Global and Site-Specific Quantitative Phosphoproteomics: Principles and Applications

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Annu. Rev. Pharmacol. Toxicol. 2009. 49:199–221

First published online as a Review in Advance on October 3, 2008

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

This article's doi: 10.1146/annurev.pharmtox.011008.145606

0362-1642/09/0210-0199$20.00

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Key Words

protein phosphorylation, cell signaling, mass spectrometry, kinase, phosphatase

Abstract

Protein phosphorylation is a key posttranslational modification, which reversibly regulates almost all processes in the living cell. Deregulated signaling is a hallmark of cancer and other diseases, and protein kinases are prominent drug targets. Phosphorylation events are commonly probed in a targeted manner by phosphorylation-specific antibodies. In contrast, advances in proteomics technology, including phosphopeptide enrichment, high-accuracy mass spectrometry, and associated bioinformatics now make it possible to analyze entire phosphoproteomes. Quantitative methods can assess the relative change in phosphorylation for several thousand sites in a single experiment. Here we review enrichment strategies and methods for mass spectrometric fragmentation and analysis of phosphopeptides. We also describe different quantitative methods and their application to problems in cell signaling and drug target discovery. Emerging phosphoproteomics technologies are becoming more comprehensive, robust, and generically applicable to a wide range of questions, including areas outside traditional eukaryotic cell signaling such as Ser/Thr/Tyr signaling in bacteria.

INTRODUCTION

Protein phosphorylation is one of the most important posttranslational modifications (PTMs) in living cells. It is involved in many regulatory functions such as cell cycle control, receptor-mediated signal transduction, differentiation, proliferation, transformation, and metabolism. The interplay of two types of enzymes, protein kinases and phosphatases, continuously controls substrate modification by reversible phosphorylation and dephosphorylation. An evolutionary highly conserved domain in kinases catalyzes phosphorylation of proteins and this reaction occurs almost exclusively with adenosine triphosphate (ATP) as donor of the phosphate group.

A hallmark of protein phosphorylation is its substoichiometric nature. Although at least one-third of all cellular proteins are estimated to be phosphorylated, their levels of phosphorylation vary widely and specific sites may be phosphorylated from less than 1% to greater than 90%.

In signal transduction, protein phosphorylation often presents an activating or deactivating switch of protein activity. Intracellular signal transduction mediated by receptor-protein tyrosine kinases (e.g., the insulin receptor or the epidermal growth factor (EGF) receptor) is initiated by time-ordered tyrosine phosphorylation of intracellular receptor chains, which immediately leads to the recruitment of adaptor proteins and other signaling molecules such as kinases and phoshophatases (1). Phosphorylation plays an equally important role in the attenuation and termination of the signal in the later stages of signal progression. Breakdown of this exquisitely balanced control system often leads to diseases, including cancer (2–4).

Although phosphorylation is easily detected and visualized in 1D- and 2D-gels by ^{32}P labeling or by western blotting with phosphosite-specific antibodies, it is far more challenging to identify novel phosphoproteins and, in particular, to localize their phosphorylation sites. In addition to simple detection of phosphorylation sites, quantitative studies of dynamic phosphorylation events are important to delineate and understand cell signaling pathways. In this regard, relative quantitation of the activities of signaling molecules involved in a specific cascade and their phosphorylation sites is of critical importance. Not all signaling proteins are turned off in the quiescent cell, but sustained at a basal level. Stoichiometry of phosphorylation activation varies significantly depending on the stimulus applied. Moreover, tight regulation of the involved kinases and phosphatases may lead to only subtle changes in the phosphorylation levels of relevant signaling proteins. Further complexities arise from the fact that phosphorylation is not only highly dynamic, but is also spatially regulated in the cell.

Mass spectrometry (MS) is an ideal detector of phosphorylation events. MS can, in principle, identify each phosphopeptide and localize the phosphorylation groups in the peptide sequence. However, only in the last few years have MS-based methods become sufficiently sensitive and robust to be used routinely and in large scale in cell signaling research. As explained in this chapter, quantitative phosphoproteomics has now become a very powerful technological platform that can be applied to almost any question in basic cell biology and drug development. We start by discussing different methods to enrich and sequence phosphopeptides before moving to illustrative applications. As a guide, **Figure 1** shows an overview of the phosphoproteomics analysis workflow.

PHOSPHOPEPTIDE ENRICHMENT STRATEGIES

The most widely used method in MS-based proteomics involves the enzymatic digestion of complex proteomes into peptide mixtures, which are then separated by nanoscale liquid chromatography and analyzed by MS (see below).

The same principle also applies to the analysis of phosphorylation sites. However, as already mentioned, site-specific phosphorylation is often substoichiometric and thus phosphopeptides

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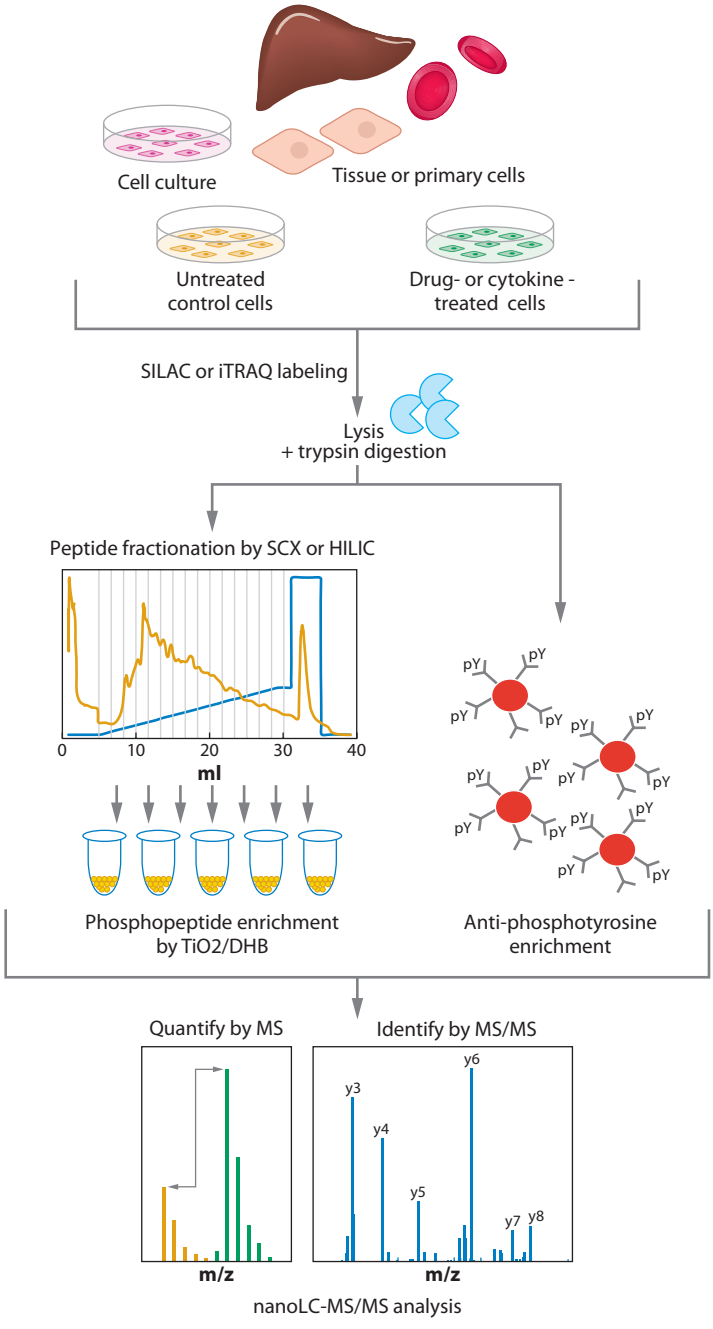

Figure 1

Typical quantitative phosphoproteomics workflow. Control and treated cells or tissues are differentially labeled by stable isotopes, lysed, and mixed together. Protein extracts are digested by trypsin, and phosphopeptides are enriched by a combination of strong cation exchange and TiO_2 chromatographies, or by immunoprecipitation with phospho-specific antibodies. Resulting enriched phosphopeptide mixtures are separated on nanoLC and directly measured in a mass spectrometer. Relative peptide quantitation is usually based on the first stage of mass spectrometry (MS), whereas peptide identification is achieved upon gas-phase fragmentation in the second stage of mass spectrometry (MS/MS).

