Mass Spectrometric Contributions to the Practice of Phosphorylation Site Mapping through 2003

A LITERATURE REVIEW

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Reversible phosphorylation of proteins is among the most important post-translational modifications, and elucidation of sites of phosphorylation is essential to understanding the regulation of key cellular processes such as signal transduction. Unfortunately phosphorylation site mapping is as technically challenging as it is important. Limitations in the traditional method of Edman degradation of $^{32}$P-labeled phosphoproteins have spurred the development of mass spectrometric methods for phosphopeptide identification and sequencing. To assess the practical contributions of the various technologies we conducted a literature search of publications using mass spectrometry to discover previously unknown phosphorylation sites. 1281 such phosphorylation sites were reported in 203 publications between 1992 and 2003. This review examines and catalogues those methods, identifies the trends that have emerged in the past decade, and presents representative examples from among these methods. Molecular & Cellular Proteomics 4:235–245, 2005.

Protein phosphorylation is vital to a host of protein functions that are important to cellular processes spanning the range from signal transduction, differentiation, and development to cell cycle control and metabolism. A primary role of phosphorylation is to act as a switch to turn “on” or “off” a protein activity or a cellular pathway in an acute and reversible manner (1). Sequencing of the human genome has highlighted the physiological importance of phosphorylation. Kinases and phosphatases, the enzymes responsible for protein phosphorylation and dephosphorylation, respectively, make up ~2% of the human genome (2, 3). Furthermore it is estimated that one of every three proteins is phosphorylated at some point in its life cycle (4).

Despite a growing knowledge of many phosphorylation consensus sequences, this post-translational modification cannot usually be predicted accurately from its translated gene sequence alone. Thus, the experimental determination of sites of phosphorylation is an important task. Various methods for protein phosphorylation site determination have been developed through the years, yet this task remains a technical challenge (5). Well established methods involving the analysis of $^{32}$P-labeled phosphoproteins by Edman degradation and two-dimensional phosphopeptide mapping have proven to be powerful but not without limitations. Beyond the inconveniences associated with radioactivity work, these traditional phosphorylation analysis methods can be time-consuming and are not well suited for the high throughput experiments required for phosphoproteome analysis.

More recently mass spectrometry has emerged as a reliable and sensitive method for the localization of protein phosphorylation sites (6–8) and is the favored method for phosphorylation analysis of signaling proteins (9). A thorough analysis of mass spectrometry-based schemes in the past decade for the analysis and determination of novel phosphorylation sites, as presented in this review, reveals that a plethora of different methods have been used, separately and in combination, for the phosphorylation analysis of proteins. The large and growing number of methods described in the literature for mass spectrometry-based phosphorylation site determination present the researcher with a variety of approaches to use. For example, the combination of $\beta$-elimination/Michael addition chemistry with mass spectrometry has been the subject of at least 20 method-oriented papers. It is not necessarily easy, however, to ascertain from the literature which of these methods is really useful in practice. Here we present a survey of methods to show which have actually proven useful for the determination of previously unknown protein phosphorylation sites.

CURRENT STATE OF THE ART IN PROTEIN PHOSPHORYLATION STUDIES

We examined peer-reviewed papers that span the time frame of 1992–2003 to highlight the methods that have been successfully used for novel phosphorylation site determination. Only the methods used to determine novel phosphorylation sites will be described here. Many other methods have been described in the literature but have not actually been used for novel phosphorylation site determination or have fallen out of use due to, for example, insufficient sensitivity.
Analysis of current methodologies in protein phosphorylation analysis was done as follows. The Current Contents Science Edition literature data base was searched with the terms “mass spectrometry” and “phosphorylation” from the years 1992 through 2003. It is possible that some relevant papers were missed due to their lack of these particular keywords in their title, abstract, or keyword list. Since Current Contents does not at present catalog papers published in Molecular & Cellular Proteomics, Medline was also searched for this particular journal. Papers were selected that clearly showed the determination of novel phosphorylation sites in which mass spectrometry played a central role in their discovery. Method papers and those that provided confirmation of known or suspected sites were not included. A total of 203 papers from 1992 to 2003, summarized in Table I and Figs. 1–3, were found to meet these criteria, and the phosphoproteins identified and methods used in each are summarized in Supplemental Tables 1 and 2. The papers were annotated with names of proteins, methods used in phosphorylation mapping excluding those methods involving the purification or digestion of the protein, whether in vivo or in vitro phosphorylation situations were investigated, what type of mass spectrometer was used, and what type of phosphorylation (Ser(P), Thr(P), or Tyr(P)) as well as how many sites were determined. Summaries of the results have also been categorized by year through 2003 as described below.

