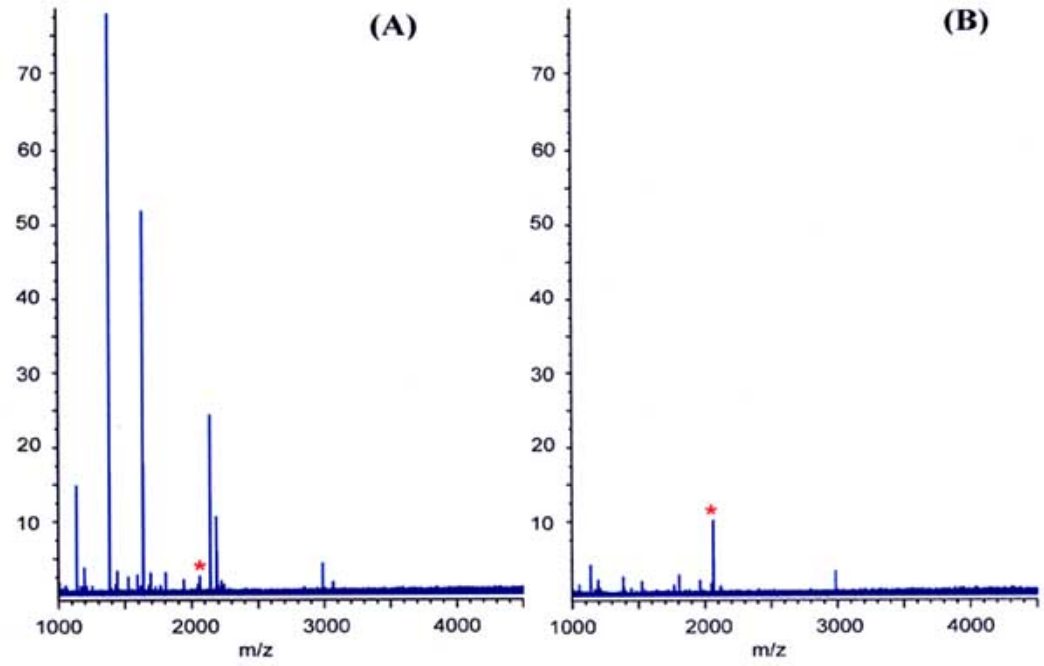

Fig. (1). On-target enrichment of 100 fmol of β-casein tryptic digest on 500 μm diameter anchor. (A) no on-plate enrichment (B) phosphopeptide enriched on iron-chelated IMAC surface. * = FQpSEEQQQTEDEFQDK Matrix = 2,4-dihydroxybenzoic acid.

Finally, Zhou *et al.* described a derivatization strategy that is applicable to all phosphopeptide species [29]. Based ultimately on the acid-catalyzed lability of phosphoramidite bonds compared to their amide counterparts, this methodology also requires several chemical protection reactions as well as a solid-phase capture and release strategy. Although the multistep nature of all the schemes described in this section creates serious limitations with respect to performing highly sensitive analyses, chemical modification enrichment strategies remain an area of active research.

Enrichment and MS-based Strategies for Kinase/ Phosphatase Profiling

Although the various combinations of sample enrichment and MS techniques described so far have been tailored towards the identification and characterization of protein phosphorylation events, similar methodologies can also be used to investigate related areas of research. For example, affinity chromatography using purvalanol B immobilized on an agarose matrix not only validated cyclin-dependant kinases as intracellular targets of such 2,6,9-trisubstituted purines but also enabled the mass spectrometric identification of several unexpected protein kinase targets [30]. Similarly, Haystead *et al*. have described the use of ATP immobilized on a surface to capture the purine-binding "sub-proteome" [31]. Functional proteomics studies can then be performed in a highly parallel fashion by treating the isolated proteins with different individual chemical species. Compounds that displace one or more members of the captured "sub-proteome" are identified as potential therapeutics, with the number of proteins displaced being indicative of the specificity, and therefore potential toxicity, of the particular compound.

In contrast to the discovery of inhibitors for individual enzymes, activity-based proteomics strives to enable the identification and comparative measurement of all the active members of a given enzyme class under different conditions [32]. Although the majority of reports to date have focused on serine and cysteine hydrolases, Lo *et al.* recently described the design of class-selective affinity probes for protein tyrosine phosphatases [33]. As shown in Fig. (**2**), these probes utilize a trapping device derived from *p*-hydroxymandelic acid that takes advantage of quinone methide chemistry, and can be synthesized with either a fluorescent or affinity-based reporting group. The probes were shown to be selectively activated by tyrosine phosphatases, resulting in their covalent labeling, while no reaction with other classes of hydrolases was observed.

Mass Spectrometric Characterization of Phosphorylation

Despite the utility of such enrichment techniques, mass spectrometric characterization of the isolated species is still required to verify the existence as well as determine the nature of the phosphorylation event. Ideally, such characterization would provide confirmation of the occurrence of phosphorylation, the location of the site of modification, and determination of its relative and absolute stoichiometry. Although several different MS-based techniques have been developed for the first two objectives, the third remains a considerable challenge especially with respect to large-scale analyses.