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represent a small proportion of all peptides present in a total cell lysate. Therefore, they need to be enriched in order to be efficiently measured in the mass spectrometer.

IMAC: immobilized metal affinity chromatography

Many different strategies for up-front selective enrichment of phosphopeptides before MS analysis have been reported. The most successful to date have been the affinity- and antibody-based methods, whereas chemical tagging methods tend to suffer from incomplete and side reactions, and are more difficult to handle. Here we discuss the most widely used methods separately. Note that for optimal analysis of the phosphoproteome these methods usually have to be combined in multi-stage enrichment strategies.

Immobilized Metal Affinity Chromatography (IMAC)

Phosphopeptide enrichment by immobilized metal affinity chromatography (IMAC) is based on the high-affinity coordination of phosphates to certain trivalent metal ions (**Figure 2*a***). Metal ions are immobilized by loading onto porous column packing material, and phosphopeptides are subsequently captured by a metal complex formed on the distant side of the immobilized metal ion. This IMAC concept was introduced by Anderson and coworkers (5), and was further developed and improved by many others. Tempst and coworkers assessed the capacity and selectivity of IMAC for phosphopeptide binding for a variety of metals, including Fe^{3+}, Ga^{3+}, Al^{3+} and Z^{r3+} (6). They observed best selectivity with iminodiacetate columns complexed with Ga^{3+}. However, they also reported increased binding of nonphosphopeptides when loading samples with pH outside the 2.0–3.5 range. Jensen and Stensballe additionally reported that excessive loading of peptide samples onto IMAC beads or columns increased the nonspecific binding of nonphosphorylated peptides (7). One challenge in IMAC is that strongly acidic peptides rich in glutamic and aspartic acid residues also coordinate well with metal complexes. Therefore, additional chemistry (e.g., methylation) or separation methods are often needed to prevent displacement of phosphopeptides by abundant acidic peptides.

The first large-scale application of IMAC in phosphoproteomics dates to 2001 when White and coworkers performed methylation of all acidic groups in peptides prior to IMAC enrichment

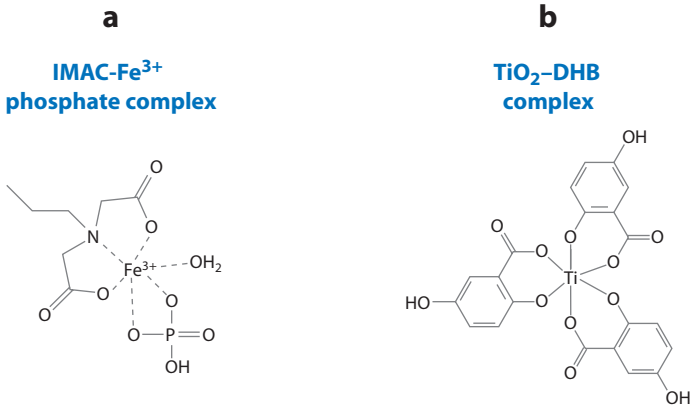

Figure 2

Compounds commonly employed in phosphopeptide enrichment. (*a*) Immobilized metal affinity chromatography (IMAC) is based on the high-affinity coordination of phosphates to certain trivalent metal ions (e.g., Fe^{3+}); (*b*) Complex of titanium dioxide (TiO_2) and 2,5 dihydroxybenzoic acid (DHB). TiO_2 adsorbs DHB with a higher affinity than acidic amino acid residues but with a lower affinity than phosphate groups, hence DHB increases the specificity of TiO_2 in phosphopeptide enrichment.

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(8). This procedure greatly increased the selectivity of IMAC for phosphopeptides and enabled the detection of more than 216 phosphopeptides and 383 phosphorylation sites in a yeast lysate using online LC-MS/MS analysis with an ion trap mass spectrometer.

SCX: strong cation exchange chromatography

Titanium Dioxide (TiO_2) Enrichment

Although IMAC enrichment strategies have been successfully applied in a number of phosphoproteomic studies, they require special expertise, and protocols were not easily standardized among laboratories. Furthermore, there was a need for enrichment methods with even higher affinity and selectivity. Titanium dioxide (TiO_2) spheres have attracted attention as an alternative column packing material to the traditional silica-based supports (**Figure 2*b***). This is because of its high chemical stability, rigidity and unique amphoteric ion-exchange properties. Efficient separation of nucleotide bases as well as phosphopeptides by TiO_2 chromatography was first reported in 1997 (9). In that study, packed TiO_2 spherical porous particles (titansphere) served as an anion-exchange column in which phosphopeptides were bound in acidified solution and separated by a gradient of an alkaline borate buffer containing NaCl. These experiments demonstrated that organic phosphates are effectively adsorbed to TiO_2 in acidic conditions and desorbed in alkaline conditions, and thus revealed the potential of TiO_2 for selective enrichment of phosphopeptides. Heck and coworkers developed TiO_2 into a selective and robust technology for a typical large-scale proteomics setting. Phosphopeptides were enriched from tryptic peptide mixtures by TiO_2 particles packed in a precolumn and online coupled to LC-MS/MS (10). However, owing to the anion-exchange properties of TiO_2 at low pH, acidic peptides rich in glutamic and aspartic acid residues were also retained. To circumvent this problem, Larsen and coworkers introduced 2,5-dihydroxy benzoic acid (DHB) as a competitive binder in the buffers used in TiO_2 enrichment of phosphopeptides (11). An excess of DHB efficiently outcompetes other carboxylic acids from adsorbing to TiO_2 while phosphopeptides are nonetheless retained, which significantly improves the selectivity of TiO_2 for phosphopeptides. However, the amount of DHB needs to be optimized in LC MS/MS applications because too much DHB will bind excessively to the separation column and introduce instability and chemical noise.

Strong Cation Exchange Chromatography

Ion exchange chromatography is another powerful approach to enrich phosphorylated peptides. Phosphopeptide enrichment by strong cation exchange (SCX) is based on the difference in the solution charge states of phosphorylated and nonphosphorylated peptides. At pH 2.7, a typical tryptic peptide has a net charge of +2 because the N-terminal amino group and the C-terminal arginine or lysine side-chains are protonated. If the same peptide is phosphorylated, the negatively charged phosphate group reduces the charge state by one; thus, phosphopeptides can be enriched via their decreased net charge. Peptides with different solution charge states can easily be separated by SCX chromatography on preparative or analytical columns using a linear salt gradient. Gygi and coworkers observed that the +1 SCX fractions containing less than 3% of the total tryptic digests were highly enriched in phosphopeptides (12). When analyzing these early SCX fractions by nanoLC-MS/MS on an ion trap, they identified more than 2000 phosphopeptides from a preparation of HeLa cell nuclei. Note that multi-phosphorylated peptides will not be retained on the SCX column under these conditions owing to their net zero or negative charge; this necessitates separate analysis of the unbound (flow-through) fraction. In fact, because this flow-through fraction is very phosphopeptide rich, in our laboratory it is analyzed several times. SCX as a

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first separation/enrichment step, followed by IMAC or TiO_2, is a powerful and robust combination that was used as the analytical strategy in several large-scale projects (13–15).

HILIC: hydrophilic interaction chromatography

Hydrophilic Interaction Chromatography

Recently, hydrophilic interaction chromatography (HILIC) was shown to be a promising method for separation of peptides (16) and phosphopeptides (17) in large-scale proteomics. In a typical experiment, peptides are loaded onto the HILIC column in an organic solvent and eluted with a shallow gradient of an aqueous solvent. Under these conditions, peptide retention is based on hydrophilicity instead of hydrophobicity. This makes the method truly orthogonal to reversed-phase chromatography, which is the standard on-line separation method before MS analysis. Annan and coworkers recently demonstrated that the hydrophilicity of the phosphate group may be used to enrich phosphopeptides by HILIC. They were able to identify more than 1000 phosphorylation sites from 300 μg of HeLa cell lysate (17). This method appears to be well suited for analyses of small amounts of material (sub mg) and to have a higher resolution than SCX. Limitations may lie in decreased solubility of longer peptides (>20 residues) in organic phase and very strong interactions of multiply phosphorylated peptides, with the stationary phase leading to difficulties with elution.

