PROTEOMICS METABOLOMICS GENOMICS INFORMATICS
GLYILEVALCYSGLUGLNALASERLEUASPARG
CYSVALLYSPROLYSPHETYRTHRLEUHISLYS

Characterization of Protein Phosphorylation Using HPLC-Chip Electron Transfer Dissociation Ion Trap Mass Spectrometry

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Abstract

Protein phosphorylation, a type of post-translational modification, plays an important role in the regulation of many cellular functions. The precise determination of phosphorylation sites within a protein is crucial to the understanding of cell regulation mechanisms. MS methods using collision-induced dissociation (CID) are often unsuitable to determine phosphorylation sites because phosphate groups easily dissociate from peptides under CID. The electron transfer dissociation (ETD) technique is an ion-molecule collision process that is much gentler than CID. The phosphate group remains attached to the amino acid during the ETD process, allowing the specific location of the phosphate to be determined. This note compares the difference between CID and ETD and demonstrates the capability of analyzing samples using both CID and ETD in the same LC/MS run. Examples of the identification of site-specific phosphorylation using an Agilent HPLC-Chip system interfaced to an Agilent 6340 Ion Trap mass spectrometer equipped with ETD technology are shown. The acquired CID and ETD spectra of phosphate-containing peptides show not only the ability of ETD to identify and map phosphorylated amino acids, but also the complementary nature of CID and ETD. ETD provides additional information that can increase the confidence in protein identification. The results of this study demonstrate that a comprehensive CID and ETD approach using the HPLC-Chip/6340 Trap MS system is a powerful method for identifying low-level PTMs.

Introduction

Post-translational modifications (PTM) of proteins, such as protein phosphorylation, play important roles in the regulation of many cellular functions. It has been estimated that a significant portion (up to one-third) of the eukaryotic proteome undergoes reversible phosphorylation (1). The precise determination of phosphorylation sites within a protein is crucial to the understanding of regulation mechanisms. Phosphorylation at different sites within the same protein may have different effects on protein function, and may also direct different cell pathway activities.

LC/MS-based methods have become the choice of many proteomics laboratories for protein identification and characterization. However, most LC/MS-based techniques are not without limitations when used on phosphoprotein digests and may produce false positive identifications due to an insufficient amount of peptide sequence MS/MS data. MS methods using collision-induced dissociation (CID) are often unsuitable to determine phosphorylation sites because phosphate groups easily dissociate from peptides under CID. In 1998, McLafferty *et al*. introduced an MS-based technique, electron capture dissociation (ECD), that provided a way to obtain MS/MS spectra complementary to CID data (2). CID involved redistribution of vibrational energy and gave b- and y-type fragment ions, while the ECD process had a different mechanism that generated primarily c and z ions. Recently, a variation of the ECD technique has been developed, referred to as electron transfer dissociation (ETD), that can provide similar results to the ECD process (3). An additional observation for the ETD technique is that the ion-molecule collision process is much gentler than the CID process. The phosphate group remains attached to the amino acid during the fragmentation process, which allows the specific location of the phosphate to be determined.

ETD provides the best available current instrumental technology for site determination of PTMs. This note compares the difference between CID and ETD, and demonstrates the capability of analyzing samples using both CID and ETD in the same LC/MS run. Examples of the identification of site-specific phosphorylation using an Agilent HPLC-Chip system interfaced to an Agilent 6340 Ion Trap mass spectrometer equipped with ETD are also shown. Finally, the performance of Spectrum Mill software for analysis and identification of phosphopeptides is demonstrated.

Experimental

Materials

The phosphopeptide TTHyGSLPQK was synthesized by SynPep (Dublin, CA). The phosphopeptide standard mixture was purchased from Invitrogen (Carlsbad, CA). Active recombinant human MAP Kinase 1 /Erk1 (extracellular signal-regulated kinase) containing an N-terminal GST (glutathione S-transferase) tag was purchased from Upstate Cell Signaling Solutions (Temecula, CA).

Sample preparation

Erk1 was denatured using 2,2,2-trifluroethanol as the denaturant (4) and followed by reduction and alkylation with iodoacetamide. The protein was then digested with trypsin at 37°C overnight. The digests were aliquoted, dried, and stored frozen until use.

Analysis

All experiments were performed using an Agilent 6340 Ion Trap LC/MS system equipped with an Agilent HPLC-Chip Cube MS interface, wellplate sampler, nanoflow pump, capillary pump, and ETD ion trap mass spectrometer.

Infusion was accomplished by using the Agilent HPLC-Chip Cube MS interface with the Infusion HPLC-Chip (Agilent Product #G4240-61002) connected to a syringe pump set at 300 nL/min.

ETD process

Figure 1 depicts the fundamental elements of the ETD process. The ETD reactant is generated by a small negative chemical ionization source (NCI) that is mounted directly to the inlet section of the second octapole ion guide on the MS ion trap. The small NCI source is filled with fluoranthene, a polynuclear aromatic hydrocarbon. The fluoranthene is sublimed and combined with methane gas in the presence of electrons emitted from a filament. The electrons are slowed by collision with the methane gas and captured by the fluoranthene molecules to make fluoranthene radical anions. From the electrospray chamber, the peptide ions are generated from the nanospray chip tip. All positive ions are allowed to enter the mass spectrometer ion inlet, through the ion optics and ultimately into the ion trap. The positive multiply-charged peptide ions are isolated and all the other ions are ejected. During this process, the flow of the reactant radical anions is closed by a voltage gating process. Alternatively, the NCI source is gated to allow negative ions to enter the ion trap as

a packet. The two packets of ions (negative and positive) exist in the ion trap at the same point in time. After tens of milliseconds, the positive peptide ions and the negative ions become interactive and the electron from the fluoranthene radical anions is transferred to the positive peptide ion. A slight 'tickle' energy is usually applied to enhance the ETD reaction. The electron transfer is rapid and sufficiently energetic, yielding a series of positive amino acid residue fragments. The ion trap is subjected to the scan process in the same manner as CID and the positive ETD product ions are scanned out of the trap yielding the ETD MS/MS spectrum.

