Analysis of phosphorylated proteins and peptides by mass spectrometry

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Phosphorylation on serine, threonine and tyrosine residues is an extremely important modulator of protein function. Therefore, there is a great need for methods capable of accurately elucidating sites of phosphorylation. Although full characterization of phosphoproteins remains a formidable analytical challenge, mass spectrometry has emerged as an increasingly viable tool for this task. This review summarizes the methodologies currently available for the analysis of phosphoproteins by mass spectrometry, including enrichment of compounds of interest using immobilized metal affinity chromatography and chemical tagging techniques, detection of phosphopeptides using mass mapping and precursor ion scans, localization of phosphorylation sites by peptide sequencing, and quantitation of phosphorylation by the introduction of mass tags. Despite the variety of powerful analytical methods that are now available, complete characterization of the phosphorylation state of a protein isolated in small quantities from a biological sample remains far from routine.

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Abbreviations

CE	capillary electrophoresis
CID	collision-induced dissociation
EGF	epidermal growth factor
ESI	electrospray ionization
HPLC	high-performance liquid chromatography
ICP	inductively coupled plasma
IMAC	immobilized metal affinity chromatography
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
Q1	first quadrupole
Q2	second quadrupole
Q3	third quadrupole
Qq	quadrupole–quadrupole
TOF	time-of-flight

Introduction

Organisms use reversible phosphorylation of proteins to control many cellular processes including signal transduction, gene expression, the cell cycle, cytoskeletal regulation and apoptosis [1,2]. Although phosphorylation is observed on a variety of amino acid residues, by far the most common and important sites of phosphorylation in eukaryotes occur on serine, threonine and tyrosine residues. Because of the central role of phosphorylation in the regulation of life, much effort has been focused on the development of methods for characterizing protein phosphorylation.

Traditional methods for analyzing *O*-phosphorylation sites [3–5] involve incorporation of ^{32}P into cellular proteins via treatment with radiolabeled ATP. The radioactive proteins can be detected during subsequent fractionation procedures (e.g. two-dimensional gel electrophoresis or high-performance liquid chromatography [HPLC]). Proteins thus identified can be subjected to complete hydrolysis and the phosphoamino acid content determined. The site(s) of phosphorylation can be determined by proteolytic digestion of the radiolabeled protein, separation and detection of phosphorylated peptides (e.g. by two-dimensional peptide mapping), followed by peptide sequencing by Edman degradation. These techniques can be tedious, require significant quantities of the phosphorylated protein and involve the use of considerable amounts of radioactivity.

In recent years, mass spectrometry (MS) has become an increasingly viable alternative to more traditional methods of phosphorylation analysis [6–8]. In this review, we outline the various mass spectrometric techniques that can be used to characterize sites of *O*-phosphorylation (Box 1). In particular, we concentrate on methods that do not involve the use of ^{32}P, although MS can certainly be used in conjunction with the classical methods of phosphoprotein and phosphopeptide analysis mentioned above (e.g. [9]).

MS can accurately provide the molecular mass of the intact phosphorylated protein. Such measurements, used in conjunction with calculation of the molecular mass of the unmodified protein [10] and/or treatment with phosphatase [11–13] allow determination of the average number of attached phosphate groups. Provided that the MS resolution is sufficiently high, it is also feasible to determine the distribution of the number of attached phosphates [14]. For more detailed analysis of the sites of phosphate attachment and stoichiometry, it is necessary to examine peptide fragments of the phosphoprotein of interest. Such fragments are usually generated by digestion of the phosphoprotein with site-specific proteases such as trypsin. Most of the work described here relates to MS measurements of phosphopeptides.

Sample preparation

Analysis of phosphopeptides presents formidable challenges to the mass spectrometrist. Ideally, every phosphorylated component of the protein should be detected. Unfortunately, MS analysis of proteolytic digests of proteins rarely provides 100% coverage of the protein sequence, and regions of interest are easily missed. In addition, negatively charged modifications can hinder proteolytic digestion by trypsin, the protease of choice in many applications. Phosphorylation is often sub-stoichiometric, such that the phosphopeptide is

Box 1

Overview of protein phosphorylation analysis by mass spectrometry.

Sample preparation
Fractionation
Use of antibodies (IP, detection after two-dimensional electrophoresis)
IMAC
Chemical introduction of affinity tags
Recognition of phosphopeptides
Peptide mapping
Post-source decay
Precursor ion scan
Neutral loss scan
Stepped skimmer potential
^{31}P detection
Identification of phosphorylated residues
Collision-induced dissociation
Post-source decay
In-source decay
Electron-capture dissociation
Quantitation of phosphorylation
Peak intensities
Isotopic labeling

present in lower abundance than other peptides from the protein of interest. Finally, the mass spectrometric response of a phosphopeptide may be suppressed relative to its unphosphorylated counterpart, and this suppression tends to be enhanced in the presence of other peptides. Analysis of phosphopeptides is therefore easier when the number of non-phosphorylated peptides has been reduced to a minimum (i.e. the phosphopeptides have been enriched). Several strategies have been developed to enrich the sample for phosphorylated peptides or phosphoproteins before analysis.

Fractionation

One obvious method for reducing the complexity of a peptide mixture is to fractionate it by HPLC. To deal with the small amounts of peptides that are normally available for these analyses, it is necessary to use capillary columns operating at low flow rates (e.g. [15,16]). Fractions containing phosphorylated peptides can be identified by prior labeling with ^{32}P coupled with detection of radioactivity in the fractions, or mass spectrometrically as described later in this review (e.g. [17,18••,19]). A simpler fractionation method has been described that uses step elution of peptides from reversed-phase beads [20]. The peptides are eluted in three fractions, which are less complex than the entire mixture. These authors assert that the addition of a phosphate group makes a peptide less hydrophobic, and care must be taken not to lose phosphopeptides entirely during the fractionation procedure.

IMAC

The most widely used method for selectively enriching phosphopeptides from mixtures is immobilized metal affinity chromatography (IMAC). In this technique, metal ions, usually Fe^{3+} or Ga^{3+}, are bound to a chelating support. Phosphopeptides are selectively bound because of the affinity of the metal ions for the phosphate moiety. The phosphopeptides can be released using high pH or phosphate buffer, the latter usually requiring a further desalting step before MS analysis. Limitations of this approach include possible loss of phosphopeptides because of their inability to bind to the IMAC column, difficulty in the elution of some multiply phosphorylated peptides, and background from unphosphorylated peptides (typically acidic in nature) that have affinity for immobilized metal ions. Two types of chelating resin are commercially available, one using iminodiacetic acid and the other using nitrilotriacetic acid. Some groups have observed that iminodiacetic acid resin is less specific than nitrilotriacetic acid [21,22], whereas another study reported little difference between the two [23]. Several studies have examined off-line MS analysis of IMAC-separated peptides (e.g. [23,24•,25•]). Direct matrix-assisted laser desorption/ionization (MALDI) analysis of phosphopeptides bound to the IMAC support has been reported [22]. IMAC has also been coupled on-line to MS analysis directly [26] or with intervening separation techniques such as HPLC [17,27] and capillary electrophoresis (CE) [28,29]. The use of IMAC to isolate phosphoproteins has not been demonstrated.

In a noteworthy example of the use of IMAC to study phosphorylation, intact thylakoid membranes from *Arabidopsis thaliana* were treated with trypsin [24•], and phosphopeptides were enriched from the resulting peptide mixture using Fe^{3+} and Ga^{3+} IMAC. The authors observed eight distinct phosphorylation sites on five different proteins, and detected changes in phosphorylation levels in response to light/dark transitions and heat shock.

Antibodies

Often, it is suspected that a protein of interest is phosphorylated under certain conditions *in vivo*. If high affinity antibodies have been raised against that protein, it can be immunoprecipitated from complex mixtures prior to further analysis (e.g. [30•,31–33]). Although effective in selectively isolating a particular phosphoprotein, this procedure requires production of a specific antibody for each protein to be analyzed.

A more generally useful tool would be an antibody able to immunoprecipitate any protein containing phosphorylated residues, and in fact non-sequence-specific antibodies directed against phosphoserine, phosphothreonine, or phosphotyrosine have been developed. However, only the anti-phosphotyrosine antibodies display binding that is tight enough to allow effective immunoprecipitation, so that antibody enrichment is presently confined to the analysis of peptides and proteins phosphorylated on tyrosine.