### Mass Spectrometry-based Approaches to Phosphorylation Site Determination

The use of mass spectrometry in protein phosphorylation site determination has increased significantly in the past few years. As shown in Table I, 58% of studies that used mass spectrometry to determine specific protein phosphorylation sites came from the years 2001–2003, 25% came from the years 1998–2000, 13% came from 1995–1997, and 4% came from 1992–1994. In the last decade, however, it is evident that in most cases multiple techniques used in combination have been necessary for protein phosphorylation site analysis and that no single combination of approaches appears to be optimal for all proteins. Among the 18 different

### Table I—continued

| Years   | ESI-MS, MS/MS | Nanospray ESI-MS, MS/MS | PSD (MALDI-TOF) | Inferred site | Edman degradation | MALDI-TOF-MS | Triple quadrupole | Ion trap | Q-TOF | FTMS or other ESI | Total papers |
|---------|---------------|-------------------------|-----------------|--------------|-------------------|--------------|-------------------|----------|-------|------------------|-------------|
| 1992–1994 | 6             | 0                       | 0               | 2            | 4                 | 6            | 0                 | 0        | 1     | 8                |             |
|         | 75%           | 0%                      | 0%              | 25%          | 50%              | 13%          | 75%              | 0%       | 0%    | 13%              | 4%          |
| 1995–1997 | 14            | 2                       | 1               | 9            | 8                 | 9            | 16                | 4        | 0     | 3                | 26          |
|         | 54%           | 8%                      | 4%              | 35%          | 31%              | 35%          | 62%              | 15%      | 0%    | 12%              | 13%         |
| 1998–2000 | 40            | 16                      | 5               | 8            | 9                 | 22           | 24                | 16       | 5     | 0                | 51          |
|         | 78%           | 31%                     | 10%             | 16%          | 18%              | 43%          | 47%              | 31%      | 10%   | 0%               | 25%         |
| 2001–2003 | 56            | 40                      | 9               | 17           | 13                | 54           | 21                | 42       | 43    | 3                | 118         |
|         | 47%           | 34%                     | 8%              | 14%          | 11%              | 46%          | 18%              | 36%      | 36%   | 3%               | 58%         |
| All     | 116           | 58                      | 15              | 36           | 34                | 86           | 67                | 62       | 48    | 7                | 203         |
|         | 57%           | 29%                     | 7%              | 18%          | 17%              | 42%          | 33%              | 31%      | 24%   | 3%               | 100%        |
nontrivial methods used as criteria for phosphorylation site analysis in this review, on average, each paper used four different methods in the search for phosphorylated residues.

**Peptide Mass Measurement in Digest Mixtures**—Phosphorylation analysis by mass spectrometry is generally accomplished by a two-step approach. The phosphoprotein of interest is proteolytically digested, usually with trypsin, and the tryptic peptides are analyzed to determine which are phosphorylated. Then those phosphopeptides are further analyzed, usually by tandem mass spectrometry (MS/MS), to determine the precise location of the phosphorylation site(s). Phosphopeptides may be identified simply by examination of the list of observed peptide masses for mass increases of 80 Da (the added mass of the phosphate group) compared with the list of expected peptide masses. Although this method is relatively straightforward, it also misses many phosphorylated peptides because 1) peptide maps are frequently incomplete even for non-phosphorylated proteins (some tryptic peptides are poorly ionized or poorly recovered), 2) the increased acidity of the phosphate group generally results in decreased ionization efficiency of a peptide (10), and 3) competition for ionization of peptides in a mixture (if no separation is performed) results in suppression of signal for some peptides. Nonetheless the ease and small amount of sample required for a simple peptide map make this method popular. Using $\beta$-elimination/Michael addition chemistry to replace the phosphate with a chemical group more conducive to efficient ionization can ameliorate some of the difficulties associated with phosphopeptides (11).

