Mapping protein post-translational modifications with mass spectrometry

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Post-translational modifications of proteins control many biological processes, and examining their diversity is critical for understanding mechanisms of cell regulation. Mass spectrometry is a fundamental tool for detecting and mapping covalent modifications and quantifying their changes. Modern approaches have made large-scale experiments possible, screening complex mixtures of proteins for alterations in chemical modifications. By profiling protein chemistries, biologists can gain deeper insight into biological control. The aim of this review is introduce biologists to current strategies in mass spectrometry–based proteomics that are used to characterize protein post-translational modifications, noting strengths and shortcomings of various approaches.

As many as 300 post-translational modifications of proteins are known to occur physiologically. Mass spectrometry (MS) is a central technology in the protein chemist's toolkit, enabling site mapping and quantification of chemical modifications on proteins, as well as detection of new types of structures. Key to analyzing post-translational modifications (PTMs) by MS is an understanding of their solution and gas-phase reactivities, given that the range in chemical behavior of amino acids and functional groups causes significant differences among peptides with variable composition.

In this review we discuss strategies and logic used in examining protein post-translational modifications by mass spectrometry, highlighting aspects of interest to biologists. We describe MS strategies used to detect common PTMs, map modified residues and estimate stoichiometries, with particular focus on protein phosphorylation as a well developed example. We also discuss selected other PTMs including acetylation, ubiquitination and cysteine oxidation, each key regulators of cell signaling. We highlight recent achievements involving large-scale studies characterizing PTMs in complex protein mixtures, as well as PTM database development, a rapidly progressing area. For an introduction to fundamental principles of mass spectrometry–based proteomics, we refer the reader to excellent reviews and web resources summarizing MS instruments, data collection and data reduction[1–4].

PHOSPHORYLATION

MS detection

Since its first characterization on glycogen phosphorylase in 1955, protein phosphorylation has been recognized as a central mechanism for cell regulation and signaling. It is estimated that one-third of eukaryotic proteins are phosphorylated, a result of carefully regulated protein kinase and phosphatase activities[5]. Protein phosphorylation events are detected by increases in amino-acid residue mass of +80 Da, which report the addition of HPO_3. Sites of phosphorylation can be identified from mass shifts in fragment ions generated by gas-phase fragmentation (MS/MS) of phosphopeptides (**Fig. 1**).

PTM ion signatures can be monitored using MS or MS/MS scanning methods tailored to specific gas-phase reactivities. For example, peptides containing phosphotyrosine can often be detected by a characteristic fragment ion of 216 Da, formed by peptide bond cleavages on both sides of the phosphotyrosine residue[6]. In addition, peptides containing phosphorylated serine and threonine often undergo cleavage of the phosphoester bond and loss of H_3PO_4 as a neutral species ('neutral loss'), yielding a product with mass lowered by 98 Da (–18 Da from the unphosphorylated species). Neutral loss often reduces additional peptide fragmentation but increases the difficulty of matching peptide sequences to the MS/MS spectrum. An 'MS^3' scanning method can be used in this

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situation, wherein the neutral loss product ion is isolated for an extra fragmentation step. This generates an MS/MS/MS spectrum where the phosphorylated serine or threonine residue is replaced by a dehydrated form (–18 Da)[7]. A related 'multistage activation' strategy fragments the neutral loss product and the parent ion simultaneously, generating a hybrid spectrum combining both MS/MS and MS/MS/MS fragmentation products[8].

Typically, MS/MS is performed using low-energy collisionally activated dissociation (CAD) in positive ion mode, in which ions commonly acquire positive charge by addition of protons. CAD of peptides mainly occurs by nucleophilic reactions; therefore sites of cleavage are strongly influenced by peptide sequences and the distribution of protons across backbone and side-chain atoms[3]. Other fragmentation methods that are becoming popular for PTM identification are electron capture dissociation (ECD) and electron transfer dissociation (ETD). These achieve fragmentation through peptide interactions with low-energy electrons (ECD) or radical anions (ETD), forming peptide radicals that rapidly undergo backbone cleavage[9,10]. ETD and ECD have advantages over CAD for detecting phosphorylation and other PTMs unstable to MS/MS, because peptide fragmentation is less influenced by peptide sequence, and neutral loss reactions are reduced. ECD and ETD are complementary to CAD, however, since they perform optimally with highly charged analytes (charge state ≥ +3) whereas CAD is more efficient with ions of lower charge[11].

MS of negatively charged ions, most commonly formed by proton removal during ionization, can be more sensitive than positive-mode MS for detecting phosphopeptides[12]. In general, negative-mode MS/MS spectra are difficult to decipher and have not been extensively investigated. However, negative-mode MS/MS of phosphorylated serine, threonine and tyrosine residues yield fragments of –79 Da (PO_3^-) or –63 Da (PO_2^-). A very sensitive method involves selective monitoring of phosphopeptide parent ions in negative mode based on their –79-Da ion signature, followed by polarity switching to obtain positive-ion MS/MS spectra[12].

