Strong Cation Exchange-based Fractionation of Lys-N-generated Peptides Facilitates the Targeted Analysis of Post-translational Modifications*

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In proteomics multi-dimensional fractionation techniques are widely used to reduce the complexity of peptide mixtures subjected to mass spectrometric analysis. Here, we describe the sequential use of strong cation exchange and reversed phase liquid chromatography in the separation of peptides generated by a relatively little explored metallo-endopeptidase with Lys-N cleavage specificity. When such proteolytic peptides are subjected to low-pH strong cation exchange we obtain fractionation profiles in which peptides from different functional categories are well separated. The four categories we distinguish and are able to separate to near completion are (I) acetylated N-terminal peptides; (II) singly phosphorylated peptides containing a single basic (Lys) residue; (III) peptides containing a single basic (Lys) residue; and (IV) peptides containing more than one basic residue. Analyzing these peptides by LC-MS/MS using an ion trap with both collision as well as electron transfer-induced dissociation provides unique optimal targeted strategies for proteome analysis. The acetylated peptides in category I can be identified confidently by both CID and ETcaD, whereby the ETcaD spectrum shows the exact location of possible phosphorylation sites and the "normal" single Lys containing peptides in category III ETcaD provides unique straightforward sequence ladders of c'-ions, from which the exact location of possible phosphorylation sites can be easily determined. The later fractions, category IV, require analysis by both ETcaD and CID, where it is shown that electron transfer dissociation performs relatively well for these multiple basic residues containing peptides, as is expected. We argue that the well resolved separation of functional categories of peptides observed is characteristic for Lys-N-generated peptides. Overall, the combination of Lys-N proteolysis, low-pH strong cation exchange, and reversed phase separation, with CID and ETD induced fragmentation, adds a new very powerful method to the toolbox of proteomic analyses. Molecular & Cellular Proteomics 8:190–200, 2009.

The enormous complexity of the proteome poses a considerable analytical challenge for global protein identification. An additional order of complexity is caused by protein post-translational modifications of which many different variants are known (1). Mass spectrometry (MS)¹ is nowadays, routinely used for such complex protein identification and characterization studies (2, 3). Common practice in high throughput global proteome analyses is to start by proteolytic cleavage of all proteins using, most often, trypsin. The so formed peptides are then separated by reversed phase (RP) nanoflow liquid chromatography (nanoLC) and subjected to tandem MS sequencing with CID as the preferred fragmentation method (3). Protein identification is then accomplished by searching the acquired peptide fragmentation data against large protein sequence databases, which is greatly aided by the availability of sequenced genomes (4). The above described general procedure for proteomics analysis provides a far from comprehensive view of the proteome and leaves room for improvement in areas such as proteolytic cleavage (5, 6), peptide enrichment and fractionation (7–18), and peptide activation and fragmentation inside the mass spectrometer (19–22). More specifically, smart combinations of proteases, peptide separations, and peptide fragmentation techniques may allow the targeted analysis of specific functional groups of peptides, enabling the analysis of low abundant proteins and peptides.

Recently, electron transfer dissociation (ETD) of peptides was introduced as an alternative peptide fragmentation method. ETD provides very unique mass spectrometry data, which allows for unambiguous peptide and protein identification (23). However, the electron transfer dissociation process is not straightforward and the acquisition of high quality ETD spectra requires specific instrument conditions (24). Furthermore, the number of available peptide fragmentation methods is limited, and in cases where peptides contain multiple basic residues, the performance of CID is very poor (25). In such cases, electron transfer-induced dissociation (ETcaD) provides unique straightforward sequence ladders of c'-ions, from which the exact location of possible phosphorylation sites can be easily determined. The later fractions, category IV, require analysis by both ETcaD and CID, where it is shown that electron transfer dissociation performs relatively well for these multiple basic residues containing peptides, as is expected. We argue that the well-resolved separation of functional categories of peptides observed is characteristic for Lys-N-generated peptides. Overall, the combination of Lys-N proteolysis, low-pH strong cation exchange, and reversed phase separation, with CID and ETD induced fragmentation, adds a new very powerful method to the toolbox of proteomic analyses. Molecular & Cellular Proteomics 8:190–200, 2009.