MS Ionization and Instrumentation

The two principal and complementary methods employed for the ionization of proteins and peptides are MALDI and

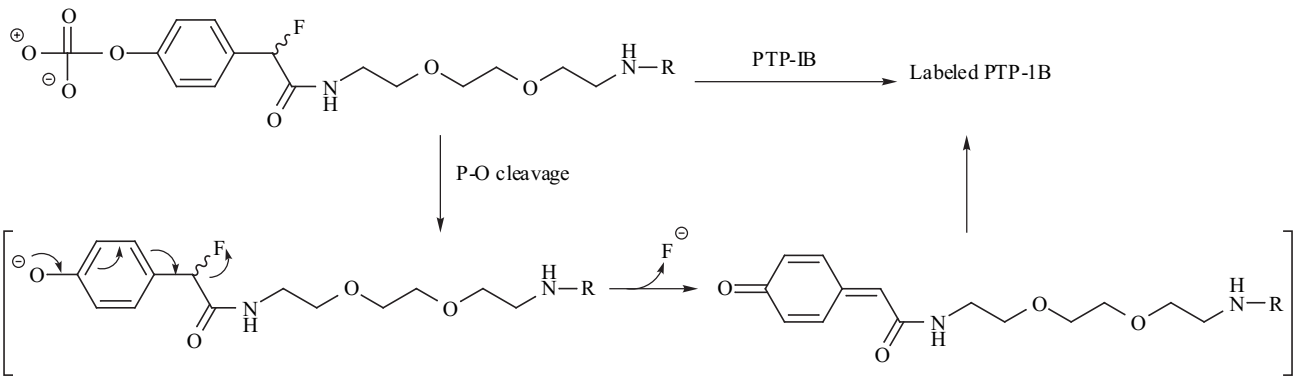

Fig. (2). Structure of activity probes specific for protein tyrosine phosphatases (PTP) and mechanism-based labeling. Modified, from reference 33.

electrospray ionization (ESI). There are numerous mass analyzers available that can be paired with either of these two ionization techniques. Each technique and detector impart their own advantages and limitations for phosphorylation analysis. For example, MALDI almost exclusively produces singly-charged ions, while ESI tends to generate multiply-charged species. Although the charge state of the analyte ion has only a limited influence on the ability to detect phosphorylation, multiply-charged ions are more advantageous for localizing the site of phosphorylation due to their more uniform backbone fragmentation compared to their singly-charged counterparts. Thus, despite the speed and simplicity of MALDI-based analyses, ESI has nearly exclusively been employed for the determination of sites of phosphorylation. In addition, although both ionization methods can in principle be used with any mass analyzer, only certain combinations have routinely been employed due to their ease of integration. Even though such limitations are slowly being addressed, to date no single analysis platform has enabled the comprehensive characterization of phosphorylation.

Detection of Phosphorylated Species

The signals of phosphopeptides in typical positive mode MALDI-based analyses are greatly attenuated due to both the differential suppression that occurs in complex mixtures as well as the inherently weaker ionization efficiency of these more acidic species. In fact, numerous reports in the literature indicate that the relative MALDI responses of phosphopeptides are significantly improved when analyzed in the inherently less sensitive negative mode [34, 35]. However, classic peptide mass fingerprinting (PMF) experiments employing MALDI time-of-flight (TOF) MS can still be used to rapidly confirm the presence of phosphorylation. In PMF, masses measured from a proteolytic digest are compared to theoretical digests of all the proteins contained in a given database using cross-correlation methods. Upon identification of the protein, the presence of phosphorylated peptides can be inferred from observed offsets of 80 Da between the observed and theoretical masses of the proteolytic fragments. Similarly, differential comparison of the PMF profiles of a tryptic digest before and after treatment with a phosphatase has also been used to confirm the presence of phosphopeptides [36-39].

In addition to such observed mass shifts, the relative instability of phosphorylated residues can also be utilized to confirm the presence of this modification. In-source fragmentation techniques have been described for the detection of phosphopeptides [40, 41], although this method requires spectra to be obtained in the linear mode with comparatively modest resolution and mass accuracy. Alternatively, post-source decay (PSD) in the field-free region of TOF instruments can also be used to produce fragment ions. These fragments are indistinguishable from their parent ions in the linear mode, but are detected in the reflectron mode as isotopic clusters of lower resolution at lower *m/z* values [42]. In addition to indicating the presence of phosphopeptides, this technique can often enable the discrimination between pS/pT- and pY-containing species. The former two often exhibit a dominant neutral loss of 98 Da due to the elimination of phosphoric acid, whereas the later typically exhibits a neutral loss of 80 Da. Improved analysis software has recently been reported to yield more confident identification of phosphopeptides using PSD in the reflectron mode [43]. Although the localization of sites of phosphorylation using PSD has been reported [42, 44], technical limitations in ion activation make other methodologies more attractive.

Recently, it has been demonstrated that MALDI can readily be performed at elevated or even atmospheric pressures. This has enabled more facile coupling of MALDI sources to mass detectors that traditionally have been employed with ESI methods nearly exclusively [45-47]. These instruments, including quadrupole TOFs (Q-TOF) and triple quadrupoles, enable several different scanning schemes for the selective detection of phosphopeptides. Higher pressure MALDI sources should also affect less fragmentation, and thus be more advantageous for the analysis of labile modifications like phosphorylation. However, the full impact of this technology remains to be seen.

Precursor ion scanning under basic conditions has extensively been used for phosphopeptide detection [48-50]. The facile loss of PO_3^- by collisionally-activated disassociation (CAD) is monitored at *m/z* 79 in the negative mode, implicating any species giving rise to this signal as a phosphorylated peptide. Although highly sensitive for the detection of phosphorylation [48, 49, 51-53], this methodology does not enable simultaneous determination of

the sites of modification. Instead, the analysis is often performed in conjunction with fraction collection. After detection of the presence of a phosphopeptide in a given fraction, that sample is then acidified and reanalyzed in the positive mode to determine the site(s) of phosphorylation. The recent introduction of robotic stations for automated nano-electrospray applications (Advion BioSciences) makes the use of such multistep procedures more attractive.

A precursor ion scanning method based on monitoring the immonium ion of pY at *m/z* 216.043 directly in the positive mode has recently been reported, enabling the selective identification of less abundant tyrosine-phosphorylated species [54, 55]. Additionally, parent ions giving rise to this signal can immediately be subjected to tandem MS in a data-dependent fashion for phosphorylation site identification. It should be noted that this method requires the use of a Q-TOF mass spectrometer in order to obtain mass measurements with sufficiently high resolution to unequivocally distinguish pY immonium ions from dozens of other possible peptide fragments ions with nominal masses of 216 Da. The performances of both triple quadrupole and Q-TOF mass analyzers have been compared for precursor ion scanning applications in both the positive and negative mode. In general, both were found to exhibit comparable sensitivities, with the exception that the Q-TOF displayed an approximately five-fold higher sensitivity during pY immonium ion scanning, which was attributed to recent instrumental improvements [55].