Antibody-Based Enrichment

Immunopurification (IP) with immobilized antiphosphotyrosine antibodies is a well-established enrichment strategy in phosphoproteomics. Pandey and coworkers used a combination of antiphosphotyrosine IP and 1D SDS-PAGE to isolate proteins that were tyrosine phosphorylated after EGF treatment (18). Nine proteins were identified by MS/MS in this pioneering study, including two novel proteins involved in EGF signaling. However, this strategy relied on quantitation by relative staining intensity in stimulated versus control lanes. Because 1D gels have low resolution and antibody staining is not strictly quantitative, many genuine targets can be overlooked and little or no site-specific quantitative information is obtained.

The antibody-based antiphosphotyrosine enrichment strategy can also be applied to peptide digests, which allows for specific enrichment and mapping of individual phosphotyrosine-containing peptides and thereby directly pinpoints tyrosine phosphorylation sites. This approach was originally described by Vandekerckhove and coworkers in 1999 (19) who mapped c-Src-induced phosphorylation sites in human gelsolin. The method has since been significantly improved and used to map thousands of tyrosine phosphorylation sites in different cancer cell lines (20, 21).

Tyrosine-phosphorylated proteins can also be affinity enriched by recombinant, tagged SH2 domains. SH2 domains recognize phosphotyrosine in a specific sequence context, and individual domains have precise sequence requirements for high-affinity binding. Using the SH2 domain of the adaptor protein Grb2 and quantitative proteomics as discussed below, Blagoev and coworkers used this approach to specifically measure proteins recruited to the EGF receptor upon ligand binding (22).

PHOSPHOPEPTIDE ANALYSIS BY MASS SPECTROMETRY

Due to the extreme complexity of phosphopeptide mixtures that have to be analyzed by mass spectrometry, phosphopeptides are usually first separated on a nanoLC column, typically filled with a reversed-phase (C_{18}) material. The effluent from the column is directly electrosprayed

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and analyzed in high-resolution mass spectrometers, which measure the mass-to-charge (m/z) ratio and intensity in a survey scan (MS^1 spectrum). At the same time, the mass spectrometer also dissociates the peptides and detects the resulting fragment ions in a so-called tandem mass spectrum (MS/MS or MS^2 spectrum) (23, 24).

MS/MS or MS^2: tandem mass spectrometry

CAD or CID: collisionally activated/induced dissociation

Precursor Ion Scanning and Reporter Ions

Mass spectrometric characterization of phosphopeptides is not a trivial task. Phosphopeptides in complex protein digests often escape detection and identification by standard MS analysis because of their low abundance, low MS response, and inadequate fragmentation patterns. Thus, several specific MS acquisition strategies have been developed to selectively fragment and sequence phosphopeptides in complex mixtures. Previously, MS strategies were mainly built on the detection of phosphopeptides by their characteristic neutral loss or reporter ions produced in MS/MS (25). Originally, a nanoelectrospray triple quadrupole (QqQ) instrument was used to selectively detect phosphopeptides by their diagnostic fragment ion at *m/z* 79 (HPO_3^-) using precursor ion scanning in the negative ion mode (26, 27). HPO_3^- precursor ion scanning, in which the mass range is continuously and exclusively scanned for ions that produce an m/z = 79 fragment, is very sensitive (subfemtomole). A weakness of this method is that MS/MS spectra in negative ion mode are generally of poor quality, necessitating manual switching to positive mode for sequence determination of the peak that gave rise to the reporter ion. An automated LC/MS/MS approach has been developed in which a novel hybrid triple quadrupole linear ion trap performs precursor ion scanning of *m/z* 79 in negative ion mode and automatically switches to positive mode to record MS and MS/MS spectra of phosphopeptide candidates (28).

Phosphorylation of tyrosine (pY) is a relatively stable modification and mass spectrometric fragmentation yields fragments with the modification stably attached (**Figure 3*a*, *b***). Furthermore, the immonium ion resulting from cleavage at either side of pY can be detected by precursor ion scanning. The phosphotyrosine immonium ion (*m/z* 216.043) is mass deficient owing to the high content of oxygen and phosphorus. Thus, high-resolution instruments such as quadrupole time-of-flight (qTOF) or Fourier transform (FT) mass spectrometers can easily discriminate between the phosphotyrosine immonium ion and other peptide fragment ions of the same nominal mass (e.g., Asn-Thr b_2-ion; *m/z* 216.098) (29). This method has been applied to map phosphorylation sites in the EGF receptor and Bcr-Abl tyrosine kinases (30, 31), and was used in combination with phosphotyrosine immunoprecipitation to identify phosphorylation sites in signaling proteins activated by overexpression of the basic fibroblast growth factor (FGF) receptor (32).

Until recently, precursor ion scanning was a low-throughput approach not easily adapted to LC-MS/MS. In addition, owing to their low mass-to-charge ratio, reporter ions could not be measured in ion traps and related hybrid instruments, which have a low mass cut-off in fragmentation mode. Newly developed fragmentation methods such as pulsed-Q dissociation (PQD) (33) and higher-energy C-trap dissociation (HCD) (34) have largely circumvented this limitation (**Figure 3*b***).

Neutral Loss–Dependent MS^n

A challenge in phosphopeptide analysis by MS is the relative lack of sequence-specific information in the MS^2 spectra of modified peptides compared with nonmodified peptides. The standard fragmentation technique in proteomics, collisionally activated/induced dissociation (CAD/CID), increases the internal energy of ions relatively slowly (on a millisecond timescale) to induce

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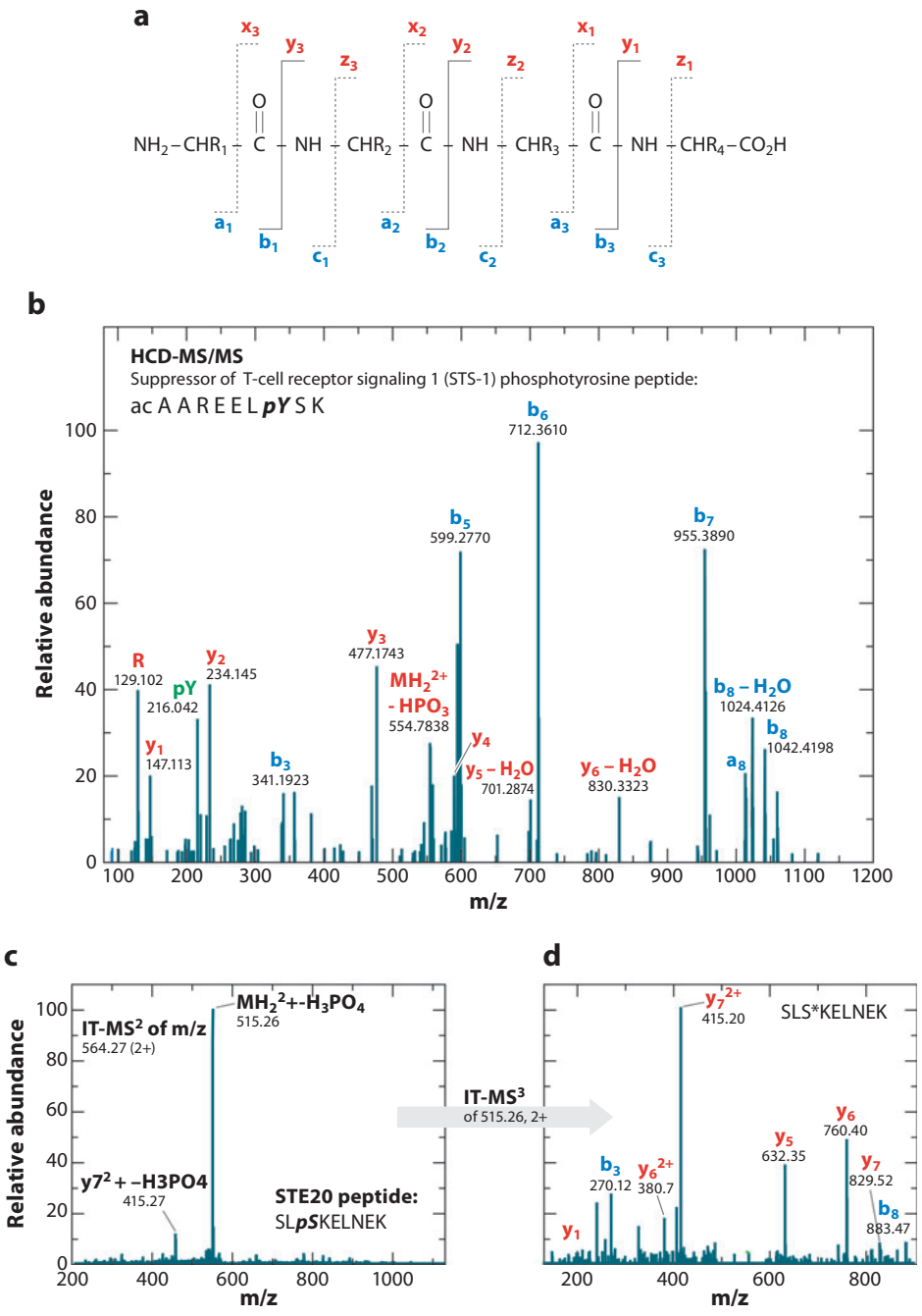
Figure 3

