Identification of an In Vitro Insulin Receptor Substrate-1 Phosphorylation Site by Negative-Ion μLC/ES-API-CID-MS Hybrid Scan Technique

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Recently, we reported a fast on-line alkaline micro-liquid chromatography/electrospray-atmospheric pressure ionization/collision-induced dissociation/mass spectrometric approach for sensitive phosphopeptide screening of a tryptic digested protein and subsequent characterization of the identified phosphopeptide. Based on this study, we now applied an improved method for the identification of phosphorylation sites in insulin receptor substrate 1, an important mediator in insulin signal transduction which was phosphorylated in vitro by protein kinase C-ζ. The approach consists of an on-line alkaline negative-ion micro-liquid chromatography/electrospray-atmospheric pressure ionization/collision-induced dissociation/mass spectrometric hybrid scan experiment using a triple-quadrupole mass spectrometer with fractionation and subsequent off-line nanoES-MS (ion trap) analysis of the phosphopeptide-containing fractions. During the liquid chromatography (LC)/ES-MS experiment, the phosphopeptides of the enzymatic digest mixture of the studied insulin receptor substrate 1 fragment were detected under high skimmer potential (API-CID) using phosphorylation-specific *m/z* 79 marker ions as well as the intact *m/z*-values of the peptides which were recorded under low skimmer potential. Subsequently, the targeted fractions were analyzed by off-line nanoES-MS/MS and MS^3. Using this approach, serine 318 was clearly identified as a major in vitro protein kinase C-ζ phosphorylation site in the insulin receptor substrate −1 fragment. Together, our results indicate that the applied strategy is useful for unequivocal and fast analysis of phosphorylation sites in low abundant signaling proteins. (J Am Soc Mass Spectrom 2003, 14, 401–405) © 2003 American Society for Mass Spectrometry

Reversible phosphorylation/dephosphorylation of proteins represents an important mechanism of cells to transduce an extracellular signal into the cell. Altered ligand-induced signaling has been shown to occur in many diseases. Particularly, numerous studies indicate that an impaired insulin signal transduction is involved in the development of peripheral insulin resistance and type 2 diabetes mellitus, a metabolic disease with high incidence of coronary heart disease [1]. Insulin receptor substrate-1 (IRS-1), one major intracellular substrate of the insulin receptor kinase, interacts with the insulin receptor at sites adjacent to the N-terminus. Recently it was found that during insulin signaling IRS-1 is phosphorylated by protein kinase C (PKC)-ζ, thereby attenuating the insulin signal transduction [2, 3]. To disclose these phosphorylation sites, we developed a mass spectrometric strategy combining two independent MS methods.

During the last few years, MALDI- and ES-MS-based techniques showed to be valuable tools for microcharacterization of post-translational modifications and an alternative or improvement to classic phosphopeptide analytical techniques [4–6]. The identification of phosphorylation sites is difficult because of the commonly low phosphorylation stoichiometry and the low abundance of phosphoproteins. Phosphopeptide analysis by MS is further complicated by generally occurring suppression effects during the ionization process [7]. Particularly, ES-MS enables sensitive and elegant strategies for the non-radioactive screening for phosphopeptides in enzymatic protein digests because it may be coupled directly to LC [8–20]. Using *m/z* 63 (PO_2^-), *m/z*

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79 (PO_3^-), and/or *m/z* 97 ($H_2PO_4^-$) phosphorylation-specific marker ions, formed by negative-ion API-CID, phosphopeptides present in proteolytic digests of proteins are selectively detected during on-line LC/ES-MS [8–15].

Based on the API-CID method described in the literature [8, 9], for the detection and identification of phosphopeptides, we describe a new electrospray MS-based approach improving our previously described alkaline μLC/ES-API-CID-MS phosphopeptide screening method [13]. First, the phosphopeptides present in the protein digest are selectively detected and fractionated during on-line alkaline (enhances ionization of phosphopeptides) negative-ion μLC/ES-API-CID-MS using a hybrid scan routine to generate a phosphorylation-specific marker ion trace (*m/z* 79) as well as full scan mass spectra (detection of molecular peptide ions). Second, the targeted fractions are analyzed off-line by nanoES-ITMS (neutral loss and MS^3). Considering the formation of a dehydroalanine (dhA) residue from a phosphoserine residue, the MS^3 spectrum can easily be interpreted using mass spectrometer data system software. This approach demonstrates an efficient procedure with high sensitivity for phosphopeptide screening which was applied for the localization of a PKC-ζ-dependent GST-IRS-1^{N2} in vitro phosphorylation site.