Enrichment of phosphorylated peptides and proteins

Low sensitivity is a frequent obstacle when analyzing phosphopeptides or phosphoproteins by MS. Substoichiometric phosphorylation often occurs, reducing phosphoanalyte abundances compared to corresponding unphosphorylated forms. In addition, phosphopeptides may show inefficient ionization or may be lost preferentially during handling by adsorption to metal or plastics. Thus, a large repertoire of techniques has been developed to enrich phosphoanalytes and improve detection sensitivity, particularly for samples of high complexity. Many of these make use of reactive chemistries for covalent coupling or affinity purification.

Covalent coupling. When PTMs are chemically reactive, they can be covalently coupled to chemical tags for selective purification. Phosphorylated serine and threonine have reactive chemistries; the β-elimination of phosphoric acid forms respectively dehydroalanine and β-methyldehydroalanine. The resulting Cα=Cβ bond is susceptible to Michael addition by various nucleophilic tags[13,14], including those containing reactive groups that can be further adapted for solid-phase capture[15–17]. Beta-eliminated products can also be used to incorporate tags that can be detected as signature fragment ions by precursor ion scanning[18]. An interesting variation of this strategy is to couple β-eliminated residues to aminoethylcysteine, generating a trypsin recognition site at previously phosphorylated residues[19].

Caveats with these methods are that Michael addition may occur at both Cα and Cβ and may racemize at either site, yielding heterogeneous products[13]. Additionally, β-elimination of other PTMs, such as O-linked oligosaccharides, may yield products misinterpreted as a signature of phosphorylation. Cysteines must be protected to prevent undesirable side reactions, and asparagine deamidation or elimination of unphosphorylated serine and threonine may occur, especially in the presence of strong base[20–22].

An alternative strategy involves the covalent reaction of phosphorylated residues with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) to produce direct linkages with the phosphate group. The resulting phosphoramidate adduct can be coupled to dendrimers for

a

Does the PTM remain stable during MS/MS fragmentation?

Is the PTM labile during MS/MS fragmentation?

	Look for characteristic mass shifts in parent and fragment ions:	Look for MS/MS signature ions formed during fragmentation:		Monitor products of labile cleavage (such as neutral loss):
Phosphorylation	+80 Da	pY pS, pT, pY	216 Da –79, –63 Da	–98 Da
Acetylation	+42 Da			
Cysteine oxidation	+16, +32, +48 Da			
Cysteine nitrosylation	+30 Da			–30 Da
Ubiquitination	+114 Da (Gly-Gly)			

b

	Is the PTM chemically reactive?	Can the PTM be selectively enriched by affinity adsorption or chromatography?
Phosphorylation	Beta-elimination followed by Michael addition of affinity tags, solid supports	Anti-pY antibody affinity purification pS, pT, pY Fe^{3+}, Ga^{3+}-IMAC, TiO_2, ZrO_2 Ion exchange chromatography
Acetylation	No reactivity	Anti-acetyllysine antibody affinity purification
Cysteine oxidation	Reduction, then alkylation or -S-S- reactivity 'biotin switch' Sulfinic acid reactivity with dimedone	
Cysteine nitrosylation	Reduction, then alkylation or -S-S- reactivity 'biotin switch'	
Ubiquitination	No reactivity	Affinity purification of tagged Ub/UBL Ub-interaction motif affinity purification Fluorous solid phase extraction for enriching branched peptides

Figure 1 | Flow chart for using MS to detect and analyze protein post-translational modifications described in this review. (**a**) Mass shifts during MS/MS fragmentation. (**b**) Enrichment strategies. pY, phosphotyrosine; pS, phosphoserine; pT, phosphothreonine; Ub, ubiquitin.

solid-phase capture[23] or captured onto functionalized glass beads[24]. Phosphoramidate chemistries have the advantage of coupling phosphorylated tyrosine as well as serine and threonine, and the method has succeeded with complex mixtures[25].

Affinity capture. Strategies for noncovalent enrichment of phosphopeptides and proteins have used affinity purification based on charge properties and antibody recognition. Widespread methods use immobilized metal affinity chromatography (IMAC), which adsorbs phosphopeptides to chelated metal ions (Fe^{3+}, Ga^{3+}) through metal-phosphate ion-pair interactions[26,27]. Titanium dioxide (TiO_2) and zirconium dioxide (ZrO_2) are also used to adsorb phosphopeptides, through bidentate interactions[28,29]. Ion exchange resins allow partial enrichment of phosphopeptides based on charge separation[7], and high selectivity has been reported with metal affinity resins in combination with ion exchange chromatography[29]. In addition, highly specific antibodies to phosphotyrosine have enabled selective immunopurification of phosphotyrosine-containing phosphopeptides as well as phosphoproteins[30–33].

All affinity capture methods suffer from phosphopeptide losses resulting from poor binding or recovery, and any method may yield biased results owing to chemical selectivity. Recovery of peptides by any one method is difficult to estimate because total numbers of phosphopeptides are usually unknown; however, analyses of simple mixtures suggest that 30–50% of peptide sequences are recovered by Fe^{3+}-IMAC[34,35]. A recent comparison of Fe^{3+}-IMAC, TiO_2 and dendrimer purifications in one laboratory showed substantial variations in the populations of identified phosphopeptides, although good reproducibility was achieved with any single method[25].