Phosphopeptide mass measurement may be achieved either by MALDI or ESI. These processes are the two most common ways to ionize peptides, yet they differ fundamentally. For this reason, one may often find some proteins ionize better by one process than the other. Of the papers analyzed, 32% used a MALDI-TOF mass spectrometer, 68% used some form of ESI mass spectrometer, and 19% used both a MALDI-TOF mass spectrometer as well as some form of ESI mass spectrometer in their phosphorylation studies. The use of MALDI-TOF mass spectrometers in phosphorylation site identification rose from 13% from 1992 to 1994 to around 33% from 1995 to 2003 indicating a higher prevalence of MALDI-TOF mass spectrometers. As shown in Table I for ESI instruments, the use of the triple quadrupole mass spectrometer has decreased from 75% between 1992 and 1994 down to 18% from 2001 to 2003 as other types of ESI mass spectrometers became available, generally with greater sensitivity. Although quadrupole ion trap mass spectrometers only became commercially available in 1995, they were used in 15% of all papers in the 1995–1997 range and increased in use to 33% between 1998 and 2003. The Q-TOF is the most recent arrival of the three ESI mass spectrometers, arriving only at the last half of the time frame analyzed, used in 10% of the papers from 1998 to 2000 and 36% from 2000 to 2003. The

![Figure 1](image_url)
newest linear ion trap instruments combine the attractive features of triple quadrupole and ion trap analyzers, and it is likely that they will become widely used for phosphorylation site determinations.

One approach to reduce the sample required for ESI measurement is to reduce the fluid flow by use of small capillary electrospray emitter tips, a process known as nanoelectrospray. Nanoelectrospray produces a constant signal for 10–30 min for a 1–μl sample, and the low flow has been shown to increase the ionization efficiency and reduce ion suppression (12, 13). Nanoelectrospray is frequently used for MS/MS studies (see below). Of the papers analyzed, 29% used nanoelectrospray. Nanoelectrospray was not present in the papers until 1997 at 8% then its use increased to 34% in 2000–2003.

Another way to reduce ion suppression phenomena is to separate the peptides prior to ionization. The common method is LC-MS, which has the added benefit of concentrating dilute samples and removing salt that interferes with the ionization process. 54% of the papers analyzed used nanoelectrospray. Nanoelectrospray was not present in the papers until 1997 at 8% then its use increased to 34% in 2000–2003. Another way to reduce ion suppression phenomena is to separate the peptides prior to ionization. The common method is LC-MS, which has the added benefit of concentrating dilute samples and removing salt that interferes with the ionization process. 54% of the papers analyzed used LC-ESI, and the usage of this method has remained steady throughout the past 12 years.

As a way to highlight the presence of phosphopeptides in a mixture or to confirm the identity of a phosphopeptide, a simple phosphatase reaction will cause a downward shift in mass of 80 Da (or multiples of 80) for each phosphopeptide (14). 17% of the analyzed papers used phosphatases. In 1992–2000, the proportion of papers using phosphatase remained between 19 and 25%, while in 2000–2003 it dipped to 14%.