Collisionally induced fragmentation of phosphopeptides. (*a*) Nomenclature of fragments produced upon peptide fragmentation (23, 87). (*b*) Higher-energy C-trap fragmentation (HCD) on an LTQ-Orbitrap mass spectrometer shows a typical fragmentation pattern of a pY-containing phosphopeptide. The reporter ion at m/z 216.043 can be used for detection of tyrosine phosphorylation in high-accuracy mass spectrometers. (*c*) A typical feature of pS- or pT-containing phosphopeptides is a neutral loss of phosphoric acid from the precursor ion and poor abundance of backbone fragment ions, which are needed for peptide identification. (*d*) In the neutral loss-dependent MS^3 measurement, abundant ion species resulting from a loss of phosphoric acid in MS/MS (**Figure 3*c***) are selected and further fragmented to produce rich peptide backbone cleavage.



fragmentation. Consequently, fragmentation occurs via the lowest-energy dissociation pathway. The O-phosphate bond in serine- and threonine-phosphorylated peptides is particularly labile during CID, which often results in a prominent neutral loss of phosphoric acid from the precursor (phosphopeptide) ion. This is a major challenge in phosphopeptide characterization by MS because tandem mass spectra may not contain sufficient sequence information to identify the peptide

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sequence in a database nor to unambiguously position the phosphorylation site. These problems can be solved by introducing consecutive stages of fragmentation. In ion traps, the neutral loss precursor ion can be further isolated, fragmented, and analyzed, a process termed MS/MS/MS (MS^3). This strategy was first used in a large-scale phosphoproteomic experiment (12) using a conventional, low resolution 3D-ion trap (LCQ, Thermo Fisher Scientific) with software-controlled neutral loss–dependent MS^3 capabilities. We have extended this approach to phosphopeptide analysis on a high-resolution, hybrid linear ion trap–Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT, Thermo Fisher Scientific). The LTQ-FT or LTQ-Orbitrap is comprised of a low-resolution linear ion trap (LTQ) in which the two stages of fragmentation can be performed while the mass and intensity of the phosphopeptide is measured with high resolution in the FT part of the instrument (35) (**Figure 3*c***).

ECD: electron capture dissociation

ETD: electron transfer dissociation

In the neutral loss–dependent MS^3 mode of operation, the diagnostic neutral loss of phosphoric acid (−98 Da) from the precursor ion in an MS/MS scan automatically triggers MS^3 fragmentation of the neutral loss precursor ion (13). Recently, this strategy has been streamlined in linear quadrupole ion trap mass spectrometers in the form of multistage activation (MSA) (36). In MSA, ion species resulting from neutral losses upon CID are consecutively dissociated without intervening scans. (In the case of phosphopeptides, these neutral loss species are at −97.97, −48.99, or −32.66 mass-to-charge units (Th) relative to the singly, doubly, or triply charged phosphorylated precursor ion, respectively.) This results in a single pseudo MS^n containing MS^2 and MS^3 information and significantly reduces analysis time. Consequently, multistage activation is routinely used in the analysis of protein phosphorylation and other labile PTMs on LTQ and LTQ-containing hybrid mass spectrometers (LTQ-FT, LTQ-Orbitrap).

Alternative Fragmentation Methods: Electron Capture Dissociation (ECD) and Electron Transfer Dissociation (ETD)

Peptides can also be sequenced with fragmentation principles different from the slow energy deposition characteristic of CID. Peptide sequencing by electron capture dissociation (ECD) (37) or the related electron transfer dissociation (ETD) (38) relies on the energy deposited in a charge neutralization reaction and is little affected by the different amide bond dissociation energies. This generally yields more indiscriminate and more extensive backbone cleavages, especially for large, highly charged peptides and those containing particularly labile bonds. The energy deposited in a peptide ion upon electron capture is not distributed along the molecule prior to dissociation as it is in CID; as a result, the ion fragments without shedding the labile phosphate groups first. In ECD, which is typically performed in the superconducting magnet of an FT-ICR instrument, the gas-phase multiply protonated polypeptides (MH_n^{n+}) react with a beam of thermal (<0.2 eV) electrons. In ETD, which is performed in an ion trap mass analyzer, the electron donors are anions produced in a separate chemical ionization source. The exothermic electron capture (ECD) or transfer (ETD) reaction leads to the formation of reduced radical ions, $MH_n^{(n-1)+\cdot}$, which are rapidly cleaved at the peptide backbone NH-C_α bond to produce series of c- and $z^\cdot$ (z dot)-type sequence ions, which are analogous to the b and y ions in CID. Both ECD and ETD were shown to fragment phosphopeptides with preservation of the phosphate moiety on all fragment ions (38–40). Because of its availability on cheaper and more sensitive ion trap mass spectrometers, ETD has a great potential in global analyses of phosphorylation, especially for multiply charged (3+ and higher) and multiply phosphorylated peptides (**Figure 4*a*,*b***). An optimal strategy is to combine CID for doubly charged peptides with ETD for more highly charged peptides (41).

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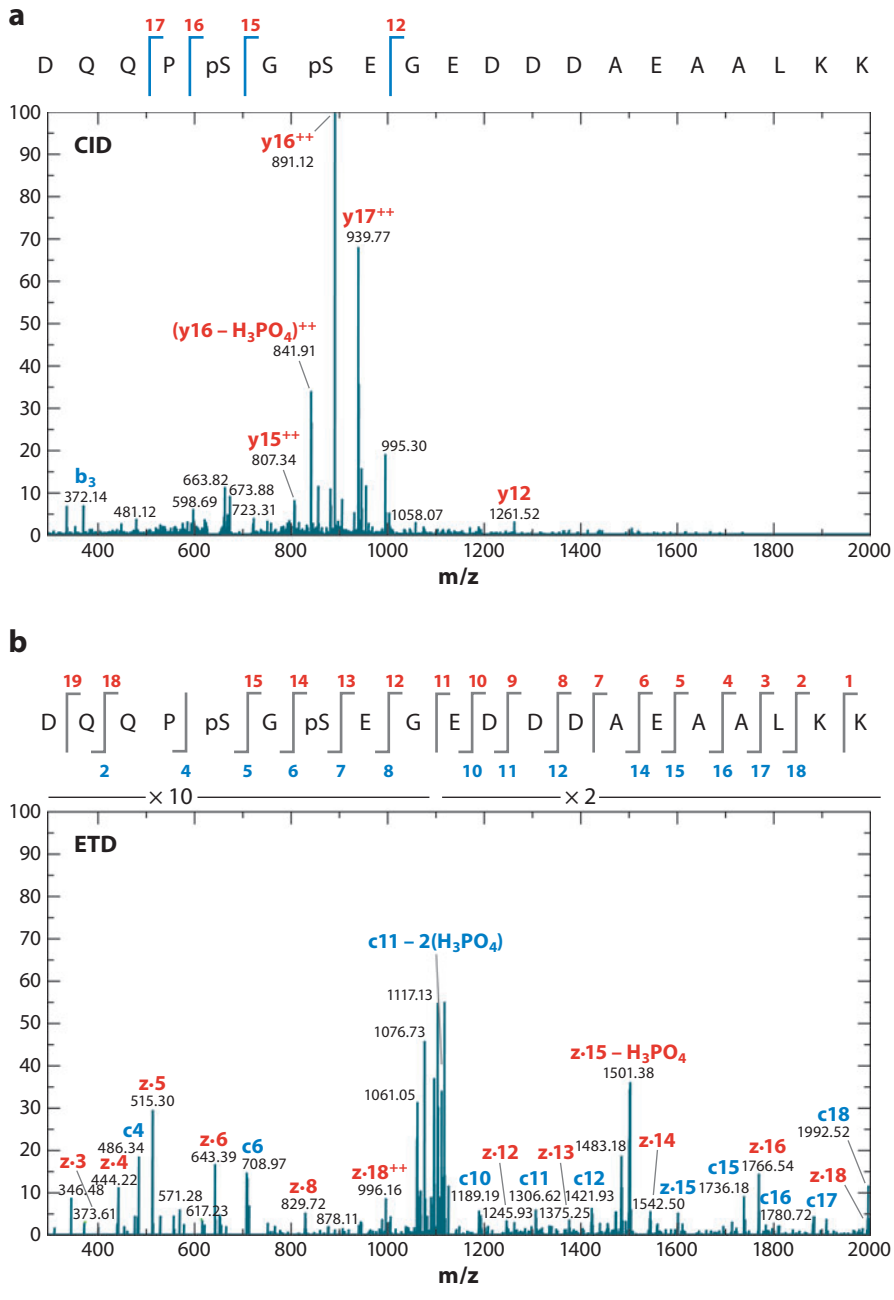
Phosphorylation Site Assignment by MS