Large-scale identification of phosphorylation sites

Studies of complex samples often yield more than 1,000 phosphopeptide identifications, and in the most comprehensive study to date, ~6,600 phosphopeptides were reported in HeLa cells by enriching phosphopeptides by ion exchange chromatography-TiO_2 and sequencing them by multistage activation using a fast scanning ion-trap mass spectrometer[36]. Two complications arise in efforts to identify phosphorylated sites (phosphosites) in large-scale experiments. First, the peptide sequence identification may be less accurate because allowing the search program to consider variable phosphate modifications at any serine, threonine or tyrosine residue increases the effective search space by ~15-fold, increasing the number of false assignments that may occur by random chance. Second, even when phosphopeptide sequences are identifiable, locating the site of modification may be difficult, and estimates suggest that as many as 25% of assigned phosphopeptides show ambiguous sites[37].

High-confidence PTM detection requires improved computational strategies to identify and localize modified residues. High-accuracy mass measurements are advantageous in phosphopeptide identifications, enabling false assignments to be removed based on stringent mass filters[36,38,39]. Search programs have also been developed to identify phosphorylation and other PTMs from MS/MS spectra. An early algorithm named SALSA (scoring algorithm for spectral analysis) scored sequence motifs in MS/MS spectra regardless of absolute positioning by detecting modifications reported by multiple product ions[40]. More recent algorithms localize sites using probability-based scores that assume a correct peptide sequence and then express the probability of phosphorylation at a particular residue using a binomial model to describe random matching of fragment ions[36,37]. Alternative approaches identify covalent modifications using spectral alignment or shared fragment ions to match the MS/MS spectra of modified peptides to their corresponding unmodified forms[41,42]. These approaches are just beginning to be applied to large-scale experiments, but have the advantage of being generalizable to other PTMs.

Accuracy of phosphopeptide identification also requires efficient fragmentation, for which ECD and ETD may present improvements over low-energy CAD, as mentioned above. One ETD-based study mapped 1,252 phosphosites in yeast tryptic digests enriched by IMAC, whereas previous CAD-based analyses by the same laboratory mapped 383 sites[43]. Negative-ion CAD has also been shown to improve site localization owing to site-specific cleavages at phosphorylated residues[44], and its application to large-scale experiments remains a future possibility.

Phosphopeptide quantification

Several approaches exist for quantifying changes in phosphopeptide abundance, which must be normalized to corresponding protein abundances to verify true changes in phosphorylation. Many methods for quantifying phosphopeptides involve some form of stable isotope labeling, in which proteins in two or more samples are labeled with isotope-derivatized moieties, samples are mixed and proteolyzed, and ratios of isotopically distinguishable peptides are quantified by MS (**Fig. 2a**)[45,46]. These approaches circumvent complications resulting from variable peptide recovery. An effective approach to stable isotope labeling (called 'stable isotope–derivatized amino acids in cell culture', SILAC)[47] involves metabolic labeling of proteins using amino acids labeled with ^{13}C versus ^{12}C, or ^{15}N versus ^{14}N. High-resolution instruments have facilitated these studies, especially in cases where ratio calculations must be corrected for incomplete labeling. Further innovations have paired $^{13}C/^{12}C$ with $^{15}N/^{14}N$ in various amino-acid combinations, allowing samples to be multiplexed. In an extensive study, two three-point multiplexed experiments were used to quantify phosphorylation events in HeLa cells stimulated with epidermal growth factor (EGF) for 0–20 min, allowing dynamics of phosphorylation to be monitored on 2,244 proteins[36].

Other strategies use chemical derivatization to couple stable isotope labels to peptides, either through phosphate-specific reactions (for example, β-elimination and addition, phosphoramidate coupling) or reactive peptide groups (for example, amine and cysteine groups; **Fig. 2a**)[16,21,48]. Such methods are particularly important with samples for which metabolic labeling is not possible, such as human fluids and tissues. Another strategy uses immobilized trypsin to catalyze O-exchange from $H_2{}^{18}O$ versus $H_2{}^{16}O$ onto C-terminal carboxylates[49]. Innovative 'iTRAQ' and 'tandem mass tag' reagents contain amine-reactive groups linked to a fragmentable tag labeled with different combinations of stable isotopes[50]. After mixing, peptide adducts with each reagent are identical in mass (isobaric), but upon fragmentation generate isotopically distinguishable tag ions with different *m*/*z* values. Ratios of phosphopeptide abundances between different samples are then measured from relative intensities of tag ions.