**Enrichment of Phosphopeptides**—Enrichment of phosphopeptides from the total digest becomes increasingly important when dealing with complex mixtures, *i.e.* large proteins or protein mixtures. Any form of peptide separation that yields isolated phosphopeptides or simpler mixtures can be helpful (e.g. reverse phase chromatography in which phosphopeptides elute earlier than their unphosphorylated counterparts or ion exchange chromatography that exploits the relative acidity of phosphopeptides). More selective approaches are 1) immunoprecipitation, 2) immobilized metal affinity chromatography, and 3) β-elimination combined with addition of an affinity tag. Immunoprecipitation uses an antibody, usually covalently attached to a resin, to bind the phosphorylated peptides after which the non-phosphorylated peptides are washed away, and the bound peptides are eluted for analysis. Antibodies specific to phosphotyrosine residues are used most commonly because a variety of high affinity, high specificity antibodies are available. To a lesser extent antibodies to Ser(P) and Thr(P) are used. As an example of this approach, Lehr *et al.* (15, 16) successfully used anti-phospho-tyrosine antibodies to identify 16 tyrosine phosphorylation sites of the human insulin receptor substrate Gab-1. The number of papers using antibodies as reagents for phosphorylation studies has increased through the last decade from 3–13% in the years 1992–1997 to around 20% in 1998–2003.
The increase may be due to improvements in the quality of antibodies against Ser(P) and Thr(P) that are useful in Western blots and the increased use of antibodies against Tyr(P) used in immunoprecipitations. Overall 17% of the analyzed papers used phosphoantibodies; 5% used them in immunoprecipitation steps to enrich for phosphopeptides.

IMAC uses Fe(III) or Ga(III) to bind to negatively charged regions of the peptides, such as the phosphate group (17–20). One pitfall of this method is that not only are phosphopeptides bound but also acidic peptides and peptides containing histidine. This problem appears to have been largely eliminated by Ficarro et al. (21) by converting carboxylic acid groups to methyl esters. Of the papers analyzed, most recently 19% use IMAC methods with the percentages increasing during the last 10 years. Ficarro and co-workers (21) found a large number of novel phosphorylation sites in proteins of Saccharomyces cerevisiae. Other peptide fractionation approaches can also assist phosphopeptide identification. Anion exchange, for example, takes advantage of the acidity of the phosphate group to enrich for phosphopeptides and has been successfully applied to the identification of 500 phosphorylation sites in the developing mammalian brain (22).

Through β-elimination/addition reactions, the phosphate group may be substituted with an affinity tag, allowing enrichment of phosphopeptides with, for example, a biotin affinity tag and an avidin column (23, 24). Only 5% of the papers analyzed used β-elimination/addition in their studies, although of the 10 papers that did use this method, three papers showed very successful studies in which large numbers of phosphorylation sites were identified (20–53 sites per study). For example, Jaffe and co-workers (25, 26) found 38 or 53 phosphorylation sites, respectively, for the high molecular weight neurofilament proteins. It is unclear why so few researchers have published phosphorylation papers using this method in light of such successes. A well known pitfall of this technique is that O-linked sugars undergo the same elimination chemistry potentially leading to glycosylation sites being incorrectly assigned as phosphorylation sites (27). One could also speculate that the chemistry requires significant effort to perform properly or that reaction yields necessitate large amounts of protein. However, the elimination/addition method has been widely used in the Edman degradation community in the past (28–30).

Detection of Phosphopeptides by Precursor Ion and Neutral Loss Scans—Phosphopeptides undergo characteristic fragmentation pathways when subjected to CID allowing them to be distinguished from non-phosphorylated peptides. In the negative ion mode, for example, phosphopeptides fragment to produce marker ions at m/z 79 (PO₃⁻) and 63 (PO₂⁻). CID of phosphoserine- and phosphothreonine-containing peptides in the positive ion mode often yields a neutral loss of H₃PO₄ via β-elimination (31). Peaks corresponding to this loss (98 from singly charged precursors, 49 from doubly charged precursors, etc.) are often the most abundant ions in the CID spectrum, although this is not invariably the case. Phosphotyrosine residues do not undergo β-elimination but do produce a characteristic immonium ion at m/z 216 (32).