Experimental

Materials

All HPLC-grade solvents and aqueous ammonia were obtained from Merck (Darmstadt, Germany). The heat shock protein fragment peptide HSP 27 [81–93; CLNRQL(pS)SGVSEIR] was purchased from Bachem (Heidelberg, Germany).

Purification and In Vitro Phosphorylation of GST-IRS-1^{N2} with PKC-ζ

The IRS-1 fragment (IRS-1^{N2}; amino acid residues 265–522; size 26.7 kDa) was ligated to glutathione *S*-transferase and cloned into the pGEX-2T vector (Amersham Pharmacia Biotech, Freiburg, Germany). The fusion protein was expressed in *E. coli* and purified by affinity chromatography. 25 pmol of the isolated GST-IRS-1^{N2} were phosphorylated in vitro by 10 mU PKC-ζ (Calbiochem, San Diego, CA) in 50 mM HEPES-NaOH, pH 7.5, 1.25 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, and 10 μM ATP. The assay was performed at 30 °C for 90 min. The samples were analyzed on a 7.5% SDS-PAGE and visualized by Coomassie Blue staining.

In-Gel Digestion

The GST-IRS-1^{N2}-band at 54 kDa was excised and in-gel-digested with 15 ng sequencing grade trypsin (Roche, Mannheim, Germany) in 50 mM ammonium bicarbonate buffer (pH 8.5) for 16 h at 37 °C. The reaction was terminated by acidification with formic acid, followed by lyophilization.

Alkaline Negative-Ion μLC/ES-API-CID-MS Hybrid Scan Experiment

For LC separation we used a gradient microLC pump (SunChrom, Friedrichsdorf, Germany), connected to a Zorbax-Extend (0.3 × 150 mm) capillary column (Agilent, Waldbronn, Germany). The flow rate was 5 $\mu l \cdot min^{-1}$. Solvent A was aqueous ammonium hydroxide (pH 10.3) and solvent B 80% acetonitrile in aqueous NH_3 (pH 10.3). Separation was performed using as gradient 3% B for 10 min followed by 3 to 60% B from 10–130 min. The sample was dissolved in 2 μl System A and directly applied to the column using a Rheodyne model 8125 injector (Rohnert Park, CA). Stable spraying conditions were achieved adding 1 $\mu l \cdot min^{-1}$ MeOH post-column to the LC-eluent using a Harvard syringe pump and a micro-tee (Upchurch, Oakharbor, WA). Using a stainless steel micro-tee (Valco, Schenkon, Switzerland), the LC eluent was split with 4 $\mu l \cdot min^{-1}$, collected in 4 μl fractions and the remaining 2 $\mu l \cdot min^{-1}$ going directly to the TSQ 700 triple quadrupole MS (Thermo Finnigan, San Jose, CA) using an in-house microES source. Ion formation was optimized using a 30 μm PicoTip (New Objective, Cambridge, MA), directly inserted into the micro-tee and positioned on-axis 2 mm in front of the MS orifice. The hybrid scan routine was written in Instrument Control Language (ICL): Negative-ion mode, +140 V API-CID offset, Q3 scanned from 75–83 *m/z* in 0.2 s (average of 20 scans); 10 V API-CID offset, Q3 scanned from 450–1500 *m/z* in 2 s (average of 3 scans). All parameters (API-CID offset, mass range, scan time) were set within this routine and are executed once per scan cycle.