Alternative approaches to quantification include 'label-free' measurements, where peptide intensities are measured in separate runs between different samples (**Fig. 2b**)[51,52]. These methods are simpler, but require stringent reproducibility in sample processing and chromatography. Thus, their application to protocols involving phosphopeptide enrichment has been limited, although one report successfully applied label-free quantification to phosphopeptides purified by IMAC[53]. A recent 'differential MS' method identified peptide changes

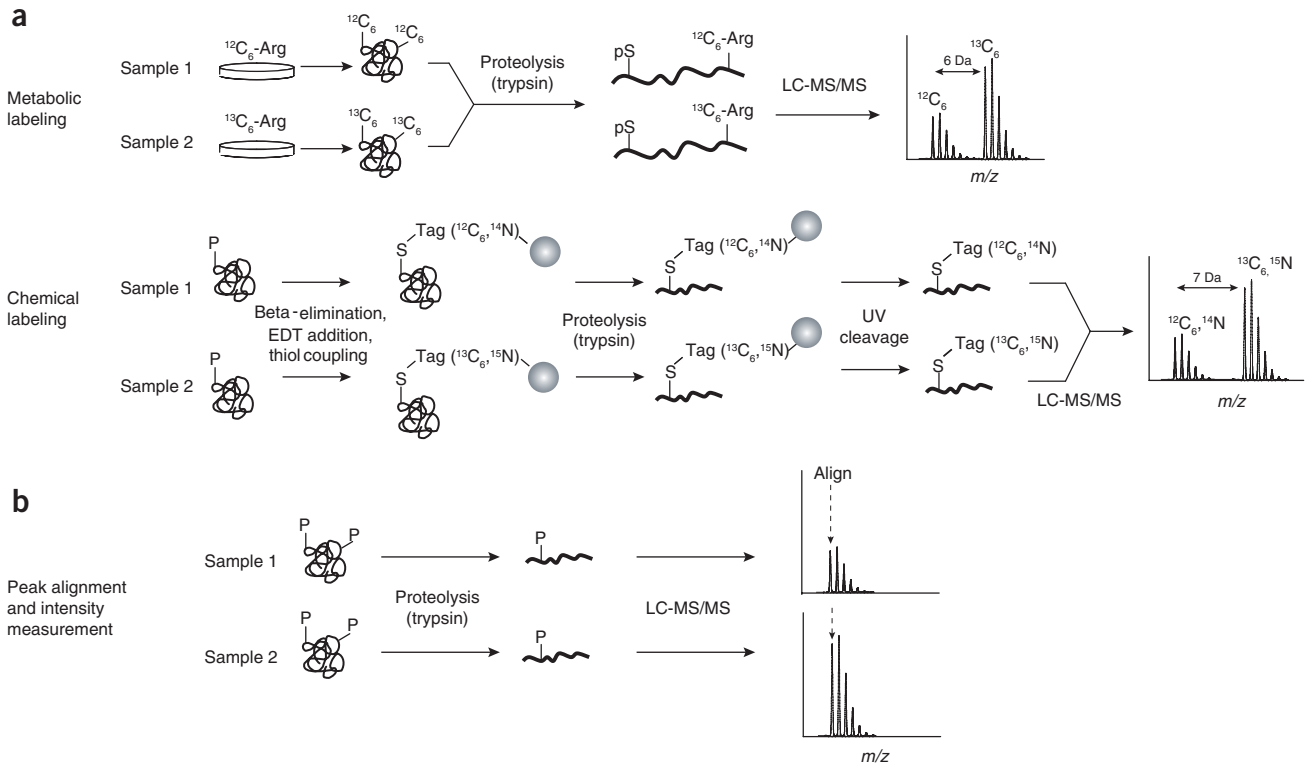


Figure 2 | Common methods used for phosphopeptide quantification. (**a**,**b**) Stable-isotope labeling (**a**) and label-free methods (**b**) both measure peak intensities to quantify phosphopeptide ratios between two or more samples.

by quantifying peak intensities and then retrospectively surveyed peaks with altered intensities by targeted MS/MS[54]. High mass-resolution instruments have improved the effectiveness of label-free methods with complex samples, and their use in quantifying phosphorylation is an area for additional exploration.

Absolute phosphopeptide abundances are not commonly quantified, but can be measured using isotopic dilution[55]. In this method, ^{12}C-labeled phosphopeptides from native samples are quantified and normalized to signal from ^{13}C-labeled synthetic analogues spiked into the samples. Using this method, phosphorylation site occupancies at multiple inhibitory sites in Cdk1 were quantified[56].

Stoichiometry

Knowledge of phosphorylation stoichiometry is often needed to understand protein regulation: for example, to assess whether phosphorylation alters enzyme-specific activity, or to assess whether it differs among cellular compartments. Label-free approaches have been used to estimate stoichiometry, by measuring ratios of summed intensities for ions corresponding to phosphorylated versus unphosphorylated forms (**Fig. 3**)[57,58]. An important assumption is that phosphorylation does not significantly alter ionization efficiency, such that peak intensities of modified and unmodified peptides can be compared. Although not true in every case, this assumption has been generally supported by many studies of phosphoproteins and phosphopeptides[57–59]. Our experience has shown that peptides with more than one phosphorylation site are often less reliably quantified.

