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Characterization of intact sialylated glycopeptides and phosphorylated glycopeptides from IMAC enriched samples by EThcD fragmentation: Toward combining phosphoproteomics and glycoproteomics

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ABSTRACT

Protein phosphorylation and glycoprotein sialylation play a major role in regulating numerous cellular and molecular processes. Several previous studies demonstrated that it is possible to combine in-depth analysis of the phosphoproteome and sialome with common phosphopeptide enrichment methods that simultaneously enrich for sialylated glycopeptides. However, earlier workflows included enzymatic release of glycans and separation of phosphopeptides and formerly glycosylated peptides before independent LC–MS/MS analyses. Here, we demonstrate that electron-transfer/higher-energy collision dissociation (EThcD) enables identification of intact sialylated N-glycopeptides from IMAC enriched samples without additional sample preparation. Furthermore, this method allows for identification of intact glycopeptide sequences modified by phosphorylated glycans such as mannose-6-phosphate (M6P). This suggests that EThcD fragmentation of IMAC enriched samples provides a relatively straightforward means of implementing sialome and M6P-proteome analysis into existing phosphoproteomic workflows.

1. Introduction

Phosphorylation and glycosylation are two of the most important and widely studied post-translational modifications (PTMs) as they regulate a variety of important biological processes and their dysregulation has been associated with disease [1–5]. However, the vast majority of studies focus on the large-scale characterization of either the glycoproteome [6,7] or phosphoproteome [8,9] as analysis of PTMs typically requires specialized sample preparation and analytical methods. MS-based characterization of PTMs relies on effective enrichment methods as modified peptides are present at substoichiometric levels and often suppress ionization efficiencies compared to unmodified peptides [10,11]. For example, immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO_2) chromatography are popular enrichments strategies that have been optimized to enable the identification of thousands or tens of thousands of phosphopeptides from biological samples by LC–MS/MS [12,13]. Phosphoproteomic techniques are relatively mature compared to glycoproteomic methods.

Glycoproteomics presents an enormous analytical challenge due to the microheterogeneity of glycan structures and the lability of glycosidic bonds [14–16]. Many glycoproteomic studies [6,7,17,18] rely on the enzymatic removal of the glycan side-chain and MS-based characterization of formerly glycosylated peptides due to a lack of MS-based fragmentation techniques and computational tools capable of sequencing intact glycopeptides. However, the analysis of intact glycopeptides provides site-specific glycan composition and microheterogeneity details unobtainable by analysis of deglycosylated peptides. Recently, the advancements of enrichments strategies [19–21], MS methods [22–26], and computational tools [27–33] have dramatically improved the analysis of intact glycopeptides.

Increasing evidence suggests the dynamic interplay between post-translational modifications (i.e., PTM crosstalk) plays a crucial

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role in regulating biochemical processes [34–37]. For example, sialylation of epidermal growth factor receptor (EGFR), a transmembrane tyrosine kinase, influences EGFR phosphorylation by attenuating the formation of EGFR dimers [38]. Recent advancements in MS-based technologies for proteomics and PTM characterization has allowed for combined analysis of multiple PTMs in a single study [39–41]. In addition to unraveling PTM crosstalk, the capability to simultaneously analyze multiple PTMs in MS-based proteomics studies yields a more complete picture of molecular and cellular processes.

Several studies have demonstrated TiO_2 chromatography and IMAC simultaneously enrich for phosphopeptides and sialylated glycopeptides [42–46]. Although these methods are more commonly used for phosphopeptide enrichment, sialylated peptides are also enriched because the negatively charged carboxylate group of the sialic acid interacts with TiO_2 and IMAC enrichment material in a similar fashion to the phosphate group of phosphopeptides. While most studies have focused on characterizing either the phosphoproteome or sialome with IMAC and TiO_2, several analytical strategies have been developed to characterize both [39,45]. For example, Larsen and coworkers developed a method that combined sequential elution from IMAC (SIMAC) enrichment, TiO_2 enrichment, enzymatic deglycosylation, hydrophilic interaction liquid chromatography fractionation, and nano-LC–MS/MS analysis to identify 10,087 phosphopeptides and 1810 previously sialylated N- glycopeptides in a comparison of human embryonic stem cells (hESCs) and neural stem cells (NSCs) [45]. Although the identification of such an impressive number of PTMs provided valuable insight into differences in hESC and NSC differentiation, a simplified sample preparation workflow for combined phosphoproteome and sialome analysis would increase the analytical throughput to allow for large-cohort studies in a reasonable amount of LC–MS/MS analysis time.

Here, we examine the potential of EThcD fragmentation for combining the analysis of phosphopeptides and intact sialylated glycopeptides. The development of EThcD [24] has been crucial for the advancement of intact glycopeptide analysis as it overcomes the limitations of HCD and ETD [15,47–49]. That is, HCD fragmentation of glycopeptides generates significant glycan sidechain fragmentation with limited peptide backbone sequence coverage. In contrast, ETD fragmentation of glycopeptides produces significant backbone fragments with limited glycan sidechain information. EThcD combines the complementary peptide and glycan sidechain fragment information from ETD and HCD to improve glycopeptide sequencing [51]. We compare two different EThcD fragmentation schemes for the characterization of phosphopeptides and glycopeptides from an IMAC enriched sample of rat smooth muscle cells (SMCs). Furthermore, we demonstrate EThcD is an effective method for identifying mannose-6-phopshote (M6P)-modified peptides. To our knowledge, this is the first large-scale characterization of phosphopeptides, sialylated glycopeptides, and M6P-modified peptides in a single LC–MS/MS analysis.

