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Review

Targeted mass spectrometry: An emerging powerful approach to unblock the bottleneck in phosphoproteomics

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ABSTRACT

Following the rapid expansion of the proteomics field, the investigation of post translational modifications (PTM) has become extremely popular changing our perspective of how proteins constantly fine tune cellular functions. Reversible protein phosphorylation plays a pivotal role in virtually all biological processes in the cell and it is one the most characterized PTM up to date. During the last decade, the development of phosphoprotein/phosphopeptide enrichment strategies and mass spectrometry (MS) technology has revolutionized the field of phosphoproteomics discovering thousands of new site-specific phosphorylations and unveiling unprecedented evidence about their modulation under distinct cellular conditions. The field has expanded so rapidly that the use of traditional methods to validate and characterize the biological role of the phosphosites is not feasible any longer. Targeted MS holds great promise for becoming the method of choice to study with high precision and sensitivity already known site-specific phosphorylation events. This review summarizes the contribution of large-scale unbiased MS analyses and highlights the need of targeted MS-based approaches for follow-up investigation. Additionally, the article illustrates the biological relevance of protein phosphorylation by providing examples of disease-related phosphorylation events and emphasizes the benefits of applying targeted MS in clinics for disease diagnosis, prognosis and drug-response evaluation.

1. Introduction

Protein phosphorylation is one of the most widespread post-translational modifications (PTM) that plays an essential role in cellular physiology. Site-specific phosphorylations affect numerous critical aspects including protein activity, sub-cellular localization, conformation, stability and binding capacity to other molecules [1–6]. Environmental cues such as mechanical stress, cell-cell interactions, growth factors or cytokines, trigger the activation of signaling networks that are mainly orchestrated by protein phosphorylation events [7–9]. It is estimated that at least one third of the proteins within a cell becomes phosphorylated at some point [10], consequently, it is assumed that virtually all cellular processes are directly or indirectly modulated by this PTM. Given the relevance of protein phosphorylation in modulating the physiology of the cell, it is not surprising that aberrant phosphorylation has been linked to a vast number of pathologies [11,12]. Hence, phosphoproteins have emerged as potential biomarkers and major efforts are being devoted to developing robust and sensitive strategies for the detection of site-specific phosphorylations within complex biological samples.

The phosphorylation status of a protein is tightly modulated by the coordinated action of kinases and phosphatases that are responsible for attaching or removing the phosphate moiety, respectively. Usually, subtle changes in the phosphorylation levels are sufficient to alter the behavior of a protein. Nevertheless, in certain cellular processes, such as mitosis, half of the phosphosites detected have been demonstrated to present occupancy of at least 75% [13]. In addition to the overall substoichiometric nature of site-specific modifications, protein phos-

Abbreviations: DDA, data dependent acquisition; IMAC, immobilized metal affinity chromatography; IP, immunoprecipitation; MS, mass spectrometry; pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine; PPSA, phospho-specific antibody; PRM, parallel reaction monitoring; PTM, post-translational modification; RT, retention time; SCX, strong cation exchange; SID, stable-isotope dilution; SIL, stable isotope labeled; SILAC, stable isotope labeling of amino acids in cell culture; SRM, selected reaction monitoring

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phorylation events are temporally modulated. Therefore, whereas a specific residue could be phosphorylated right after a specific stimulus, it might become unmodified after a while and vice versa [14,15]. Precisely, among the three phosphorylable amino acids (Ser, Thr and Tyr) tyrosines are the less abundant but frequently regulated residues [14,16]. Additionally, the regulation of phosphoproteins can be restricted to certain cellular compartments [13,17,18]. All these challenges are further complicated when multiple phosphorylation sites are present on the same phosphopeptide. In this scenario, it is crucial to determine the specific residue or residues that are modified in order to characterize the biological significance of such phosphorylation.

Traditionally, protein phosphorylation has been investigated using various biochemical approaches that rely on the use of radioactivity (^{32}P), phospho-specific dyes or *in vitro* kinase assays [19–21]. Although the contribution of these types of studies is invaluable, they do not provide information about the precise site or sites that are modified within the phosphoprotein. Genetic approaches such as site-directed mutagenesis have greatly expanded our knowledge about biological role of the site specific phosphorylations occurring in proteins [22–24]. The increasing number of commercially available phosphosite-specific antibodies also allows detecting relatively easily whether a protein is phosphorylated or not on a certain residue that has been previously described. Nevertheless, phospho-site recognition is often hampered due to the antibodies' specificity and sensitivity issues. At present, mass spectrometry (MS) offers the most suitable approach for mapping phosphorylation sites and discovering novel modifications. The gain of mass resulting from the presence of the phosphate moiety is used to assign the specific residue subjected to modification. Indeed, breakthrough developments in the field of MS-based phosphoproteomics allow the detection and quantification of thousands of phosphosites simultaneously, which has greatly expanded our knowledge of site-specific protein modification as never before [25,26]. The amount of novel information regarding phosphosites increases so rapidly that none of the classical techniques mentioned above can cope with the speed. Yet again, another MS-based strategy called targeted phosphoproteomics has emerged as a powerful resource to systematically monitor site-specific protein phosphorylation with high precision and sensitivity [27].