These diagnostic ions can be selectively detected in several instrumental methods. Triple quadrupole mass spectrometers are capable of precursor ion scanning and neutral loss scan-
Phosphorylation Site Mapping from 1992 to 2003

ning modes. In precursor ion scans, also known as parent ion scans, only those peptides that fragment to produce the chosen marker ion, e.g. \( m/z \) 79, produce peaks in the spectrum, screening out all other species. Likewise scanning for the neutral loss of \( \text{H}_3\text{PO}_4 \) can detect peptides containing phosphoserine and phosphothreonine, although care must be taken to match the charge state of the precursor ion to the mass of the neutral lost. Other instruments such as hybrid Q-TOF mass spectrometers can perform analogous experiments, although in the strictest sense they are not scanning but rather acquiring CID spectra of many precursor ions and extracting the desired information after the fact. Some loss of sensitivity is incurred, but the high resolution and mass accuracy of these instruments can help discriminate the true phosphopeptides from non-phosphorylated peptides that coincidentally produce product ions or neutral losses with the same nominal mass. The phosphotyrosine immonium ion at \( m/z \) 216.043, for example, is close in mass to several two-amine acid combinations but can be unambiguously assigned given sufficient resolution (32). Of the papers analyzed, 18% used precursor ion scans or neutral loss scans. From 1992 to 1997, 11% used these methods, while from 1998 to 2003 it increased to 19%.

Precursor ion and neutral loss scans can be incorporated into various experimental schemes. Their simplest application involves direct infusion of sample and electrospray ionization on triple quadrupole or Q-TOF instruments. Static nanospray is advantageous because it allows extended acquisition time with signal averaging (13). Sample introduction by MALDI is also possible but does not provide a consistent supply of ions, which is problematic for scanning experiments. The traditional application of precursor ion and neutral loss scans is in conjunction with liquid chromatography; separation of ions, which is problematic for scanning experiments. The clear favorite of these methods is MS/MS, used in 57% of the papers, while PSD seems to be rarely used with only 7% adopting this method.

Inference of the site when a phosphopeptide contains only one possibility for the phosphorylation of given residues, e.g. a singly phosphorylated peptide containing only one Ser, Thr, or Tyr, was reported in 18% of the papers. The use of Edman degradation in combination with some form of mass spectrometry takes place in 17% of the papers analyzed. The decreased use of this method in general, however, is apparent when looking throughout the past 12 years with 50% of the papers from 1992 to 1994 using this method and a steady decreasing usage throughout the following years, down to 11% from 2000 to 2003. (Note that our paper selection criteria did not include papers that describe Edman degradation experiments without the use of mass spectrometry.)

Along with a steady increase throughout the past 12 years in papers describing phosphorylation by mass spectrometry, it follows that the number of sites determined has steadily increased year by year. As shown in Fig. 3, there was a spike in the number of total phosphorylation sites determined in the year 2002. This is due to one notable paper by Ficarro et al. (21) that determined 303 Ser(P) sites and 61 Thr(P) sites from a whole-cell lysate of \( S. \) cerevisiae. Although fewer total sites were determined in 2003, a trend followed with generally more sites determined per paper. Although the ratio of Ser(P):Thr(P):Tyr(P) sites naturally found in proteins is ~90:10:1, it is shown that in many cases the determination of Tyr(P) sites is greater than one would suspect according to their prevalence. This may be due to the presumption of greater importance of Tyr(P) sites in signaling pathways and thus a greater effort to discern these sites on signaling proteins.

** MASS SPECTROMETRY IN COMBINATION WITH TRADITIONAL APPROACHES **

Radioactive Labeling of Phosphoproteins with \( ^{32}\text{P} \)—A favored method for studying phosphorylation has been the incorporation of \( ^{32}\text{P} \) via the \( \gamma \) phosphate of ATP into the phosphoprotein (35–41). This can be done either in the in vivo context of a tissue or cell line or the in vitro context of kinase reaction with a substrate or recombinant protein autophosphorylation. The in vivo phosphorylation, while more physiologically relevant by avoiding the possibility of nonspecific phosphorylation, also requires working with significantly larger amounts of radioactivity due to the extensive cellular consumption of phosphate in the form of phosphoproteins,
nucleic acids, phospholipids, etc. Radioactive proteins, fractionated by chromatography or gel electrophoresis, can be sequenced by Edman degradation (36), assuming the phosphorylated residue is close to the N terminus; or peptides from proteolyzed proteins can be separated by HPLC or two-dimensional thin layer chromatography (two-dimensional peptide mapping) (36) followed by Edman degradation. In addition, one can perform complete hydrolysis of the radioactive protein for determination of phosphoamino acid content.