Database search

Protein database searches were performed with the Agilent Spectrum Mill protein identification software updated to extract and search ETD data. CID and ETD data were searched simultaneously. A customized database was created to search the synthetic phosphopeptide mixture. Protein digest data were searched against the SwissProt database. An iterative searching strategy was employed for protein digest searches: (1) Trypsin specificity, identity mode, missed cleavage 2, maximum charge 4; (2) Validation, subset database created from valid hits; (3) Trypsin specificity, homology multi-mqsty against the subset database from Step 2, missed cleavage 5, maximum charge 5; (4) Validation; (5) No-enzyme specificity against saved results from Step 2; (6) Validation.

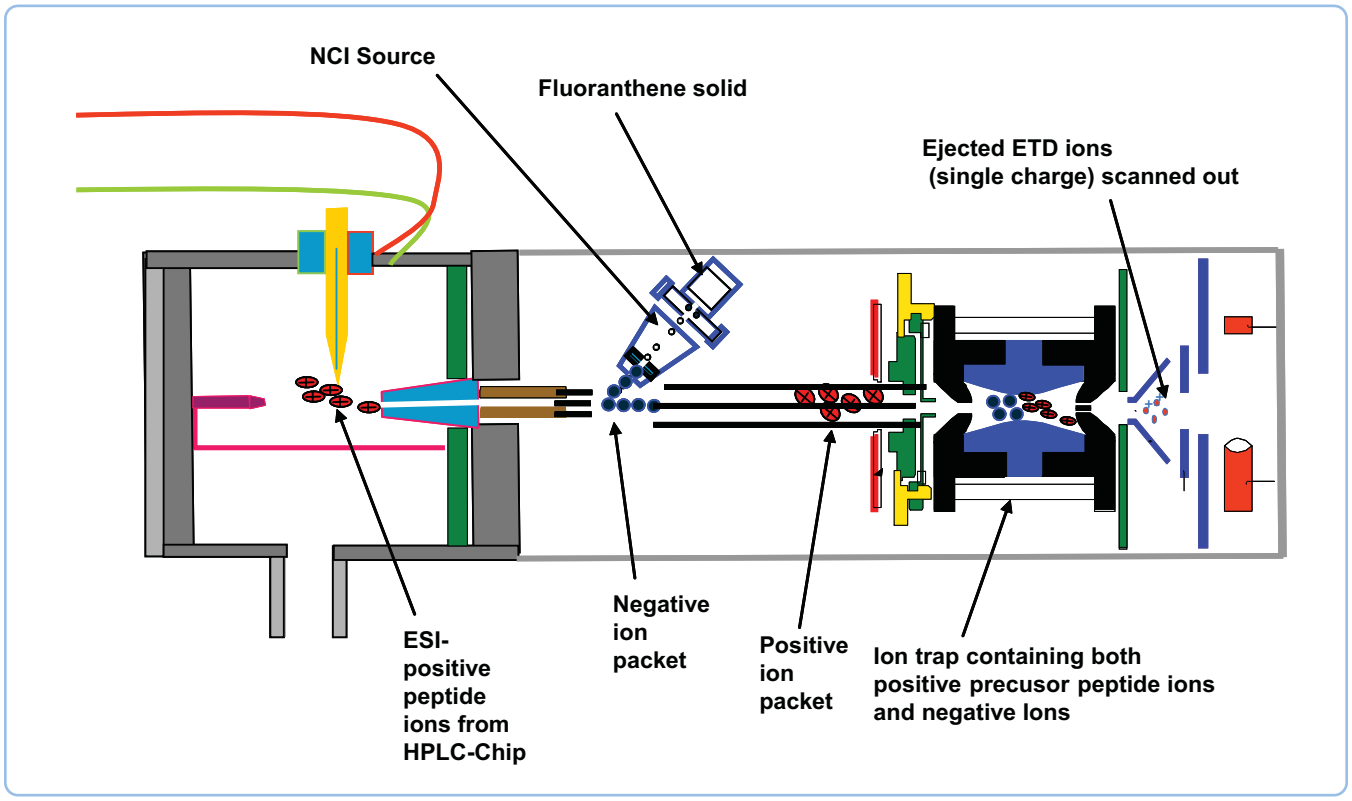

Figure 1. Diagram of the Agilent 6340 ion trap mass spectrometer with ETD module.

Instrument conditions for rapid phosphopeptide analysis (9 minute cycles)

HPLC-Chip conditions

Enrichment column:
ZORBAX 300SB-C18, 0.300 x 5 mm, 5 µm
Analytical column: ZORBAX 300SB-C18, 0.075 x 43 mm, 3.5 µm
Loading flow rate: 4 µL/min
Loading mobile phase: 0.1% trifluroacetic acid in H2O
Flow rate: 600 nL/min
Mobile phase: A = 0.1% formic acid in water;
B = 0.1% formic acid in 90% acetonitrile
Gradient:
- 3% B at 0 min
- 80% B at 7 min

Injection volume: 100 nL

MS conditions

Ionization mode: Positive nanospray
Drying gas flow: 4 L/min
Drying gas temperature: 325°C
Capillary voltage: –1800 V
Skimmer 1: 30 V
Capillary exit: 100 V
Trap drive: 85
Scan : *m/z* 300 – 2200
Scan mode:Standard enhanced
Averages: 2
ICC: On
- Maximum accumulation time: 200 ms
- Target: 300000