Here we review the contribution of MS-based phosphoproteomics to globally decipher phosphorylation in the cell, reviewing the ground covered from shotgun proteomics to targeted proteomics; we discuss the advantages and challenges of the targeted methodology and its promising applications in phosphosite quantification-based diagnosis of diseases.

2. Shotgun phosphoproteomics: an explosion on the identification of site-specific phosphorylation

The field of phosphoprotemics experienced a great revolution upon the development of strategies to enrich phosphopeptides and phosphoproteins prior MS analysis, thus overcoming the challenging substoichiometric nature of this transient PTM.

One of the most common phospho enrichment techniques is the immobilized metal affinity chromatography (IMAC) which uses metal ions such as Fe^{3+}, Ga^{3+}, Ti^{4+} or Zr^{4+} to bind the negatively charged phosphopeptides. Although the potential of IMAC to isolate phosphorylated species is long known [28,29] it is only a decade ago that is was efficiently applied to study the global phosphoproteome of *Saccharomyces cerevisiae* and of *Arabidopsis thaliana* which resulted in the identification of a few hundred phosphopeptides [30,31]. Another method, namely strong cation exchange (SCX) fractionation, which is performed under strong acidic conditions so that negatively charged resin captures positively charged peptides, is also one of the first strategies used to enrich phosphorylated peptides. In 2004 Beausoleil and co-workers identified a total of 2002 phosphosites on 967 distinct proteins using SCX-based enrichment of the nuclear fraction of HeLa cell lysates demonstrating the suitability of this approach [32]. Later, Gruhler and co-workers combined SILAC labeling with SCX and IMAC to dissect the yeast pheromone signaling pathway. This study revealed 700 phosphopeptides, including unreported phosphosites corresponding to proteins involved in cellular polarization and mating tip formation [33]. More recently, SCX/IMAC-based phosphoproteomic studies resulted in the identification of more than 13,000 phosphorylation sites in fly embryos [34], over 13,000 in the parasite *Toxoplasma gondii* [35] and nearly 36,000 unique phosphosites in 9 mouse tissues [36].

Antibody-based immunoprecipitation (IP) of tyrosine phosphorylated (pY) proteins or peptides followed by LC–MS/MS analysis is routinely used to study in detail phosphorylation events occurring specifically on tyrosine residues, which play a pivotal role in signal transduction. Salomon and colleagues combined pY IP with methyl esterification and IMAC of tryptic peptides to study on a global scale the tyrosine phosphorylation pattern of distinct subsets of stimulated immune cells. This pioneer study resulted in the identification of 32 proteins containing 64 unique pY sites of which half were not documented before in the literature [37]. Blagoev and colleagues were first dissecting the pY-dependent signaling networks in EGF-stimulated HeLa. Combining two triple SILAC experiments with pY-containing protein IP they generated a five-point dynamic profile of 81 signaling proteins including 31 novel effectors [7]. Alternatively, Zhang and co-workers deciphered the signal transduction downstream EGFR by studying changes in specific pY sites. They combined 4plex-iTRAQ labeling with anti-pY antibody-based enrichment of pY-containing peptides followed by IMAC prior to LC–MS/MS analysis and disclosed the dynamics of 78 pY sites on 58 proteins, of which 5 pY were novel [15]. Since then numerous signaling pathways have been elucidated by LC–MS/MS analysis of peptides or proteins enriched by high affinity anti-pY IP protocols [38–43]. Large-scale phosphoproteomic studies focused on pY-dependent molecular events have proven fruitful and have greatly contributed to our current knowledge of the effectors and site-specific phosphorylation events that govern distinct signaling networks.

Another milestone in the field was the application of TiO_2 for phosphopeptide enrichment [44,45] which exploits the same principle as IMAC to isolate modified peptides. It should be mentioned that both strategies have been proven to be complementary and hence have been combined in numerous studies [46,47]. Additionally, TiO_2-based enrichment in combination with SCX and distinct fractionation strategies has proven useful to detect 6600 phosphosites in HeLa cells [16], over 3000 [48] and 23,000 [14] in human embryonic and mesenchymal stem cells, respectively, and recently over 50,000 distinct phosphorylated peptides in a single human cancer cell line [49].

As a result of the extensive large-scale phosphoproteomic studies that are routinely performed, the amount of information regarding site-specific phosphorylation is growing exponentially, which is actively incorporated in the different specialized datasets including Phosida [50,51], PhosphoELM [52–54], PhosphoPEP [55,56] andPhosphoSitePlus [57]. For instance, in 2008 the later database (http://www.phosphosite.org) listed more than 30,000 phosphorylation sites [58] and at present contains over 250,000 phosphosites. Validation of the data derived from MS-based quantitative phosphoproteomic screening has traditionally relied on antibody-based detection, i.e. Western blotting analysis. Although some phospho-specific antibodies (PPSA) allow a sensitive, specific and rapid detection of site-specific protein phosphorylation events, others cannot distinguish between similar phosphoepitopes on distinct proteins. For instance, Cell Signaling, the most reliable antibody company according to a survey done to the scientific community in 2012, commercialize a PPSA that recognizes pY319 on Zap70 but also pY352 on Syk. Additionally, despite steadily increasing, the current panel of commercially available PPSA only covers a minor fraction of the site-specific phosphorylations that are described in literature [59]. Noteworthy to mention that novel phos-