Although phosphopeptides can be effectively fragmented and thereby identified by MS/MS and MS^3, it is often difficult to pinpoint the position of the phosphorylation site with single–amino acid resolution, especially for multiply phosphorylated peptides. For example, in a peptide with consecutive serines, the fragments between each of them have to be identified in the MS/MS



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spectrum to unambiguously place the phosphorylation on the correct one. Therefore, sequence information in MS fragment spectra may not be sufficient to automatically assign the phosphorylation site by standard database search engines, and manual inspection of each spectrum may become necessary. However, in large-scale phosphoproteomic experiments involving hundreds or thousands of sites, this is not feasible.

To address this issue, we have separated the peptide identification problem from the phosphorylation site–localization problem. The spectrum of an identified peptide is compared with theoretical spectra in which the phosphate group is placed at each possible sequence position. The probability scores for each position (35) are normalized, and phosphosites are classified according to this localization score (14). This algorithm is incorporated into the open source program MSQuant (**http://msquant.sourceforge.net**) as well as into the MaxQuant software suite (**www.maxquant.org**). A similar approach, also based on the same probability score as in Reference 35, has been taken in Reference 42 and termed A-score.

The localization score provides a statistical means for assigning individual phosphorylation sites within the identified peptide sequences, and can also be applied to the analysis of other PTMs. Note that even with an ambiguous localization score, the phosphosite is still unambiguously confined to the sequence of the identified peptide.

Phosphopeptide Databases and Prediction Tools

Until a few years ago, known phosphorylation sites came exclusively from targeted biological investigations. This has completely changed, and many proteomics laboratories now generate data for hundreds or thousands of sites. To make these sites useful for the community, specialized databases have been developed.

Three prominent databases are Phospho.ELM (**http://phospho.elm.eu.org**), PhosphoSite (**www.phosphosite.org**), and Phosida (**www.phosida.com**). Each captures large-scale phosphoproteomics data, and also incorporates the sites contained in universal databases such as UniProt (**www.uniprot.org**). Most of these databases, as well as UniProt itself, in principle accept all published data. However, the amount of phosphoproteomic data that will soon be produced threatens to overwhelm phosphosite databases. We suggest that databases should contain only specifically produced data of exceptionally high quality. Otherwise, even a small proportion of false positive identifications in each submitted dataset and the inability to distinguish high-quality from low-quality data will soon make data repositories ineffective.

ScanSite (**http://scansite.mit.edu**) and NetPhos (**www.cbs.dtu.dk/services/NetPhos**) are phospho-prediction tools based on matrix motifs and neural networks, respectively. The unprecedented amount of in vivo phosphorylation data from MS-based proteomics presents new

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Figure 4

Comparison of collisionally induced dissociation (CID) and electron transfer dissociation (ETD) fragmentation of a doubly phosphorylated, triply charged peptide. (*a*) CID fragmentation leads to dissociation of the most labile bonds, notably the O-phosphate bond (loss of H_3PO_4) and the peptide bond on the C-terminal side of proline (y16). Although relatively few fragment ions were produced, this was enough for a reliable identification of the peptide in the database search and for localization of phosphorylation sites. (*b*) ETD fragmentation occurs faster than CID and leads to a more complete backbone cleavage, especially in multiply charged peptides. Neutral losses of phosphoric acid, visible in this spectrum, occurred because of additional activation of ions performed in the linear ion trap mass spectrometer in order to increase signal intensity. Note that ETD cannot be used for analysis of singly charged ions, and that CID is the method of choice for dissociation of doubly charged ions.

opportunities for in silico prediction of phosphorylation sites and their corresponding kinases. For example, Phosida contains a support vector machine (a machine learning technique), which is trained on more than 6000 in vivo phosphorylation sites (43).

iTRAQ^{TM}: tandem mass tags

SILAC: stable isotope labeling by amino acids in cell culture

Phosphoproteomics alone does not directly address kinase-substrate relationships. In a recent study, Pawson and coworkers have shown that these can be inferred by bioinformatic mining of linear sequence motifs as well as by combination with other large-scale data (44).

MS-based phosphoproteomics is not the only technology employed in high-throughput discovery of new phosphoproteins and sites. In recent years, several large-scale protein- and antibody-array phosphoproteomic studies have been reported (45, 46). These studies have provided a wealth of information about kinases and their substrates, and their findings can be validated by the in vivo data provided by MS-based phosphoproteomics using bioinformatics tools.

Quantitative Proteomics Using Stable Isotope Labeling

Although MS has proven to be an extraordinary tool for protein characterization, measurement of peptide intensities alone does not immediately provide quantitative information. Peptide ion responses (signals) in the mass spectrometer are extremely variable from peptide to peptide because the ionization efficiency is highly dependent on their chemical structure. There are several approaches to overcome this problem (47). For highest accuracy, stable isotope atoms such as deuterium (^{2}H), carbon-13 (^{13}C), nitrogen-15 (^{15}N), or oxygen-18 (^{18}O) can be incorporated into peptides derived from one of the proteomes to be analyzed by MS. The chemical and physical characteristics and properties of the heavy stable isotope–labeled peptides remain the same as the corresponding light versions, except for the difference in the mass introduced by the isotope label, which makes the two forms distinguishable in the mass spectrometer. After mixing, peptides from two cell states are analyzed simultaneously and appear as pairs (doublets) in the mass spectra. The relative intensities of the doublet directly reflect the relative quantity of the peptide in the two proteomes to be compared.

In the past decade, many techniques based on stable isotope labeling for protein quantitation by MS have been developed, but only a few have found more than sporadic application. These include isotope-coded affinity tags (ICAT) (48), metabolic labeling by ^{15}N-incorporation (49), stable isotope labeling by amino acids in culture (SILAC) (50), enzymatic ^{18}O-labeling (51) and the recently introduced chemical labeling by tandem mass tags (known as iTRAQ^{TM}) (52). SILAC and iTRAQ are currently the most frequently used techniques in quantitative MS-based phosphoproteomics.

Metabolic Labeling

Unlike other areas of biology, metabolic labeling in MS requires stable isotopes (no radioactivity) and complete labeling. There are several different ways to introduce the label during cell growth. In one approach, mainly used for microorganisms, all instances of the isotope used for labeling are replaced with their heavy counterparts, typically ^{15}N for ^{14}N or ^{13}C for ^{12}C. This methodology requires well-defined cell culture systems, in which the cells of interest are capable of synthesizing or obtaining all the necessary amino acids from the isotope-labeled compound. Langen introduced this method and applied it to *Escherichia coli* (*E. coli*) bacteria and yeast cells (53), and Chait et al. quantified phosphopeptides in yeast using ^{15}N labeling (49). Heck and coworkers reported a two-step approach to ^{15}N-encode higher-multicellular organisms. They labeled *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster* (*D. melanogaster*) by growing them on completely ^{15}N-labeled *E. coli* or yeast cells (54). Even small mammals have been labeled with the ^{15}N method (55).