Alternatively, a chemical labeling strategy that replaces the phosphate group on pS/pT residues with a positively charged sulfenic acid moiety has also been shown to facilitate precursor ion scanning for these phosphorylated species in the positive mode [56]. This chemical modification is introduced using a β-elimination/Michael addition reaction scheme as described earlier followed by an oxidation procedure. The resulting sulfenic acid moiety behaves like a phosphate group under CAD conditions in that it undergoes a gas phase β-elimination. However, the resulting ion instead carries a positive charge that is detectable at *m/z* 122 Da without interference from other potential immonium ions. Thus, species identified as phosphopeptides can immediately be subjected to tandem MS characterization. Although advantageous, this methodology is subject to the same limitations of multistep chemical labeling schemes as described earlier, with in-gel conversion rates of approximately 60% being reported.

Neutral loss scanning methods for the selective detection of phosphopeptides [50, 57-59] are based on the characteristic losses of 98 (H_3PO_4) and 80 Da (HPO_3) exhibited by these species, although this phenomenon is greatly attenuated for pY-containing peptides. Employing a triple quadrupole mass spectrometer, this methodology requires the simultaneous scanning of both mass selectors offset from each other by the expected *m/z* loss. However, given the nature of the ESI technique typically employed, this *m/z* value will vary based on the charge state of the individual ions. This information is typically not available at the outset of an analysis, explaining the scarcity of neutral loss scanning application in protein phosphorylation analysis [57].

Neutral loss scanning can also be performed in an automated fashion using Q-TOF instrumentation. The instrument changes collisional energies between different data acquisitions, and analyses the resulting spectra to identify all differential neutral losses as well as the charge state of their associated ions. A data-dependent MS/MS scan is performed on those species showing the appropriate losses in order to identify the peptide and localize the site of modification. Although this feature is currently available and has been utilized in the authors' laboratory, the authors are unaware of any publication describing the implementation of this methodology.

The use of inductively coupled plasma-mass spectrometry (ICP-MS) to indicate the presence of phosphorylation by monitoring elemental ^{31}P has also been described [60]. Detection limits of 100 fmol as well as the ability to determine the average phosphorylation content of proteins by simultaneously monitoring ^{31}P and ^{32}S have been reported [61]. The same group used this methodology in combination with tandem MS to both identify the phosphorylation sites in the polo-like kinases Plx1 and Plx2 as well as quantitate the extent of their phosphorylation [62].

Localization of the Site of Phosphorylation

The determination of the exact site(s) of phosphorylation of a protein can be a challenging endeavor. Under fortuitous circumstances, a confirmed phosphopeptide possesses only one possible site of modification, obviating the need for further sequencing experiments. However, nature is rarely so accommodating, and ironically, the MS behavior of phosphopeptides that is often so instrumental in identifying their presence is highly detrimental to localizing the site of modification. Specifically, tandem MS experiments performed on phosphopeptides often yield limited sequence information due to the preferred loss of the phosphate group, resulting in spectra dominated by peaks corresponding to this loss.

Fig. (**3**) shows the tandem MS spectrum of the serine-phosphorylated peptide KGpSEQESVKEFLAK that results from the tryptic digestion of PKA protein. The spectrum is dominated by two fragments arising from the losses of H_3PO_4 as well as H_3PO_4 and water, while a much smaller but still informative series of y-ions (as expected from the site of phosphorylation) [50, 63] is also observed. Daughter ions resulting from the fragmentation of the β-eliminated species will contain dehydroalanine (in the case of serine) or β-methyldehydroalanine residues (in the case of threonine) that serve to identify the original site of phosphorylation. However, the observed ratio of β-eliminated species to sequence-informative fragment ions is different for each peptide, and numerous species provide little or no useful fragmentation data. By comparison, pY-containing peptides exhibit significantly higher resistance to the β-elimination reaction, and often exhibit pY-containing fragments upon tandem MS.

In light of these issues, numerous strategies have been described that seek to facilitate phosphorylation site determination by varying the nature of the phosphopeptide under study. For example, multiple sequences containing the

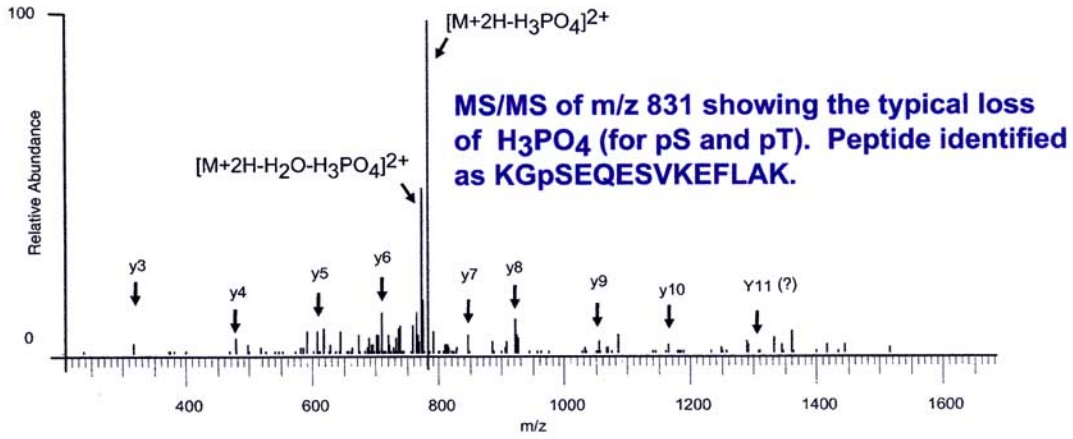

Fig. (3). Tandem MS spectra of $[M+2H]^{2+}$ ion of the peptide KGpSEQESVKEFLAK recorded on a ThermoFinnigan LCQ DECA ion trap MS.

modification can be generated by employing a variety of enzymes with different cleavage specificities [57,64]. Additionally, the phosphate group can chemically be transformed into a more fragmentation-resistant moiety using some of the strategies described earlier [65].

