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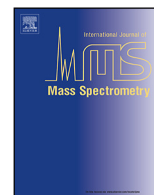

Uncovering dynamic phosphorylation signaling using mass spectrometry

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ABSTRACT

Dynamic phosphorylation is a critical regulatory mechanism that controls many aspects of cell biology. To gain insight into the regulation of kinases in health and disease it is essential to understand phosphorylation signaling both qualitatively and quantitatively. Recent advances in mass spectrometry based methods have made it possible to identify and quantify thousands of phosphorylation events across many different cellular conditions. Furthermore, these quantitative phosphoproteomics methods facilitate the relative, absolute quantification and determination of phosphorylation stoichiometry. This review summarizes mass spectrometric based strategies that have been developed to uncover phosphorylation signaling and describes examples of mass spectrometry driven analyses that have led to the understanding of signaling dynamics in mammalian cells.

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1. Dynamic phosphorylation signaling

Dynamic protein phosphorylation plays a crucial role in regulating key signaling pathways involved in controlling many cellular processes such as cell differentiation, proliferation, apoptosis, and cell survival. Phosphorylation is the process whereby a phosphate group is transferred to specific amino acids, namely to serine, threonine and tyrosine residues. The negative charge of the phosphate group has the potential to cause conformational changes of a substrate protein and can significantly alter the activity of the protein. The presence of a phosphate group can also form a recognition site, facilitating the binding of effector proteins. For instance, the presence of tyrosine phosphorylation often forms docking sites for Src Homology 2 (SH2) and phosphotyrosine binding (PTB) domains which allow the propagation of signal throughout the cell, *i.e.* from the receptor tyrosine kinase (RTK) to effector proteins and downstream kinases [1]. Phosphorylation is catalyzed by the 518 known protein kinases (representing ~2% of all human genes) [2]. There are 428 serine/threonine kinases and 90 tyrosine kinases (of which 58 are RTKs) that are responsible for all cellular phosphorylation [2]. Phosphorylation events can occur rapidly and to high stoichiometry; thus a key mechanism in the regulation of signaling pathways is the removal of phosphate from proteins. Interestingly, while there are 107 protein-tyrosine phosphatases (PTPs) [3], there are only 44 known serine/threonine phosphatases (PSPs) [4,5]. This limited group of PSPs are responsible for the dephosphorylation of thousands of serine/threonine phosphorylated substrates [4]. Conversely, the disproportionately large number of protein tyrosine phosphatases highlights the tight regulation of tyrosine phosphorylation in the cell. As a consequence, unlike many serine and threonine phosphorylation events, tyrosine phosphorylation events are incredibly dynamic due to the PTPs that rapidly dephosphorylate phosphotyrosine residues. As an example, phosphotyrosine sites on the epidermal growth factor receptor (EGFR)

Abbreviations: AEX, anion exchange chromatography; CID, collision induced dissociation; DHB, 2,5-dihydroxybenzoic acid; ECD, electron capture dissociation; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EGFRvIII, epidermal growth factor receptor variant III; ERLIC, electrostatic repulsion–hydrophilic interaction chromatography; ETD, electron transfer dissociation; FDR, false discovery rates; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FT-ICR, Fourier-transform ion cyclotron resonance; HCD, higher energy collisional dissociation; HGF, hepatocyte growth factor; HILIC, hydrophilic interaction chromatography; HMEC, human mammary epithelial cells; hSAX, hydrophilic strong anion exchange; IDA, iminodiacetic acid; IMAC, immobilised metal affinity chromatography; iTRAQ, isobaric tags for relative and absolute quantification; LC, liquid chromatography; MARQUIS, multiplex absolute regressed quantification with internal standards; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MuDPiT, Multi-Dimensional Protein identification Technology; NTA, nitriloacetic acid; pI, isoelectric point; RP, reverse phase; PSPs, serine/threonine phosphatases; PTB, phosphotyrosine binding; PTPs, protein-tyrosine phosphatases; RTK, receptor tyrosine kinase; SILAC, stable isotopically labeled amino acids in cell culture; SIMAC, sequential elution from IMAC; SH2, Src Homology 2; SRM, selected reaction monitoring; TCR, T-cell receptor (TCR); TiO_2, titanium dioxide chromatography (TiO_2); Ti^{4+}-IMAC, titanium IMAC; TMT, tandem mass tags.

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have short half-lives of only a few seconds [6]. It is thought that this rapid regulation is responsible for the low levels of cellular phosphotyrosine (which constitutes <1% of all cellular phosphorylation events, while serine phosphorylation accounts for 90% and threonine phosphorylation 10%) [7]. However, despite the presence of PTPs and PSPs it has been estimated recently that ~70% of the proteins in the proteome can be phosphorylated in response to different extracellular stimuli [8]. While many serine and threonine phosphorylation sites have been shown to be present at high stoichiometry (>75%) during the mitotic phase of the cell cycle, the majority of the remaining serine, threonine and tyrosine residues were shown to be phosphorylated to low stoichiometry (<25%) [8].