Isotope labeling methods are also appropriate for stoichiometry determinations. In one method, half the protein sample is dephosphorylated by phosphatase treatment, then both halves are proteolyzed and the carboxyl groups are methylesterified with isotopically distinguishable methyl groups[60]. The two halves are then mixed and analyzed by LC-MS/MS, quantifying ratios of phosphorylated peptides to unphosphorylated cognates in phosphatase-treated samples, to normalize for differences in ionization efficiency. This may be complicated in certain cases when phosphate removal alters the proteolytic cleavage pattern.

Databases. Large-scale studies have greatly increased the numbers of entries in public databases of observed phosphorylation sites (**Box 1**). Results should be treated with caution, however, because there are difficulties in localizing phosphosites with high confidence. For example, phosphosites with borderline search scores are sometimes accepted when they match sites previously identified in other studies; in some cases this might reflect systematic biases in experimental or search methods. Data mining suggests that the majority of primary sequence motifs match those previously determined, with some evidence for new determinants of protein kinase recognition[31].

How much of the phosphoproteome has so far been mapped is still a matter of speculation. Recent large-scale experiments showed overlap of ~40% of sites between studies[36], which may increase as more cell types are examined. An in-house study of ~600 phosphosites in human melanoma cells found 50% present in either of the two most comprehensive databases, PhosphoSite and SwissProt (W.M.O. *et al.*, unpublished data). This suggests that half the sites in the human phosphoproteome remain to be identified. Such estimates, however, are subject to change as the sensitivity of MS methods increases. For example, a recent MS analysis of the cytoskeletal protein cortactin showed 23 phosphosites, far greater than the 5 sites identified in four

earlier studies[61]. Thus, the total number of phosphosites could expand manyfold as databases reach saturation.

ACETYLATION

Acetylation at lysine ε-amino or N-terminal groups is stable to peptide fragmentation by CAD, and it can be detected by its characteristic mass shift of +42.01 Da from unmodified forms. Although trimethylated lysine (42.04 Da) is similar in mass to acetyllysine, these structures can be distinguished by high-resolution mass spectrometers[62]. Trypsin cleavage at acetyllysine residues is usually blocked owing to charge neutralization, so the acetylated peptides are detected as 'missed cleavage' products differing in sequence from their unmodified forms.

Enrichment of acetylated peptide residues is difficult, because acetylated amines do not readily undergo derivatization in solution. Thus, studies have generally characterized protein acetylation on partially purified mixtures such as histones, where the key involvement of acetylation in regulating chromatin structure has been extensively studied. This perspective has changed with a recent large-scale screen for protein acetylation. A wide range of acetyllysine sites were mapped by enriching acetylated peptides using resin-coupled antibodies to acetyllysine[63]. Remarkably, 68% of acetyllysines were found on mitochondrial proteins, suggesting a mechanistic link between deacetylase inhibition and metabolic control by caloric restriction, both of which have been shown to prolong lifespan in model organisms.

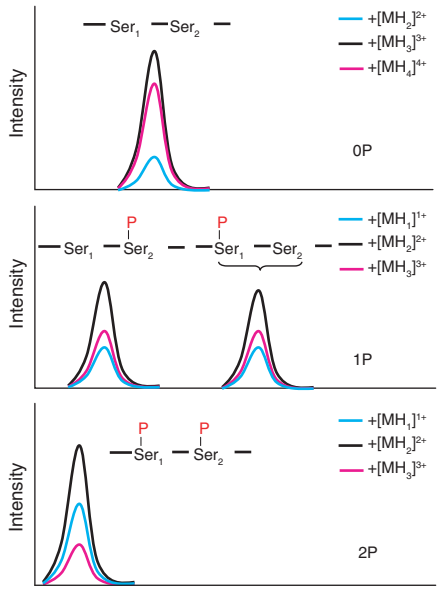
New chemistries for protein acetylation have emerged from recent proteomics studies. O-acetylated serine and threonine residues were discovered as products of group transfer from acetyl coenzyme A in reactions catalyzed by the *Yersinia pestis* effector YopJ (ref. 64). This event esterifies key serine residues in MAP kinase kinases, interfering with phosphorylation and kinase activation. In addition, recent studies have reported propionylation and butyrylation of lysine residues in histones and other targets of lysine acetylation, catalyzed by known histone acetyltransferases[65]. This revealed that chemical modifications at lysine are more heterogeneous than previously recognized.

UBIQUITIN AND UBIQUITIN-LIKE PROTEINS

Ubiquitin and ubiquitin-like proteins (UBLs) are well known regulators of protein stability, activity, cellular localization and degradation. Ubiquitin is covalently coupled by E3 ligases to target proteins through isopeptide linkages between ubiquitin C-terminal carboxyl groups and target protein lysine ε-amino groups[66]. These modifications pose unique challenges for MS identification, because upon proteolysis, C-terminal residues on ubiquitin and UBLs remain covalently attached to lysine residues on target peptides. Trypsin proteolysis of ubiquitin releases a C-terminal Gly-Gly dipeptide, creating a signature mass shift of 114.1 Da[67,68]. However, trypsin cleavage can be suppressed at Gly-Gly–coupled lysine[68], resulting in missed-cleavage products that can be larger than the mass range ideal for CAD. ECD and ETD are being explored to improve fragmentation of large peptides formed from proteins modified by ubiquitin or UBLs[69,70].