Electron capture dissociation [66] (ECD) is a relatively new fragmentation method for peptide and protein sequencing. This method appears to be extraordinarily useful for the localization of labile post-translational modifications, as the nonergodic fragmentation process employed produces abundant backbone fragmentation without concomitant loss of the modifications[67]. Among other applications, this technique has been applied to the sequencing of singly- and multiply-phosphorylated peptides [68] and proteins [69]. It has been demonstrated that ECD generally produces more sequence data than CAD and other fragmentation methods, although often the data obtained using different methods is complementary [69]. Fig. (**4**) shows a representative example of the CAD and ECD fragmentation patterns of the same species. Although powerful, this methodology has only been implemented with a Fourier transform ion cyclotron resonance mass spectrometer, therefore limiting its general availability. Additionally, the technique can only be performed with multiply-charged ions, restricting its use with MALDI-based systems.

Determination of the Stoichiometry of Phosphorylation

Several methods for quantifying the relative and absolute levels of phosphorylation at individual site(s) have been developed in an effort to understand the competitive biological processes acting on a protein. Traditional techniques for phosphorylation quantitation include phosphoamino acid analysis and Edman degradation [4, 70], but these methods are relatively tedious and are typically performed only after a phosphorylation event has already been identified. Given the ever increasing importance of MS in protein characterization, it is not surprising that researchers are also investigating its ability to simultaneously affect quantitation. However, a direct comparison of the relative strengths of different peptide signals cannot be used to accurately perform quantitation, since the ionization efficiencies of peptides vary widely both as a function of their individual sequences as well as the experimental conditions employed. Wind *et al.* [62] reported a method based on the combination of ICP- and ESI-MS, and demonstrated its utility by characterizing the extent of phosphorylation at several sites in Plx1 and Plk1.

Recently, differential quantitation methods based on stable isotope incorporation and MS detection have garnered significant attention. Perhaps the simplest method to affect the incorporation of stable isotopes into proteins is to directly grow cell cultures in isotopically enriched or depleted media. Thus, Oda *et al.* [71] differentially labeled two cell cultures grown under different condition using ^{14}N and ^{15}N enriched media. Equal numbers of cells from the two cultures were combined, and all subsequent processing steps and mass spectrometric analyses were performed on the mixture. Due to the incorporation of the different isotopes, each peptide species appears as a pair of peaks in the mass spectra, with the ratio of the peaks reflecting the relative abundance of each species in the original cultures. This relative quantitation takes advantage of the fact that in effect, each peptide serves as its own internal standard. Using this methodology, phosphorylation sites in the PAK-related yeast Ste20 protein kinase that specifically depend on the presence of G1 cyclin Cln2 were identified by significant changes in their relative ratio. Although simple to implement, this approach is obviously limited to the exploration of systems that can be grown in cell culture, and provides no enrichment of the phosphorylated species.

Alternatively, chemical labeling can also be used to introduce stable isotopes into protein samples. Often, the same methodologies described previously to affect sample enrichment can readily be adapted to also perform this function. For example, Weckwerth *et al.* described the use of ethanethiol and its deuterated analog in the β-elimination/ Michael addition reaction scheme to differentially quantitate serine and threonine phosphorylation [72]. Similarly, phosphorylation specific variations of the isotope coded affinity tag [73, 74] approach have also been reported [26, 75]. Although highly versatile, performing multiple chemical transformations both quantitatively and selectively on highly complex mixtures remains a formidable challenge.