Phosphotyrosine-specific antibodies have been used to enrich phosphopeptides from proteolytic digests of proteins phosphorylated *in vitro* [34] or *in vivo* [30•]. The phosphopeptides were analyzed by MALDI time-of-flight (TOF) MS while still bound to the antibodies. A mixture

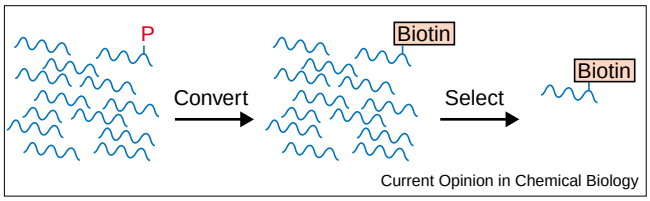
of two anti-phosphotyrosine antibodies was used to isolate phosphorylated proteins from HeLa cells treated with epidermal growth factor (EGF) [35••]. Comparison by one-dimensional electrophoresis of this sample with a similar immunoprecipitation from untreated cells resulted in identification by MS of seven known EGF kinase substrates and one novel substrate. The same technique was later applied to starved cells [36] to identify another novel protein in the EGF signaling pathway.

Although antibodies used for immunoprecipitation must have relatively high affinity for their substrates, lower-affinity antibodies may still be quite effective for western-blot analysis. Thus, antibodies directed against phosphoserine and phosphothreonine can be used for this purpose. For example, anti-phosphoserine and anti-phosphotyrosine antibodies were used to explore changes in phosphorylation of fibroblast proteins after stimulation with platelet-derived growth factor [37]. 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 two-dimensional electrophoretic spots because of differing phosphorylation states, the requirement for in-gel digestion to identify the proteins of interest, and the reliability of the non-sequence-specific antibodies used to detect phosphoserine and phosphothreonine. In addition, when phosphoproteins are merely detected and not enriched by antibodies, the presence of unrelated proteins co-migrating with the protein of interest may lead to false conclusions.

Chemical tagging of phosphorylation sites

Recently, two methods have been developed to specifically isolate phosphopeptides or phosphoproteins from complex mixtures [38••,39••,40] (Figure 1) (see also Update). Oda *et al.* [38••] and Goshe *et al.* [40] exposed mixtures of peptides or proteins to high pH in the presence of ethanedithiol, causing loss of H_3PO_4 from phosphoserine and phosphothreonine residues by β-elimination. The resulting double bond was attacked by the ethanedithiol, leading to the net replacement of the phosphate group by a thiol tag. Biotin was attached to the thiol via a sulfhydryl-reactive group, and the tagged peptides/proteins were isolated by chromatography on avidin resins. Care must be taken to properly block the thiol groups of cysteine residues before performing these reactions. For this purpose, performic acid oxidation [38••] is preferred over alkylation [40] because alkylated cysteine residues may undergo β-elimination in a fashion similar to phosphoserine and phosphothreonine. Using an alternative chemistry, Zhou *et al.* [39••] modified phosphopeptides by attachment of cysteamine (1-amino-2-thioethane) to the phosphate moiety using a carbodiimide condensation reaction. The modified peptides were subsequently immobilized by covalent attachment to iodoacetyl resin, washed, and released by treatment with acid. Features of the first method are its ability to isolate both phosphopeptides and phosphoproteins and its inability to enrich phosphotyrosine-containing species. Enrichment of phosphoproteins using the second approach has not yet been demonstrated, but the method can be applied to phosphotyrosine residues in addition to phosphoserine and phosphothreonine. The applicability of these new chemistries for exploring signaling and control pathways remains to be established.

Figure 1



Enrichment of phosphopeptides from complex mixtures by chemical introduction of an affinity tag. Blue wavy lines represent proteins or peptides in a mixture. The wavy line with 'P' designates a phosphoprotein or phosphopeptide. The affinity tag (in this case, biotin) allows selective isolation of the species of interest. Figure adapted from [38••].

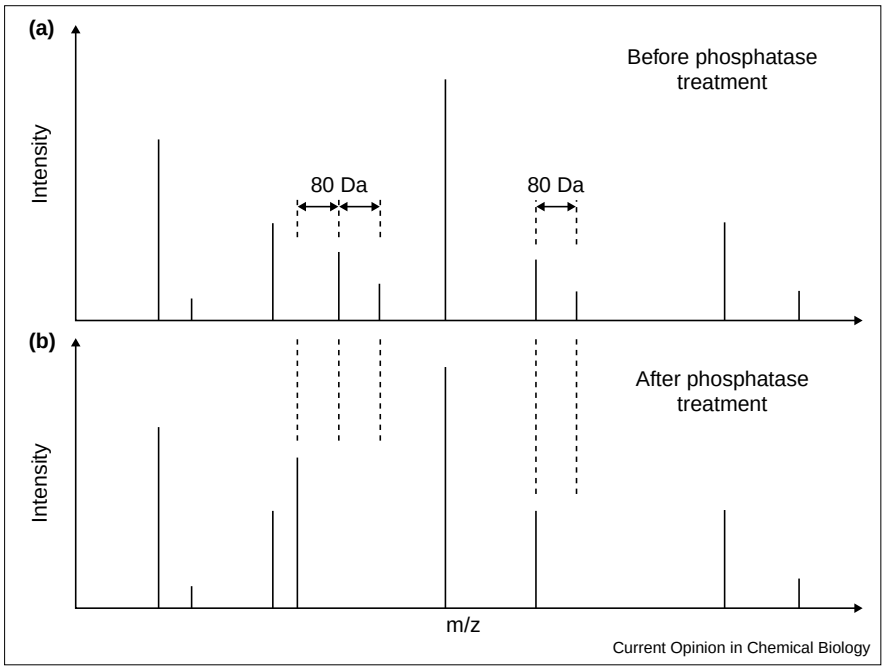
Recognition of phosphopeptides

After the sample has been prepared, any peptides that bear phosphate groups must be identified and distinguished from all other peptides present. A number of techniques based on MS have been developed to accomplish this goal.

Peptide mapping and phosphatase treatment

Peptide mapping involves proteolytic digestion of a protein of interest, often purified by one-dimensional or two-dimensional electrophoresis, followed by MALDI-TOF-MS to determine the masses of the peptides [18••,41•]. If the identity of the protein being analyzed is known or can be deduced by MS analysis of the proteolytic fragments, then examination of the peptide map for peptides shifted by multiples of 80 Da (HPO_3 = 80 Da) can be effective in identifying phosphopeptides. Differences in the peptide map before and after treatment with phosphatase can further aid in the analysis (Figure 2). This strategy has recently been evaluated [41•], and the authors concluded that at least 1 pmol of protein must be present in a gel band for reliable characterization of the site(s) of phosphorylation. This level of sensitivity, which is at least one order of magnitude lower than that required to simply identify a protein band, is the result of the need to obtain high coverage of the protein in the MS analysis. Other contributing factors to this reduced sensitivity include selective loss of phosphopeptides during sample handling, suppression of phosphopeptide signals in complex mixtures, and sub-stoichiometric phosphorylation. In attempts 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 [41•,42,43]. Another strategy that minimizes sample handling involves treatment of the peptides in a reactor cell containing immobilized phosphatase coupled on-line to HPLC–MS or CE–MS [44].

Figure 2



Phosphopeptide identification by MALDI-TOF-MS mapping combined with alkaline phosphatase treatment. (a) The MALDI-TOF-MS spectrum of a proteolytic digest. Phosphopeptides are indicated by peaks shifted by multiples of 80 Da (HPO_3 = 80 Da) relative to predicted unphosphorylated peptide masses. (b) The disappearance of such peaks upon treatment with a phosphatase confirms their identity as phosphopeptides.

Phosphopeptides have also been detected by MALDI ion trap MS [45,46]. Pairs of peaks 98 units apart, arising because of facile loss of the elements of H_3PO_4 from the phosphopeptides, provide a signature for these species. Ambiguities caused by the possible presence of proteolytic peptides of 98 mass units less than the putative phosphopeptide are easily resolved by isolating the species in the ion trap and subjecting it to collision-induced dissociation (CID). Phosphopeptides readily lose 98 mass units under these conditions [46]. Unfortunately, MALDI ion trap mass spectrometers are not yet commercially available.