The continuing importance of traditional $^{32}$P labeling is clear from its consistent usage. As a tool for visualizing low amounts of phosphoproteins and low stoichiometric phosphorylation, the sensitivity of $^{32}$P is unparalleled. Of the papers analyzed, 51% used $^{32}$P either in the context of two-dimensional thin layer chromatography or autoradiography or both together. The percentage of papers using $^{32}$P remained the same at roughly 50% in all time frames analyzed. Despite the dramatic rise in use of mass spectrometric methods for phosphorylation site determination, it is noteworthy that the use of $^{32}$P methods in combination with mass spectrometry has not declined.

In Vivo Versus In Vitro Approaches—The choice by an investigator to study in vivo versus in vitro phosphorylation events depends on many factors. These include the following: ease of purifying the protein of interest in an in vivo situation, the stoichiometry of phosphorylation in vivo, the molecular biology capabilities to easily express or overexpress a protein in a cell system that is compatible with the native physiological state of the protein, and whether the phosphorylating kinase has been determined for in vitro phosphorylation.

Although there are differing opinions of what constitutes an in vivo versus an in vitro situation, for the purpose of this review, an in vivo phosphorylation event will be thought of as phosphorylation occurring in the cell, whether cells are from either a living organism or cultured cells, without the addition of supplementary kinases (although one in vivo paper co-expressed their protein and kinase in the cell), and conversely an in vitro phosphorylation event will be considered as phosphorylation occurring after the addition of kinase(s) outside the cell or, in the case of autophosphorylation, the addition of ATP outside of the cell. In the past 12 years, of the papers analyzed, 61% looked at in vivo phosphorylation, 55% looked at in vitro phosphorylation, and 15% looked at both situations. Although in vivo phosphorylation is more relevant from a physiological stance, such studies are generally more technically demanding, so as a result, over the last 12 years, the number of research papers looking at either situation was roughly equivalent. The advantage of studying in vitro phosphorylation is the theoretical attainment of 100% phosphorylation stoichiometry, while the big disadvantage is the burden of proof that the phosphorylation sites found are real and relevant physiologically. The most widely accepted way to do this is by site-directed mutagenesis.

Site-directed Mutagenesis—As molecular biology methodologies have evolved in the past decade, becoming commonplace in most life science laboratories, the use of site-directed mutagenesis in the study of protein function and characterization has increased, and it is becoming a staple in phosphorylation analysis. Site-directed mutagenesis can be used as a confirmation tool for experimentally derived phosphorylation sites or ab initio if domain homologies indicate a discrete number of phosphorylation possibilities. Almost a quarter of the papers analyzed (24%) used site-directed mutagenesis in their studies. The astonishing fact in this number is that 95% of the papers using site-directed mutagenesis were published between the years 1998 and 2003. The increases in site-directed mutagenesis may reflect increased availability of molecular biology kits making these manipulations easier and quicker.

EMERGING TRENDS

Phosphopeptide Sequencing—Although Edman degradation and MS/MS using CID are currently the methods of choice for sequencing phosphopeptides, alternatives have recently been demonstrated. Tandem mass spectrometry using electron capture dissociation was described in 1998 (42) and subsequently applied to phosphorylation site mapping (43). This nonergodic technique favors fragmentation of peptides along the peptide backbone; Ser(P) and Thr(P) residues retain their phosphates, greatly facilitating sequencing of the peptide and locating the site(s) of phosphorylation. Electron capture dissociation is implemented on FTICR mass spectrometers and has only recently seen use for novel site determination (44). In an attempt to realize the advantages of electron capture dissociation on ion trap mass spectrometers Syka et al. (45) applied principles of negative ion chemical ionization in a technique dubbed electron transfer dissociation. In this technique, externally formed negative ions interact with multiply protonated peptides in the electric field of a linear ion trap. Efficient fragmentation of phosphorylated and unphosphorylated peptides was achieved on chromatographic time scales with fragmentation patterns similar to those observed for electron capture dissociation. Increased use of FTICR instruments or commercialization of electron transfer dissociation on the relatively inexpensive ion traps could lead to significant improvements in the sequencing of phosphopeptides.