Off-Line NanoES-ITMS Analysis

Off-line nanoES-MS mass spectra (MS, MS^2, and MS^3) were acquired on an Esquire3000^+ ion trap mass spectrometer, equipped with an off-line nanoES source (Bruker-Daltonics, Bremen, Germany). The targeted peptide fractions were lyophilized, reconstituted in 3 μl methanol/water (1:1, vol/vol), containing 1% formic acid, and 1 μl was introduced into the nanoES needle (BioMedical Instruments, Zöllnitz, Germany). To sequence the phosphopeptides, a full scan mass spectrum was first acquired in the negative-ion mode (+650 V spray voltage); the polarity was then switched to positive mode (−750 V) and MS^2 and MS^3 experiments were performed on mass-selected ions in the ion trap mass spectrometer using standard isolation and excitation procedures. Auxiliary nitrogen gas was supplied at 3 l/min at a temperature of 150 °C.

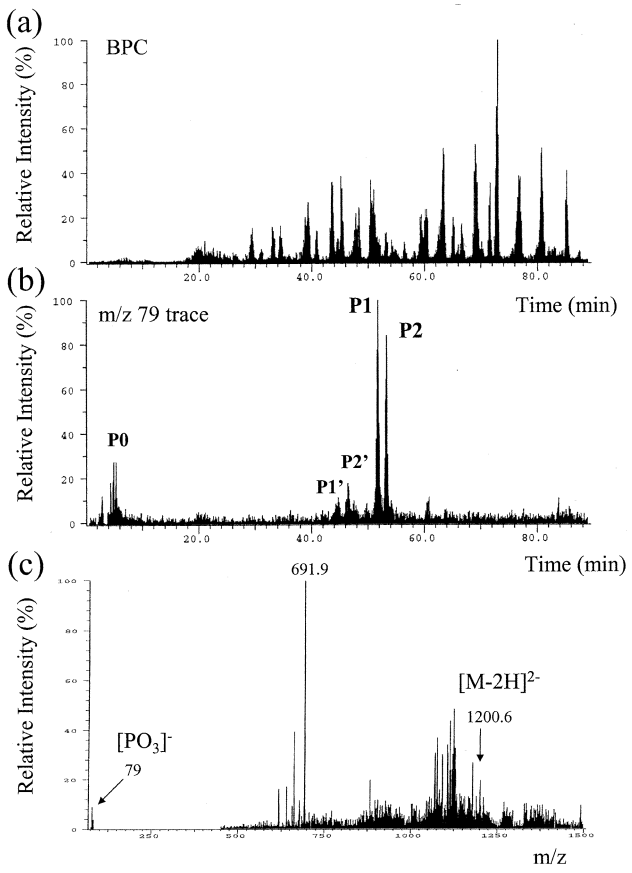

Figure 1. On-line alkaline negative-ion μLC/ES-API-CID-MS hybrid scan analysis (TSQ 700) of tryptic in-gel-digested GST-IRS-1^{N2} fusion protein (25 pmol). Prior to analysis the fusion protein was phosphorylated in vitro by protein kinase C-ζ. (**a**) Negative-ion base peak chromatogram (BPC). (**b**) Phosphorylation-specific *m/z* 79 (PO_3^-) marker trace. (**c**) Averaged negative-ion mass spectrum acquired during the elution of phosphopeptide P2.

Results and Discussion

Figure 1 shows the alkaline (pH 10.3) negative-ion μLC/ES-API-CID-MS hybrid scan analysis of the tryptic in-gel-digested GST-IRS-1^{N2} fusion protein (25 pmol) which was in vitro-phosphorylated by PKC-ζ. The separation profile of the base peak chromatogram (BPC) demonstrates the complexity of the digest mixture (Figure 1a). Using the *m/z* 79 (PO_3^-) marker ion trace (Figure 1b), generated during high API-CID offset (+140 V), two intense peaks (noted as P1 and P2; signal-to-noise ratios >20) and three less intense peaks (P0, P1', P2'; signal-to-noise ratio <5) were detected. Although the eluting compounds led to the formation of intensive signals in the *m/z* 79 trace, only weak corresponding signals in the BPC were observed. In order to detect the corresponding molecular phosphopeptide ion(s), the full scan mass spectrum over the BPC signal of P2 was averaged. Only a weak signal at *m/z* 1200.6 (2 times above noise) could be obtained, possibly corresponding to the $[M - 2H]^{2-}$-molecular ion of a monophosphorylated tryptic peptide. To estimate the sensitivity of the phosphopeptide screening procedure, we used a synthetic serine phosphorylated heat shock peptide fragment HSP 27 [CLNRQL(pS)SGVSEIR]. Injection of 125 fmol resulted in a signal-to-noise ratio peak of 3:1 (data not shown).