¹ The abbreviations used are: MS, mass spectrometry; SCX, strong cation exchange; RP, reversed phase; nanoLC, nanoflow liquid chromatography; ETD, electron transfer dissociation; ESI, electrospray ionization; ETcaD, electron transfer collisional-activated dissociation; HEK, human embryonic kidney; HPLC, high pressure liquid chromatography; ACN, acetonitrile; LC-MS/MS, liquid chromatography tandem mass spectrometry; FA, formic acid; CID, collision induced dissociation.

method. ETD cleaves peptides at the N-C\(_\text{\textendash}}\) bond producing c\(^{\text{-}}\) and z-type ions (23–25). From the early work it is clear that ETD can be complementary to CID because it prefers larger and more basic peptides, which attain multiple charges during electrospray ionization (ESI) (26, 27). Reduced fragmentation efficiency of ETD for doubly charged peptides is compensated by the use of supplemental collisional activation (ETcaD) (28). Interestingly, ETD (and ETcaD) leaves post-translational modifications largely intact on the peptide backbone during fragmentation thus providing, potentially, simpler spectra in which the site of modification can be easily annotated (29). Recently, we explored and introduced the use of a relatively little known metallo-endopeptidase for digestion of proteins in combination with ETD (30). This metallo-endopeptidase, termed Lys-N, has enzymatic cleavage specificity for lysine residues with cleavage occurring at the N-terminal side (31, 32). Lys-N was shown to be as sensitive and selective as currently used proteases and can be used for both in-gel and in-solution digestion experiments (30). Our preliminary data indicated that the resulting proteolytic peptides are favorable for ETD sequencing, with respect to peptide size and the number of charges after ESI, with the added advantage of the lysine residue being situated at the peptide N terminus. A large proportion of these proteolytic Lys-N peptides do not contain any other basic residue leaving only two basic entities each residing at the N terminus. We argued and demonstrated that the strong basic nature of the N-terminal side of Lys-N proteolytic peptides attracts the proton providing the final fragment ion charge before/after electron transfer, causing the observed fragments to be almost exclusively c\(^{-}\)-type ions. Utilizing this unique fragmentation behavior of Lys-N peptides under ETcaD conditions could markedly reduce the dependence on sequenced genomes and open up a complete new window for de novo sequencing and potentially the analysis of post-translational modifications in a facile manner (30). In the accompanying paper we show that this unique feature is not solely a characteristic of ETD fragmentation, as the same category of peptides provides straightforward peptide sequence ladders in MALDI-CID-MS/MS fragmentation.\(^2\)

Here, we further explore the potential of this protease in proteomics, focusing on the analysis of post-translational modifications. We developed a method for global protein analysis of whole cell lysates using a combination of Lys-N proteolytic cleavage followed by low-pH strong cation exchange (SCX) fractionation and RP-nanoLC-ETcaD-MS analysis and RP-nanoLC-CID-MS analysis. Low-pH SCX chromatography has shown to be a valuable tool for phosphopeptide enrichment after tryptic digestion, although often they still co-elute with (more abundant) acidic and N-acetylated peptides (8, 34, 35). We show that the combination of Lys-N and low-pH SCX is an ideal combination for global proteome and phosphoproteome analysis, as well as for the selective enrichment and analysis of protein N-terminal peptides (36, 37). We argue that the benefits of this approach are multiple because we take full advantage of the proteolytic peptide properties after Lys-N digestion in both SCX enrichment and ETcaD analysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Protease inhibitor mixture was obtained from Roche Diagnostics. Metallo-endopeptidase from Grifola Frondosa (Lys-N) was obtained from Seikagaku Corporation (Tokyo, Japan). Iodoacetamide and sodium orthovanadate were obtained from Sigma-Aldrich. Dl-Dithiothreitol was obtained from Fluka Biochemical (Steinheim, Germany). Human embryonic kidney (HEK) 293 cells were provided by Dr. Pantelis Hatzis and Dr. Tokameh Mahmoudi from the Netherlands Institute for Developmental Biology, Hubrecht Institute, The Netherlands. HPLC-S gradient grade acetonitrile was purchased from Biosolve ( Valkenwaard, The Netherlands). Acetic acid was obtained by MERCK KGaA (Damstadt, Germany) and high purity water obtained from Milli-Q system (Millipore, Bedford, MA).