Phosphoproteome Analysis—As large scale protein identification (proteomic) studies become increasingly common, so do searches for constituents of the proteome that become phosphorylated, namely the phosphoproteome, also known as the phosphorylome. Phosphoproteome studies aim to comprehensively analyze protein phosphorylation by identification of the phosphoproteins, exact localization of the residues that are phosphorylated, and preferably quantitation of the phosphorylation (46, 47). Most phosphoproteome studies have been published since 2001. The phosphoproteome pa-
pers from Table I analyzed capacitated human sperm (48), rat brain membrane proteins (49), MFC-7 (human breast cancer cell line) proteins (50), S. cerevisiae proteins (21), and Arabidopsis thaliana thylakoid membrane proteins (51).

Due to low stoichiometry, heterogeneity, and low abundance, enrichment of phosphopeptides is crucial for phosphoproteome studies. The largest number of novel phosphorylation sites identified in these studies, 365, were from S. cerevisiae proteins (21) and used a combination of methods including IMAC (Fe$^{3+}$) in which the hydrolysate had been methylated to block the binding of unphosphorylated aspartate- and glutamate-containing peptides, phosphatases, and LC-MS/MS on an ion trap. Some may prefer a more directed phosphoproteome profiling approach of steady-state versus dynamic phosphorylation such as that by Metodiev et al. (52) who showed by statistical analysis that the method by Ficarro et al. (21) produced biases in the data generated toward abundant and multiply phosphorylated phosphopeptides. This prompted them to separate intact phosphoproteins from unphosphorylated proteins with either high affinity phosphoantibodies or a phosphoprotein purification kit from Qiagen followed by gel electrophoresis and MALDI-MS analysis of signal-induced changes. Another new development in phosphorylome analysis is the Pro-Q® Diamond phosphoprotein gel stain from Molecular Probes that is suitable for the fluorescence detection of phosphoserine-, phosphothreonine-, and phosphotyrosine-containing proteins directly in SDS-polyacrylamide gels (53, 54); this stain recently helped to identify 672 phosphorylated proteins from Chinese hamster ovary cells (55).

Phosphorylation Site Stoichiometry—Techniques for the discovery of phosphorylation sites have been the focus of this review without regard to site stoichiometry except insofar as low occupancy represents an analytical challenge. Most published studies of phosphorylation site mapping are likewise concerned with the identification of sites rather than quantifying stoichiometries. But phosphorylation in signal transduction pathways is a dynamic process in which successive phosphorylation and dephosphorylation events at multiple sites are responsible for biological activity in these highly regulated systems.

Mass spectrometric approaches to phosphorylation site stoichiometry generally use stable isotope dilution whereby two samples are differentially labeled with mass-encoded tags such that the samples can be mixed and analyzed simultaneously. Each phosphopeptide thus appears as two peaks in the mass spectrum, and the relative abundances of the peaks reflect the amount of the phosphopeptide in each sample. This can be accomplished by metabolic labeling of proteins in cell culture (56, 57) or subsequent chemical labeling of functional groups such as peptide N termini or C termini (58–60). These methods are equally applicable to phosphorylated and unphosphorylated peptides. Methods developed specifically for phosphopeptides either make use of the β-elimination/Michael addition chemistry to introduce the mass tag (61, 62) or treatment with phosphatase in conjunction with isotopic labeling. In the latter approach, the stoichiometry of phosphorylation in individual samples is measured by dividing a sample in two, labeling each with the light/heavy forms of a mass tag and treating one sample with phosphatase before recombining the fractions (63, 64). Alternatively synthetic phosphopeptides incorporating heavy isotopes can be doped into the sample at defined concentrations, allowing absolute quantitation of phosphorylation levels (65).

