High-sensitivity determination of tyrosine-phosphorylated peptides by on-line enzyme reactor and electrospray ionization mass spectrometry

LAWRENCE N. AMANKWA, KENNETH HARDER,
FRANK JIRIK, AND RUEDI AEBERSOLD

Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

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Abstract

We describe a simple, fast, sensitive, and nonisotopic bioanalytical technique for the detection of tyrosine-phosphorylated peptides and the determination of sites of protein tyrosine phosphorylation. The technique employs a protein tyrosine phosphatase micro enzyme reactor coupled on-line to either capillary electrophoresis or liquid chromatography and electrospray ionization mass spectrometry instruments.

The micro enzyme reactor was constructed by immobilizing genetically engineered, metabolically biotinylated human protein tyrosine phosphatase β onto the inner surface of a small piece of a 50- μ m inner diameter, 360- μ m outer diameter fused silica capillary or by immobilization of the phosphatase onto 40–90- μ m avidin-activated resins. By coupling these reactors directly to either a capillary electrophoresis column or a liquid chromatography column, we were able to rapidly perform enzymatic dephosphorylation and separation of the reaction products. Detection and identification of the components of the reaction mixture exiting these reactors were done by mass analysis with an on-line electrospray ionization mass spectrometer.

Tyrosine-phosphorylated peptides, even if present in a complex peptide mixture, were identified by subtractive analysis of peptide patterns generated with or without phosphatase treatment. Two criteria, namely a phosphatase-induced change in hydrophathy and charge, respectively, and a change in molecular mass by 80 Da, were used jointly to identify phosphopeptides.

We demonstrate that, with this technique, low picomole amounts of a tyrosine-phosphorylated peptide can be detected in a complex peptide mixture generated by proteolysis of a protein and that even higher sensitivities can be realized if more sensitive detection systems are applied.

Keywords: capillary electrophoresis; electrospray ionization mass spectrometry; liquid chromatography; micro enzyme reactor; protein phosphorylation; protein tyrosine phosphatase

Many, if not most, proteins are edited after their translation by proteolytic processing or modification of selected functional groups (Uy & Wold, 1977; Wold, 1981; Wold & Moldave, 1984). Frequently, such posttranslational processing has been found

to affect the activity and specificity of proteins. Consequently, there is growing interest in the characterization of posttranslationally modified proteins at the level of their covalent structure. Much of the interest and effort in this regard is directed toward the identification of sites of protein phosphorylation. Protein phosphorylation is now recognized as a major mechanism for information relay and processing within the microcosm of the eukaryotic cell (Hunter, 1987; Pelech et al., 1990) and as an effective modulator of protein three-dimensional structure and function (Johnson & Barford, 1993). Protein phosphorylation on tyrosine residues, in particular, is a key event in the regulation of numerous cellular processes, including proliferation and response to external stimuli (Schlessinger & Ullrich, 1992; Bolen, 1993; Fry et al., 1993).

The techniques currently used for identification and localization of sites of protein phosphorylation either involve metabolic

Reprint requests to Ruedi Aebersold at his present address: Department of Molecular Biotechnology, University of Washington, FJ-20, Seattle, Washington 98195; e-mail: ruedi@u.washington.edu.

Abbreviations: ER, enzyme reactor; CE, capillary electrophoresis; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC/MS, liquid chromatography–mass spectrometry; ESI/MS, electrospray ionization mass spectrometry; CID, collision-induced dissociation; TIC, total ion current; RT, retention time; HPTP, human protein tyrosine phosphatase; TBS, tris-buffered saline; TBST, tris-buffered saline/Tween-20; peptide PDGF-R, synthetic phosphotyrosine peptide corresponding to PDGF-R B-chain residues 851–863; IPTG, isopropyl thiogalactoside; i.d., inner diameter; o.d., outer diameter; DTT, dithiothreitol; OD, optical density; TFA, trifluoroacetic acid.