Compared to the known API-CID-based methods [8–12, 14], ionization of the phosphopeptides is enhanced using alkaline LC-conditions, as reported in our previous study [13]. Spraying in the negative mode under alkaline conditions (1) led to increased yield of molecular anions of the phosphopeptides [16], and (2) spraying in negative-ion mode under alkaline conditions prevented the suppression of negatively-charged ions normally occurring during negative ionization under acidic conditions ("wrong-way-round" [21]). Additionally, the sensitivity was further enhanced by the in-house micro ES-source which utilizes a 30 μm spray-tip, thereby generating smaller droplets and leading to an improved formation of the peptide ions.

The phosphopeptide-containing fractions were subsequently analyzed off-line by nanoES-ITMS (Figure 2). In the negative-ion full scan spectrum of fraction P2 (Figure 2a), the $[M - 2H]^{2-}$-molecular ion signal at *m/z* 1200.9 corresponds to the theoretical mass of the monophosphorylated peptide sequence SRTESITATSPASMVGGKPGSFR. Switching to the positive mode to sequence the phosphopeptide, neither the corresponding $[M + 2H]^{2+}$-signal nor the higher charged $[M + 3H]^{3+}$-molecular ion was detected (data not shown). Entering the *m/z* value of the triple-charged molecular phosphopeptide ion, the signal at *m/z* 802.0 could be isolated in the trap and subsequently fragmented (Figure 2b). The set CID conditions led to a neutral loss of 32.2 Thompson (98/3, H_3PO_4), i.e., the phosphopeptide-specific formation of a $[M + 3H - H_3PO_4]^{3+}$-neutral loss fragment ion at *m/z* 769.8. According to DeGnore and Qin [22] this $[M + 3H - H_3PO_4]^{3+}$-fragment ion was isolated and sequenced in a MS^3 experiment (Figure 2c). Compared to the MS^2 spectrum, the MS^3 spectrum contained more characteristic fragment ions due to an increased fragmentation along the peptide backbone. The ion trap mass spectrometer was utilized for the following reasons: (1) In an ion trap mass spectrometer the ions can be accumulated, hence achieving higher sensitivity, which is particularly necessary for MS^2 of low abundant peptide ions, (2) in contrast to a triple quad- or a Q-TOF-MS, fragment ions generated by CID in an ion trap can not be activated since the fragment ions fall out of resonance activation frequency, and thus phosphopeptides normally produce very intense neutral loss fragment ions during IT-MS^2 without further fragmentation, (3) an IT-MS enables MS^3 experiments, thus the created neutral loss fragment ions from phosphopeptides can be further fragmented, leading to significantly enhanced fragmentation along the peptide backbone, enabling an unambiguous identification of the phosphorylation site.

The phosphate group was localized at serine 318 using the BioTool software (Bruker-Daltonics) consid-