Post-source decay

In MALDI-TOF mass spectrometers, loss of HPO_3 and the elements of H_3PO_4 occur post-source as a result of metastable decomposition and/or CID, giving rise to fragments having the same velocity as their parent ions but with reduced energy. As a result, the fragment ions are not distinguishable from their parents in spectra collected in linear mode, but are detected at a lower apparent mass in reflector mode [47]. Peptides phosphorylated on serine or threonine are distinguished in some cases from those phosphorylated on tyrosine because the former more readily lose H_3PO_4, whereas the latter are more likely to lose HPO_3 [47–49]. The use of infrared laser wavelengths rather than the more typical ultraviolet wavelengths may reduce the loss of phosphate by post-source decay, thereby enhancing the phosphopeptide signal in reflector mode [50].

Precursor ion scan

This method utilizes the detection of phosphate-specific fragments to signal the presence of a phosphorylated peptide [51,52,53•]. To date, this approach has been applied exclusively to ions produced by electrospray ionization (ESI). One variation of the precursor ion scan uses the ready loss of phosphate from phosphopeptides under CID conditions [54,55]. In a typical experiment, a proteolytic digest is desalted, made basic and infused directly into a triple quadrupole mass spectrometer (although a quadrupole–quadrupole [Qq]-TOF spectrometer can also be used [56]; see also Update). The first quadrupole (Q_1) is scanned over the full mass range of the instrument and CID is induced in the second quadrupole (Q_2) (Figure 3). The third quadrupole (Q_3) is set to selectively pass only m/z 79^- ions (i.e. PO_3^-). The point during the Q_1 scan at which a 79^- signal is detected yields the mass of any phosphopeptide present. This method is useful because of the sensitive MS detection of the PO_3^- anion. Synthetic peptides have been characterized at concentrations as low as 10 fmol/μl [51,52], whereas a phosphopeptide from β-casein was detected after in-gel digestion of 100 fmol of protein using nano-ESI [20]. Precursor ion scanning has been successfully applied to *in vivo* modified proteins in a number of cases (e.g. [31,33,57,58]). However, in the absence of fractionation, phosphopeptides present in low abundance might not be detected in complex mixtures. In principle, on-line HPLC fractionation reduces this difficulty, but negative ion precursor scans have compromised sensitivity at low pH making their use in conjunction with on-line HPLC difficult.

In an attempt to overcome these limitations, a multidimensional approach to the analysis of mixtures containing phosphorylated peptides has been developed [59•]. First, the peptides are applied to an HPLC column. A portion of the flow from the column is split off, electrosprayed into a

triple quadrupole mass spectrometer and subjected to CID in the high-pressure region close to the skimmer. The phosphate-derived anions at m/z 63 and 79 are selectively monitored to flag the presence of phosphopeptides. This is a sensitive means for detecting the presence of phosphopeptides because it does not involve scanning the mass analyzers. The remainder of the HPLC output is collected as fractions. A portion of each fraction containing the putative phosphopeptides is rendered basic and their molecular masses are determined using the precursor ion (79^-) scan method. The remainder of the HPLC fraction is analyzed under acidic conditions in the positive ion mode to determine the phosphorylation site by MS/MS (see below). The authors state that the current sensitivity of the procedure is about 5 pmol of phosphopeptide, the primary limitation being the relatively wide diameter of the HPLC column used in their study.

In a variation of the precursor scanning methodology that does not utilize the elimination of the phosphate moiety, phosphotyrosine-containing peptides have been detected by measuring the phosphotyrosine immonium ion [53•]. This technique requires high resolution MS (e.g. Qq-TOF) to resolve the phosphotyrosine immonium ion at m/z 216 from interfering background ions at the same nominal mass. The authors report the detection and site mapping of phosphotyrosine-containing peptides from 100 fmol of a phosphoprotein in a gel. This technique does not appear to be applicable to phosphoserines and phosphothreonines because of their high tendency to lose phosphate.

Neutral loss scanning

This method uses tandem MS (e.g. with a triple quadrupole analyzer) to detect the neutral loss of the elements of H_3PO_4 (98 Da) after CID [12,60]. Q_1 scans the entire mass range, while Q_2 is used as the collision cell (Figure 3). Q_3 then scans in parallel to Q_1, but at m/z 98/*n* lower, to look for phosphopeptides carrying a charge of +*n*. Only peptides losing H_3PO_4 in Q_2 will be able to pass through Q_3. Usually, when coupled to HPLC, it is only practical to scan at most two charge states during a single run. It is noteworthy that 98/*n* neutral loss scanning has not seen much use for determination of unknown samples [60].

Stepped skimmer potential

It has been observed that application of a high potential to the skimmer region of an ESI triple quadrupole spectrometer results in loss of phosphate groups from phosphopeptides [61,62]. This property can be exploited to detect phosphopeptides by raising the skimmer potential during the low part of a negative ion mass scan. Observation of signals at m/z 63 (PO_2^-) and 79 (PO_3^-) indicates the presence of a phosphopeptide. As the scan continues to higher masses, the skimmer potential is lowered allowing determination of the masses of the peptides present. A drawback of this method is that the higher masses are recorded in the negative ion mode, which often has lower sensitivity than does the positive ion mode. This problem can be overcome by changing the ion polarity during the second part of the scan [63]. These authors reported a detection limit of ~100 fmol of phosphopeptide in a model peptide mixture. Like neutral loss scanning, the stepped skimmer potential method has not seen much use for determination of unknown biological samples.

Figure 3

	Q_1	q_2	Q_3
Precursor ion	Scanned	CID	Fixed
Neutral loss	Scanned	CID	Scanned
Product ion	Fixed	CID	Scanned

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Techniques for analysis of phosphopeptides in a triple quadrupole mass spectrometer. Three types of experiments are shown. The precursor ion and neutral loss scans are used to detect phosphopeptides, and the product ion scan is used to obtain sequence information about a peptide.

^{31}P detection

Specific detection of phosphopeptides during HPLC separation has been described [64•] by monitoring the presence of ^{31}P with inductively coupled plasma (ICP)-MS. The masses of the putative phosphopeptides are measured in a separate LC–ESI-MS run. The authors report a detection limit of 100 fmol of synthetic peptide injected onto the HPLC column. Although this technique requires an ICP mass spectrometer with sufficient resolution (about 2500) to distinguish between ^{31}P and background ions, it has the advantage that the ^{31}P detection can be made quantitative (as long as inorganic phosphate background can be properly accounted for). Recently, these same authors demonstrated that the average phosphate content of proteins can be determined using ICP-MS to measure the ratio of ^{31}P to ^{32}S [14].

Identification of phosphorylation sites

After a phosphopeptide has been identified, one usually desires to determine which residue carries the modification. If the sequence of the protein is known, the peptide may be identified by its mass. In fortuitous cases, the peptide will have only one serine, threonine or tyrosine residue, making assignment of the site of phosphorylation easy. In most cases, fragmentation of the peptide and characterization of the fragments is required.

Collision-induced dissociation

The most common method of identifying sites of phosphorylation is CID of samples produced by ESI. Fragment ions can be measured in a triple quadrupole instrument (e.g. [20,59•]) (Figure 3), an ion trap instrument (e.g. [19,65]) or a hybrid Qq-TOF instrument (e.g. [66,67]). Loss of phosphate as HPO_3 or H_3PO_4 is a favored fragmentation event, and usually dominates over the backbone cleavages that are useful for sequence determination. The preferred loss of

phosphate is particularly dominant when the parent ion is singly charged. However, even in cases where the loss of phosphate is dominant, informative fragmentation information can be obtained provided that sufficient statistics can be accumulated to discern the weaker backbone fragment ions. Loss of 98 Da (H_3PO_4) from a phosphorylated fragment ion is indistinguishable from loss of 18 Da (H_2O) from a non-phosphorylated fragment ion, sometimes making the interpretation of a CID spectrum challenging. Derivatization of the amino terminus of the phosphopeptide may result in a simpler spectrum in which many of the fragments retain the phosphate group [68].