**HEK 293 Cells**—Approximately 6 \times 10\(^{\text{6}}\) HEK 293 cells were harvested by centrifugation for 5 min at 1200 rpm and after removal of the medium, cells were resuspended in 50 mM ammonium bicarbonate containing 25 mM sodium phosphate, 1 mM potassium fluoride and 1 mM sodium orthovanadate. After centrifugation at 1200 rpm and removal of the supernatant ice-cold lysis buffer (50 mM ammonium bicarbonate containing 8 M urea, protease inhibitor, 5 mM sodium phosphate, and 1 M potassium fluoride and 1 M sodium orthovanadate) was added to the cell pellet and the cells were lysed on ice for 30 min. Subsequently, centrifugation at 20000 \times g in a tabletop centrifuge (Eppendorf, Hamburg, Germany) at 4 °C separated the soluble and insoluble protein fractions. The soluble fraction was collected and the protein concentration was determined by a Bradford assay to be 12.3 mg.

**Lysate In-solution Digestion**—One mg of lysis was reduced with 45 mM dithiothreitol (50 °C, 15 min) followed by alkylation using 100 mM iodoacetamide (dark, RT, 15 min) and digested with Lys-N. Lys-N was added at a ratio of 1:85 (w/w) and the sample was incubated over night for 37 °C. The digest was dried in a vacuum centrifuge and resuspended in 0.05% formic acid (FA).

**SCX Chromatography**—SCX was performed using an Agilent 1100 HPLC system (Agilent Technologies) with two C18 Opti-Lynx (Optimized Technologies, Oregon OR) guard columns and a polysulfoethyl A SCX column (PolyLC, Columbia, MD; 200 mm × 2.1 mm inner diameter, 5 \(\mu\)m, 200-Å). The digested cell lysate was dissolved in 0.05% formic acid, and 750 \(\mu\)g was loaded onto the guard column at 100 \(\mu\)L/min and subsequently eluted onto the SCX column with 80% acetonitrile (ACN) and 0.05% formic acid. SCX buffer A was made of 5 mM KH\(_{2}\)PO\(_4\), 30% ACN and 0.05% FA, pH 2.7; SCX buffer B consisted of 350 mM KCL, 5 mM KH\(_{2}\)PO\(_4\), 30% ACN and 0.05% FA, pH 2.7. The gradient was performed as follows: 0% B for 10 min, 0–85% B in 35 min, 85–100% B in 6 min and 100% B for 4 min. A total of 49 fractions were collected and dried in a vacuum centrifuge.

**ETD Experiments**—The 49 dried SCX fractions were diluted in 100 \(\mu\)L of 10% formic acid, and 1/20 (5 \(\mu\)L) of all the SCX fractions were subjected to nanoscale liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) analysis, performed on an Agilent 1100 HPLC system (Agilent technologies) connected to a LTQ XL Linear Ion Trap Mass Spectrometer with an ETD source at the back from Thermo Fisher Scientific, Inc. (Waltham, MA).