Automatic MS/MS:
- Number of parents: 3
- Isolation width: 4 *m/z*
- CID fragmentation amplitude: 1.3 V
- Smart Frag: On, 30 – 200%
- Active Exclusion: On, 2 spectra, 0.5 min
- Precursor preference: None, exclude singly charged
- ETD source: On
- Temperature: 55°C
- Ionization energy: 85 eV
- Emission current: 4.0 mA
- Ionisation chamber: 6.0
- Gate lens offset:
  - Pass: 2.0
  - Block: –10
- Hex DC offset: 8.2
- Focus lens: –4.4
- Partition: –2.4
- Oct1 DC: –12.2
- Oct2 DC: –1.7
- Oct RF:
  - Pass: 75
  - Block: 0
- Trap drive: 30
- Reactant accumulation time: 30 ms
- Reaction time: 80 ms

Results and Discussion

Infusion of the phosphopeptide TTHyGSLPQK

To first illustrate the differences between CID and ETD fragmentation, the synthetic phosphopeptide TTHyGSLPQK was infused at 100 fmol/µL. Both CID and ETD spectra were acquired to compare the different fragmentation techniques (Figure 2). ETD gave almost complete sequence coverage with 90% of the peptide backbone cleavage, while CID produced only 30% of the peptide backbone cleavage. With cleavage at nearly every amino acid, ETD could be an extremely useful tool for *de novo* sequencing. Most of the CID fragments were doubly-charged and located in the lower mass region, making the interpretation of the MS/MS spectrum more difficult. The CID spectrum exhibited only a few dominant fragment ions. The base peak was produced by cleavage adjacent to a proline residue, which is known to be one of the most labile bonds to the CID process. ETD proceeds through a different mechanism and generally occurs randomly over the peptide backbone without sequence dependence. No evidence for the existence of the phosphate group was found in the CID spectrum. However, the mass difference between z_7 and z_6 fragments in this ETD spectrum was 247 amu, which clearly indicated the existence and location of the phosphotyrosine residue.

LC/MS analysis of the synthetic phosphopeptide mixture

Next, a synthetic phosphopeptide mixture was analyzed by a short nine-minute LC/MS run to demonstrate the capability of switching between CID and ETD on-the-fly. The commercially available phosphopeptide mixture consisted of four phosphorylated and three unphosphorylated peptides. Two phosphopeptides were identified from the mixture by both CID

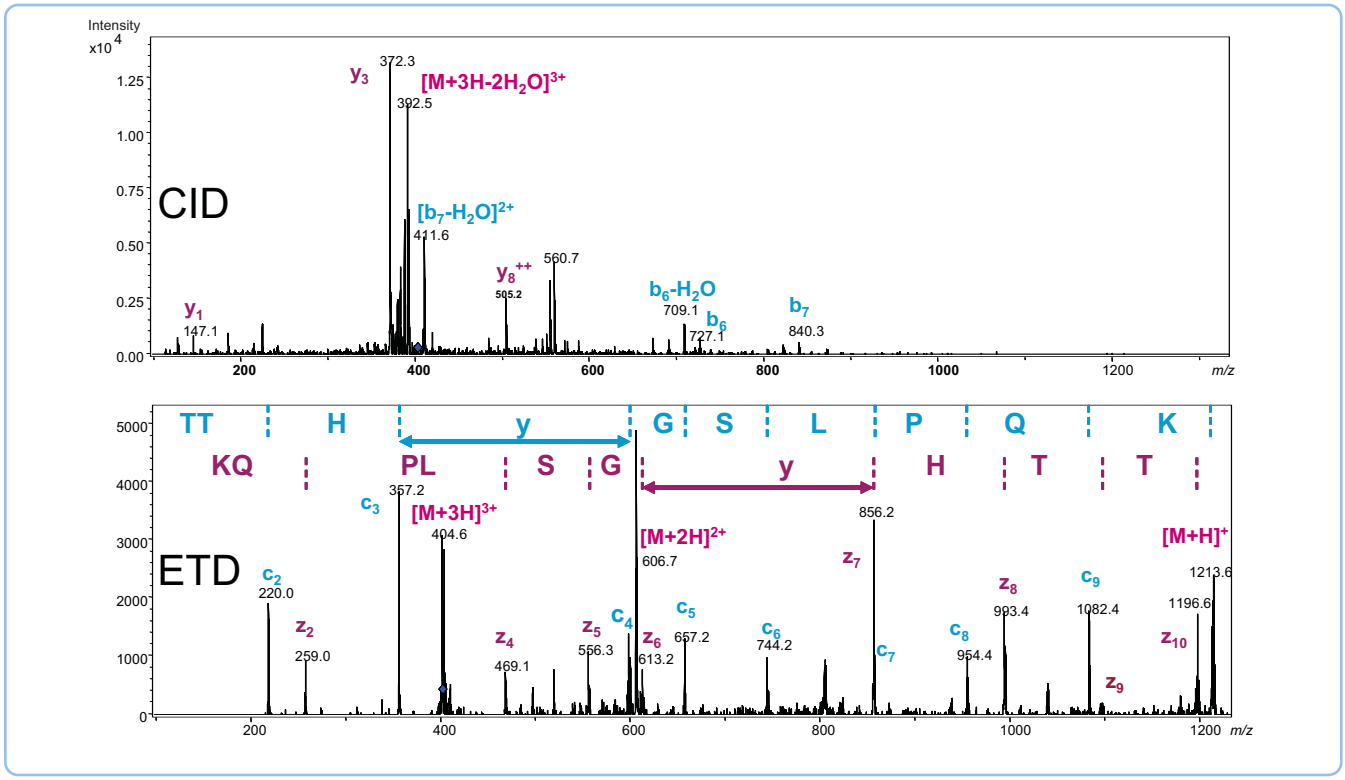

Figure 2. Comparing CID and ETD of the phosphopeptide TTHyGSLPQK (m/z = 404.7) using HPLC-Chip nanospray infusion. CID has very few fragment ions, while ETD produced nearly complete sequence coverage with indication of the phosphotyrosine location.

and ETD. The base peak chromatogram (BPC) and extracted ion chromatograms (EIC) detected five peptides; two phosphopeptides (labeled as peaks 1 and 2) are shown in Figure 3. The CID and ETD spectra of these two phosphopeptides are shown in Figures 4 and 5. For the phosphopeptide containing phosphothreonine, loss of phosphate dominated the CID spectrum, while the ETD spectrum had many more fragments, including fragments indicating the phosphothreonine location (Figure 4). Similarly, the CID and ETD spectra of the phosphotyrosine-containing peptide show not only the ability of ETD to identify and map the phosphorylated amino acid, but also the complementary nature of CID and ETD (Figure 5). ETD provided additional information that can increase the confidence in protein identification.

