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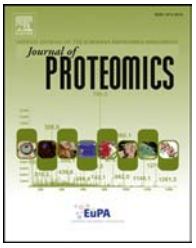

Technical note

Finding the same needles in the haystack? A comparison of phosphotyrosine peptides enriched by immuno-affinity precipitation and metal-based affinity chromatography

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ABSTRACT

Analysis of tyrosine (Tyr) phosphorylation by mass spectrometry (MS)-based proteomics remains challenging, due to the low occurrence of this post-translational modification compared to serine and threonine phosphorylation events in mammalian systems. Conventional metal-based affinity chromatography methods used to enrich phosphopeptides can nowadays isolate over 10,000 phosphopeptides. However, these approaches are not particularly suitable for the selective enrichment of low abundant Tyr phosphorylated peptides as the higher abundant co-enriched serine (Ser) and threonine (Thr) phosphorylated peptides typically obscure their detection. Therefore, a more targeted approach based on immuno-affinity precipitation at the peptide level has been introduced for the specific analysis of Tyr phosphorylated species. This method typically leads to the detection of a few hundreds of phosphopeptides, albeit typically over 70% of those are Tyr phosphorylated. Here, we evaluated and compared phosphotyrosine peptides enriched by a phospho-Tyr immuno-affinity enrichment (employing pY99 antibodies) and a multidimensional approach consisting of metal affinity based enrichment (Ti^{4+}-IMAC) followed by hydrophilic interaction liquid chromatography (HILIC) fractionation. Our aim was to assess differences and similarities in the set of Tyr phosphorylated peptides detected by each approach. Our data suggest that both strategies are not redundant but complementary and should ideally be combined for a more comprehensive view at phosphotyrosine signaling.

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Biological significance

Here we evaluated enabling tools for the global analysis of phosphotyrosine phosphorylation. Phosphotyrosine phosphorylation is a key protein modification driving cellular response also involved in disease/cancer molecular pathways.

1. Introduction

Protein phosphorylation is a ubiquitous post-translational modification (PTM) involved in several key intracellular processes including metabolism, secretion, homeostasis, transcriptional and translational regulation, cellular signaling and cell–cell communication [1,2]. It is a reversible and dynamic process that typically induces changes in conformation, activity and interaction partners of a protein [3]. Phosphorylation is catalyzed by different protein kinases and mostly occurs in mammalian systems on serine (S) and threonine (T) residues and, to a lesser extent, on tyrosine (Y) residues. Mass spectrometry (MS)-based phosphoproteomics is to-date the most powerful tool to analyze large-scale protein phosphorylation events in a variety of biological samples [4,5]. However, significant analytical barriers still hamper the routine application of phosphoproteomics. Since protein phosphorylation is typically present at substoichiometric levels, the detection of phosphopeptides by MS can be impaired by low ionization efficiency and signal suppression in the presence of non-phosphorylated species [6]. Therefore, the success of phosphoproteomic experiments greatly relies on the use of selective enrichment strategies, which decrease the number of unphosphorylated peptides in the sample, improving phosphopeptide identification by MS/MS sequencing.

There are several phosphoproteomic enrichment strategies that are typically performed after proteolytic digestion. The most widely applied method is based on chemical coordination by affinity chromatography, such as immobilized metal-ion affinity chromatography (IMAC) [7] and metal oxide affinity chromatography (MOAC) [8]. One of the issues associated with these techniques is the predominant enrichment of phospho-serine (pS) and -threonine (pT) peptides, as these are, by far, the most frequent phosphorylated species, whereby in mammalian systems only 1–2% of the detected phosphopeptides originate from tyrosine phosphorylated species. Another challenge is the unspecific binding of non-phosphorylated peptides in highly complex peptide mixtures (e.g. cell lysate digest), reducing the selectivity of the method.

Enrichment techniques are generally more efficient when the complexity of the sample is decreased, for instance in combination with a fractionation step prior to the enrichment. Several methods based on liquid chromatography (LC) are especially suited for this purpose, such as ion exchange chromatography or hydrophilic interaction liquid chromatography (HILIC) [9]. In large-scale phosphoproteomic studies, strong cation exchange (SCX) is by far the most common fractionation technique applied prior to either IMAC or TiO_2 chromatography [10–14], separating peptides by their solution charge state. In the last years, HILIC has been employed in phosphoproteomic studies, either before or after the enrichment step [15], since it is well suited for polar compounds [15–18]. Recently, we described a multidimensional (2D) approach where HILIC was used to fractionate phosphopeptide samples enriched by IMAC [19]. Although we achieved a high phosphoproteome coverage (over 20,000 phosphorylated species), only 1–2% (i.e. a few hundreds) was tyrosine phosphorylated (pY) peptides.

To overcome the underrepresentation issue, the investigation of tyrosine phosphorylation events has relied mostly on using specific antibodies for targeted immuno-affinity purification (IP). Initially, selective anti-phosphotyrosine antibodies were successfully employed for the enrichment of phosphotyrosine proteins from whole cell lysate digests [20,21]. Nowadays, IP is more often performed at the peptide level as this seems to be more efficient, whereby up to around 1000 pY peptides can be readily detected from a cellular lysate [22,23]. Nevertheless, some of the major disadvantages of using IP strategies at this stage are the large amounts of protein starting material required, namely mg of protein sample, and the irreproducibility of the IP, partly caused by the batch-to-batch variability of the available antibodies [24,25].

In this study, we set out to evaluate and systematically compare the pools of pY peptides enriched either by an antibody-based immunoaffinity enrichment (using pY99 antibodies) or by a multidimensional approach (2D) based on Ti^{4+}-IMAC affinity enrichment in combination with HILIC fractionation. Here, we aim to determine the preference (or bias) of both methods toward specific subsets of pY peptides and to understand if the bias introduced by each enrichment is an intrinsic property of the employed method or is a cell-dependent effect. Thus, we first chose to perform several antibody based immuno-affinity enrichments on two different cell lines (i.e. pervanadate-treated HeLa and K562 cells), generating relatively large pools of pY peptides. Then, we compared these identified pY peptides with those obtained from the aforementioned 2D strategy, and assessed differences in pY peptide sequences and other peptide physico-chemical properties that may affect the selectivity of each method. We find that both strategies are not redundant but more complementary and should be combined for a comprehensive view at phosphotyrosine signaling.

2. Materials & methods

2.1. Cell culture & treatment

HeLa cells and K562 cells were grown in either Dulbecco's modified Eagle's medium (HeLa) or RPMI medium (K562) supplemented with 10% fetal bovine serum, 10 mM Glutamine and 5% Penicillin/Streptomycin (Lonza, Belgium) at 37 °C in the

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