The site and stoichiometry at which phosphorylation occurs has a specific impact on functional effects within the cell. Changes in phosphorylation stoichiometry of a single phosphorylation site on a protein kinase can significantly alter its activity. With this in mind, it is not surprising that amplification, overexpression and mutation of a significant proportion of protein kinases have been implicated in the progression of cancer [9]. Due to their major role in pathogenesis and their enzymatic activities, protein kinases have emerged as a major class of drug targets [10]. Thus, when studying the effect of specific components in signaling pathways it is important to identify and quantify the specific sites of phosphorylation, followed by functional analyses to gather a better understanding of the biological effects of phosphorylation events. Currently the method of choice for identifying phosphorylation sites and quantifying their abundance across different conditions is mass spectrometry (MS). With improvements in mass spectrometric instrumentation over the last 2 decades it is now possible to (i) qualitatively characterize thousands of phosphorylation sites within a cellular system, (ii) quantitatively identify the dynamic changes that occur across thousands of phosphorylation sites, (iii) identify the absolute quantity of a given phosphorylation site across many conditions, and (iv) quantify the stoichiometry of phosphorylation. This review will highlight some of the key aspects of phosphorylation analyses from the identification and site localization of phosphorylation events through to quantification (both relative and absolute) and describe examples where mass spectrometry has been fundamental in understanding aspects of dynamic cellular signaling.

2. Sample preparation

Selective detection of phosphorylated species is an important task in proteomics, but with phosphorylation events often present at sub-stoichiometric levels [11], it is essential to use a highly sensitive and specific method to enrich phosphorylated species prior to mass spectrometric analysis. To facilitate the site specific identification of phosphorylation sites, proteins are typically enzymatically digested into peptides, using a protease, prior to enrichment and liquid chromatography (LC)–tandem mass spectrometry (MS/MS). This approach generates peptides that contain fewer numbers of serine, threonine and tyrosine residues which facilitates phosphorylation site identification. Proteolytic digestion can be carried out on proteins that have been separated by polyacrylamide gel electrophoresis (PAGE) or in a solution containing suitable protease compatible buffer (such as ammonium acetate or ammonium bicarbonate). The most commonly used protease for proteomics is trypsin which cleaves at the carboxyl-terminus of arginine and lysine residues. This approach allows the generation of short amino acid sequences (typically 500–3000 Da) that are readily detected and fragmented by LC–MS/MS to reveal sequence information. There are also other proteases that are extensively used for the analysis of proteins and post-translational modifications. These include Lys-C, which cleaves at the carboxyl-terminus of lysine and Lys-N, which cleaves at the amino-terminus of lysine residues. As a result, the peptides generated by Lys-N and Lys-C are longer than those generated by trypsin; furthermore Lys-N is capable of cleaving methylated lysine residues [12]. Glu-C [13] cleaves specifically at the carboxyl-terminus side of glutamic acid residues and Asp-N [14] cleaves proteins at the amino terminus of aspartic acid and cysteic acid residues. When using proteases to cleave proteins prior to LC–MS/MS it is essential to generate 100% cleavage efficiency to avoid splitting the MS signal between fully cleaved and miss cleaved peptides which can hamper quantification of phosphorylated peptides. The identification of phosphorylated peptides can also be complicated as negatively charged phosphorylated residues can inhibit proteolysis if in close proximity to the cleavage site [15,16]. To reduce the numbers of miss-cleaved peptides, serial digestion with Lys-C and trypsin can be used to achieve complete proteolysis [17]. Due to the sequence specificity of each protease and the varied amino acid sequences of the proteins expressed in the proteome, only a portion of the phosphoproteome can be captured with any single protease [18,19]. To increase the coverage of the phosphoproteome it is important to use a combination of proteases with complementary residue cleavage preferences. For instance, the use of three proteases (Lys-N, Lys-C and trypsin) led to a 72% increase in the number of identified phosphorylation sites when compared to trypsin alone [20].

3. Phosphopeptide enrichment strategies

There are a number of available methods that can be used to selectively enrich for phosphorylated peptides in complex biological mixtures. Phosphorylated peptides or proteins are typically enriched for using methods that take advantage of the unique chemical properties of the negatively charged phosphate group attached to the protein amino acid sequences. The most commonly used methods are: immobilized metal affinity chromatography (IMAC) [21], titanium dioxide chromatography (TiO_2) [22–24], and phosphotyrosine immunoprecipitation [25] which are discussed in the following sections. Alternative methods for the enrichment of phosphorylated proteins and peptides and combinations of enrichment strategies that have gained in popularity over recent years are also highlighted.

3.1. *Immobilized metal affinity chromatography*

IMAC utilizes the specific interactions of phosphate groups with metal ions bound to a solid phase ligand. To characterize global phosphorylation events that occur in the cell, phosphopeptides can be enriched from whole cell lysates using this approach. To generate a phosphopeptide yield required to identify a significant proportion of the phosphoproteome, typically micrograms (100–1000 μg) of protein derived from cell lysate is used as starting material. To selectively enrich for phosphorylated peptides, complex peptide samples are acidified and loaded onto an IMAC column. Phosphopeptide enrichment using IMAC has been demonstrated for many different metal ions, for instance Fe^{3+} [26], Ga^{3+} [27], Zr^{4+} [28] and Ti^{4+} [29,30]. Due to its extensive testing and comparative high specificity for phosphate groups, Fe^{3+} is the most commonly used metal ion conjugated to either iminodiacetic acid (IDA [31]) or nitriloacetic acid (NTA [21]). During IMAC phosphorylated peptides specifically bind to immobilized metal ions and the column can be rinsed with a low pH/high organic solvent solution to remove non-phosphorylated peptides and impurities [21]. Phosphorylated peptides are then eluted from the column in a high pH buffer such as ammonium hydroxide prior to LC–MS/MS [21]. There are a couple of potential pitfalls with this approach. Firstly, as metal ions are not covalently bound to the ligand, they can leach from the column during the enrichment protocol. This can lead to the loss

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