Characterization of the phosphorylation in active MAP kinase 1/Erk1

Finally, we performed a phosphopeptide analysis of an activated kinase. The extracellular signal-regulated kinase (ERK) pathway is essential to transmitting signals from many extracellular agents to regulate cellular process such as proliferation, differentiation and cell cycle progression (5).

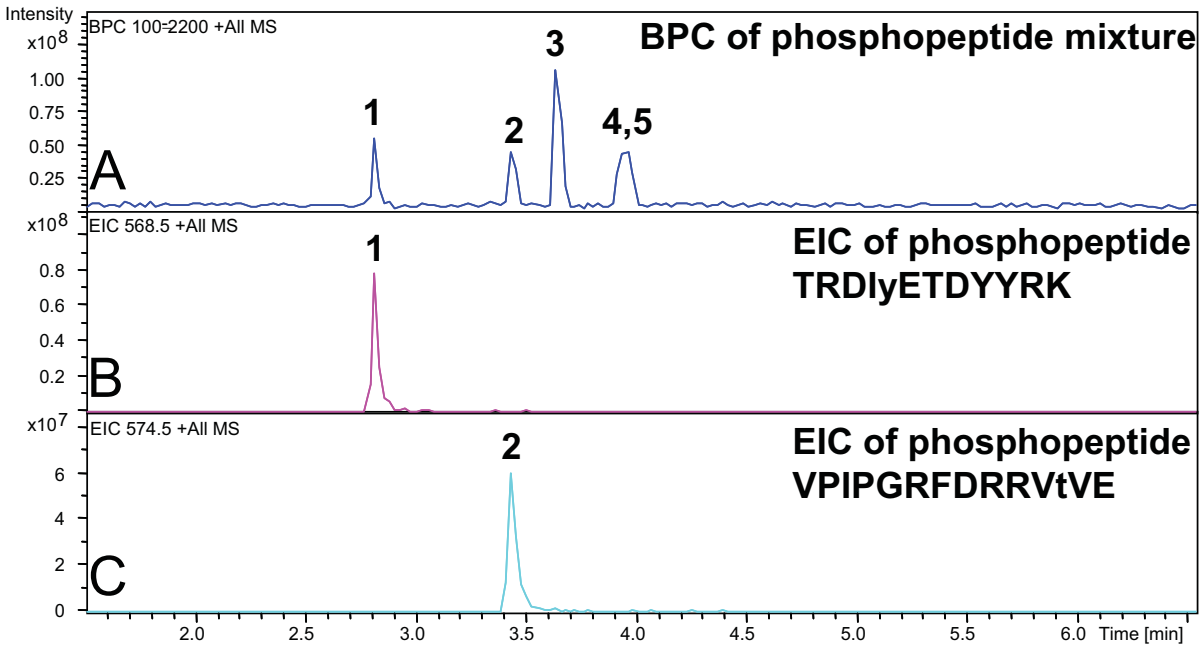

Figure 3. Synthetic phosphopeptides mixture analyzed by Agilent's HPLC-Chip/6340 Trap with ETD. A: BPC of phosphopeptide mixture. Five peptides were identified. B: EIC of phosphopeptide TRDIyETDYYRK *m/z* = 568.5. C: EIC of phosphopeptide VPIPGRFDRRVtVE *m/z* = 574.5.

The signaling via the ERK cascade is mediated by sequential phosphorylation and activation of protein kinases within a cascade pathway. To determine the system's ability to detect phosphorylation of ERK, recombinant human Erk1 containing an N-terminal GST tag was expressed in *E. coli* and activated with MEK1. The active Erk1 was denatured, reduced, alkylated, and digested with trypsin as described in the Experimental section. Approximately 600 fmol of Erk1 digests were injected on the HPLC-Chip/ 6340 ion trap MS system (Figure 6). Both CID and ETD fragmentations were performed on the digest. The LC/MS/MS data was searched against the full SwissProt database using Spectrum Mill software and three proteins were identified as Erk1, GST, and trypsinogen (Figure 7a). Twenty distinct peptides were identified from Erk1, which gave 53% sequence coverage. One peptide was identified that was phosphorylated at two different amino acids (Figure 7b). The two phosphorylation sites were phosphothreonine at 202 and phosphotyrosine at 204 positions. This was consistent with what has been reported in the literature (6).