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In SILAC, proteins from two cell populations to be compared are isotope encoded with an essential amino acid such as $^{13}C_6$-Arg. This makes them distinguishable by MS from proteins of another cell population cultured in media containing normal $^{12}C_6$-Arg (56). SILAC allows lysates from the two SILAC-encoded populations to be combined before any protein separation, minimizing quantitation errors that are otherwise introduced in parallel purification procedures. SILAC results in easily interpretable isotope patterns, and the number of labeled amino acids (usually arginines and lysine when trypsin is the proteolytic enzyme) can easily be deduced. SILAC is particularly well suited to study PTMs such as phosphorylation changes (13, 57). So far, SILAC has been limited to mainly cell culture systems, but it has recently been extended to in vivo experiments in mice (58).

Chemical Labeling

Chemical methods for quantitative proteomics are based on reacting isotope encoded forms of a mass tag with cysteines, amino groups, or carboxylic groups (59). The reaction can occur on the protein level, but is more typically applied to the peptides resulting from proteolytic digestion. iTRAQ is a recently developed protein quantitation method that utilizes isobaric amine-specific tandem mass tags and quantitation in the MS/MS rather than the MS spectra (52). Each tag consists of a reporter and balance group, which is prone to fragmentation. In MS spectra, the differentially labeled versions of a peptide combine in a single signal (peak) and are indistinguishable. However, upon fragmentation, in the MS/MS spectra each tag generates a unique reporter ion (immonium-like ion). Protein quantitation is then achieved by comparing the intensities of the reporter ions in the MS/MS spectra. Mass tags enabling comparison of four or eight different conditions to be analyzed in one experiment are commercially available. Although not as accurate as SILAC, chemical labeling can be directly applied to tissue and patient samples.

QUANTITATIVE PHOSPHOPROTEOMICS TO DELINEATE SIGNALING PATHWAYS

Protein phosphorylation is dynamically regulated in almost all known cell signaling pathways and has a profound influence on propagation of the signal inside the cell. Therefore, global and site-specific phosphoproteomics is opening new perspectives in studies of complex biological signaling networks. Here we discuss recent applications of quantitative phosphoproteomics in several key signal transduction pathways.

Yeast Pheromone Response: A Prototypical Signaling Pathway

Classical biochemical analysis of haploid yeast cells and their response to peptide mating pheromones has produced important advances in the understanding of G protein and MAP kinase signaling mechanisms (60). The yeast alpha-factor pheromone response pathway is one of the most well studied cell signaling pathways, and we use it here to illustrate the potential of quantitative MS-based phosphoproteomic technology (13).

A double auxotroph yeast strain was SILAC encoded, with $^{13}C_6$-lysine and $^{13}C_6$-arginine serving as a control, and mixed with light labeled yeast cells, which were treated with alpha factor pheromone for two hours. Whole yeast cell protein extracts were converted to peptides by trypsin digestion, and phosphorylated peptides were enriched by a combination of SCX chromatography and IMAC. Phosphopeptides were sequenced and quantified using online nanoLC and two consecutive stages of fragmentation on a high resolution hybrid instrument (LTQ-FT MS). We

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identified more than 700 unique phosphorylation sites from approximately 500 different yeast proteins, many of them known to be associated with pheromone signaling. However, only a small subset of the phosphorylation sites had been described previously in the literature. Importantly, the quantitative information inherent in the SILAC experiment immediately allowed for the distinguishing of sites involved in general functions (unchanging sites) from those specifically involved in the pheromone response (changing sites with a SILAC ratio different from one to one). The latter included many novel regulatory phosphorylation sites on proteins already known to be involved in the pheromone signaling pathway, including the pheromone receptor (STE2), components of the MAP kinase pathway, transcription factors, proteins involved in cell polarization, and proteins that participate in the assembly of mating projections.

This unbiased phosphoproteomics study identified many pheromone-regulated phosphorylation sites on RNA-processing and RNA-transport proteins, which suggests that the pheromone pathway has a previously unappreciated role in regulating mRNA metabolism (61). This study demonstrates the power of a quantitative phosphoproteomic approach to add specific knowledge to known cellular signaling mechanisms, as well as to make connections to unsuspected areas of cell biology. A subsequent study using similar technology has found additional phosphorylation sites in the yeast pheromone pathway (62).

Receptor Tyrosine Kinase Signaling

The human genome encodes more than 500 kinases, of which 91 are tyrosine kinases (63). These are further divided into the plasma membrane–spanning receptor-protein tyrosine kinases (RTKs) and the intracellular nonreceptor tyrosine kinases (NRTKs). RTKs are receptors for secreted polypeptide growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and nerve growth factor (NGF). Peptide growth factor binding induces dimerization of the receptors, which leads to transactivation in which one receptor tyrosine kinase phosphorylates the other. This creates binding sites for other signaling molecules, and thereby recruits and initiates a downstream signaling cascade. We have applied SILAC-based phosphoproteomics to study proteins phosphorylated in response to FGF in a human 293T cell line transfected with the FGF receptor 1(FGFR1) (57). Phosphoproteins were isolated by immunoprecipitation with the 4G10 antibody to phosphotyrosine. Of more than 800 proteins nonspecifically copurifying in the immunoprecipitates, 28 showed response to basic FGF (FGF2). A number of these were proteins not previously implicated in FGF signaling, including IRS4, GIT1, and GIT2 (GTPase-activating proteins for the Arf small GTPases), SHIP2 (SH2 domain–containing inositol phosphatase), annexins VII and XI, liver-specific bHLH-Zip transcription factor (LISCH7), and WDR6, a protein with WD40 repeats that functions in protein interactions. Additionally, we identified 17 FGF-regulated tyrosine phosphorylation sites on eight proteins: FGFR1, PLC gamma, FAK, IRS4, Paxillin, p130CAS, LISCH7, and Fyn.

Insulin receptor substrate 4 (IRS4) was identified as a novel component of FGF signaling, and we established interactions of IRS4 with other components of the signaling cascade. The adaptor protein Grb2, PLC gamma, and PI3-kinase were found to be direct binders of the novel tyrosine phosphorylation site (pY915) in IRS4 that we had identified. In these interaction studies, SILAC was used to distinguish specifically between binders to the phosphorylated IRS4 peptide sequence and the nonphosphorylated species (64, 65).

SILAC encoding of three cell states and multiplexed experiments allow reconstruction of the kinetics of signaling pathways. This was demonstrated by antibody-based enrichment of tyrosine-phosphorylated proteins after different times of EGF stimulation (66, 67). Kinetics for more than

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100 effector proteins were obtained simultaneously, in principle allowing time ordering of this growth factor pathway. The insulin signaling pathway has been similarly investigated by SILAC-based phosphoproteomics, yielding a number of novel components in this well-studied pathway (68).

White and coworkers have employed phosphoproteomics (using the iTRAQ method) to quantitate hundreds of tyrosine phosphorylation sites after EGF and insulin treatment in a time-resolved manner (69, 70). In an innovative systems biology approach, they recorded these profiles for a variety of cellular perturbations and connected them to different cellular outcomes. In this way, the relevance of specific phosphorylation sites for cellular decision making can in principle be uncovered. Kratchmarova et al. used phosphoproteomics to determine the cellular control point for differentiation of adult stem cells into bone cells (66, 67). Both PDGF and EGF were found to elicit substantial tyrosine phosphorylation, but only EGF stimulated differentiation. Unbiased, quantitative phosphoproteomics pinpointed the PI3K pathway as differentially regulated between EGF and PDGF. Indeed, chemical inhibition of PI3K converted the PDGF signal into a bone-forming signal, exactly like EGF.

To study the temporal changes of regulated tyrosine, serine, and threonine phosphorylation sites in HeLa cells induced by EGF, we collected SILAC-encoded cells at multiple time points of EGF treatment, followed by enrichment of phosphorylated peptides by SCX and TiO_2, and finally by subsequent identification and quantitation via online nanoLC-MS.