2. Experimental procedures

2.1. Sample digestion

Aortic smooth muscle cells (SMCs) were collected from male Sprague-Dawley rats. Cells were dissolved in 8 M urea, 50 mM Tris HCl, 30 mM NaCl, 5 mM $CaCl_2$,1 protease inhibitor tablet (Roche, Mannheim, Germany), 1 phosphatase inhibitor tablet (Roche, Mannheim, Germany), pH 8 buffer and lysed using a probe sonicator. Protein concentration was measured by a BCA assay kit (Thermo Fisher Scientific, San Jose, CA) and 2 mg was used for tryptic digestion protocol. Disulfide bonds were reduced with 5 mM dithiothreitol (DTT) for 1 h, alkylated with 15 mM iodoacetamide for 30 min in the dark, and alkylation was quenched with 5 mM DTT. Urea was diluted to 0.9 M and sequencing grade trypsin (Promega, Madison, WI) was added at 50:1 Protein:Trypsin and incubated at 37 °C for 18 h. Tryptic peptides were desalted with a C18 SepPak (Waters, Milford, MA).

2.2. Fe^{3+}-IMAC enrichment

IMAC enrichment protocol was adapted from Rose et al. [50]. Ni-NTA magnetic agarose beads (Qiagen, Hilden, Germany) were washed with H_2O (3x), incubated with 40 mM EDTA (pH 8) for 30 min on a vortex mixer, washed with H_2O (3x), incubated with 100 mM $FeCl_3$ on a vortex mixer for 30 min, and washed with 0.15% trifluoroacetic acid (TFA) 80% ACN (5x). SMC digest was reconstituted in 0.15% TFA 80% ACN and incubated with beads on a vortex mixer for 30 min. Beads were washed with 0.15% TFA 80% ACN (3x) to remove unbound peptides. Enriched peptides were eluted with 0.7% NH_4OH 50% ACN into 4% FA and immediately dried by vacuum centrifugation. Samples were stored at −80 °C and reconstituted in 20 μl 0.1% formic acid (FA) before LC–MS/MS analysis.

2.3. NanoLC–MS/MS analysis

Samples were analyzed with an Ultimate 3000 nanoLC coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). 2 μl of sample was injected onto a 75 μm × 15 cm homemade column with integrated emitter tip and packed with 1.7 μm, 130 Å, BEH C18 material from a Waters UPLC column (Waters, Milford, MA). Peptides were separated at a flow rate of 0.3 μl min^{-1} with a gradient that ramped from 3% solvent B (0.1% FA in ACN) to 30% solvent B over 80 min and from 30% solvent B to 70% solvent B over 20 min.

We tested two different MS methods in this study. An EThcD method optimized for phosphopeptides and a HCD-product-dependent-EThcD (HCD-pd-EThcD) method optimized for glycopeptides. EThcD method is a top 15 data-dependent acquisition (DDA) in which all MS/MS experiments use EThcD fragmentation. HCD-pd-EThcD method is a product-dependent method [23] in which HCD fragmentation is performed with top speed (3 s) DDA and EThcD fragmentation is triggered by measurement in the HCD scan of product ions at m/z 138.0545 (HexNAc marker), 204.0867 (HexNAc), and 366.1396 (HexNAcHex). HCD activation was performed with 35% collision energy and fragments were measured in the OT with 30,000 resolution and 1.0e5 target automatic gain control (AGC). In general, EThcD parameters are similar between both methods with a few exceptions. Both methods use charge state optimized ETD reaction times of 50 ms, 20 ms, and 10 ms for charge states 2+, 3–4+, and 5+, respectively [51]. ETD reactions time for charge states >6+ were 9 ms for EThcD method and calibrated charge dependent ETD parameters were used for HCD-pd-EThcD method. Supplemental HCD activation energy is 33% for both methods. MS survey scans were 300–1500 at orbitrap (OT) resolution of 60,000 for EThcD. Whereas MS survey scans were 400–1800 at orbitrap (OT) resolution of 120,000 for HCD-pd-EThcD. AGC target for EThcD orbitrap scans was 5.0e4 and 3.0e5 for the EThcD and HCD-pd-EThcD methods, respectively.

2.4. Data analysis

Mass spectra were analyzed using the Byonic (Protein Metrics, San Carlos, CA) node in Proteome Discoverer 2.1 (Thermo Fisher Scientific, San Jose, CA). Raw files were searched against *Rattus norvegicus* protein database of reviewed (Swiss-Prot) and unreviewed (TrEMBL) sequences downloaded from Uniprot on 03/28/17. Trypsin was selected as the digestion enzyme with a