The instrument was equipped with a 20 mm × 100 \(\mu\)m inner diameter. Aqua C18 trap column (Phenomenex, Torrance, CA) and a...
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200 mm × 50 μm inner diameter Reprosil C18 RP analytical column (Dr. Maisch, Ammerbuch-Entringen, Germany). Trapping was performed at a flow of 5 μl/min for 10 min, and the fractions were eluted using a 75-min linear gradient from 0 to 40% solvent B (0.1% acetic acid in 80% ACN (v/v), in which solvent A was 0.1% acetic acid, 40 to 100% solvent B in 2 min, and 100% B for 2.5 min. The flow rate from the LC was passively split from 0.360 ml/min to 100 nl/min. The column effluent was directly introduced into the ESI source of the MS using a standard coated fused silica emitter (New Objective, Woburn, MA) (outer diameter 360 μm, tip inner diameter 10 μm) biased to 1.7 kV. The mass spectrometer was operated in positive ion mode, from 350 to 1500 m/z in MS mode, and with an automatic gain control value of 1.00e+ 05 and a max injection time of 50 ms. Parent ions were isolated for a more accurate measurement by performing a single ion monitoring scan and fragmented by CID and ETD in data-dependent mode with an automatic gain control value of 3.00e+ 04 and a max injection time of 500 ms. Ions were fragmented using CID with normalized collision energy of 35 and 30 ms activation time. ETD fragmentation was performed with supplemental activation; fluorescence was used as reagent anion, and ion/ion reaction in the ion trap was taking place for 100 ms.

Protein Identification—Raw MS data were converted to peak lists using Bioworks Browser software, version 3.1.1. Spectra were searched against the International Protein Index Human database version 3.36 (69012 sequences; 29002682 residues) using Mascot software version 2.2.0, with Lys-N cleavage specificity. The database search was made with the following parameters set to consider a peptide tolerance of ±0.5 Da, a fragment tolerance of ±0.6 Da, allowing two missed cleavages, Carbamidomethyl (C) as fixed modification, Oxidation (M), Phosphorylation (ST), Phosphorylation (Y), and Acetylation (N-terminal) as variable modifications. Tandem mass spectra assigned with a Mascot Score ≥30 (p value ≤0.05) were accepted providing a false discovery rate for the CID data of 4.68% and 2.53% for ETD, determined using a decoy database. Mascot interpretation was accepted for N-terminal acetylation and phosphorylation site assignment. All data are stored in the public depository PRIDE under accession numbers, 3746–3843, and the project description: “A simple strategy for straightforward proteome analysis by a combination of Lys-N, strong cation exchange and electron transfer dissociation”.

Lys-N Fragment Ion Statistics—For the calculation of the frequency of occurrence of c- and z-type ions ETD MS/MS fragmentation spectra of unique peptides with a minimum Mascot Score of 30 and with a Lysine at the N terminus (this does not apply for N-acetylated peptides) were considered. The Mascot identification of the spectra was used for automated peak fragment ion counting. For ETD spectra, exceptions occurred when c- or z-type ions (or related ions with ammonia/water losses) were assigned to the same isotope cluster in which case the most appropriate assignment (e.g. based on mass accuracy trend, mono isotopic peak etc) was chosen in an automated fashion.

RESULTS

We explored a method for global protein analysis of whole cell lysates using a combination of Lys-N proteolytic cleavage followed by low-pH SCX fractionation and RP-nanoLC-MS/MS analysis using both CID and ETD. After in-solution digestion of a whole HEK 293 cell lysate with Lys-N, the resulting peptides were separated into 49 fractions using a low-pH SCX separation. SCX chromatography separates peptides primarily based on their charge state in-solution, which is governed by the protonation and deprotonation of the basic and acidic residues, and thus the pH of the solution. We hypothesized that low-pH SCX chromatography of Lys-N proteolytic peptides would be an ideal tool for the separation and enrichment of acetylated protein N termini, phosphorylated and unphosphorylated single lysine containing peptides, as illustrated schematically in Fig. 1. Many of the Lys-N-generated peptides will contain a single basic lysine residue at the N terminus of the peptides and therefore carry two positive charges in solution (30). Similarly, a significant number of the Lys-N-generated peptides with acetylated N termini (i.e. mostly protein N termini) will not contain any basic group and therefore be uncharged. Singly phosphorylated Lys-N peptides with no additional basic residues will carry a single charge in solution and should thus potentially be separated from the unphosphorylated single lysine containing peptides and, more importantly, from the acetylated protein N-terminal proteolytic peptides described above. Naturally, in the final SCX fractions peptides will be found that contain multiple basic residues, for instance because of the presence of arginine and histidine or more than one lysine residues because of miss-cleavages. This clear separation as represented in Fig. 1 will allow prior knowledge of each fraction composition (i.e. “peptide must be N-terminally acetylated” or “peptide must contain a single lysine and one phosphorylated residue”) that can be used in the database analysis. This can lead to the removal of certain peptides that are false positives leaving behind a dataset with a higher overall confidence.