Fractionation methods such as HPLC [18••] or CE [28,29] may be coupled on-line to a mass spectrometer to improve the CID analysis of complex proteolytic mixtures. This analysis is usually carried out by repeated MS scanning of the chromatographic peaks, and subsequent selection of the most intense ion(s) from a given scan for CID — generating fragmentation data for many peptides (including phosphopeptides) in the sample. However, in such an experiment the quality of the fragmentation spectrum is limited by the short time period over which the peptide is eluted. Furthermore, when two or more peptides co-elute there may not be enough time to obtain MS/MS spectra of each species. Therefore, on-line methods may not always enable characterization of a phosphorylation site. To remedy these problems, one may conduct HPLC [69] or CE [70] separations in a data-dependent manner such that the flow rate is reduced as a peptide elutes. This 'peak parking' procedure increases the number of fragmentation spectra that can be collected from a given peak. To our knowledge, analysis of phosphopeptides by peak parking has been used only in conjunction with CE [70,71], and not with HPLC. Although highly effective in the developers' laboratories, this approach has not yet been widely adopted presumably because of the high degree of skill required.

If the mass of a phosphopeptide has already been determined by mass mapping (usually by MALDI-TOF), then CID of this single selected ion during on-line LC–ESI ion trap analysis is a powerful tool for obtaining sequence information [18••]. In this approach, the mass spectrometer is programmed to specifically retain and fragment species of a particular m/z value, ignoring all other peptides. Although analysis of each phosphopeptide requires a separate HPLC run, the method has been used to characterize a number of *in vivo* phosphorylation sites (e.g. [18••,32,72]). An advantage of this method is its ability to obtain informative MS/MS spectra of phosphopeptides whose ESI-MS response is weak.

Because sequence determination by CID generally becomes more difficult as the peptide size increases, one group has proposed the use of the low-specificity protease elastase instead of more traditional high-specificity proteases such as trypsin to generate fragments for MS analysis [60]. The authors state that the resulting increase in the number of isobaric peptides is compensated for by the increased ease of sequence determination of the smaller peptides arising from elastase digestion. This claim should be balanced with the difficulties in interpreting fragmentation data produced by CID of a mixture of isobaric peptides.

Some workers [73–75] have facilitated the identification of phosphorylation sites using β-elimination chemistry similar to that described above. CID was performed after conversion of phosphoserine or phosphothreonine to *S*-ethylcysteine or β-methyl-*S*-ethylcysteine residues by addition of base and ethanethiol. Because the labile phosphate group was removed, the modified peptides fragmented more evenly within the peptide backbone, giving more complete sequence information.

The foregoing discussion has dealt only with CID of ions produced by ESI. The development of instruments in which a MALDI source is coupled to a Qq-TOF analyzer [76,77] has enabled effective CID fragmentation of peptide ions generated by MALDI. Characterization of phosphopeptides in such instruments has not yet been widely reported, although standard compounds have been investigated [78,79]. These authors report significant prompt loss of HPO_3 or the elements of H_3PO_4 from phosphopeptides, resulting in a characteristic pair or series of peaks in the MALDI-MS spectrum. In the analysis of a multiply phosphorylated peptide, the fully dephosphorylated ion formed by this prompt loss was observed to produce a more easily interpretable CID fragmentation pattern than that produced from the fully phosphorylated form [78]. In this case, the formerly phosphorylated residues were identified by the presence of dehydroalanine residues in place of serine residues, as previously described for peptides undergoing loss of H_3PO_4 [55,65].

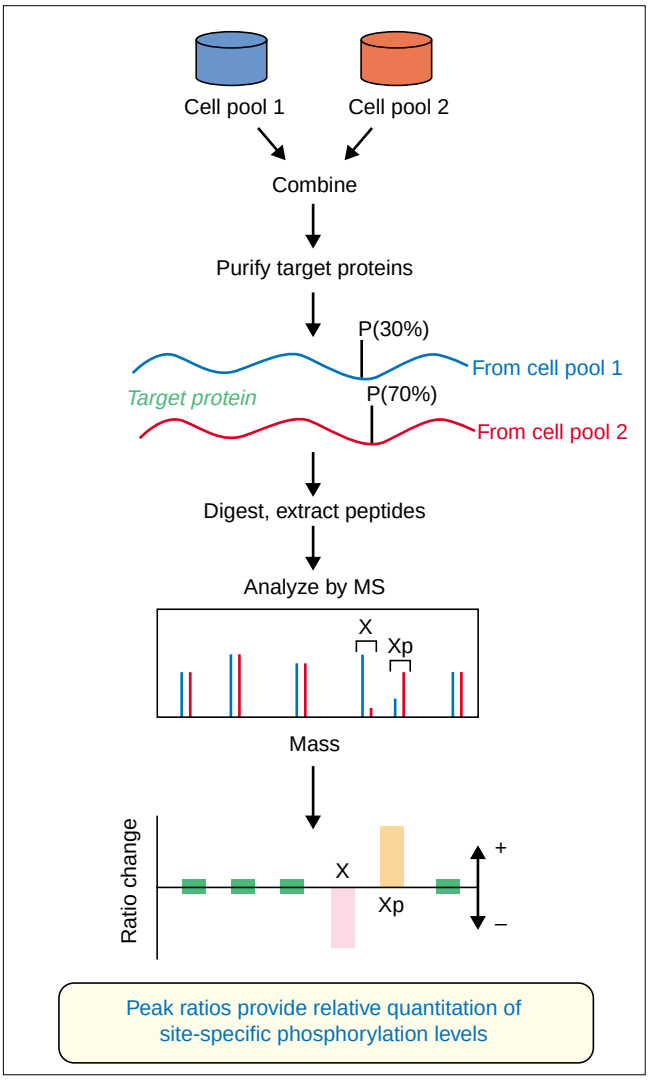
Post-source decay

Although phosphate loss is the dominant metastable and CID fragmentation pathway for phosphopeptides in MALDI-TOF instruments, phosphopeptides may also fragment along their backbones. Although identification of sites of phosphorylation by this method has been demonstrated [21,24•,47,49], the yield of fragment ions can be low and the collection and interpretation of these spectra can be challenging. When the phosphorylation site is immediately to the amino-terminal side of a proline residue, cleavage of the intervening amide bond is highly preferred, making characterization much easier [80]. This preferred cleavage is of interest because serine–proline and threonine–proline-containing sequences are common targets for proline-directed kinases.

In-source decay

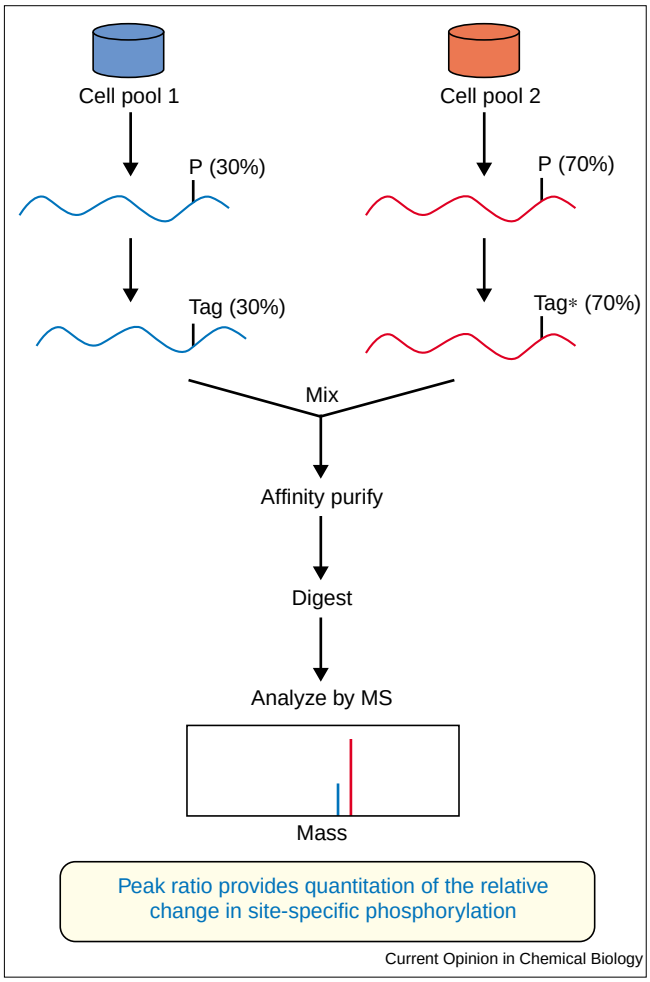
Prompt and metastable fragment formation that occurs during the delay between ion desorption and acceleration into the flight-tube of a MALDI-TOF mass spectrometer is termed in-source decay. This fragmentation method has been used to sequence synthetic phosphopeptides in low

Figure 4



Quantitation of phosphorylation using labeling via the growth medium. For illustration, protein expression in the two pools is assumed to be equal. (In practice, proteins in the two cell pools may not be present in equal abundance, and the ratios of the unphosphorylated peptide peaks will be a constant value that is not equal to one.) The level of phosphorylation of peptide X is assumed to change from 30% (pool 1) to 70% (pool 2), leading to a decrease in the measured intensity ratio of unphosphorylated peptide X and an increase for phosphorylated peptide Xp. Figure adapted from [88•].