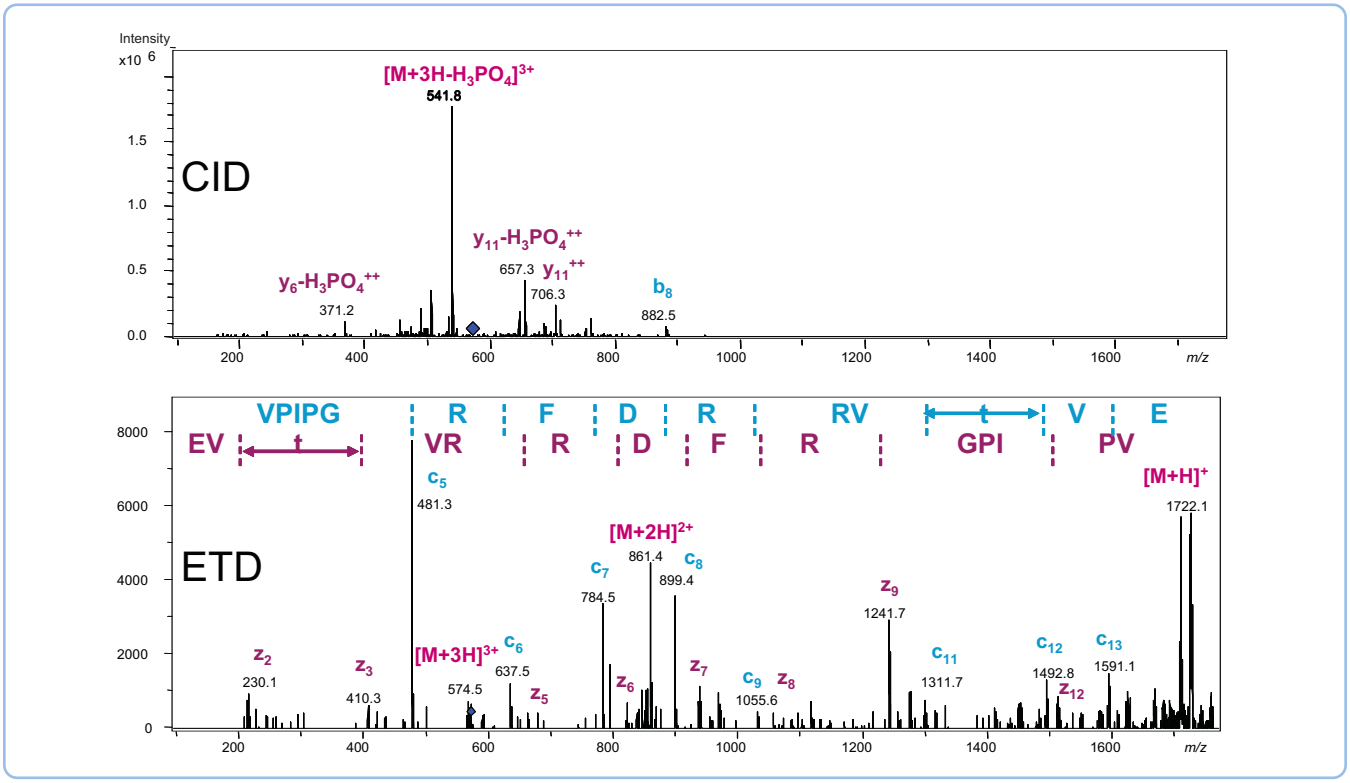

Figure 4. Comparison of CID and ETD of the phosphopeptide VPIPGRFDRRVtVE *m/z* = 574.5. The most prominent peak in CID corresponds to the loss of phosphate group. In the ETD spectrum, the mass differences between c_{11} and c_{12}, z_2 and z_3 are 181 amu, which indicates the existence and location of the phosphothreonine residue.

The two phosphorylation sites were identified on the same tryptic peptide IADPEHDHTGFLtEyVATR with a quadruply-charged precursor *m/z* = 584.24. The ETD spectrum of this peptide obtained a high score of 27.71 from Spectrum Mill, while the CID spectrum of the same precursor ion only scored 8.14 (Figure 7b). The ETD and CID spectra of this phosphopeptide are shown in Figure 8. The corresponding c, z, b, and y ions were labeled as identified by Spectrum Mill. ETD gave a nearly complete c and z series of fragments. In the ETD spectrum, c_{12} and c_{13} fragments (as well as z_6 and z_7 fragments) demonstrated a 181 amu difference in mass, which identified the phosphothreonine amino acid. Similarly, c_{14} and c_{15} (along with z_4 and z_5) fragments had a mass difference of 247 amu, which indicated a phosphotyrosine residue. Another