We hypothesized that ionization of peptides from the early eluting SCX fractions by ESI could be less efficient because of the lack of potential basic protonation sites. If these peptides would attain just a single charge in the ESI process, analysis with ETD would not be possible because ion-neutralization will occur by the invoked charge reduction. To probe this effect we decided to conduct alternating ETcaD and CID fragmentation experiments on the same precursors. Each of the 49 SCX fractions was analyzed using RP-nanoLC-MS/MS using in each single run alternating ETcaD and CID activation and fragmentation. The resulting spectra were searched against the International Protein Index human database using the Mascot search engine, taking a Mascot Score ≥30 as threshold. We classified, for each of the SCX fractions, all identified peptides into 8 different categories, based on the number of basic residues (from 1 to 6) present in the peptide. Additionally, categories were defined for acetylated N-terminal peptides and phosphorylated peptides containing only a single lysine residue.

As can be seen in Fig. 2, low-pH SCX separation of the Lys-N-digested peptides results in a clear fractionation of all these above-mentioned categories of peptides. Specifically, the enrichment of N-acetylated and singly phosphorylated peptides is outstanding. Nearly all of the acetylated N-terminal peptides are distinctly separated from the singly phosphorylated peptides, both for the peptides identified by ETD (Fig. 2A) and CID (B). Statistical analysis of the data (Fig. 2) show
that the fractions 7 till 18 contain predominantly acetylated protein N-terminal peptides (92% in ETD and 80% in CID) and only a very small number of phosphopeptides (4% in both ETD and CID). The following 8 SCX fractions (19–27) contain only ~3% acetylated protein N-terminal peptides compared with more than 75% singly phosphorylated peptides, illustrating nicely the separation power of the combination of Lys-N digestion and low-pH SCX separation. However, in the later SCX fractions (24–27) also several non-phosphorylated peptides were identified, which predominantly contained just a single lysine basic residue. Closer inspection of the fractions containing the N-acetylated protein termini resulted in the observation that most of these peptides contained a single basic residue. The acetylated N-terminal peptides without basic residues are present in the very early fractions (i.e. 4–6) whereas the following fractions contain almost exclusively peptides with one basic residue. This observation explains why we identify so many of the N-terminal peptides in the ETcaD tandem mass spectra, we observed that the majority of the doubly charged acetylated protein N-terminal peptide precursor ions lead to clear MS/MS spectra mainly consisting of z-fragment ions (as illustrated in Fig. 3, A and C). We believe that this preference for the formation of z'-ions is caused by the acetylation of the N terminus and the presence of a basic residue close to the C terminus, which directs the remaining proton to the C terminus. In agreement with this hypothesis, N-acetylated peptides with no basic residues lead to the

**Fig. 1. Expected SCX separation scheme for peptides from a Lys-N digest.** The horizontal axis shows an increase in salt concentration in the eluted sample, and the vertical axis shows an increase in intensity of the eluted peptide fractions. The uncharged Lys-N peptides are by theory expected to elute first from an SCX column and thereafter the singly charged Lys-N peptides and so forth. Using Lys-N, acetylated, and phosphorylated peptides should be separated on the SCX column, as they possess different charge states in-solution.
abundant formation of both c’- and z-fragment ions (see supplemental dataset present in PRIDE, accession numbers 3746–3843). As demonstrated in Fig. 4, the dominant presence of Z-ions is consistent over the whole range of SCX fractions from 9 to 17, containing primarily the N-acetylated protein N termini with a single basic residue. In Fig. 3 (B and D), the associated CID spectra of the same peptides are shown. Although the CID spectra are more fragment ion-rich, they do provide a less clear picture of the peptide sequence when compared with the ETD spectra given in Fig. 3, A and C.