The experimental approaches most favorable for mapping phosphorylation sites are generally different from those that are suitable for assaying site stoichiometry under biologically relevant conditions. For example, in vitro kinase reactions, endogenous phosphorylation of overexpressed proteins, and the addition of phosphatase inhibitors to live cells to accumulate phosphorylated species are all helpful in the initial discovery of phosphorylation sites but are, to a greater or lesser degree, artificial conditions. Furthermore, since phosphorylation takes place in the context of pathways involving multiple kinases, phosphatases, and their substrates, it is desirable to assay changes in the phosphorylation state of multiple proteins simultaneously, whereas the initial mapping of phosphorylation sites is most successfully done through the exhaustive analysis of single proteins. Targeted detection of phosphopeptides can use any of the mass spectrometric quantitation methods described above applied in ways that maximize speed and sensitivity. Selected reaction monitoring of isotopically labeled peptides, for example, can be used to assay multiple peptides in a single MALDI or LC-MS/MS experiment, quantifying peptides of interest out of complex mixtures. This approach has been applied to phosphopeptides and unphosphorylated peptides alike and can be used for relative or absolute quantitation (14, 65, 66).

Ultimately, however, mass spectrometry may not be the most powerful tool for rapid, sensitive quantitation of phosphorylation levels. ELISAs and other antibody-based detection methods still outstrip mass spectrometry in sensitivity and are compatible with high throughput screens. Phosphorylation site-specific antibodies are already commercially available from over 30 vendors and target several hundred known sites (67). This approach requires significant initial development to raise antibodies and validate their specificity, but the benefits appear substantial, and protein microarrays with phosphospecific antibody detection may become a favored method for phosphorylation site quantitation (68, 69).

CONCLUSIONS

Phosphorylation Site Mapping from 1992 to 2003

The use of mass spectrometry for the determination of protein phosphorylation sites has increased rapidly throughout the past 12 years. A comprehensive analysis of methods used in combination with mass spectrometry indicates there is no one method applicable for determination of all phosphorylation sites, and indeed the majority of identifications have relied on an ensemble of methods. In fact, the lack of a clearly
superior or generally accepted approach is rather surprising, considering the large number of studies evaluated. Only the use of ESI is widely accepted (87% of all studies; although not at the exclusion of MALDI-TOF, which is used in 51% of all studies). Tandem mass spectrometry is also used in more than half the studies (57%) for determination of the precise site(s) of the phosphorylation. In terms of mass spectrometers, trends show the introduction of the Q-TOF mass spectrometer contributed to phosphorylation site analysis in the middle of the time frame analyzed, and there has been an increase throughout the years in the use of the ion trap and to a lesser extent the MALDI-TOF mass spectrometer. In contrast, the use of the triple quadrupole mass spectrometer has decreased rapidly in phosphorylation site determination. Although other mass spectrometers such as the FTICR mass spectrometer are even more sensitive, the limited availability and high cost of these machines has translated into few papers actually using them for novel site determination. Edman degradation chemistry has been used for decades, but its use in combination with mass spectrometry has decreased significantly in recent years. Comprehensive phosphorylation site analyses have steadily relied on the tried and true use of 32P labeling methods and increasingly on the use of mutagenesis methods in combination with mass spectrometry. Difficulties inherent in phosphorylation site analysis demand that phosphopeptides or phosphoproteins be enriched prior to analysis. Affinity enrichment using techniques such as IMAC, β-elimination, and phosphorylation-specific antibodies increase the likelihood of success, but the choice of which technique is most appropriate must be balanced with various parameters such as quantity of protein available, which residues are expected to be phosphorylated, and the degree of sample purification.

Many challenges remain in the identification and localization of phosphorylation sites in proteins. Not least is the discovery of serine, threonine, and tyrosine sulfation as post-translational modifications with nominal masses isobaric with phosphorylation, which represents a pitfall for mass spectrometric approaches to phosphorylation site mapping (70, 71). Other challenges include global analysis of dynamic versus steady-state phosphorylation in phosphoproteomic studies, stoichiometric determinations of low abundance proteins, the development of better and more sensitive instrumentation and enrichment techniques, and real time system analysis to better understand the biological processes in which protein phosphorylation is involved.

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