We chose EGF stimulation for 0, 1, 5, 10, and 20 minutes. From this study, activation profiles for 6600 unique phosphorylation sites on 2244 proteins were determined (14). Regulated tyrosine, serine, and threonine phosphorylation sites were identified from many known as well as unknown members of the EGF receptor signaling pathway, including the EGF receptor and downstream signaling molecules such as Shc, STAT5, Ymer, ERK1, and ERK2. More than one thousand protein phosphorylation sites responded more than twofold to EGF stimulation. The growth factor signal quickly spread to virtually all parts and processes of the cell, and the experiment documented EGF-induced phosphorylation of more than forty transcriptional regulators within 20 minutes (**Figure 5**). Similar quantitative studies of the phosphoproteome have since been undertaken by other laboratories and applied to important biological questions such as the ATR/ATM-dependent DNA damage response (71).

Kinome Phosphorylation Analysis and Identification of Novel Kinase Inhibitor Targets

Quantitative proteomics and phosphoproteomics have also proven to be powerful tools for characterizing the mechanisms of kinase inhibitors, an important class of drug targets. Two groups have pioneered the use of immobilized kinase inhibitors with broad specificity to enrich a substantial subset of protein kinases from total cell lysates followed by quantitative mass spectrometry. Daub et al. developed a kinase inhibitor pulldown technique in combination with phosphoproteomics to map and quantify more than one thousand phosphorylation sites on human protein kinases arrested in S- and M-phase of the cell division cycle (72). Researchers at Cellzome employed Kinobeads™ to enrich protein kinases and then performed competition-based assays using specific kinase inhibitor drugs such as imatinib (Gleevec), dasatinib (Sprycel) and bosutinib in bcr-abl positive K562 cells (73). Specific kinase inhibitor targets and their respective K_d values were determined by mass spectrometric quantitation of all binders at different concentrations of the individual drugs with four iTRAQ labels (**Figure 6**). Known targets of analyzed drugs, including ABL and SRC family kinases, were confirmed and novel targets of imatinib, such as the receptor tyrosine kinase DDR1 and the oxidoreductase NQO2, were identified.

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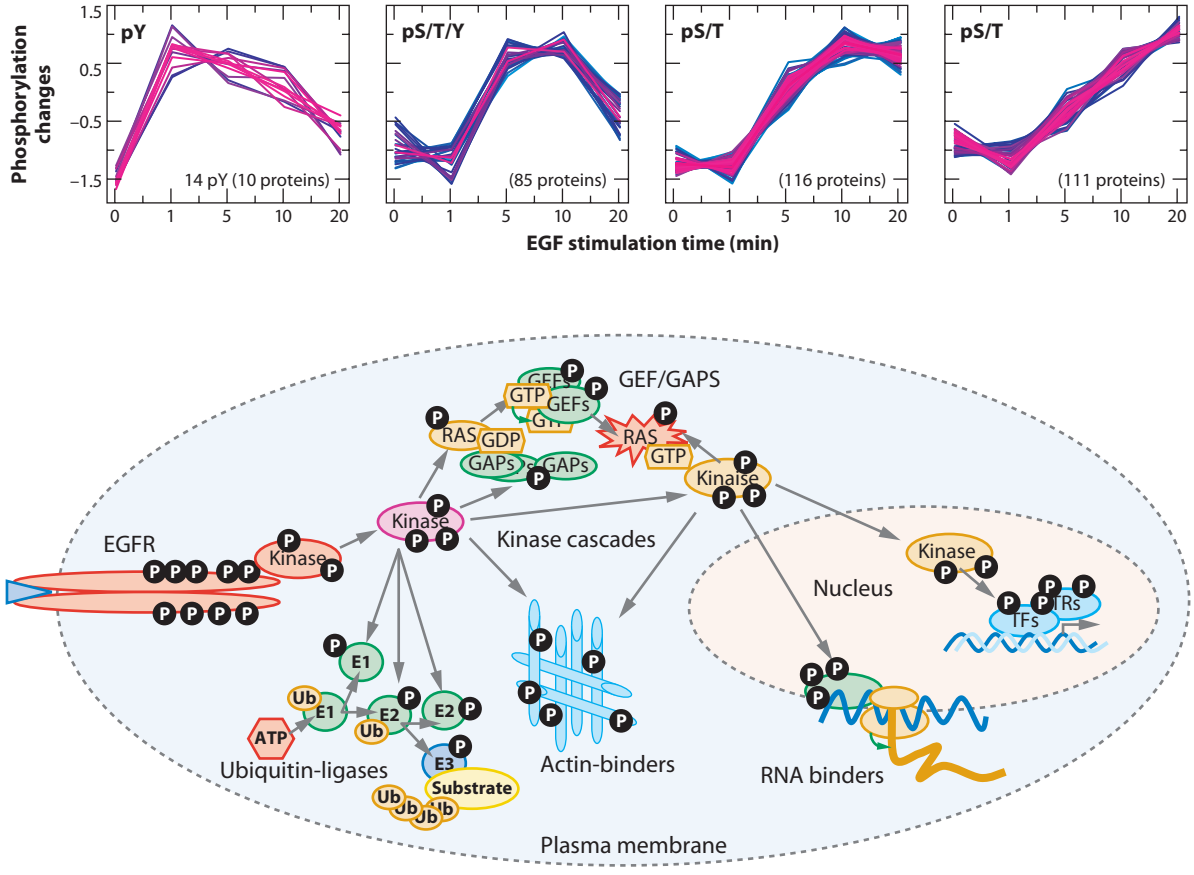

Figure 5

Quantitative phosphoproteomics applied to temporal analysis of EGF signaling. Different combinations of stable isotope-labeled amino acids (SILACs) enabled analysis of phosphoproteome changes in HeLa cells after 1, 5, 10, 15, and 20 minutes of treatment with EGF. In total, dynamics of more than 6600 phosphorylation sites from more than 2200 phosphoproteins were measured, and progression of the signal from the EGF receptor to virtually all major compartments and processes of the cell was detected. Clustering of activation profiles revealed different global dynamics of pY and pS/T phosphorylation (inserts).

In another application of chemical biology in phosphoproteomics, Pan et al. have recently determined the effect of phosphatase inhibitors on the phosphoproteome. This study showed that tyrosine phosphatase inhibitors are indeed very general as shown by the boosting of the majority of tyrosine phosphorylation sites in a quantitative proteomics experiment. Surprisingly, commonly used Ser/Thr phosphatase inhibitors turned out to be much less broad. They changed the level in only less than half of all detected phosphorylation sites (74).

New Insights in Bacterial Ser/Thr/Tyr Phosphorylation

Phosphorylation on serine, threonine, and tyrosine (Ser/Thr/Tyr) has long been considered exclusive to eukaryotes, especially metazoans, and either not present or not functionally significant in bacteria. Instead, the two-component signaling system involving histidine and aspartate phosphorylation is the paradigm of bacterial signal transduction (75).