Figure 5



Quantitation of phosphorylation using isotope affinity tagging. Proteins from two pools of cells are obtained. For illustration, protein expression in the two pools is assumed to be equal, while the level of phosphorylation changes from 30% in the blue pool to 70% in the red pool. An affinity tag is introduced by phosphate-directed chemistry. In one pool, the tag is unlabeled, whereas in the second the tag contains an isotopic label such as deuterium (indicated by the asterisk). The proteins can be isolated using the affinity tag and then digested prior to MS analysis. Alternatively, the proteins can be digested before affinity purification. Pairs of peaks in the mass spectrum indicate formerly phosphorylated peptides; comparison of the peak intensities within each pair gives the relative amount of a particular phosphopeptide that was present in the two pools. Note that this method does not distinguish between an increase in expression of a given protein and an increase in its phosphorylation at a specific site.

pmol amounts [81,82]. The resulting spectra were relatively easy to interpret, giving rise primarily to c, y and z+2 ions. In this case, the presence of the phosphate on a given residue was inferred by the 80 Da shift in the residue mass.

Electron capture dissociation

A relatively new technique for dissociation of proteins and peptides into fragments involves irradiation of ESI-produced multiply charged ions with a stream of subthermal electrons in a Fourier transform ion cyclotron resonance mass spectrometer [83]. When applied to phosphopeptides, the formation of extensive c and $z^{\cdot}$ ion series was observed with retention of the phosphate group by the fragment ions [84••], making identification of the site(s) of phosphorylation simpler than for CID analysis. Furthermore, the method has the potential for localizing phosphorylation site(s) within intact small proteins, without the need for enzymatic digestion [85]. So far, electron capture dissociation has only been applied to known phosphopeptides at concentrations in the low-micromolar range.

Quantitation of phosphorylation

Once a site of phosphorylation has been determined, one would like to know its stoichiometry (i.e. the ratio of phosphorylated to unphosphorylated peptide). An early quantitation method involved HPLC separation of the phosphopeptide from its unphosphorylated counterpart (as identified by MS), quantitative amino acid analysis, and integration of the two peaks in the HPLC trace [86]. Subsequently, others have estimated the extent of phosphorylation by monitoring the relative strengths of the peptide signals by ESI-MS [24,52,87]. Because of our poor understanding of how sequence context might affect the ion formation efficiency of phosphorylated versus unphosphorylated peptides, comparison of the peak intensities of the two forms gives an estimate of phosphorylation stoichiometry that is rough at best.

In many biological studies, it proves more relevant to quantitate changes in the level of phosphorylation. Such information can be obtained by isotope labeling, either via the growth medium or by the introduction of labeled tags. In the former method [88•], two pools of cells are grown under different conditions, and one of the pools is labeled with ^{15}N introduced through the growth medium (Figure 4). The two pools of cells are mixed and the protein of interest is isolated, proteolytically digested, and analyzed by MS. A pair of peaks representing each proteolytic fragment arises because of the incorporation of ^{14}N and ^{15}N, respectively, into proteins from the two pools. The ratios between the intensities of the peaks in each pair provide a measure of the relative levels of protein expression under the two conditions. A difference in the level of phosphorylation between the two pools of cells is reflected by a change in the ratio of the unphosphorylated peptide labeled with ^{15}N to its unlabeled counterpart. This technique is limited to situations where cells may be grown on labeled media.

An alternative method for quantitating changes in the level of phosphorylation involves introduction of a mass tag [40,89]. One variation of this approach is illustrated in Figure 5. Two separate samples containing either phosphopeptides or phosphoproteins are treated with base in the presence of a nucleophile, causing β-elimination of the phosphate group and Michael addition of the nucleophile to the resulting carbon–carbon double bond. In one sample, the nucleophile is unlabeled, whereas in the second the nucleophile is deuterated. The two samples are mixed and an affinity tag [40] may be added to the modified residue and used to specifically isolate formerly phosphorylated species as described earlier. Proteolytic fragments are analyzed by MS. Each proteolytic peptide that was previously phosphorylated gives rise to a pair of peaks because of the different masses of the nucleophilic tags. The ratio of the peak intensities of the normal and deuterated peptides gives the stoichiometry of phosphorylation, assuming that the amount of protein present under the two conditions is unchanged. As an alternative to isotopic labeling, nucleophiles differing in mass by, for example, an ethylene group may be used. Although very promising, this technique has only been successfully applied to the analysis of protein standards.

Conclusions and future directions

As described above, a variety of powerful MS methods for detecting and characterizing phosphoproteins and phosphopeptides is available. Despite this formidable armamentarium, complete characterization of the phosphorylation state of a protein isolated in small quantities from a biological sample remains far from routine. The following is a list of analytical tools and methods that will be required to make comprehensive phosphorylation analyses routine:

1. A method for selectively enriching/purifying phosphoproteins and/or phosphopeptides that ensures recovery of all phosphorylated species and gives a low background of unphosphorylated species. Improved phospho-directed antibodies and chemistry hold much promise in this regard.

2. A method to ensure that all sites of phosphorylation are detected. Although phospho-directed chemistry and MS detection based on the lability of the phosphate moiety appear to be worthwhile avenues of pursuit, exhaustive detection of all sites of phosphorylation remains a most challenging analytical task.

3. A method for pinpointing the sites of phosphorylation. Current CID MS/MS methods are already reasonably effective, and newer methods such as electron capture dissociation hold much promise for further improvements.

4. A universal method for measuring changes in phosphorylation *in vivo*. Metabolic labeling and phosphorylation site labeling with stable isotopes appear to be viable approaches for attaining this goal.

In addition to the study of individual phosphoproteins, it remains critical to develop methodologies for elucidating the phosphorylation state of collections of proteins at an organelle-wide or cell-wide level. Such global techniques promise to provide a new window into the inner workings of the cell.

Update

A potentially powerful tool to identify protein kinase substrates has recently been demonstrated [90]. The technique involves radiolabeling cell extracts with a kinase of interest followed by blotting and MS identification of phosphorylated bands. Two studies have observed that phosphopeptides have increased signal intensity relative to other peptides in MALDI-TOF spectra recorded in negative ion mode as opposed to positive ion mode [91,92]. This increased signal intensity of phosphopeptides in negative ion mode may provide a useful means of detecting phosphopeptides in a mixture. The β-elimination/ethanedithiol addition chemistry has been used to attach a disulfide-linked biotin

group to phosphoproteins [93]. The use of an easily reversible link to attach the biotin allows more efficient recovery of the formerly phosphorylated peptides. The effectiveness of Q-TOF and triple quadrupole analyzers for detecting phosphopeptides using precursor ion scanning has been compared [94]. The two types of instrument were found to have similar sensitivity for monitoring loss of PO_3^- ions, whereas the Q-TOF analyzer was more sensitive and more selective for detecting loss of the phosphotyrosine immonium ion. The same group has also reported that sulfation and phosphorylation of tyrosine residues (both of which cause a mass increase of 80 Da) can be distinguished by the higher stability of the phosphotyrosine moiety under CID conditions [95]. Signal intensity of phosphopeptides detected in negative mode by ESI Fourier transform ion cyclotron resonance MS was shown to depend on the base used to adjust the pH of the sample solution [96]. In analogy to fragmentation observed under CID conditions, facile loss of the elements of H_3PO_4 from phosphopeptides was observed by these authors upon irradiation of the sample with an infrared laser pulse.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Marks F (Ed): *Protein Phosphorylation.* New York: VCH; 1996.

2. Hunter T: Signaling — 2000 and beyond. *Cell* 2000, 100:113-127.

3. Yan JX, Packer NH, Gooley AA, Williams KL: Protein phosphorylation: technologies for the identification of phosphoamino acids. *J Chromatogr A* 1998, 808:23-41.

4. van der Geer P, Luo K, Sefton BM, Hunter T: Phosphopeptide mapping and phosphoamino acid analysis on cellulose thin-layer plates. In *Protein Phosphorylation: a Practical Approach, edn 2*. Edited by Hardie DG. Oxford: Oxford University Press; 1999:97-126.