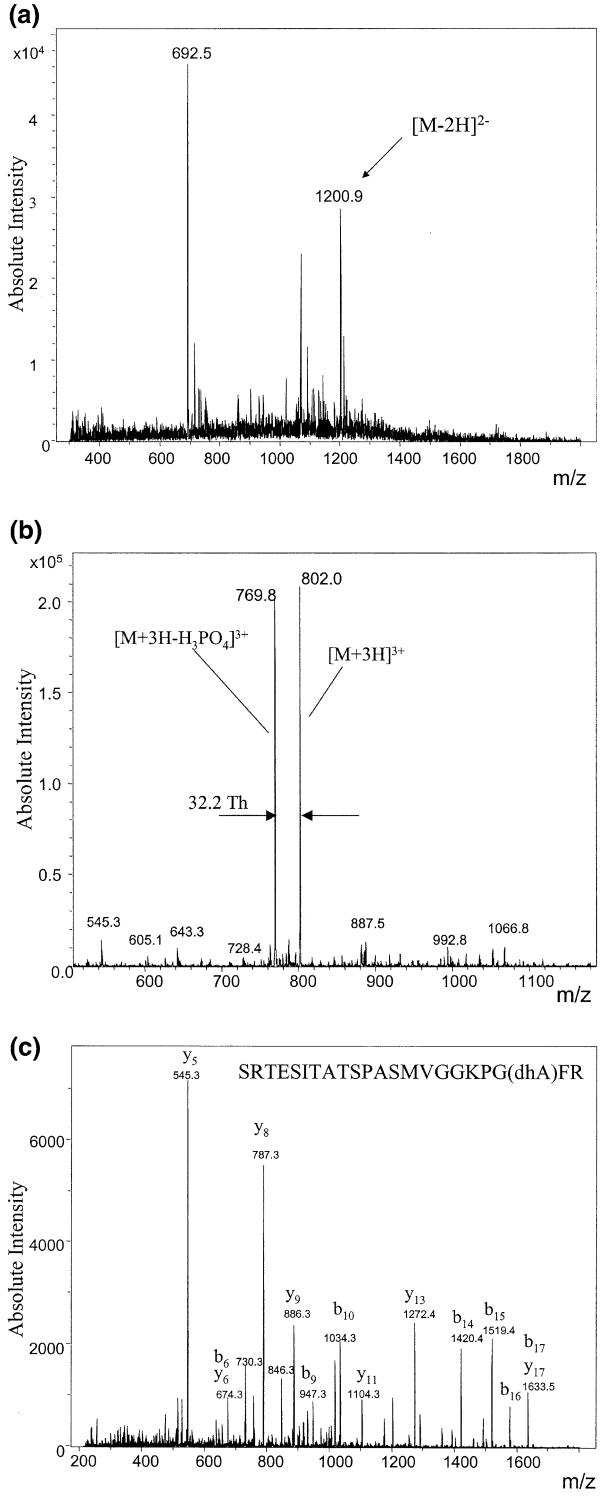

Figure 2. Off-line nanoES-ITMS analysis of fraction P2 from Figure 1. (**a**) Negative-ion mass spectrum of fraction P2. (**b**) Positive-ion MS^2 spectrum of the $[M + 3H]^{3+}$-phosphopeptide ion at *m/z* 800.2. As a result of gas phase β-elimination of H_3PO_4 from the precursor ion (loss of 98/3 Da), an intense $[M + 3H - H_3PO_4]^{3+}$-fragment ion at *m/z* 769.8 was formed. Th = Thompson. (**c**) Product ion spectrum of the gas phase dephosphorylated $[M + 3H - H_3PO_4]^{3+}$-fragment ion at *m/z* 769.8 (MS^3) allowing the localization of Ser 318 within the peptide SRTESITATSPASMVGGKPG(S*)FR as being originally phosphorylated. The former phosphoserine residue was clearly identified as dehydroalanine (dhA) based on a residue mass of 69 Da.

ering the dehydroalanine residue [22–24], formed by the loss of the H_3PO_4 from the phosphoserine residue during gas phase dephosphorylation. By mutating serine 318 to alanine in IRS-1, this site was not phosphorylated by PKC-ζ (unpublished observation), thus the results of the applied procedure were independently verified. Analysis of fraction P1 (51.7 min; Figure 1b) was performed in the same manner and led to the detection of the excepted cleaved tryptic phosphopeptide sequence TESITATSPASMVGGKPG(pS)FR; furthermore, the minor fractions P1' (44 min) and P2' (46.5 min) could be identified as oxidized methionine species of P1 and P2, respectively (data not shown). The identification of the phosphopeptides in these minor peaks further documents the high sensitivity of the applied method. The detailed analysis of phosphopeptide P0 is currently at work. These data clearly demonstrate that PKC-ζ phosphorylates this IRS-1 fragment (amino acid residues 265–522) preferentially at Ser 318 containing 74 potential Ser/Thr phosphorylation sites. This new identified phosphorylation site may be relevant for the attenuation of the insulin signal in metabolic diseases.