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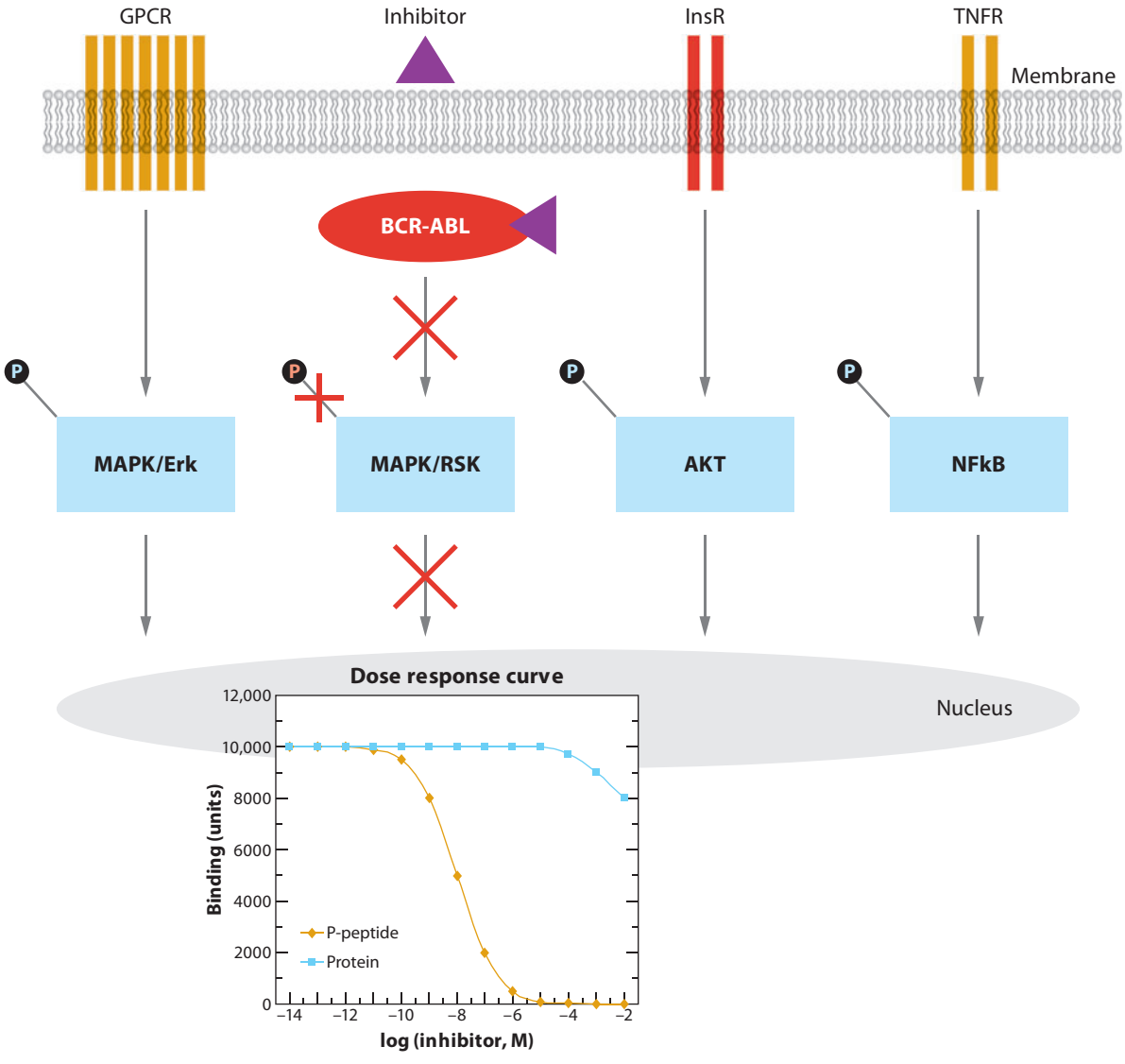

Figure 6

Quantitative phosphoproteomics in discovery of novel kinase inhibitor targets. Immobilized kinase inhibitors were used to capure their specific targets. The binding constants were determined by tandem mass tags (iTRAQ^{TM})-based MS quantitation of bound proteins at different inhibitor concentrations. Global and quantitative phosphorylation analysis of bcr-abl positive K562 cells upon treatment with kinase inhibitors imatinib, dasatinib, and bosutinib, revealed decreased phosphorylation on their known and novel downstream targets (73).

Over the past 20 years, evidence has accumulated that Ser/Thr/Tyr phosphorylation is indeed present in bacteria, and that it can have important regulatory roles (76). Signaling via Ser/Thr/Tyr is implicated in the regulation of bacterial virulence (77), and can interfere with eukaryotic signal transduction, rendering the host more prone to infection (78). Bacteria possess protein kinases and phosphatases similar to those in eukaryotes (79), but also some with no known homologs in other phyla (80). A recent metagenomic study revealed that microbial eukaryotic-like protein kinases

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outnumber histidine kinases, traditionally considered typical microbial kinases (81). Despite the fact that even early 2D SDS-PAGE studies indicated the presence of hundreds of Ser/Thr/Tyr phosphorylated proteins in bacteria, the number of identified kinase substrates and especially phosphorylation sites remained very small, and almost no in vivo sites had been reported.

We recently applied the qualitative global peptide-based phosphoproteomics workflow described above to study Ser/Thr/Tyr protein phosphorylation in the model bacteria *Bacillus subtilis* (82), *Escherichia coli* (83), and *Lactococcus lactis* (84). This approach allowed us to analyze the bacterial phosphoproteome at the phosphorylation site level and to detect approximately 100 phosphorylation events in each analyzed bacterium.

The number of phosphoproteins and phosphorylation sites detected in bacteria is much lower than in eukaryotes, where there is evidence for more than 10,000 phosphosites. However, essential proteins and enzymes involved in carbon metabolism and sugar transport were found to be significantly overrepresented among detected phosphoproteins, supporting the emerging concept of Ser/Thr/Tyr phosphorylation as an important regulatory mechanism in the bacterial cell. Almost all glycolytic and tricarboxylic acid (TCA) cycle enzymes were found to be phosphorylated, and regulation of some of these enzymes by phosphorylation is already known (85). Interestingly, bacterial phosphoproteins and phosphorylated residues are significantly more conserved than their nonphosphorylated counterparts. A number of potential phosphorylation sites are conserved from *Archaea* to humans, pointing to the likely presence of this regulatory modification since the earliest stages of cellular life.

Given the rapid increase of antibiotic resistance among pathogenic species, there is an urgent need for identification of alternative regulatory pathways in microorganisms. However, development of new antibiotics is limited by robust bacterial metabolism networks (86). It will be interesting to investigate Ser/Thr/Tyr phosphorylation as a potential avenue to disrupt bacterial growth.

CONCLUSIONS AND PERSPECTIVES

Proteomics in general and phosphoproteomics in particular are becoming the methods of choice for discovery-driven protein analysis at a systems level. As we have shown here, phosphoproteomics is now able to map signaling pathways in considerable depth, to delineate cellular control points, and via its unbiased nature, to deliver unexpected biological connections.

However, future progress in MS-based proteomics and phosphoproteomics necessitates further instrumental developments, as significant improvements are needed before we can comprehensively and routinely analyze whole proteomes or phosphoproteomes. Peptide identification capacity in LC-MS/MS analysis is limited by the scan rate, sensitivity, and dynamic range of the mass spectrometer used, as well as by the chromatographic performance of the LC system such as its peak capacity and separation efficiency. If nearly all peptides elute within a 90-minute time frame, and if the mass spectrometer can perform up to four MS/MS events per second, the identification capacity is maximally 21,600 peptides (90 min $\times$ 60 s $\times$ 4). However, if a mammalian cell expresses roughly 10,000 proteins at any given time, and these have an average molecular weight of 40 kDa, it will give rise to more than 100,000 tryptic peptides within the m/z range detectable by LC-MS (without considering missed cleavages, peptide modifications and redundant sequencing). This clearly suggests the need for faster instruments and better MS software to ensure optimal peptide selection for MS/MS. Furthermore, the dynamic range of phosphoproteomics should be increased in order to determine substoichiometric phosphorylation sites on low-abundance proteins in the presence of high-abundance phosphopeptides. Increasing sensitivity will be helpful in enabling direct analysis of patient material such as tumor biopsies.

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For the biologist dealing with phosphoproteomics data reported in the literature, it is very important to critically examine the evidence because large-scale MS-based experiments inherently produce false-positive identifications, individual researchers have MS equipment of different sophistication and, more importantly, different criteria for accepting protein identifications.

Leading journals in the field (e.g., *Molecular and Cellular Proteomics*) are establishing criteria that have to be fulfilled for proteomic data to be accepted. Stringent publication standards will hopefully ensure that only high-quality proteomic data sets will be published in the future.

In the past few years, MS-based proteomics has evolved from producing mere lists of protein identifications to producing highly sophisticated strategies indispensable in the analysis of complex biological systems. In this regard, quantitative methods like SILAC have significantly improved the value of proteomic studies, in particular in phosphoproteomics. Considering the importance of reversible phosphorylation as a signal transduction mechanism, phosphoproteomics is likely to play a major and indispensable part in the future study of signal transduction pathways.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We thank members of the Department of Proteomics and Signal Transduction at Max Planck Institute for Biochemistry for helpful discussions and comments on the manuscript. We also thank Dr. Bernhard Kuster for kindly providing **Figure 6**. Work on phosphoproteomics in the Department of Proteomics and Signal Transduction is supported by an EU research directorate sixth framework grant (Interaction Proteome Grant LSHG-CT-2003-505520).

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