5. Haystead TAJ, Garrison JC: Study of protein phosphorylation in intact cells. In *Protein Phosphorylation: a Practical Approach, edn 2*. Edited by Hardie DG. Oxford: Oxford University Press; 1999:1-31.

6. Resing KA, Ahn NG: Protein phosphorylation analysis by electrospray ionization-mass spectrometry. *Methods Enzymol* 1997, 283:29-44.

7. Quadroni M, James P: Phosphopeptide analysis. *Exs* 2000, 88:199-213.

8. Aebersold R, Goodlett DR: Mass spectrometry in proteomics. *Chem Rev* 2001, 101:269-295.

9. Ogueta S, Rogado R, Marina A, Moreno F, Redondo JM, Vázquez J: Identification of phosphorylation sites in proteins by nanospray quadrupole ion trap mass spectrometry. *J Mass Spectrom* 2000, 35:556-565.

10. Carmel G, Leichus B, Cheng X, Patterson SD, Mirza U, Chait BT, Kuret J: Expression, purification, crystallization, and preliminary X-ray analysis of casein kinase-1 from ***Schizosaccharomyces pombe***. *J Biol Chem* 1994, 269:7304-7309.

11. Ge H, Zhao Y, Chait BT, Roeder RG: Phosphorylation negatively regulates the function of coactivator PC4. *Proc Natl Acad Sci USA* 1994, 91:12691-12695.

12. Hunter AP, Games DE: Chromatographic and mass spectrometric methods for the identification of phosphorylation sites in phosphoproteins. *Rapid Commun Mass Spectrom* 1994, 8:559-570.

13. Weijland A, Neubauer G, Courtneidge SA, Mann M, Wierenga RK, Superti-Furga G: The purification and characterization of the catalytic domain of Src expressed in ***Schizosaccharomyces pombe***. Comparison of unphosphorylated and tyrosine phosphorylated species. *Eur J Biochem* 1996, 240:756-764.

14. Wind M, Wesch H, Lehmann WD: Protein phosphorylation degree: determination by capillary liquid chromatography and inductively coupled mass spectrometry. *Anal Chem* 2001, 73:3006-3010.

15. Arnott D, Henzel WJ, Stults JT: Rapid identification of comigrating gel-isolated proteins by ion trap-mass spectrometry. *Electrophoresis* 1998, 19:968-980.

16. Davis MT, Lee TD: Rapid protein identification using a microscale electrospray LC/MS system on an ion trap mass spectrometer. *J Am Soc Mass Spectrom* 1998, 9:194-201.

17. Watts JD, Affolter M, Krebs DL, Wange RL, Samelson LE, Aebersold R: Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70. *J Biol Chem* 1994, 269:29520-29529.

18. •• Zhang X, Herring CJ, Romano PR, Szczepanowska J, Brzeska H, Hinnebusch AG, Qin J: Identification of phosphorylation sites in proteins separated by polyacrylamide gel electrophoresis. *Anal Chem* 1998, 70:2050-2059.
The authors describe a robust strategy for analysis of phosphoproteins after in-gel digestion. Phosphopeptides were identified by MALDI-TOF mass mapping with and without alkaline phosphatase treatment. Phosphopeptides detected in this manner were then selectively sequenced by LC–ESI-MS/MS to determine the sites of phosphorylation.

19. Zarling AL, Ficarro SB, White FM, Shabanowitz J, Hunt DF, Engelhard VH: Phosphorylated peptides are naturally processed and presented by major histocompatibility complex class I molecules ***in vivo***. *J Exp Med* 2000, 192:1755-1762.

20. Neubauer G, Mann M: Mapping of phosphorylation sites of gel-isolated proteins by nanoelectrospray tandem mass spectrometry: potentials and limitations. *Anal Chem* 1999, 71:235-242.

21. Neville DC, Rozanas CR, Price EM, Gruis DB, Verkman AS, Townsend RR: Evidence for phosphorylation of serine 753 in CFTR using a novel metal-ion affinity resin and matrix-assisted laser desorption mass spectrometry. *Protein Sci* 1997, 6:2436-2445.

22. Zhou W, Merrick BA, Khaledi MG, Tomer KB: Detection and sequencing of phosphopeptides affinity bound to immobilized metal ion beads by matrix-assisted laser desorption/ionization mass spectrometry. *J Am Soc Mass Spectrom* 2000, 11:273-282.

23. Posewitz MC, Tempst P: Immobilized gallium(III) affinity chromatography of phosphopeptides. *Anal Chem* 1999, 71:2883-2892.

24. • Vener AV, Harms A, Sussman MR, Vierstra RD: Mass spectrometric resolution of reversible protein phosphorylation in photosynthetic membranes of ***Arabidopsis thaliana***. *J Biol Chem* 2001, 276:6959-6966.
In a step toward organelle-wide characterization of phosphorylation, the authors treated intact thylakoid membranes with protease and used IMAC to selectively enrich the phosphopeptides from the resulting mixture. MALDI-TOF post-source decay was used to confirm that isolated peptides were phosphorylated, and LC–ESI-MS/MS was used to identify the sites of phosphorylation. Changes in phosphorylation were observed in membranes isolated from plants grown under different conditions.

25. • Stensballe A, Andersen S, Jensen ON: Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. *Electrophoresis* 2001, 22:207-222.
The authors describe an optimized strategy for analysis of phosphoproteins involving in-gel digestion, small-scale IMAC to isolate phosphopeptides, mass mapping by MALDI-TOF MS with and without alkaline phosphatase treatment, and nano-ESI-MS/MS to determine sites of phosphorylation.

26. Nuwaysir LM, Stults JT: Electrospray ionization mass spectrometry of phosphopeptides isolated by on-line immobilized metal-ion affinity chromatography. *J Am Soc Mass Spectrom* 1993, 4:662-669.

27. Wu X, Ranganathan V, Weisman DS, Heine WF, Ciccone DN, O'Neill TB, Crick KE, Pierce KA, Lane WS, Rathbun G *et al.*: ATM phosphorylation of Nijmegen breakage syndrome protein is required in a DNA damage response. *Nature* 2000, 405:477-482.

28. Cao P, Stults JT: Phosphopeptide analysis by on-line immobilized metal-ion affinity chromatography-capillary electrophoresis-electrospray ionization mass spectrometry. *J Chromatogr A* 1999, 853:225-235.

29. Cao P, Stults JT: Mapping the phosphorylation sites of proteins using on-line immobilized metal affinity chromatography/capillary electrophoresis/electrospray ionization multiple stage tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2000, 14:1600-1606.

30. • Kalo MS, Pasquale EB: Multiple ***in vivo*** tyrosine phosphorylation sites in EphB receptors. *Biochemistry* 1999, 38:14396-14408.
Non-sequence-specific anti-phosphotyrosine antibodies were used to isolate phosphopeptides from an enzymatic digest of a receptor phosphorylated *in vivo*. The phosphopeptides, still bound by the antibodies, were identified by MALDI-TOF MS.

31. Watty A, Neubauer G, Dreger M, Zimmer M, Wilm M, Burden SJ: The ***in vitro*** and ***in vivo*** phosphotyrosine map of activated MuSK. *Proc Natl Acad Sci USA* 2000, 97:4585-4590.

32. Okamura H, Aramburu J, García-Rodriguez C, Viola JP, Raghavan A, Tahiliani M, Zhang X, Qin J, Hogan PG, Rao A: Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol Cell* 2000, 6:539-550.

33. Knotts TA, Orkiszewski RS, Cook RG, Edwards DP, Weigel NL: Identification of a phosphorylation site in the hinge region of the human progesterone receptor and additional amino-terminal phosphorylation sites. *J Biol Chem* 2001, 276:8475-8483.

34. De Corte V, Demol H, Goethals M, Van Damme J, Gettemans J, Vandekerckhove J: Identification of Tyr438 as the major ***in vitro*** c-Src phosphorylation site in human gelsolin: a mass spectrometric approach. *Protein Sci* 1999, 8:234-241.