Conclusion

Herewith, we present a novel electrospray mass spectrometry-based phosphopeptide mapping strategy combining triple quadrupol and ion trap MS methods. Our results demonstrate the advantage of the applied strategy: (1) Selective screening for low abundant phosphopeptides using on-line negative-ion μLC/ES-API-CID-MS hybrid scan technique under alkaline conditions (phosphorylation marker trace and masses of the peptide molecular ions in one run simultaneously); (2) significantly enhanced fragmentation of the targeted peptide fractions by MS^3 using off-line nanoES-ion trapMS, facilitating the interpretation of the fragment ion spectrum and thus the unequivocal localization of the phosphorylated amino acid. The sensitivity of the described application was assessed to be in the subpicomole range using a synthetic phosphopeptide. In conclusion, our data show that the applied MS strategy is particularly useful for unequivocal and fast analysis of phosphorylation sites in low abundant signaling transduction proteins.

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References

1. Zierath, J. R.; Wallberg-Henriksson, H. From Receptor to Effector: Insulin Signal Transduction in Skeletal Muscle From

Type II Diabetic Patients. *Ann. N.Y. Acad. Sci.* **2002,** *967,* 120–134.
2. Liu, Y. F.; Paz, K.; Hershkowitz, A.; Alt, A.; Tenenbaum, T.; Sampson, S.; Ohba, M.; Kuroki, T.; LeRoith, D.; Zick, Y. Insulin Stimulates PKC-zeta-Mediated Phosphorylation of Insulin Receptor Substrate-1 (IRS-1): A Self-Attenuated Mechanism to Negatively Regulate IRS Proteins Function. *J. Biol. Chem.* **2001,** *276,* 14459–14465.
3. Ravichandran, L. V.; Esposito, D. L.; Chen, J.; Quon, M. J. PKC-zeta Phosphorylates IRS-1 and Impairs Its Ability to Activate PI 3-Kinase in Response to Insulin. *J. Biol. Chem.* **2001,** *276,* 3543–3549.
4. Quadroni, M.; James, P. Phosphopeptide Analysis. *In Proteomics in Functional Genomics: Protein Structure Analysis;* Jolles, P.; Jörnvall, H., Eds.; Birkhäuser-Verlag: Basel, Boston, Berlin, 2000.
5. Larsen, M. R.; Sorensen, G. L.; Fey, S. J.; Larsen, P. M.; Roepstorff, P. Phospho-Proteomics: Evaluation of the Use of Enzymatic De-Phosphorylation and Differential Mass Spectrometric Peptide Mass Mapping for Site Specific Phosphorylation Assignment in Proteins Separated by Gel Electrophoresis. *Proteomics* **2001,** *1,* 223–238.
6. Aebersold, R.; Goodlett, D. R. Mass Spectrometry in Proteomics. *Chem. Rev.* **2001,** *101,* 269–295.
7. Mann, M.; Ong, S. E.; Gronborg, M.; Steen, H.; Jensen, O. N.; Pandey, A. Analysis of Protein Phosphorylation Using Mass Spectrometry: Deciphering the Phosphoproteome. *Trends Biotechnol.* **2002,** *20,* 261–268.
8. Huddleston, M. J.; Annan, R. S.; Bean, M. F.; Carr, S. A. Selective Detection of Phosphopeptides in Complex Mixtures by Electrospray Liquid Chromatography/Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **1993,** *4,* 710–717.
9. Ding, J.; Burkhart, W.; Kassel, D. B. Identification of Phosphorylated Peptides from Complex Mixtures Using Negative Ion Orifice-Potential Stepping and Capillary Liquid Chromatography/Electrospray Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **1994,** *8,* 94–98.