Elution of phosphorylated peptides in SCX overlaps marginally with peptides containing a single lysine residue and no post-translational modifications, as can be seen in Fig. 2. The phosphorylated peptides were mainly observed as doubly charged ions after ESI and contained exclusively a single N-terminal lysine as the only basic residue. ETcaD fragmentation of these doubly charged phosphorylated peptides results in “clean” straightforward interpretable spectra consisting predominantly of c’-type ions, as observed and reported previously for their non-phosphorylated counterparts (30). Utilizing Mascot annotation we calculated the frequency of the various fragment ions observed, and we found that over 90% of the ions were N-terminal in nature (i.e. c-type ions), as illustrated in Fig. 4. Interestingly, these ETcaD spectra require very little effort to interpret because clear sequence ladders are formed with the phosphor group left intact on the peptide backbone (Fig. 5), facilitating site-specific phosphopeptide identification. As can be seen in Fig. 5D, tyrosine phosphorylation could be easily determined from the sequence ladders. The single lysine-containing peptides are strongly enriched in mainly three SCX fractions (28–30), which contain almost 85% of the single lysine peptide population (Fig. 2), calculated from both the ETcaD and CID analyses. In agreement with our earlier reported data (30) ETD analysis of these fractions resulted in MS/MS spectra almost exclusively consisting of c-type ions in which c’-ions dominate, providing very simple to interpret sequence ladders (Fig. 6 A). For comparison, in Fig. 6B is shown the complementary CID spectrum of the same precursor peptide, which reveals again a rich, but less clear fragmentation spectrum.

In the ETcaD experiments the number of generated c-type ions decreases as the charge state increases; however, still more than 65% of the ions observed in the fractions containing peptides with three or more basic residues are c-type ions (see supplemental dataset present in PRIDE accession numbers, 3746–3843). Here, the relative contribution of c-type ions
ions is determined by the position of the additional basic residue, i.e. an additional lysine, arginine, or histidine. If the extra basic residue is positioned close to the C terminus both c- and z-ions will be formed, but when the basic residue is closer to the N-terminal site c-ions are dominant, as illustrated by some examples in Fig. 6. Obviously, these higher charged peptides are still very useful in the analysis of the whole cell lysate because ETD efficiency increases with increasing charge state. When comparing the global SCX profiles of the ETcaD and the CID experiments, CID performs relatively well in the SCX fractions that contain peptides with up to maximally two basic residues. For peptides containing more than two basic residues, which attain more charges in the ESI process, a rapid decrease in the absolute and relative number of peptides identified by CID is observed. For these peptides containing multiple basic residues ETcaD performs relatively better largely because of the increased efficiency of the electron transfer process. We believe that in the current
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Fig. 5. ETcaD and CID MS/MS spectra of phosphorylated doubly charged ions originating from Lys-N generated peptides from HEK 293 cells. A, ETcaD MS/MS spectrum of serine phosphorylated KLTG\textsubscript{pS}TSSLN (m/z: 544.11, 2\textsuperscript{+}) from EXOC1 isoform 1 of Exocyst complex component 1. From the sequence of c\textsuperscript{-}ions it is easy to determine the phosphorylation site. B, CID MS/MS spectrum of serine phosphorylated KLTG\textsubscript{pS}TSSLN (m/z: 544.08, 2\textsuperscript{+}). Compared with the ETcaD spectrum a clear sequence for interpretation of the phosphorylation site is not observed. C, ETcaD MS/MS spectrum of serine phosphorylated KNSSLL\textsubscript{pS}FDNEDENE (m/z: 910.68, 2\textsuperscript{+}) from the uncharacterized protein ENSP00000307425. Nearly the entire sequence is fragmented into c\textsuperscript{-}ions by the ETcaD process. The phosphate group is not lost in the fragmentation process. D, ETcaD MS/MS spectrum of tyrosine phosphorylated KIGEGT\textsubscript{pY}GVVY (m/z: 633.13, 2\textsuperscript{+}) CDK3 Cell division protein kinase 3. By interpretation of the peptide fragment ions in the spectrum the sequence and site of phosphorylation can be determined, as only c\textsuperscript{-}ions are generated, and the phosphate group is not lost in the fragmentation process.