35. •• Pandey A, Podtelejnikov AV, Blagoev B, Bustelo XR, Mann M, Lodish HF: Analysis of receptor signaling pathways by mass spectrometry: identification of vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors. *Proc Natl Acad Sci USA* 2000, 97:179-184.
The authors used non-sequence-specific anti-phosphotyrosine antibodies to immunoprecipitate proteins from HeLa cells that had been either treated with EGF or left untreated. Comparison of the proteins isolated from both types of cells allowed MS identification of proteins that were phosphorylated on tyrosine in response to EGF treatment.

36. Pandey A, Fernandez MM, Steen H, Blagoev B, Nielsen MM, Roche S, Mann M, Lodish HF: Identification of a novel immunoreceptor tyrosine-based activation motif-containing molecule, STAM2, by mass spectrometry and its involvement in growth factor and cytokine receptor signaling pathways. *J Biol Chem* 2000, 275:38633-38639.

37. Soskic V, Görlach M, Poznanovic S, Boehmer FD, Godovac-Zimmermann J: Functional proteomics analysis of signal transduction pathways of the platelet-derived growth factor beta receptor. *Biochemistry* 1999, 38:1757-1764.

38. •• Oda Y, Nagasu T, Chait BT: Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat Biotechnol* 2001, 19:379-382.
Phosphoserine-specific and phosphothreonine-specific chemistry was used to affinity purify phosphoproteins and phosphopeptides from complex mixtures via the attachment of a biotin tag. LC–ESI-MS/MS was used to identify the formerly phosphorylated proteins and localize the sites of phosphorylation.

39. •• Zhou H, Watts JD, Aebersold R: A systematic approach to the analysis of protein phosphorylation. *Nat Biotechnol* 2001, 19:375-378.
Chemistry directed against phosphorylated residues was used to affinity purify phosphopeptides from complex mixtures via the attachment of a biotin tag. LC–ESI-MS/MS was used to identify the phosphorylated peptides and localize the sites of phosphorylation.

40. Goshe MB, Conrads TP, Panisko EA, Angell NH, Veenstra TD, Smith RD: Phosphoprotein isotope-coded affinity tag approach for isolating and quantitating phosphopeptides in proteome-wide analyses. *Anal Chem* 2001, 73:2578-2586.

41. • Larsen MR, Sørensen GL, Fey SJ, Larsen PM, Roepstorff P: Phospho-proteomics: evaluation of the use of enzymatic de-phosphorylation and differential mass spectrometric peptide mass mapping for site specific phosphorylation assignment in proteins separated by gel electrophoresis. *Electrophoresis* 2001, 22:223-238.
The authors evaluate the utility of mass mapping by MALDI-TOF using alkaline phosphatase treatment to identify phosphopeptides.

42. Liao PC, Leykam J, Andrews PC, Gage DA, Allison J: An approach to locate phosphorylation sites in a phosphoprotein: mass mapping by combining specific enzymatic degradation with matrix-assisted laser desorption/ionization mass spectrometry. *Anal Biochem* 1994, 219:9-20.

43. Jensen ON, Larsen MR, Roepstorff P: Mass spectrometric identification and microcharacterization of proteins from electrophoretic gels: strategies and applications. *Proteins* 1998, Suppl 2:74-89.

44. Amankwa LN, Harder K, Jirik F, Aebersold R: High-sensitivity determination of tyrosine-phosphorylated peptides by on-line enzyme reactor and electrospray ionization mass spectrometry. *Protein Sci* 1995, 4:113-125.

45. Jonscher KR, Yates JR III: Matrix-assisted laser desorption ionization/quadrupole ion trap mass spectrometry of peptides. Application to the localization of phosphorylation sites on the P protein from Sendai virus. *J Biol Chem* 1997, 272:1735-1741.

46. Qin J, Chait BT: Identification and characterization of posttranslational modifications of proteins by MALDI ion trap mass spectrometry. *Anal Chem* 1997, 69:4002-4009.

47. Annan RS, Carr SA: Phosphopeptide analysis by matrix-assisted laser desorption time-of-flight mass spectrometry. *Anal Chem* 1996, 68:3413-3421.

48. Schnölzer M, Lehmann WD: Identification of modified peptides by metastable fragmentation in MALDI mass spectrometry. *Int J Mass Spectrom Ion Proc* 1997, 169/170:263-271.

49. Metzger S, Hoffmann R: Studies on the dephosphorylation of phosphotyrosine-containing peptides during post-source decay in matrix-assisted laser desorption/ionization. *J Mass Spectrom* 2000, 35:1165-1177.

50. Cramer R, Richter WJ, Stimson E, Burlingame AL: Analysis of phospho- and glycopolypeptides with infrared matrix-assisted laser desorption and ionization. *Anal Chem* 1998, 70:4939-4944.

51. Wilm M, Neubauer G, Mann M: Parent ion scans of unseparated peptide mixtures. *Anal Chem* 1996, 68:527-533.

52. Carr SA, Huddleston MJ, Annan RS: Selective detection and sequencing of phosphopeptides at the femtomole level by mass spectrometry. *Anal Biochem* 1996, 239:180-192.

53. • Steen H, Küster B, Fernandez M, Pandey A, Mann M: Detection of tyrosine phosphorylated peptides by precursor ion scanning quadrupole TOF mass spectrometry in positive ion mode. *Anal Chem* 2001, 73:1440-1448.
Phosphotyrosine-containing peptides were identified by detection of characteristic phosphotyrosine immonium fragment ions in a high resolution Qq-TOF mass spectrometer. Unlike precursor ion scans that detect PO_3^-, the experiment is performed in the positive ion mode, facilitating the sequencing of the parent phosphopeptides.

54. Covey T, Shushan B, Bonner R, Schröder W, Hucho F: LC/MS and LC/MS/MS screening for the sites of post-translational modification in proteins. In *Methods in Protein Sequence Analysis*. Edited by Jörnvall H, Höög JO, Gustavsson AM. Basel: Birkhäuser Verlag; 1991:249-256.

55. Tholey A, Reed J, Lehmann WD: Electrospray tandem mass spectrometric studies of phosphopeptides and phosphopeptide analogues. *J Mass Spectrom* 1999, 34:117-123.

56. Borchers C, Parker CE, Deterding LJ, Tomer KB: Preliminary comparison of precursor scans and liquid chromatography-tandem mass spectrometry on a hybrid quadrupole time-of-flight mass spectrometer. *J Chromatogr A* 1999, 854:119-130.

57. Verma R, Annan RS, Huddleston MJ, Carr SA, Reynard G, Deshaies RJ: Phosphorylation of Sic1p by G1 Cdk required for its degradation and entry into S phase. *Science* 1997, 278:455-460.

58. Beuvink I, Hess D, Flotow H, Hofsteenge J, Groner B, Hynes NE: Stat5a serine phosphorylation. Serine 779 is constitutively

phosphorylated in the mammary gland, and serine 725 phosphorylation influences prolactin-stimulated ***in vitro*** DNA binding activity. *J Biol Chem* 2000, 275:10247-10255.

59. • Annan RS, Huddleston MJ, Verma R, Deshaies RJ, Carr SA: A multidimensional electrospray MS-based approach to phosphopeptide mapping. *Anal Chem* 2001, 73:393-404.
The authors outline a strategy for characterizing phosphopeptides that combines phosphopeptide detection during HPLC using the PO_2^- and PO_3^- marker ions, subsequent mass determination by precursor ion scanning, and localization of the site of phosphorylation by CID.

60. Schlosser A, Pipkorn R, Bossemeyer D, Lehmann WD: Analysis of protein phosphorylation by a combination of elastase digestion and neutral loss tandem mass spectrometry. *Anal Chem* 2001, 73:170-176.

61. Huddleston MJ, Annan RS, Bean MF, Carr SA: Selective detection of phosphopeptides in complex mixtures by electrospray liquid chromatography/mass spectrometry. *J Am Soc Mass Spectrom* 1993, 4:710-717.

62. Ding J, Burkhart W, Kassel DB: Identification of phosphorylated peptides from complex mixtures using negative-ion orifice-potential stepping and capillary liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* 1994, 8:94-98.

63. Jedrzejewski PT, Lehmann WD: Detection of modified peptides in enzymatic digests by capillary liquid chromatography/electrospray mass spectrometry and a programmable skimmer CID acquisition routine. *Anal Chem* 1997, 69:294-301.