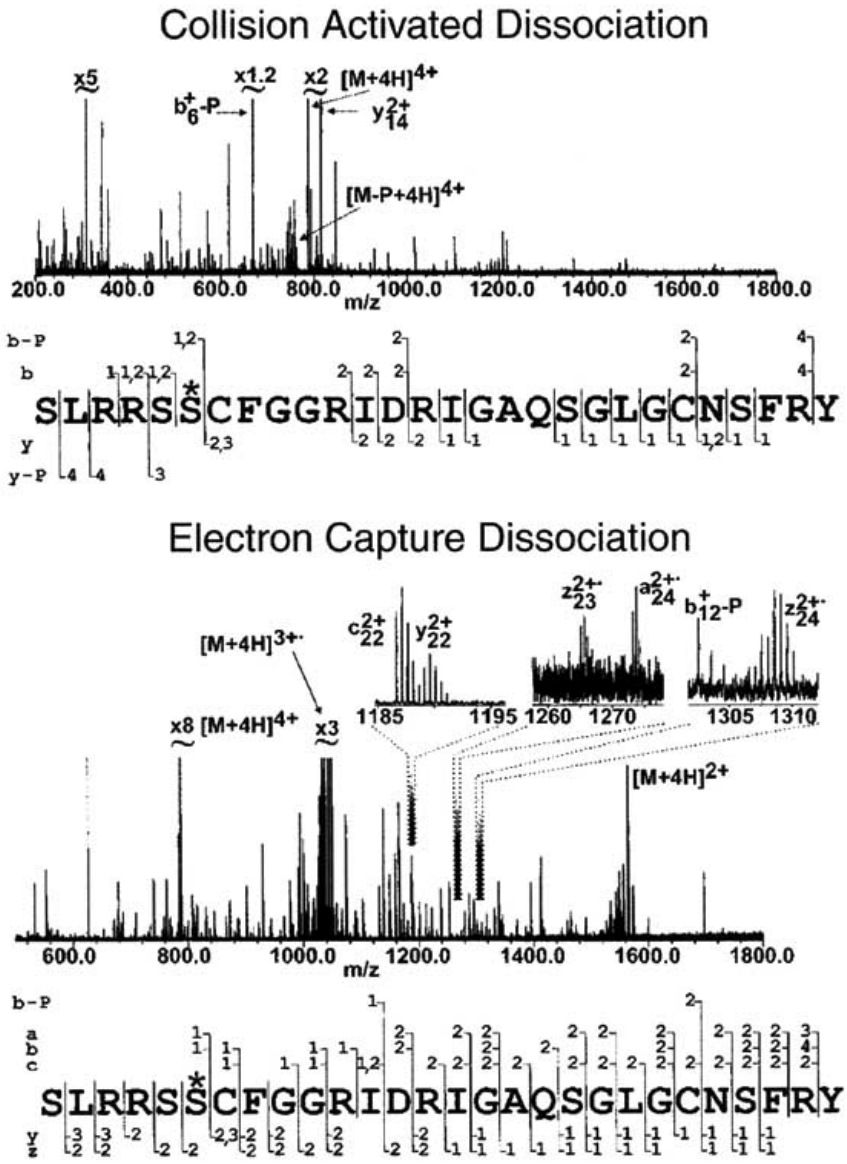

Fig. (4). Comparison of sequence information obtained from CAD (top) and ECD (bottom) of the $[M+4H]^{4+}$ ion of the phosphopeptide atrial natriuretic peptide substrate. Modified, from reference 69.

Zhang *et al.* recently reported a clever strategy for determining the absolute stoichiometry of phosphorylation of individual sites [76]. As outlined in Fig. (**5**), the sample of interest is first digested and all lysine residues are selectively guanidated. The sample is then split into two equal portions. The first portion is dephosphorylated by treatment with a phosphatase and then *N*-terminally labeled with propionic anhydride, while the other half is only *N*-terminally labeled with the deuterated analog. The two portions are recombined and analyzed by MS, with each phosphopeptide giving rise to three distinct isotopic clusters compared to only two for all other species. The stoichiometry of phosphorylation is directly calculated from the peak intensities of the differentially labeled dephosphorylated peptides with errors of less than 10%.

PROGRESS TOWARDS PROFILING CELLULAR SIGNALING NETWORKS

To date, the majority of phosphorylation studies have been performed on individual proteins. Specifically, after confirming the presence of this modification in a given protein, the species is first purified to homogeneity and experiments are then performed to identify the phosphorylated amino acid(s) [77, 78]. Although such studies are clearly useful as starting points in defining the regulatory effects of this modification, they are unable to assess the dynamic interplay of phosphorylation that occurs on multiple proteins as signals are transduced from receptors to downstream effectors.

In recognition of the highly interconnected nature of such regulatory pathways, researchers have begun to develop