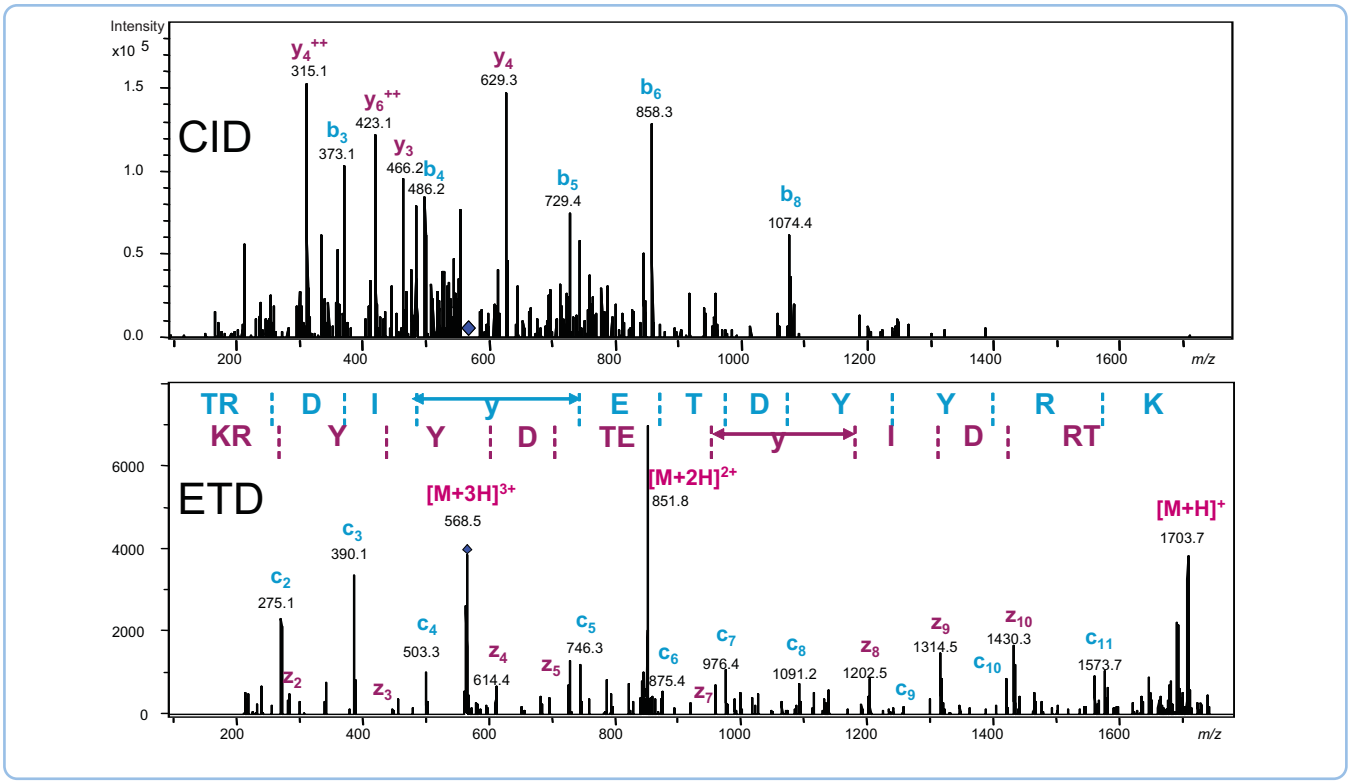

Figure 5. Comparison of CID and ETD of the phosphopeptide TRDIyETDYYRK *m/z* = 568.5. In the ETD spectrum, the mass differences between c_4 and c_5, z_7 and z_8 are 247 amu, which indicates the existence and location of the phosphotyrosine residue.

threonine residue at position 199 was also observed in this peptide. This threonine was demonstrated to be unphosphorylated by fragments c_8 and c_9, z_{10} and z_{11} with a mass difference of 101 amu between each pair. The CID spectrum was dominated by the neutral loss fragment, which was known to be the weakest bond in the CID process. The loss of phosphate made the localization of the phosphorylation difficult, especially between T202 and T199. These data demonstrate the advantage of ETD over CID for protein phosphorylation site determination.

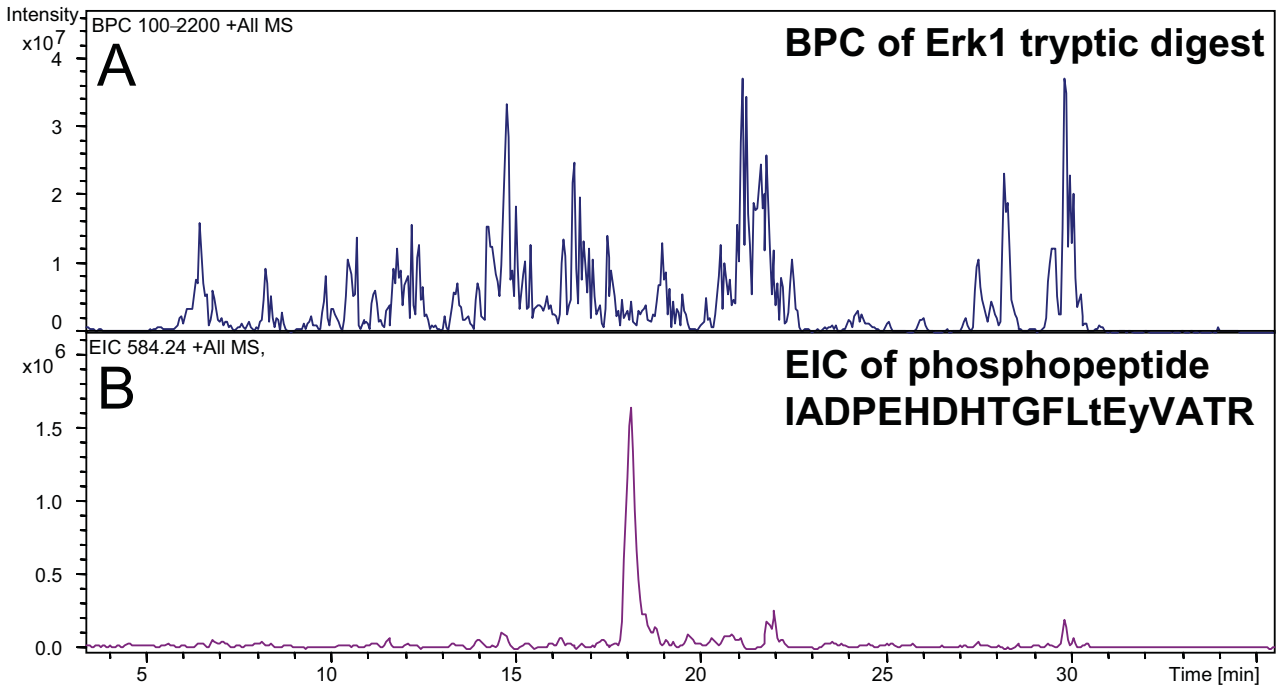

Figure 6. Erk1 analyzed by the HPLC-Chip/6340 Trap MS with ETD. A: BPC of Erk 1 tryptic digest. B: EIC of phosphopeptide IADPEHDHTGFLtEyVATR *m/z* = 584.24.