NEDD8 and ISG15 share C-terminal Gly-Gly sequences with ubiquitin, requiring the use of proteases other than trypsin to distinguish these three from one another[71]. Other UBLs release larger C-terminal polypeptides. For example, mammalian SUMO1 and SUMO2/3 yield distinct isopeptide adducts (+2,155 and +3,570 Da, respectively) that upon fragmentation superimpose extra product ions onto target peptide fragments[72]. This complicates the resulting MS/MS spectra, introducing problems for sequencing. Recent strategies search first for signature fragments that report UBL C-terminal product ions and then exclude these from further searching. This simplifies the spectrum, facilitating identification of the target peptide sequence and site of modification from the remaining fragments[72]. Because SUMOylated peptides often show inefficient cleavage owing to cross-linking and larger peptide size, strategies to improve coverage have used combinations of proteases as well as C-terminal SUMO mutants with shorter C-terminal tags[73,74].

Large-scale analysis of protein ubiquitination. Several sites are often ubiquitinated within proteins, and mutational studies show that not all are required for proteasome recognition[75]. This effectively dilutes the regulatory sites, requiring enrichment to efficiently detect the functionally targeted sites in large-scale studies. Expression of His_6-ubiquitin by homologous recombination has been used to affinity enrich and identify ubiquitin targets in *S. cerevisiae* and mice[68,76]. Similarly, stable expression of His_6-Flag-SUMO was effective in identifying SUMOylation targets in yeast[71]. Alternative enrichment methods bypass the use of ubiquitin affinity tags—for example, by using affinity enrichment with immobilized ubiquitin interaction motifs[77]. Selective enrichment of branched peptides formed by proteolysis of ubiquitinated proteins has also been achieved by tagging N termini of peptides with perfluorinated alkyl moieties using fluorous functionalized silica resins[78]. Databases of modifications by ubiquitin and UBLs are emerging as valuable tools as more sites are identified. Currently,



$$\text{Stoichiometry (site}_1\text{ or site}_2\text{, mol/mol)} = \frac{I_{1P(site1\text{ or site }2)} + I_{2P}}{I_{0P} + I_{1P(site\ 1)} + I_{1P(site\ 2)} + I_{2P}}$$

Figure 3 | Determining stoichiometry of phosphorylation. A phosphopeptide modified at two serine residues may show up to four forms: unphosphorylated (0P), monophosphorylated (1P) at site 1 or site 2, and diphosphorylated (2P). In this example, each phosphoform elutes with different *m/z* and retention time and can be quantified by summing intensities of all charge states. Note the typical shift in charge state distribution to lower values upon phosphate addition. Phosphorylated and unphosphorylated forms of peptides often show similar ionizations, allowing mol/mol stoichiometry at any site to be estimated as shown (I = summed intensities for three charge states); otherwise, each form must be quantified against separate calibration standards.

400 proteins are listed in the UbiProt database, along with annotations of experimental evidence for PTM type and modified residues[79].

CYSTEINE OXIDATION

Widespread responses of cells to oxidative stress have been demonstrated in many disease states, in many cases involving nonenzymatic protein oxidation catalyzed by reactive oxygen species. Oxidation of cysteine yields sulfenic acid (R-SOH), sulfinic acid ($R\text{-}SO_2H$) and sulfonic acid ($R\text{-}SO_3H$), respectively revealed by mass shifts of +16, +32 and +48 Da. Reactivity is facilitated by protein environments that reduce the pK to enable thiolate formation at neutral pH. Examples include peroxidases and tyrosine phosphatases, which are inactivated by H_2O_2 through oxidation of active-site cysteine residues[80–82].

Cysteine also forms S-nitrosylated species by reacting with nitric oxide (NO), nitrogen dioxide (NO_2) or dinitrogen trioxide (N_2O_3)[83]. These nitrogen species are produced enzymatically by nitric oxide synthase, yielding the highly diffusible and short-lived NO second messenger[84]. Thus, it is assumed that localized production of NO confers spatial selectivity of protein nitrosylation, which has been confirmed with fluorescent NO biosensors[85].

MS analysis of endogenously S-nitrosylated proteins has been challenging, because the SNO modification is chemically labile and found

BOX 1 DATABASES OF PROTEIN POST-TRANSLATIONAL MODIFICATIONS

Observed phosphorylation sites

PhosphoSite, http://www.phosphosite.org/. Developed by Cell Signaling Technologies. Contains 26,884 sites on 8,736 proteins from various vertebrates (mainly human and mouse), combining data from literature curation, published large-scale studies and in-house screens. High-confidence data lists 7,393 phosphoserine, 2,069 phosphothreonine and 7,866 phosphotyrosine sites in 5,325 human proteins. Each record contains references and orthologous residues in other species.

