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Enhancing Phosphotyrosine Proteome Coverage using a Combined ETD and CID Approach on a LTQ Orbitrap XL ETD

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Key Words

- LTQ Orbitrap XL ETD
- Electron Transfer Dissociation, ETD
- Data-Dependent Decision Tree, DDDT
- Proteome Discoverer Biosoftware Suite
- Phospho-Proteome Analysis

Introduction

Reversible tyrosine phosphorylation plays important roles in numerous cellular processes such as growth, differentiation and migration. Phosphotyrosine signaling is tightly controlled by the balanced action of protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). In multiple cancers, aberrant tyrosine phosphorylation has been suggested to be the underlying course[1]. Therefore the detection and site specific localization of tyrosine phosphorylation has emerged over the past years. Low abundances and the dynamic nature of tyrosine phosphorylation cause detection of this modification to be problematic. The situation is exacerbated by the higher abundance of non-phosphorylated peptides since these non-modified peptides are preferentially selected for fragmentation by mass spectrometers. In addition, large scale phosphoproteomic approaches based on, for example, immobilized metal affinity chromatography (IMAC) or TiO_2 generally reveal only low amounts of tyrosine phosphorylation sites due to the more dominant peptides with serine and threonine phosphorylation[2-4].

A variety of strategies ranging from mass spectrometric (MS) based methods to phosphopeptide enrichment prior to MS have been developed to overcome the underrepresentation of tyrosine phosphorylation[5, 6, 8]. Immuno-affinity enrichment by antibodies directed against the tyrosine phosphorylated residue can be used for both tyrosine phosphorylated proteins and peptides. Currently, immuno-affinity purification (IP) of tyrosine phosphorylated peptides has demonstrated a high success rate for detection and site localization of tyrosine phosphorylation[5-7]. Despite recent advances, the tyrosine phosphoproteome is far from comprehensive and therefore ongoing method development is still essential. Here, we utilized the Thermo Scientific LTQ Orbitrap XL ETD to identify the site of phosphorylation from a peptide immuno-affinity purification of pervanadate treated HeLa cells.

Mass Spectrometric Strategy

The analytical strategy is based on the use of the two discrete and yet complimentary dissociation techniques collision induced dissociation (CID) and electron transfer dissociation (ETD) for the identification of the peptide as well as for the unambiguous determination of the tyrosine phosphorylation site.

We will compare two approaches: One is the fragmentation of every peptide by both CID and ETD, the other approach is the use of the most efficient dissociation technique depending upon the peptide's property such as mass-to-charge ratio *m/z* and charge state *z* (so-called data-dependent decision tree, DDDT)[9]. The LTQ Orbitrap XL ETD™ is capable of acquiring spectra with high resolution and accuracy in the orbitrap detector and uses the linear ion trap for either CID or ETD fragmentation. High resolution is the prerequisite for the unambiguous on-the-fly charge state determination of precursor ions. Using CID and ETD on the same precursor ions can improve the probability of the sequencing success but might be too time consuming for the analysis of complex samples. The DDDT therefore uses the information of the full scan spectra to make *a priori* decisions in real time about which fragmentation method to apply to increase the probability of efficient fragmentation for all precursors.

Results and Discussion

HeLa cells were treated with 1mM pervanadate for 10 minutes and proteins were denatured and extracted using 8M urea. Enzymatic degradation of the protein pool was achieved followed by peptide immuno-affinity purification by an anti-phosphotyrosine antibody. The peptides eluted from the immuno-affinity resin were analyzed in two LC-MS/MS runs. In the first run, we performed both CID and ETD on the same precursor (classical). In the second run we used the data dependent decision tree to get optimal sequencing of the peptide precursor (DDDT).

Both runs were processed with the Thermo Scientific Proteome Discoverer software suite. ETD and CID spectra were processed independently, using the non-fragment filter for ETD spectra. This filter removes all typical non-sequence diagnostic fragment ion peaks from the spectra such as peaks corresponding to unreacted precursor, the charge reduced species and their neutral losses. Removal of the non-fragment ion peaks decreases the possibility of false positives and increases the search specificity. The processed spectra (DTA files) were merged into a single text file before database searching using the Mascot™ search engine. In the initial, standard experiment we identified 154 unique tyrosine phosphorylated peptides (Mascot score ≥ 20). The majority of sites (56%) were identified from both CID and ETD spectra (Figure 1).