7a

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Protein MW (Da)	Database Accession #	Protein Name
1	39	20	289.22	53	1.84e+006	43135.8	P27361	Mitogen-activated protein kinase 3 (EC 2.7.1.-) (E
2	16	8	112.47	28	2.90e+005	25498.8	P08515	Glutathione S-transferase 26 kDa (EC 2.5.1.18) (GS
3	2	2	22.27	13	1.39e+005	25424.9	P00760	Trypsinogen, cationic precursor (EC 3.4.21.4) (Bet
Totals:	57	30						

7b

Run #	Run Name	Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
1	ERK_CIDETD_45MIN	1	39	20	289.22	53	1.84e+006	P27361	Mitogen-activated protein kinase 3 (EC 2.7.1.-) (E

#	Frag Mode	Score	SPI (%)	Spectrum Intensity	Sequence	Modifications	m/z Measured (Da)	MH+ Matched (Da)
1	ETD	27.71	98.7	4.39e+005	(R)IADPEHDHTGFLtEyVATR(W)	t:Phosphorylated T y:Phosphorylated Y	584.24	2172.036
2	ETD	25.14	89.5	5.10e+005	(R)LKELIFQETAR(F)		450.04	1347.763
3	ETD	23.68	82.6	4.86e+005	(K)SDSKALDLLDR(M)		411.98	1232.648
4	CID	21.70	97.0	5.65e+005	(R)DVYIVQDLMETDLYK(L)		923.03	1844.899
5	CID	16.94	93.1	1.60e+006	(R)RTEGVQPGVPGEVEMVKGQPFDVGPR(Y)		899.39	2694.367
6	CID	16.89	96.9	3.38e+006	(R)DLKPSNLLINTTCDLK(I)	C:Carbamidomethylation	923.44	1844.979
7	CID	16.85	96.5	1.37e+006	(R)LKELIFQETAR(F)		674.38	1347.763
8	CID	17.13	91.8	7.22e+006	(R)YTQLQYIGEGAYGMVSSAYDHVR(K)		870.42	2608.214
9	ETD	16.70	65.2	1.37e+006	(R)LKELIFQETAR(F)		674.38	1347.763
10	CID	15.57	91.5	4.88e+005	(K)SDSKALDLLDR(M)		411.98	1232.648
11	CID	14.94	93.7	2.40e+006	(K)ELIFQETAR(F)		554.01	1106.584
12	CID	14.90	85.0	1.06e+006	(R)DLKPSNLLINTTCDLK(I)	C:Carbamidomethylation	923.07	1844.979
13	CID	14.70	87.0	3.68e+005	(R)YTQLQYIGEGAYGMVSSAYDHVRK(T)		912.49	2736.309
14	CID	14.26	90.8	5.52e+005	(K)SDSKALDLLDR(M)		616.84	1232.648
15	CID	13.70	81.5	1.20e+005	(R)MLTFNPNKR(I)		560.75	1120.593
16	CID	13.50	83.2	2.63e+006	(R)LKELIFQETAR(F)		450.04	1347.763
17	CID	13.39	93.8	2.07e+006	(R)TEGVQPGVPGEVEMVK(G)		793.13	1584.794
18	CID	12.19	93.4	2.11e+005	(R)YTQLQYIGEGAYGMVSSAYDHVR(K)		870.82	2608.214
19	ETD	11.70	61.3	4.98e+004	(R)WYRAPEIMLNSKGYTK(S)		489.43	1957.000
20	CID	11.67	96.9	7.48e+005	(R)RTEGVQPGVPGEVEM(V)		757.44	1513.732
21	ETD	10.60	57.8	5.83e+005	(R)DVYIVQDLMETDLYK(L)		923.03	1844.999
22	CID	10.49	85.8	3.46e+005	(Y)DPTDEPVAEEPFTFAMELDDLPKER(L)		984.28	2891.329
23	CID	10.19	96.9	2.91e+006	(K)ALDLLDR(M)		408.05	815.462
24	ETD	10.01	51.2	5.52e+005	(K)SDSKALDLLDR(M)		616.84	1232.648
25	CID	9.53	73.5	6.68e+006	(R)RTEGVQPGVPGEVEMVKGQPFDVGPR(Y)		674.94	2694.367
26	CID	9.22	70.6	3.88e+006	(R)IADPEHDHTGFLIEYVATR(W)	t:Phosphorylated T	778.39	2172.036
27	CID	8.39	86.7	2.58e+005	(R)RTEGVQPGVPGEVEMVK(G)		671.46	1340.895
28	CID	8.37	84.5	1.20e+007	(R)LKELIFQETAR(F)		450.57	1347.763
29	CID	8.31	96.4	1.09e+005	(R)ITVEEALAHPYLEQ(Y)		806.94	1612.922
30	CID	8.30	85.2	4.81e+006	(K)ALDLLDR(M)		408.88	815.462
31	ETD	8.29	45.8	1.43e+006	(K)ALDLLDR(M)		408.05	815.462
32	CID	8.14	45.9	4.39e+005	(R)IADPEHDHTGFLtEyVATR(W)	t:Phosphorylated T y:Phosphorylated Y	584.24	2172.036
33	CID	7.42	79.7	2.72e+006	(K)ELIFQETAR(F)		554.44	1106.584
34	CID	7.18	85.8	2.46e+005	(R)WYRAPEIMLNSK(G)		754.75	1507.773
35	ETD	7.11	48.9	1.25e+005	(R)MLTFNPNKR(I)		560.75	1120.593
36	CID	6.12	81.7	3.63e+004	(R)EIQILLR(F)		443.41	884.556
37	CID	5.72	88.9	2.11e+005	(R)YTQLQYIGEGAYGMVSSAYDHVR(K)		870.46	2608.214
38	CID	5.71	54.0	6.33e+006	(R)IADPEHDHTGFLTEYVATR(W)	t:Phosphorylated T	752.00	2172.036
39	CID	5.25	83.2	2.95e+005	(K)ICDFGLAR(I)	C:Carbamidomethylation	952.14	951.472

Figures 7a and 7b. Spectrum Mill database search results of Erk1 analyzed by CID and ETD. A: List of three proteins identified. B: Distinct peptides identified from Erk1 including the phosphopeptides. As indicated by the arrows, the phosphopeptide IADPEHDHTGFLtEyVATR was identified by ETD with a high score of 27.71, while the same peptide was identified by CID with a low score of 8.14.