SwissProt, http://ca.expasy.org/sprot/. One of the most comprehensive databases for all post-translational modifications, reporting 8,801 phosphoserine, 1,724 phosphothreonine and 1,212 phosphotyrosine sites from 3,613 human proteins. Data include literature phosphosites, including those from large-scale screening. Sites are linked to entries in the RESID database, which contains detailed structural and chemical information for each modification. Approximately one-fifth of entries are inferred from protein orthologs with similar sequences.

PhosIDA, http://www.phosida.com/. Data are derived from screens carried out by Matthias Mann's laboratory (Max Planck Institute, Martinsreid, Germany) in *Bacillus subtilis* and in EGF-treated HeLa cells. Data from four large-scale studies of EGFR-stimulated cells published by other laboratories are also included, providing a comparison resource for this pathway.

Phospho.ELM, http://phospho.elm.eu.org/. Formerly Phosphobase; contains 13,613 phosphosites on 3,674 phosphoproteins from various eukaryotic organisms. Each site is annotated (where known) with associated kinases, phosphosite binding interaction domains, and links to three-dimensional structures.

Human Protein Reference Database (HPRD), http://www.hprd.org/. HPRD contains 5,080 PTM sites (not limited to phosphorylation) in 1,556 human proteins. Data are manually curated from various public literature sources.

More phosphorylation site resources

mtcPTM, http://www.mitocheck.org/cgi-bin/mtcPTM/search/. A database of mouse and human phosphorylation sites from published sources and from the Mitocheck project, with associated experimental data.

Phospho3D, http://cbm.bio.uniroma2.it/phospho3d/. Three-dimensional structures of phosphoproteins.

PlantsP, http://plantsp.genomics.purdue.edu/html/. Plant protein phosphorylation site database.

ProMEX, http://promex.mpimp-golm.mpg.de/home.shtml. Reference mass spectra of tryptic peptides from plant proteins and phosphoproteins.

Scansite, http://scansite.mit.edu/. Enables protein sequences to be searched for phosphorylation motifs recognized by many kinases, as well as motifs involved in protein and phospholipid binding.

Other post-translational modifications

dbPTM, http://dbPTM.mbc.nctu.edu.tw/. Database of sites, including those from PTM prediction programs.

UbiProt, http://ubiprot.org.ru/. Database of ubiquitinated proteins.

O-Glycbase, http://www.cbs.dtu.dk/databases/OGLYCBASE/. O- and C-linked glycosylation sites.

DSDBASE, http://caps.ncbs.res.in/dsdbase/dsdbase.html. Database of disulfide bonds in proteins.

Known protein post-translational modifications and chemical derivatives: masses and elemental composition

UniMod, http://www.unimod.org. A community-supported database of modifications for mass spectrometry applications, listing 531 entries. Includes mass changes due to amino-acid substitutions and masses of products from metabolic and chemical isotopic labeling experiments.

RESID, http://www.ebi.ac.uk/RESID/. A resource from the European Bioinformatics Institute, listing 428 entries. Includes chemical structures, literature citations and information on biological function. Cross-references Swiss-Prot, PIR, PDB, GO, COMe and PubMed databases.

Delta Mass, http://www.abrf.org/index.cfm/dm.home. A resource from the Association of Biomolecular Resource Facilities, listing 351 entries. Includes extensive compilations of derivatives encountered during chemical synthesis of peptides.

in low abundance. Current methods use an indirect 'biotin-switch' approach in which unmodified cysteines are first protected by methylthiolation, S-nitrosylated cysteine residues are reduced, and the resulting free thiols are reacted with a biotin-tagged alkylating reagent[86]. Affinity-enriched SNO-conjugated proteins are then trypsinized and identified by MS. Proteins can also be digested before affinity purification[87]. The biotinylated peptides are then identified by mass shift (for example, +428 Da for Cys-*S*-biotin)[88].

Large-scale studies of cysteine oxidation are emerging with the development of methods for selective enrichment. SDS-PAGE separation and MS were used to confirm the identities of newly discovered S-nitrosylated proteins enriched by avidin-biotin purification[89]. Likewise, affinity probes are being developed that selectively react with oxidized cysteine. One approach traps sulfenic acid as a stable thioether by oxidation with dimedone, generating a residue mass shift of 138 Da. Biotinylated dimedone analogs can be used to label and identify sulfenic acid–modified proteins from H_2O_2-treated tissues[90].

BIOLOGICAL DISCOVERY

As MS instrumentation achieves greater sensitivity for in-depth profiling, the bottleneck shifts toward confirming PTM sites and validating their function. New experimental and computational strategies are needed to ask: what cellular events are regulated by PTMs, and how does one rank the importance of several PTMs occurring on a single protein? How do PTMs respond to spatial and temporal information? How can low-level information about individual modified sites be used to infer higher-level properties of biological systems?

Molecular function of PTMs. An important insight emerging from phosphosite databases is that very large numbers of phosphorylation events may occur on a single protein, sometimes reaching dozens of modified sites. Standard approaches to probe function test phenotypes induced by blocking PTMs, either by interfering with upstream enzymes that catalyze these modifications, or by mutating PTM sites and testing whether the mutants are able to rescue phenotypes in cells deficient in wild-type forms. Controls are needed to test deleterious side effects of mutations, due to compromised protein stability or induction of new adventitious PTMs.