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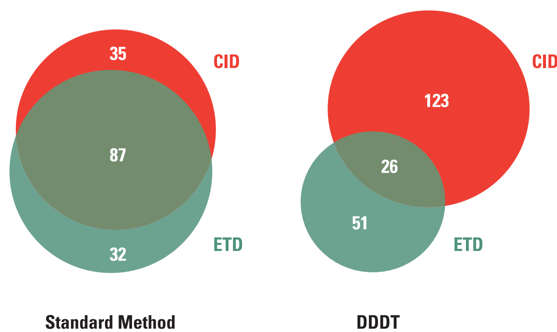

Figure 1: Identified tyrosine phosphorylated peptides from 'classic' and DDDT method.
a) Overlap between identified phosphopeptides identified from CID and ETD in the same run using the classic method, systematically performing CID and ETD fragmentation on the same peptide precursor.
b) Overlap between identified phosphopeptides identified from CID and ETD in the same run using the DDDT method, choosing the optimal dissociation method for each peptide precursor.

However, 35 phosphopeptides were exclusively identified with CID fragmentation, whereas 32 phosphopeptides were exclusively identified with ETD fragmentation. Although we performed specific enrichment of tyrosine phosphorylated peptides, the LC-MS/MS runs can still be complex due to the presence of contaminating non-phosphorylated peptides (data not shown). Complex samples, such as phosphotyrosine peptide IPs would benefit from the application of the optimal dissociation method for each precursor, rather than doubling the experiment time by performing both CID and ETD on every precursor. Therefore, we used a data dependent decision tree (DDDT), to make real-time decisions on which dissociation method to use. In the DDDT experiment, a total number of 200 unique tyrosine phosphorylated peptides was identified. Only 13% of the peptides (26 phosphopeptides) were identified from both CID and ETD spectra (Figure 1). 128 phosphopeptides were identified exclusively after CID fragmentation and 51 phosphopeptides were identified exclusively after ETD fragmentation. This resulted in a 30% increase in the total number of tyrosine phosphopeptide identifications in the DDDT method, clearly indicating that this method is superior to the 'classic' method.

In the gas-phase phosphorylation is typically a labile modification which undergoes facile neutral loss of phosphoric acid under CID conditions although this issue is much reduced for pY. The tendency to lose the phosphate moiety decreases in the order pS > pT >> pY [10]. ETD preserves labile modifications and is therefore suited for phosphorylation analysis. ETD is particularly successful with multiply charged peptides, i.e. with peptides with three or more charges. Doubly charged peptides do not fragment efficiently in ETD due to the initial charge reduction but usually result in identifiable spectra. The DDDT method takes this behaviour into account by subjecting all doubly charged peptide precursor ions to CID and use ETD and CID on the higher charged peptides depending on the *m/z* of the peptide precursor ions.

Enzymatic degradation typically produces quite short peptide stretches which predominantly ionize doubly charged in electrospray ionization. This explains why the number of identified peptides with CID is considerably higher in the DDDT method compared to the classical method. The example spectra in Figure 2 show the complementary character of CID and ETD for the identification of tyrosine phosphorylated peptides and the determination of the phosphorylation sites. Figure 2a shows a doubly tyrosine phosphorylated peptide that was identified by Mascot with a high score for the ETD spectrum and a low score for the CID spectrum. Although the peptide only contains two tyrosine residues, the series of z-type ions allows the unambiguous determination of those two tyrosine residues as phosphorylated excluding the two serine and three threonine residues in this peptide. This peptide is an example for the often observed trend that longer peptides with higher precursor charge states are more successfully identified by ETD. Nearly all bonds were cleaved by ETD (19 out of 21 possible peptide bonds). The two remaining bonds are associated to proline residues which ETD cannot cleave due to the cyclic character of proline (indicated by the red dotted line). Thus, ETD generated a comprehensive coverage of this peptide opening the way to allow *de novo* sequencing. In contrast, CID only produced fragment ions below *m/z* 1100. The yellow marked areas in the ETD spectrum indicate the *m/z* areas of the precursor, the charge reduced species and the neutral loss from the charge reduced precursors that are deleted from the mass list before submission to the search engines by the "Non-Fragment Filter" node in Proteome Discoverer.