64. • Wind M, Edler M, Jakubowski N, Linscheid M, Wesch H, Lehmann WD: Analysis of protein phosphorylation by capillary liquid chromatography coupled to element mass spectrometry with ^{31}P detection and to electrospray mass spectrometry. *Anal Chem* 2001, 73:29-35.
The authors report an MS-based method of detecting phosphopeptides that does not rely on the lability of the phosphate moiety. HPLC coupled on-line to inductively coupled plasma MS was used to identify phosphopeptides based on detection of ^{31}P. Peptide mass information was obtained in parallel LC–ESI-MS experiments.

65. DeGnore JP, Qin J: Fragmentation of phosphopeptides in an ion trap mass spectrometer. *J Am Soc Mass Spectrom* 1998, 9:1175-1188.

66. Merrick BA, Zhou W, Martin KJ, Jeyarajah S, Parker CE, Selkirk JK, Tomer KB, Borchers CH: Site-specific phosphorylation of human p53 protein determined by mass spectrometry. *Biochemistry* 2001, 40:4053-4066.

67. Polson AG, Huang L, Lukac DM, Blethrow JD, Morgan DO, Burlingame AL, Ganem D: Kaposi's sarcoma-associated herpesvirus K-bZIP protein is phosphorylated by cyclin-dependent kinases. *J Virol* 2001, 75:3175-3184.

68. Sadagopan N, Malone M, Watson JT: Effect of charge derivatization in the determination of phosphorylation sites in peptides by electrospray ionization collision-activated dissociation tandem mass spectrometry. *J Mass Spectrom* 1999, 34:1279-1282.

69. Davis MT, Lee TD: Variable flow liquid chromatography-tandem mass spectrometry and the comprehensive analysis of complex protein digest mixtures. *J Am Soc Mass Spectrom* 1997, 8:1059-1069.

70. Figeys D, Corthals GL, Gallis B, Goodlett DR, Ducret A, Corson MA, Aebersold R: Data-dependent modulation of solid-phase extraction capillary electrophoresis for the analysis of complex peptide and phosphopeptide mixtures by tandem mass spectrometry: application to endothelial nitric oxide synthase. *Anal Chem* 1999, 71:2279-2287.

71. Gallis B, Corthals GL, Goodlett DR, Ueba H, Kim F, Presnell SR, Figeys D, Harrison DG, Berk BC, Aebersold R *et al.*: Identification of flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *J Biol Chem* 1999, 274:30101-30108.

72. Herbst R, Zhang X, Qin J, Simon MA: Recruitment of the protein tyrosine phosphatase CSW by DOS is an essential step during signaling by the sevenless receptor tyrosine kinase. *EMBO J* 1999, 18:6950-6961.

73. Resing KA, Johnson RS, Walsh KA: Mass spectrometric analysis of 21 phosphorylation sites in the internal repeat of rat profilaggrin, precursor of an intermediate filament associated protein. *Biochemistry* 1995, 34:9477-9487.

74. Lapko VN, Jiang XY, Smith DL, Song PS: Posttranslational modification of oat phytochrome A: phosphorylation of a specific serine in a multiple serine cluster. *Biochemistry* 1997, 36:10595-10599.

75. Jaffe H, Veeranna, Pant HC: Characterization of serine and threonine phosphorylation sites in beta- elimination/ethanethiol addition-modified proteins by electrospray tandem mass spectrometry and database searching. *Biochemistry* 1998, 37:16211-16224.

76. Krutchinsky AN, Zhang W, Chait BT: Rapidly switchable matrix-assisted laser desorption/ionization and electrospray quadrupole-time-of-flight mass spectrometry for protein identification. *J Am Soc Mass Spectrom* 2000, 11:493-504.

77. Shevchenko A, Loboda A, Ens W, Standing KG: MALDI quadrupole time-of-flight mass spectrometry: a powerful tool for proteomic research. *Anal Chem* 2000, 72:2132-2141.

78. Lee CH, McComb ME, Bromirski M, Jilkine A, Ens W, Standing KG, Perreault H: On-membrane digestion of beta-casein for determination of phosphorylation sites by matrix-assisted laser desorption/ionization quadrupole/time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 2001, 15:191-202.

79. Baldwin MA, Medzihradszky KF, Lock CM, Fisher B, Settineri TA, Burlingame AL: Matrix-assisted laser desorption/ionization coupled with quadrupole/orthogonal acceleration time-of-flight mass spectrometry for protein discovery, identification, and structural analysis. *Anal Chem* 2001, 73:1707-1720.

80. Hoffmann R, Metzger S, Spengler B, Otvos L Jr: Sequencing of peptides phosphorylated on serines and threonines by post-source decay in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J Mass Spectrom* 1999, 34:1195-1204.

81. Lennon JJ, Walsh KA: Locating and identifying posttranslational modifications by in-source decay during MALDI-TOF mass spectrometry. *Protein Sci* 1999, 8:2487-2493.

82. Kinumi T, Niwa H, Matsumoto H: Phosphopeptide sequencing by in-source decay spectrum in delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Anal Biochem* 2000, 277:177-186.

83. Zubarev RA, Kelleher NL, McLafferty FW: Electron capture dissociation of multiply charged protein cations. A nonergodic process. *J Am Chem Soc* 1998, 120:3265-3266.

84. •• Stensballe A, Jensen ON, Olsen JV, Haselmann KF, Zubarev RA: Electron capture dissociation of singly and multiply phosphorylated peptides. *Rapid Commun Mass Spectrom* 2000, 14:1793-1800.
The authors demonstrate the use of electron capture dissociation to fragment phosphopeptides. Fragment ion series produced by this method were more complete than those produced by collision-induced dissociation, and loss of the phosphate moiety was not observed, resulting in more informative MS/MS spectra.

85. Shi SD, Hemling ME, Carr SA, Horn DM, Lindh I, McLafferty FW: Phosphopeptide/phosphoprotein mapping by electron capture dissociation mass spectrometry. *Anal Chem* 2001, 73:19-22.

86. Cohen P, Gibson BW, Holmes CF: Analysis of the ***in vivo*** phosphorylation states of proteins by fast atom bombardment mass spectrometry and other techniques. *Methods Enzymol* 1991, 201:153-168.

87. Tsay YG, Wang YH, Chiu CM, Shen BJ, Lee SC: A strategy for identification and quantitation of phosphopeptides by liquid chromatography/tandem mass spectrometry. *Anal Biochem* 2000, 287:55-64.

88. • Oda Y, Huang K, Cross FR, Cowburn D, Chait BT: Accurate quantitation of protein expression and site-specific phosphorylation. *Proc Natl Acad Sci USA* 1999, 96:6591-6596.
Mass spectrometry in combination with whole-cell stable-isotope labeling was used for simultaneous identification and quantitation of individual proteins and for determining changes in the level of phosphorylation at specific sites.

89. Weckwerth W, Willmitzer L, Fiehn O: Comparative quantification and identification of phosphoproteins using stable isotope

labeling and liquid chromatography/mass spectrometry. *Rapid Commun Mass Spectrom* 2000, 14:1677-1681.

90. Knebel A, Morrice N, Cohen P: A novel method to identify protein kinase substrates: eEF2 kinase is phosphorylated and inhibited by SAPK4/p38delta. *Embo J* 2001, 20:4360-4369.

91. Janek K, Wenschuh H, Bienert M, Krause E: Phosphopeptide analysis by positive and negative ion matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun Mass Spectrom* 2001, 15:1593-1599.

92. Ma Y, Lu Y, Zeng H, Ron D, Mo W, Neubert TA: Characterization of phosphopeptides from protein digests using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and nanoelectrospray quadrupole time-of-flight spectrometry. *Rapid Commun Mass Spectrom* 2001, 15:1693-1700.

93. Adamczyk M, Gebler JC, Wu J: Selective analysis of phosphopeptides within a protein mixture by chemical modification, reversible biotinylation and mass spectrometry. *Rapid Commun Mass Spectrom* 2001, 15:1481-1488.

94. Steen H, Kuster B, Mann M: Quadrupole time-of-flight versus triple-quadrupole mass spectrometry for the determination of phosphopeptides by precursor ion scanning. *J Mass Spectrom* 2001, 36:782-790.

95. Rappsilber J, Steen H, Mann M: Labile sulfogroup allows differentiation of sulfotyrosine and phosphotyrosine in peptides. *J Mass Spectrom* 2001, 36:832-833.

96. Flora JW, Muddiman DC: Selective, sensitive, and rapid phosphopeptide identification in enzymatic digests using ESI-FTICR-MS with infrared multiphoton dissociation. *Anal Chem* 2001, 73:3305-3311.