Experimental set-up: the number of peptides identified by ETcaD in the lower charged fractions is relatively somewhat underrepresented because of the suboptimal weighting in the search algorithm for the “unique” c\textsuperscript{-}-fragment ion ladder sequences observed for the single lysine peptides. Improving the ion scoring algorithms for these peptides might improve the number of identifications.

DISCUSSION

Generally the first step in a typical global proteomics analysis workflow is fractionation of a tryptic digest to reduce the complexity of the peptide mixtures subjected to RP-nanoLC-MS/MS. The reduction of complexity is vital to the outcome of the experiment because existing mass spectrometers lack the speed to be able to sequence every peptide of a complex sample eluting from the LC column. Fractionation is often accomplished by SCX chromatography of peptides formed by in-solution digestion. An additional benefit of SCX separation is the enrichment into distinct fractions of similar peptide properties before MS analysis. This advantage can be further exploited by performing the SCX separation at low pH, where after tryptic digestion most of the phosphopeptides are found in the early fractions of the SCX separation because of their reduced charge states in solution because glutamates and aspartates will be neutralized and the phosphate group will possess a negative charge (8, 34). Here, we show the added value of using the metallo-endopeptidase Lys-N to digest the proteins into peptides in combination with low-pH SCX fractionation and ETcaD-MS and CID-MS analysis. Digestion with Lys-N yields proteolytic peptides with the lysine at their N terminus, resulting in an increased basic entity caused by the lysine and N-terminal amines. In the subsequent low-pH SCX separation distinct fractionation profiles could be observed in which peptides from different functional categories were extremely well separated. The four categories we were able to separate well are (I) acetylated N-terminal peptides, (II) singly phosphorylated peptides containing a single basic (Lys) residue, (III) peptides containing a single basic (Lys) residue, and (IV) peptides containing more than one basic residue. Because the SCX gradient was optimized for the enrichment of acetylated protein N-termini and phosphorylated peptides, the separation of the later eluting multiple charged peptides (i.e. containing more than one basic residue) was somewhat compromised. Still, with the current SCX set-up the single lysine containing peptides elute in a very clean pool spread only over mainly three fractions. The enrichment of post-
translational modified peptides is relatively high in our current experiments when taking into account that no additional enrichment method is applied and that solely in-solution charge state separation has been used.

Most interestingly the proposed set-up results in a fractionation of peptides whereby there is almost no overlap between the N-acetylated and phosphorylated peptides. N-terminal acetylation, and less so propionylation, of proteins is a common and important process in cellular biology, which is for instance linked to protein stability, protecting the proteins from attack by aminopeptidases (35, 38–40). Because ~90% of cellular proteins in eukaryotic cells contain blocked N termini, they make up a significant portion of the peptide pool and are of great interest with respect to the examination of N-terminal protein processing or alternatively terminated protein isoforms (33, 35). Our data show that the Lys-N-proteolyzed acetylated protein N termini in category I form primarily z'-ions in ETcaD fragmentation (~70%; Figs. 3 and 4) caused by the blockage of the N terminus and the presence of a basic residue.

![SCX-based Fractionation of Lys-N-generated Peptides](image)