Figure 2b shows an example of a monotyrosine phosphorylated peptide with a higher Mascot score for the CID spectrum. This is a typical example where CID fragments doubly charged precursor ions more efficiently compared to ETD. The fragmentation efficiency of doubly charged precursor ions with ETD is typically low as the ETD process is initiated by one charge neutralization resulting in only one charge available as charge for the fragment ions. In addition in this example, the peptide contains four proline residues. The peptide contains only one possible phosphorylation site and an almost complete y-ion series result in a confident identification.

The third example in Figure 2c shows an example for a monotyrosine phosphorylated peptide that has been identified with high confidence with both CID and ETD although doubly charged precursor ions have been fragmented. Almost complete y- and z-type fragment ion series allow unambiguous identification and phosphorylation site determination.

Summary

We have shown that CID and ETD are truly complimentary fragmentation techniques and their combined use greatly enhances the phosphoproteome coverage compared to MS methods that are solely based on CID. We have shown that a significant number of phosphopeptides can only be identified by ETD and the intelligent use of CID and ETD maximizes the outcome of our phosphoproteomics experiment.

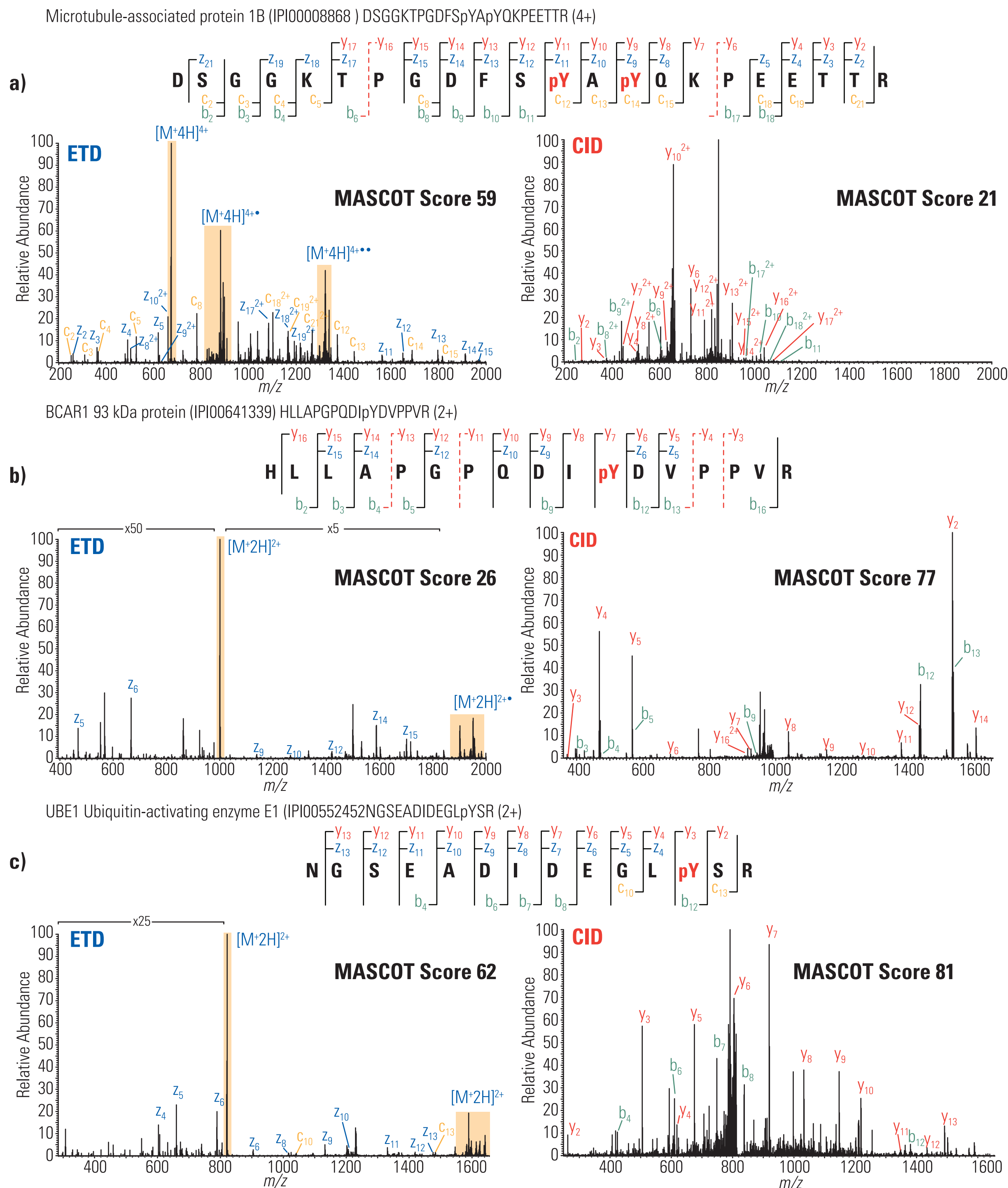

Figure 2: Example spectra of identified phosphotyrosine peptides.
a) ETD of the quadruply charged peptides results in a higher Mascot score compared to CID.
b) CID of the doubly charged peptide results in a higher Mascot score compared to CID.
c) Both CID and ETD of the doubly charged peptide result in high Mascot scores.

Materials and Methods

HeLa cells were grown in confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 0.05 mg/ml penicillin / streptomycin (Invitrogen). Cells were placed in serum-free medium 16 h before pervanadate treatment. Cells were treated with 1 mM pervanadate for 10 min. Cells were washed with cold phosphate buffered saline (PBS) and lysed. Cells were lysed in 8 M urea / 50 mM ammonium bicarbonate 5 mM sodium phosphate, 1 mM potassium fluoride, 1 mM sodium orthovanadate and EDTA-free protease inhibitor cocktail (Sigma, St. Louis, MO, USA). Samples were reduced with dithiothreitol (DTT) at a final concentration of 10 mM at 56°C. Subsequently samples were alkylated with iodoacetamide at a final concentration of 55 mM at room temperature. The samples were diluted to 2 M urea / 50 mM ammoniumbicarbonate and degradation enzymes (1:100) was added. Digestion was performed overnight at 37°C.

Immuno-Affinity Purification