One approach to prioritizing PTMs is to examine sequences of protein orthologs. Modifiable residues that are conserved between species are often used to increase confidence that a PTM is functionally important. However, only as databases reach saturation will we know how true this assumption is and to what extent regulatory function is species dependent. Stoichiometry is another metric that may be useful in prioritizing PTMs, although it is often difficult to measure. For example, knowledge of stoichiometry obviously affects the importance of cysteine oxidation. Because reactive cysteines are often important for enzymatic catalysis, mutation of these sites disrupts activity and cannot be used to probe regulatory functions of oxidation. Measurements of ratios of modified to unmodified proteins can be correlated with activity loss and used to judge whether oxidation levels are sufficient to compromise function[91].

Spatial and temporal regulation of PTMs. Association of PTMs with specific cell compartments, protein interactions or temporal windows has motivated systematic analyses of PTM changes with respect to space and time. For example, organelle separations have been used to reduce sample complexity while interrogating PTM compartmentalization. In one MS study, five ubiquitin-modified lysine residues were identified within the kinase domain of the EGF receptor (EGFR)[92]. Mutating each lysine individually reduced ubiquitination and turnover, and only the wild-type receptor could be found in lysosomes, showing that EGFR ubiquitination directs degradation through lysosome targeting of receptors.

PTM measurements have also been used to infer temporal order of regulatory mechanisms. For example, monitoring events in the first 60 s after EGF stimulation showed that phosphorylation at only three of eight observed phosphotyrosine sites in EGFR increased significantly within the first 5 s, two of which are known to be binding sites for the adaptor molecule SHC (ref. 93). This indicates that receptor transphosphorylation is ordered, directing sequential activation of downstream effectors. Likewise, fibroblast growth factor receptor analyses showed that autophosphorylation occurred in a sequential and ordered manner, implying that receptor tyrosine kinases differentially control downstream signaling events by the temporal order of autophosphorylation[94].

PTM interactions and higher-level properties. Future expansion of PTM databases should improve the understanding of sequence specificity. For example, large-scale experiments have failed to define consensus motifs for ubiquitin modification, suggesting that E3 ligases do not recognize local sequence for isopeptide bond formation, but instead recognize secondary or tertiary structure. In contrast, a consensus SUMOylation motif (ψ-Lys-X-Glu, where ψ is a hydrophobic amino acid and X is any amino acid) has been observed in ~75% of SUMOylation sites reported from individual protein analyses[95].

An area for future exploration is the occurrence of hierarchical or reciprocal regulation between different chemistries that target the same residues. A simple case can be seen when PTMs compete for the same residue, as exemplified by *O*-linked *N*-acetylglucosamine and O-acetylation, which preemptively block protein phosphorylation at regulatory serine and threonine residues[64,96]. Serine phosphorylation of IκBα facilitates subsequent lysine ubiquitination, targeting the protein for degradation. SUMOylation of the same lysines protects IκBα from degradation, without requiring previous phosphorylation[97]. Therefore, phosphorylation and SUMOylation independently control IκBα stability through reciprocal control of ubiquitination.

Pathways may also interact combinatorially to regulate intracellular proteins, through mechanisms that are in many cases still obscure. Global profiling and clustering of PTMs have been used to monitor hierarchical coregulation between signaling pathways. In a screen for phosphorylation events responding to CD3 and CD28 signaling in T cells, phosphotyrosine phosphorylation sites on Vav-1 and WASP were observed upon stimulation of CD3 plus CD28, but were absent in response to either stimulus alone[98]. This demonstrates pathway synergy and signal convergence at specific effectors, providing new insight into how multiple pathways control biological processes.

PTM interactions that confer reciprocal, hierarchical or combinatorial regulation are likely to be very common. An excellent illustration is the 'histone code' whereby different combinations of histone modifications produce distinct states controlling transcriptional activation and silencing. Defining the histone code requires monitoring all PTMs at once within single polypeptides, to elucidate the heterogeneity of distinct modified states. In order to examine similar mechanisms in other proteins, methods are needed to monitor covariation between PTMs on single polypeptides, potentially spanning the entire protein sequence. 'Top-down' MS methods for sequencing large polypeptides may be the best route to achieve this, and they remain an important future goal for PTM research[99].

Ultimately, global interrogation of protein modifications should be possible as PTM databases reach saturation through large-scale MS efforts. Thus, there is an increasing need for improved technologies that enable quick and routine assay of known PTM chemistries. For example, a method called multiple reaction monitoring allows multiplexed detection of analytes with specific chemical signatures, as demonstrated for phosphopeptides[100]. In addition, new materials to better discriminate between PTMs with high specificity and sensitivity would hasten development of protein arrays and increase the speed at which individual proteins can be analyzed in focused studies. Solutions to these problems will greatly affect future capabilities for PTM discovery as well as diagnostics.

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