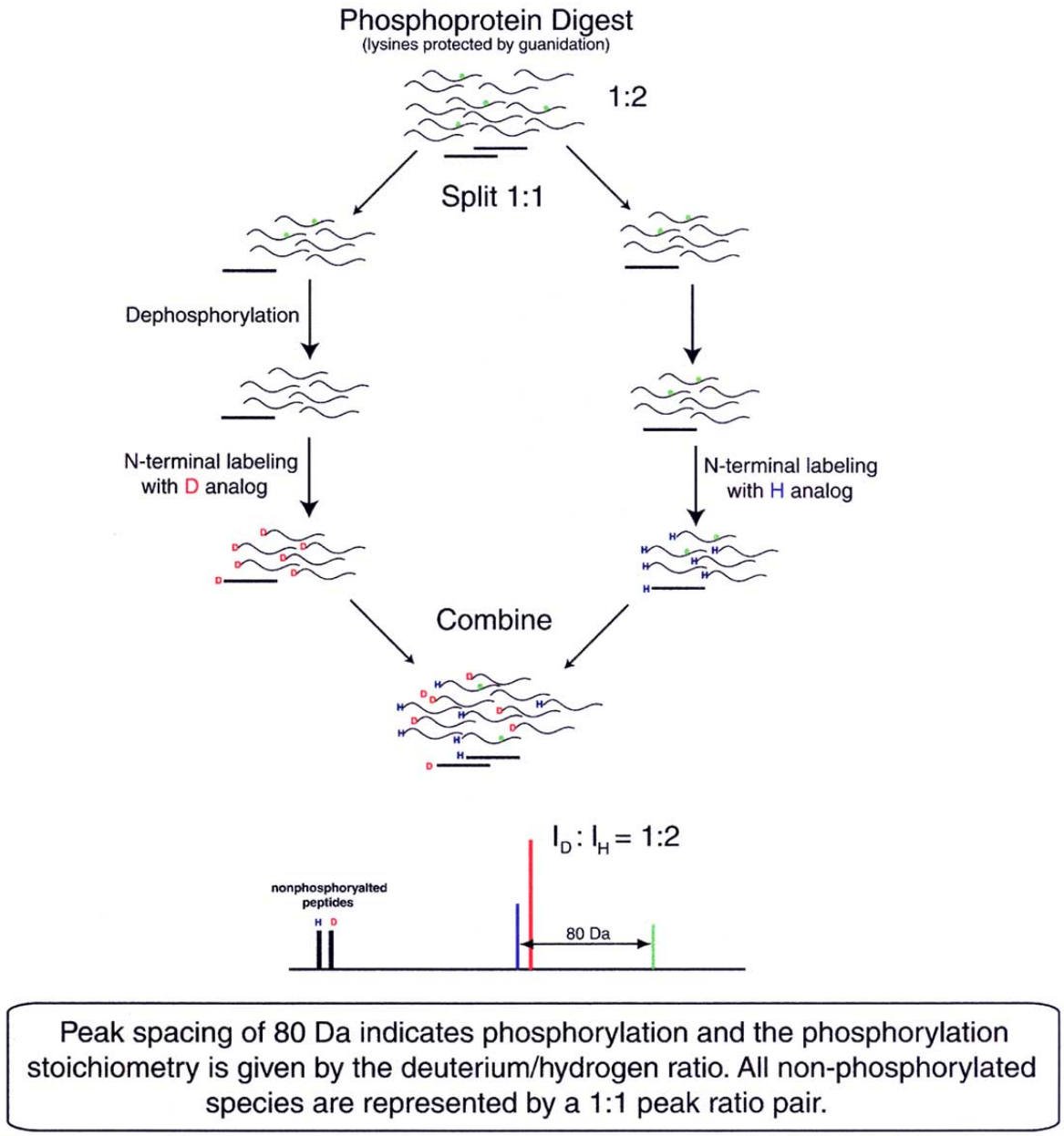

Fig. (5). Scheme for the determination of absolute phosphorylation stoichiometry. A lysine-protected phosphoprotein digest is split equally into two fractions. Fraction 1 (left) is dephosphorylated by phosphatase treatment. Fraction 2 (right) is incubated with inactivated phosphatase (no dephosphorylation). Fractions 1 and 2 are N-terminally propionylated with D_5 and H_5 propionic anhydride, respectively. The combined fractions are hydroxylamine treated to remove acyl-tyrosines, followed by MS analysis. Modified, from reference 76.

methods that enable the profiling of signaling networks and cellular phosphorylation on a broader scale. However, most of these approaches utilize the protein resolving power of 2D gel electrophoresis to primarily identify proteins involved in various signaling pathways rather than specifically localize the sites of modification. Typically, differently treated cell samples are lysed and the resulting proteins are resolved by 2D gel electrophoresis. After comparison of the resulting 2D images, spots exhibiting a differential display pattern are excised and analyzed by mass spectrometry. In an elegant series of experiments, Ahn and co-workers utilized this approach to identify novel members of the MKK/ERK pathway [79]. Employing combinations of phorbol ester treatment, transfection of active mutant kinases, and inhibitors of the MKK1/2 kinases, the authors identified 25 members of the MKK/ERK signaling cascade, 20 of which were previously not reported. Although most of the observed changes in the 2D protein patterns were probably due to variations in post-translational modifications including phosphorylation, this study did not specifically confirm the presence of phosphorylated amino acids in any given protein.

In order to further focus such studies towards proteins regulated by phosphorylation, Western blotting with phosphospecific antibodies has been employed after 2D gel

electrophoresis. Soskic *et al.* used this approach to identify proteins involved in platelet-derived growth factor β receptor (PDGFR) signaling [14]. The authors reported that nearly 100 spots showed strong intensity changes after PDGF stimulation as visualized by Western blotting with α-phosphoserine or α-phosphotyrosine antibodies. Known members of the PDGFR signaling pathway such as ERK1, akt, and syp were identified, as well as a plexin-like protein previously not associated with this pathway. However, only nineteen of the protein bands were positively identified by mass spectrometry, and among these, only two sites of phosphorylation were defined. In a similar fashion, Marcus *et al.* employed 2D gel electrophoresis followed by blotting with α-phosphotyrosine antibodies to identify pY-containing proteins in platelets [80]. They observed 29 putative tyrosine phosphorylated proteins, and identified 28 of them. However, no sites of phosphorylation were localized, with the authors stating that the phosphopeptides were present at levels below their detection limit.

In studies such as these employing unfractionated cell lysates, phosphorylated proteins represent only a small fraction of the total sample applied to the gel, further decreasing their likelihood of detection. To address these limitations, several groups have reported performing 2D gel electrophoresis studies on samples that were first enriched by immunoprecipitation with α-phosphotyrosine antibodies. For example, Maguire *et al.* studied changes in the tyrosine phosphorylation of platelet proteins in response to thrombin stimulation [17]. 67 proteins were found to be unique in the thrombin-activated platelet sample compared to resting platelets, and ten of these were identified by Western blotting and MALDI-TOF MS, including the known signaling proteins FAK, Syk, and MAPKKK. Similarly, this methodology was used to create subproteome maps of tyrosine phosphorylated proteins in B-lymphoblasts [81]. Five proteins were found to be specifically expressed in Scott syndrome phenotype lymphoblasts while four were unique to the control cells.

Other approaches for profiling signaling pathways instead use only a single dimension of electrophoresis. For example, Kerr and co-workers reported that 2D gel electrophoresis of proteins from cytokine-treated cells followed by Western blotting gave irreproducible results. Thus, several different techniques were used to isolate protein subsets, and these fractionated samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [16]. The use of SDS-PAGE has the advantage of detecting high molecular weight and/or hydrophobic proteins not amenable to 2D gel electrophoresis [12, 82]. Immunoprecipitation followed by SDS-PAGE has been utilized to identify tyrosine phosphorylated proteins in the epidermal growth factor receptor (EGFR) signaling pathway [12, 83], components of the T-cell antigen receptor complex [82], and serine and threonine phosphorylated proteins [13] using appropriate antibodies.

Although these approaches enable the identification of multiple signaling cascade components at the protein level, it should be noted that in the overwhelming majority of cases they are unable to discern the actual site(s) of phosphorylation. Such analyses typically require the use of previously described methodologies for the selective enrichment and/or detection of phosphopeptides. For example, Steen *et al.* utilized a pY-specific precursor ion scanning technique to identify phosphorylation sites from an immunoprecipitated protein sample fractionated by SDS-PAGE [84]. Using this phosphotyrosine immonium ion scanning technique, they identified five novel phosphorylation sites on proteins involved in EGFR signaling.