Peptides were desalted, dried down and re-dissolved in immuno-affinity purification buffer. The peptide mixture was added to the pY99 agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubation was performed overnight at 4°C. Beads were washed three times and peptides were eluted by adding 0.15% TFA for 20 min at room temperature. Eluted peptides were desalted and concentrated on Stage-tips.

On-Line Nanoflow Liquid Chromatography

Nanoflow LC-MS/MS was performed by coupling an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) to a Thermo Scientific LTQ Orbitrap XL ETD mass spectrometer as described previously[2]. Dried fractions were reconstituted in 10 µl 0.1 M acetic acid and delivered to a trap column (AQUA™ C18, 5 µm, Phenomenex, Torrance, CA, USA); 20 mm × 100 µm ID, packed in-house) at 5 µl/min in 100% solvent A (0.1 M acetic acid in water). Subsequently peptides were transferred to an analytical column (ReproSil-Pur™ C18-AQ, 3 µm, Dr. Maisch GmbH, Ammerbuch, Germany; 40 cm × 50 µm ID, packed in-house) at ~100 nl/min in a 3 hr gradient from 0 to 40% solvent B (0.1 M acetic acid in 8/2 (v/v) acetonitrile/water). The eluent was sprayed via distal coated emitter tips (New Objective, Woburn, MA, USA), butt-connected to the analytical column.

Methods CID and ETD

The LTQ Orbitrap XL ETD performed a full MS scan (RP 60,000 FWHM) followed by data-dependent CID and ETD MS/MS scans with detection of the fragment ions in the linear ion trap. Target values were 5e5 for FTMS full scans and 3e4 for ion trap MS^n scans. Anion target value was 1e6. ETD activation time was 100 msec. Supplemental activation was used for all ETD MS/MS scans. The decision tree was set up to fragment all doubly charged ions, all triply charged ions above *m/z* 650, all quadruply charged ions above *m/z* 900 and all higher charged ions above *m/z* 950 with CID and all other precursor ions with ETD.

Data Analysis with Proteome Discoverer

Data was analyzed using the Proteome Discoverer™ 1.0 software suite. ETD spectra were preprocessed using the non-fragment filter[11].

Data Analysis

Runs were searched using an in-house licensed Mascot search engine (Mascot (version 2.1.0) software platform (Matrix Science, London, UK)) against the Human IPI database version 3.36 (63012) with carbamidomethyl cysteine as a fixed modification. Oxidized methionines and phosphorylation of tyrosine, serine and threonine were set as variable modifications. For the proteolytic enzyme up to two missed cleavages were allowed. The mass tolerance of the precursor ion was set to 5 ppm and that of fragment ions was set to 0.6 Da.

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