Instrument conditions for Erk1 analysis

HPLC-Chip conditions

Enrichment column:
ZORBAX 300SB-C18, 0.300 x 5 mm, 5 µm
Analytical column: ZORBAX 300SB-C18, 0.075 x 43 mm, 3.5 µm
Loading flow rate: 4 µL/min
Loading mobile phase: 0.1% trifluroacetic acid in H_2O
Flow rate: 300 nL/min
Mobile phase: A = 0.1% formic acid in water; B = 0.1% formic acid in 90% acetonitrile

Gradient:

- 3% B at 0 min
- 3% B at 2 min
- 5% B at 2.1 min
- 45% B at 42.1 min
- 90% B at 42.5 min
- 90% B at 44.5 min
- 3% B at 45 min

Injection volume: 1 µL

MS conditions are the same as described for phosphopeptide analysis.

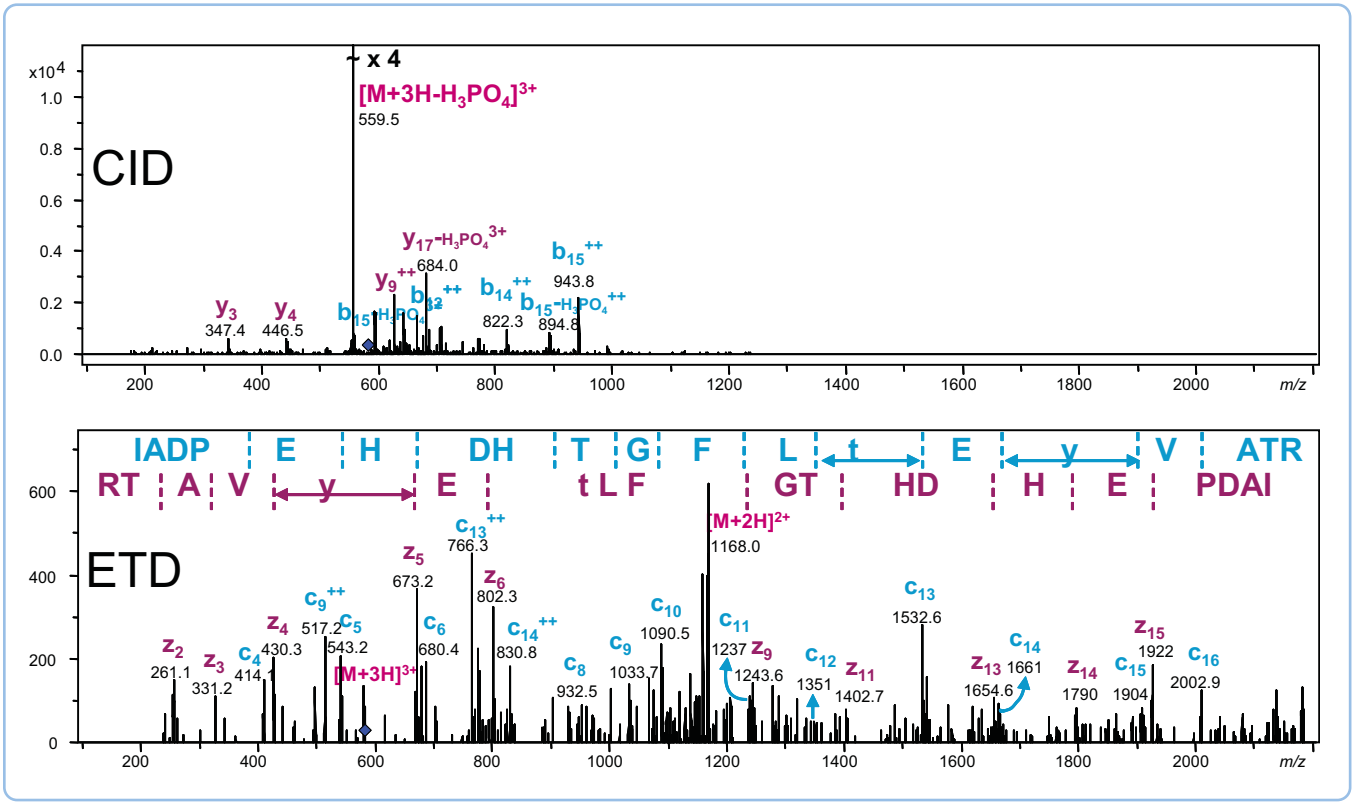

Figure 8. Comparison of CID and ETD of phosphopeptide IADPEHDHTGFLtEyVATR *m/z* = 584.24. The most prominent peak in CID corresponds to the loss of phosphate group. ETD produces nearly complete sequence coverage. The cleavage sites next to the phosphorylated amino acids help to determine the phosphorylation sites.

Conclusions

Effective PTM detection and identification requires sensitivity, accuracy, and reproducibility. In this study, a fully integrated approach is described that demonstrates all these attributes. The Agilent HPLC-Chip/6340 Trap MS system provides sensitivity and reproducibility for low-level peak detection and reliable results. The results of this study demonstrate that a comprehensive CID and ETD approach using the 6340 Trap MS system is a powerful method for identifying low-level PTMs. ETD and CID are complementary fragmentation techniques that can be used to provide more complete and accurate information on protein sequence and modification. Most importantly, the ETD process preserves the phosphorylation on the amino acid backbone and allows determination of the modification sites. In addition, the almost complete sequence tags generated by ETD potentially make it an extremely useful tool for *de novo* sequencing.

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