Recently, methods have emerged that do not employ any electrophoretic separations but that still enable the identification of numerous phosphorylation sites using liquid chromatography coupled to MS. For example, Zarling *et al.* reported a method for profiling the phosphorylation of peptides presented by major histocompatibility complex (MHC) class I molecules [85]. Immunoprecipitation of individual MHC class I alleles followed by IMAC enrichment and RP-HPLC/MS enabled the detection of approximately 100 phosphorylated species, of which 15 were successfully sequenced by tandem MS. The identification of such MHC class I phosphopeptides unique to cancer cells could serve to identify potential targets for immunotherapy approaches.

Several groups have recently reported the identification of multiple phosphorylation sites directly from complex protein mixtures without any initial fractionation. As shown in Fig. (**6A**), Aebersold and co-workers described a six step method for the isolation and analysis of phosphorylated peptides [29]. Based ultimately on the acid-catalyzed lability of phosphoramidite bonds compared to their amide counterparts, this methodology also requires several chemical protection reactions as well as a solid-phase capture and release strategy. The authors identified 24 phosphopeptides from abundant glycolytic proteins in a yeast total protein extract digest, and could assign the phosphate group to a single residue in 14 cases.

An alternative strategy that also utilizes the chemical derivatization of phosphopeptides to facilitate their enrichment was reported by Ficarro *et al.* [23]. As shown in Fig. (**6B**), peptides derived from a yeast total protein extract digest were converted to their corresponding methyl esters and subjected to IMAC enrichment. This derivatization greatly increased the selectivity of the IMAC column for phosphopeptides, enabling the detection of more than 1,000 distinct phosphorylated species. Of these, 216 phosphopeptides could readily be sequenced by tandem MS, leading to the unequivocal localization of 383 sites of phosphorylation. Over sixty phosphopeptides arose from proteins having a codon bias less than 0.1, demonstrating that this method can be used to define sites of phosphorylation even for proteins of low abundance. In addition to facilitating phosphopeptide enrichment by IMAC, methyl ester derivatization has also shown utility for peptide quantitation using stable isotope dilution [86]. Visconti and co-workers applied this methodology in conjunction with IMAC enrichment to quantify changes in phosphorylation that occur during sperm cell maturation [87]. In addition, the authors identified over 60 phosphorylation sites on proteins from human sperm, including 5 sites of tyrosine phosphorylation.

Although such methods for wide-scale phosphorylation profiling are promising, they typically result in the

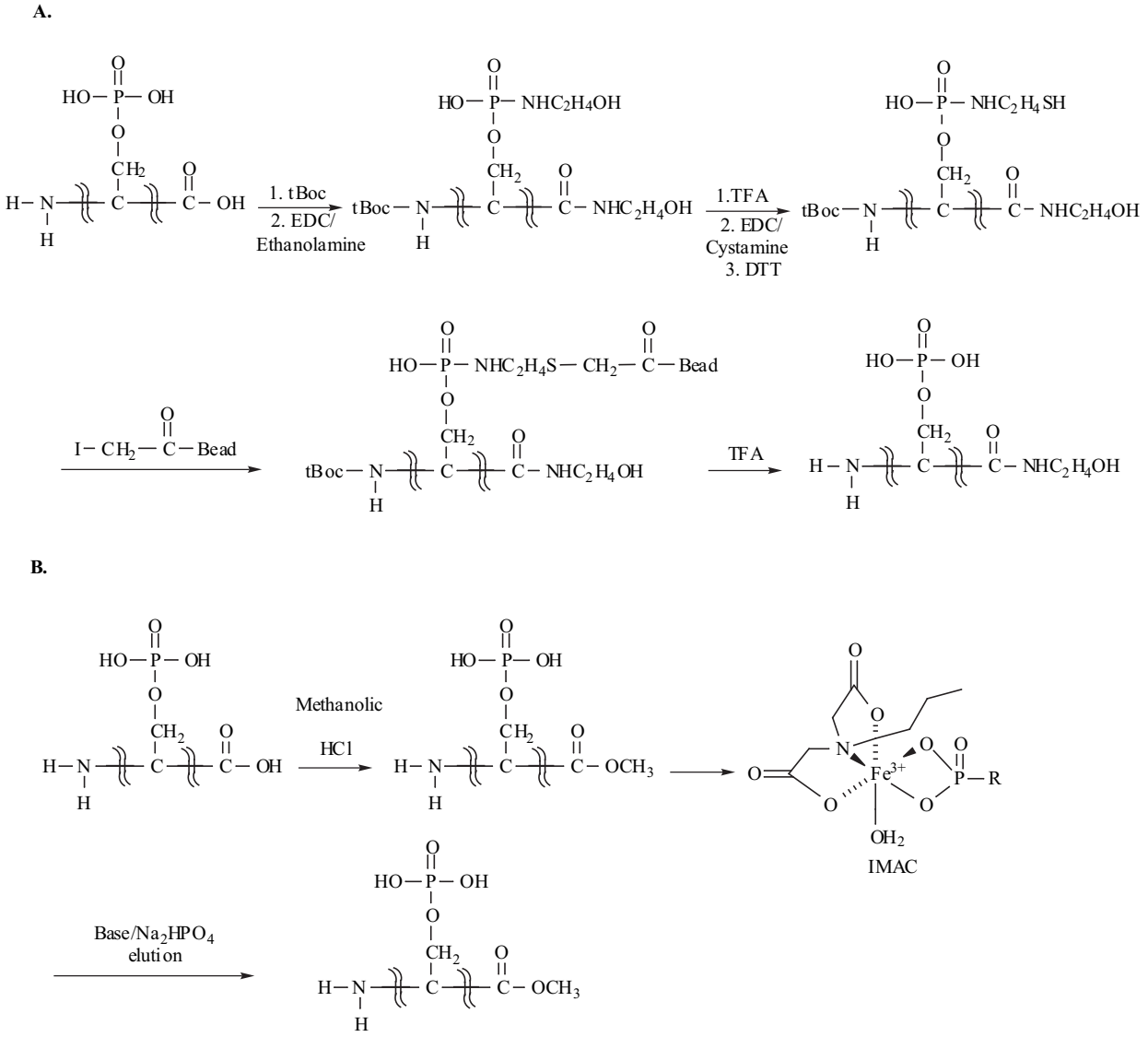

Fig. (6). Schemes for wide-scale identification of phosphorylation sites from complex mixtures. (A) Zhou *et al.* [29] procedure for phosphopeptide enrichment utilizes a carbodiimide activation to selectively couple thiol tags to phosphate functional groups. Tagged peptides are captured on iodoacetamide-functionalized beads, released by treatment with TFA, and analyzed by RP-HPLC/MS. (B) Ficarro *et al.* [23] method for enriching phosphorylated peptides using immobilized metal affinity chromatography. Peptides were first methyl ester modified to limit non-specific binding of carboxylic acid groups to the IMAC column. Enriched peptides were then analyzed by RP-HPLC/MS.