**Fig. 6.** ETcaD and CID MS/MS spectra of double- and triple-charged ions originating from Lys-N-generated peptides from HEK 293 cells. A, ETcaD spectrum of doubly charged peptide KOAFDDAIAELDNLNEDSY (m/z: 1079.34, 2+) from YWHAH 14–3-3 protein eta. Protons/charges in Lys-N-generated peptides, with a single basic residue, will be preferentially located at the N terminus because of the presence of two free amine groups, which results in the exclusive generation of c'-type fragment ions. B, CID spectrum of doubly charged peptide KOAFDDAIAELDNLNEDSY (m/z: 1079.36, 2+) from YWHAH 14–3-3 protein eta. CID fragmentation does not result in the exclusive generation of N-terminal fragment ions. C, ETcaD spectrum of triple-charged peptide KNTGVILANDANAEKR (m/z: 566.82, 3+) from NOL1 94 kDa protein. Lys-N-generated peptides, with a basic residue contiguous to the c-terminal, result in the formation of almost equally numbers of c'- and Z-ions. D, CID spectrum of doubly charged KNTGVILANDANAEKR (m/z: 849.86, 2+) from NOL1 94 kDa protein. In CID the same trend is not observed as mainly y-ions are generated. E, ETcaD spectrum of doubly charged peptide KRGEIIIEQLDVTSEYE (m/z: 1097.45, 2+) from HSPD1 60 kDa heat shock protein, mitochondrial precursor. Lys-N-generated peptides, with a second basic residue adjacent to the N-terminal lysine, will result in the exclusive generation of c'-type fragment ions. F, ETcaD spectrum of triple-charged peptide KPWLATFSYGRALQASAL (m/z: 660.53, 3+) from ALDOA fructose-bisphosphate aldolase A. Lys-N-generated peptides, with a second basic residue in the center of the peptide sequence, will result in the generation of c'- and z-type fragment ions.
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An additional advantage of the almost complete separation
is that the fractions containing acetylated protein N termini are
removed as “contamination” from the phosphorylated frac-
tions, allowing enrichment and targeted analysis of the phos-
phoproteome. ETcaD analysis of the purified phosphopep-
tides (category II) results in clear c'-ion ladder sequences of

Fig. 7. General overview describing the dominance of particular fragmentation patterns observed in ETcaD analysis of (A) peptides with an acetylated N terminus containing a single basic residue (Group I). B, phosphorylated peptides containing a single N-terminal lysine (Group II). C, peptides containing a single N-terminal lysine (Group III). D, peptides with at least two basic residues in their sequence (Group IV).
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the peptides and allows a simple read out of the location of the phosphorylation. Peptides containing a single lysine and no other basic residues (category III) can be also be purified via low pH SCX. These peptide ions can be preferentially analyzed by ETcaD as with this activation method simple c- ion sequence ladders are generated as illustrated in Fig. 6A. These sequence ladders can potentially facilitate de novo sequencing of peptides from species with unsequenced genomes or unknown isoforms, circumventing the need for the availability of databases containing known protein molecular sequences.

A general overview of the fragmentation patterns observed in ETcaD analysis of the peptides seen in the four different categories is shown in Fig. 7. Fig. 7A shows the preferred ETcaD fragmentation of the acetylated protein N terminus peptides (category I). Because the N terminus is blocked, the remaining proton after the ETcaD analysis will preferentially go to the C terminus and in the resulting spectra z'-ions will dominate. Both the phosphorylated and unphosphorylated single lysine peptides (Fig. 7, B and C) (category II and III, respectively) will produce spectra dominated by c'-ions after ETcaD analysis because of the favored protonation of the N terminus. Finally, the peptides containing multiple basic residues, category IV, form both c' and z'-ions. Here a preference for c-type ions is only observed when the basic residues are close to the N terminus (also see Fig. 6E), and equal occurrence of c- and z-type ions, when the basic residue is close to the C terminus (also see Fig. 6C), as also shown for tryptic peptides with electron capture dissociation.

In summary, the use of the metallo-endopeptidase Lys-N in combination with SCX described here in detail is a powerful proteomics method. It allows the separation to near completion of (I) acetylated N-terminal peptides (with and without a single basic residue), (II) singly phosphorylated peptides containing a single basic (Lys) residue, (III) peptides containing a single basic (Lys) residue, and (IV) peptides containing more than one basic residue. Analyzing these peptides by LC- MS/MS with both CID as well as ETcaD provides unique optimal targeted strategies for proteome analysis of these classes of peptides. Strikingly, ETcaD provides a facile method for site localization of phosphorylated peptides in category II and facilitates a database independent method for sequencing of “normal” single lysine containing peptides. Overall, the combination of the use of the Lys-N protease, with SCX and RP separation, and CID- and ETD-induced fragmentation, adds a new very powerful method to the toolbox of proteomic analyses enabling also facile analysis of peptides and their post-translational modifications.

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