10. Hunter, A. P.; Games, D. E. Chromatographic and Mass Spectrometric Methods for the Identification of Phosphorylation Sites in Phosphoproteins. *Rapid Commun. Mass Spectrom.* **1994,** *8,* 559–570.
11. Jedrzejewski, P. T.; Lehmann, W. D. Detection of Modified Peptides in Enzymatic Digests by Capillary Liquid Chromatography/Electrospray Mass Spectrometry and a Programmable Skimmer CID Acquisition Routine. *Anal. Chem.* **1997,** *69,* 294–301.
12. Annan, R. S.; Huddleston, M. J.; Verma, R.; Deshaies, R. J.; Carr, S. A. A Multidimensional Electrospray MS-Based Approach to Phosphopeptide Mapping. *Anal. Chem.* **2001,** *73,* 393–404.
13. Beck, A.; Deeg, M.; Möschel, K.; Schmidt, E. K.; Schleicher, E. D.; Voelter, W.; Lehmann, R. Alkaline Liquid Chromatography Electrospray-Ionization Skimmer Collision-Induced Dissociation Mass Spectrometry for Phosphopeptide Screening. *Rapid. Commun. Mass Spectrom.* **2001,** *15,* 2324–2333.
14. Zappacosta, F.; Huddleston, M. J.; Karcher, R. L.; Gelfand, V. I.; Carr, S. A.; Annan, R. S. Improved Sensitivity for Phosphopeptide Mapping Using Capillary Column HPLC and Microionspray Mass Spectrometry: Comparative Phosphorylation Site Mapping from Gel-Derived Proteins. *Anal. Chem.* **2002,** *74,* 3221–3231.
15. Hunter, A. P.; Games, D. E. Chromatographic and Mass Spectrometric Methods for the Identification of Phosphorylation Sites in Phosphoproteins. *Rapid. Commun. Mass Spectrom.* **1994,** *8,* 559–570.
16. Carr, S. A.; Huddleston, M. J.; Annan, R. S. Selective Detection and Sequencing of Phosphopeptides at the Femtomole Level by Mass Spectrometry. *Anal. Biochem.* **1996,** *239,* 180–192.
17. Wilm, M.; Neubauer, G.; Mann, M. Parent Ion Scans of Unseparated Peptide Mixtures. *Anal. Chem.* **1996,** *68,* 527–533.
18. Neubauer, G.; Mann, M. Mapping of Phosphorylation Sites of Gel-Isolated Proteins by Nanoelectrospray Tandem Mass Spectrometry: Potentials and Limitations. *Anal. Chem.* **1999,** *71,* 235–242.
19. Stensballe, A.; Jensen, O. N.; Olsen, J. V.; Haselmann, K. F.; Zubarev, R. A. Electron Capture Dissociation of Singly and Multiply Phosphorylated Peptides. *Rapid Commun. Mass Spectrom.* **2000,** *14,* 1793–1800.
20. Shi, S. D. H.; Hemling, M. E.; Carr, S. A.; Horn, D. M.; Lindh, I.; McLafferty, F. W. Phosphopeptide/Phosphoprotein Mapping by Electron Capture Dissociation Mass Spectrometry. *Anal. Chem.* **2001,** *73,* 19–22.
21. Mansoori, B. A.; Volmer, D. A.; Boyd, R. K. "Wrong-Way-Round" Electrospray Ionization of Amino Acids. *Rapid Commun. Mass Spectrom.* **1997,** *11,* 1120–1130.
22. DeGnore, J. P.; Qin, J. Fragmentation of Phosphopeptides in an Ion Trap Mass Spectrometer. *J. Am. Soc. Mass Spectrom.* **1998,** *9,* 1175–1188.
23. Tholey, A.; Reed, J.; Lehmann, W. D. Electrospray Tandem Mass Spectrometric Studies of Phosphopeptides and Phosphopeptide Analogues. *J. Mass Spectrom.* **1999,** *34,* 117–123.
24. Reid, G. E.; Simpson, R. J.; O'Hari, R. A. Leaving Group and Gas Phase Neighboring Group Effects in the Side Chain Losses from Protonated Serine and Its Derivatives. *J. Am. Soc. Mass Spectrom.* **2000,** *11,* 1047–1060.