identification of only a very limited number of tyrosine phosphorylation sites [23, 87]. This effect is due to the significantly lower abundance of pY compared to pS/pT as well as the fact that some methodologies specifically target serine or threonine phosphorylation [25, 26]. However, tyrosine phosphorylation is disproportionately important with respect to its abundance due to its involvement in numerous pathways that regulate cellular proliferation and differentiation. Salomon *et al.* [88] described a method for profiling tyrosine phosphorylation sites from whole cell lysates, and utilized it to detect phosphorylation changes that occur over time either during the activation of T cells or in response to chemical inhibition of the BCR/Abl tyrosine kinase in leukemia cells. The procedure involved an initial immunoprecipitation step with an α-phosphotyrosine antibody, followed by digestion of the isolated proteins with trypsin. The resulting peptides were methyl esterified, and subjected to IMAC followed by RP-HPLC/MS. This strategy resulted in the assignment of over 60 sites of tyrosine phosphorylation, many of which were previously unknown. In addition, time course experiments enabled the temporal organization of various phosphorylation events to be discerned. For example, Fig. (**7**) clearly demonstrates the increased phosphorylation of ZAP-70 residues Y315 and Y319 in Jurkat cells upon stimulation for one minute with anti-CD3 and anti-CD4 antibodies. The single ion chromatograms (SIC), or plots of ion current vs. time, for *m/z* 990 corresponding to the $(M+3H)^{3+}$ ion of the methyl ester modified peptide PMPMDTSVpYESPpYSDPEELKD KK derived from ZAP-70 show dramatic changes between the unstimulated (Fig. **7B**) and 1 minute stimulated (Fig. **7F**) Jurkat cell samples. An identical pattern is seen in the SICs for *m/z* 742.6 corresponding to the $(M+4H)^{4+}$ ion of the same peptide. Importantly, the SICs for *m/z* 686

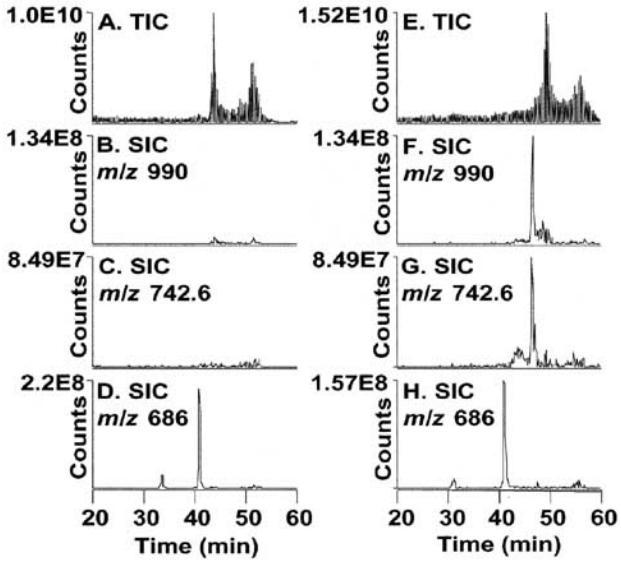

Fig. (7). Application of method by Salomon *et al.* [88] revealed increased phosphorylation of ZAP-70 residues Y315 and Y319 in Jurkat cells stimulated for one minute with anti-CD3 and anti-CD4 antibodies. Total ion chromatograms recorded during analysis of phosphopeptides from (A) unstimulated and (E) 1 minute stimulated Jurkat cells. Single ion chromatogram (SIC) for *m/z* 990 corresponding to the $(M+3H)^{3+}$ ion of the methyl ester modified peptide PMPMDTSVpYESPpYSDPEELKDKK for (B) unstimulated and (F) 1 minute stimulated Jurkat cells. SIC for *m/z* 742.6 corresponding to the $(M+4H)^{4+}$ ion of the same peptide for (C) unstimulated and (G) 1 minute stimulated Jurkat cells. SIC for *m/z* 686 corresponding to the $(M+2H)^{2+}$ of the control peptide LIEDNEpYTAR for (D) unstimulated and (H) 1 minute stimulated Jurkat cells.

corresponding to the $(M+2H)^{2+}$ ion of the control peptide LIEDNEpYTAR that was spiked into both samples before IMAC show nearly identical signals, indicating that the observed changes in the reported phosphopeptides were not simple artifacts of the sample preparation method employed.

CONCLUSION

Bioanalytical mass spectrometry is revolutionizing the ability of researchers to study protein phosphorylation, enabling the investigation of ever more complex systems. However, despite the numerous advances of the past five year, it must be emphasized that these methodologies are not nearly sophisticated enough to truly characterize a system's phosphoproteome. Every technique described has its own strengths and weaknesses, and issues regarding protein solubility, choice of proteolytic enzyme, derivatization, enrichment, separation, ionization mode, tandem MS method, and sensitivity must all be considered with respect to the desired analysis. In the end, it is unlikely that any single technique could provide all the desired information in a single analysis, and that effective phosphoprofiling of complex mixtures will require the combination of several analytical methods.

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