radiolabeling in vivo using ^{32}P -phosphate or enzymatic phosphorylation by an in vitro kinase reaction using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, followed by enzymatic digestion of the labeled protein for subsequent peptide mapping either by HPLC, gel electrophoresis, or two-dimensional (electrophoresis, thin-layer chromatography) peptide separation. Purified radiolabeled peptides are subjected to phosphoamino acid analysis or sequencing to identify the type and the site of the phosphorylated amino acid residue (Aebersold et al., 1991; Wettenhall et al., 1991). In spite of the very high sensitivity of assays used to monitor radioactivity, analysis of protein phosphorylation by this technique has several drawbacks and limitations. Incorporation of ^{32}P is effective only by in vitro phosphorylation of proteins and peptides. Identification of in vivo protein phosphorylation sites is at present very difficult. Even using large amounts of radiolabel of very high specific activity (typically 5–10 mCi of carrier-free $^{32}\text{PO}_4^{2-}$ [Palczewski et al., 1992]), only very small amounts of radioactivity are incorporated into phospho amino acids. Poor transport of the phosphate through the cell membrane and competition with nonradioisotopic phosphate for incorporation into the cellular ATP pool dramatically reduce the ^{32}P specific activity. Also, the use of radioactive reagents at the dose required for these experiments exposes researchers to radiation hazards. Most importantly, detection of the separated phosphopeptides by autoradiography or Cerenkov counting does not provide any structural information on the phosphopeptides.

During the past few years, several methods involving mass spectrometry and tandem MS have been developed for the characterization of phosphopeptides (Carr & Biemann, 1984; Covey et al., 1991; Di Luca et al., 1992; Yip & Hutchens, 1992; Hou et al., 1993; Huddleston et al., 1993; Nuwaysir & Stults, 1993). Covey et al. (1991) developed a technique that uses both LC/MS and coupled LC/MS/MS to screen for the collision-induced loss of a neutral phosphate group for identification of sites of phosphorylation in peptides. Under the collision conditions used, phosphotyrosine was refractory to elimination of the phosphate group. The method was therefore suitable for localizing sites of serine and threonine phosphorylation only and also required an expensive tandem mass spectrometer. More recently, Huddleston et al. (1993) developed an LC/ESI/MS/MS technique for selective detection of phosphopeptides based on CID and detection of generated negative ions, which was also compatible with tyrosine phosphate. Both techniques require multiple picomoles of substrate and are therefore not compatible with the analysis of in vivo phosphorylated samples.

In this report we describe an integrated analytical system consisting of a protein tyrosine phosphatase micro enzyme reactor, coupled to either a capillary electrophoresis instrument or to a capillary liquid chromatograph and an electrospray ionization mass spectrometer for fast (within minutes) on-line identification of low picomole amounts of phosphopeptides in peptide mixtures. The ER specifically dephosphorylates phosphotyrosyl peptides and proteins. The products of the enzyme reaction are then separated by either CE or LC and detected by ESI/MS. Confirmation as to whether a peptide was originally phosphorylated at a tyrosine residue is not only provided by the presence of a peptide whose mass is reduced by 80 Da per phosphate compared to the original peptide but also by the high specificity of the enzyme and the characteristic increase in electrophoretic mobility or decrease in polarity as detected by a change in chromatographic retention time. The ER was constructed by immo-

bilization of a fusion protein expressed in *Escherichia coli*, consisting of a quantitatively biotinylated 12.5-kDa subunit of the transcarboxylase complex from *Propionibacterium shermanii* and human protein tyrosine phosphatase- β (Krueger et al., 1990; Wang & Pallen, 1992; Harder et al., 1994) onto the inner surface of a 50- μm i.d. fused silica capillary or onto avidin-modified methacrylate resin. In each case, immobilization was afforded by binding the single biotin group per fusion protein to an avidin ligand attached to the fused silica or the resin. Enzyme immobilization was performed simply by the perfusion of a small volume of the crude lysate of overexpressing *E. coli* cells through an avidin-modified matrix.

This sensitive, nonisotopic, rapid, and conclusive approach to the identification of tyrosine-phosphorylated peptides is easily adaptable to the characterization of different posttranslational processing or modification events and therefore represents a useful technological solution to a common problem in protein biochemistry.

Results

Expression and characterization of biotinylated HPTP- β

The expression system used in this work was the PinPoint™ Xa-2 Vector DNA, which expresses cloned genes as fusion proteins consisting of a naturally and quantitatively biotinylated 12.5-kDa subunit of the transcarboxylase complex from *P. shermanii*, attached to the N-terminus of the protein to be expressed (Cronan, 1990; Cress et al., 1993). This system was used to provide a means for affinity purification as well as affinity detection and immobilization of the expressed protein via the high-affinity biotin-avidin interaction. In addition, the specificity of metabolic biotinylation assured that the modification did not interfere with phosphatase catalysis, thereby avoiding a common problem in chemical biotinylation of purified enzymes. Figure 1 demonstrates the inducible expression of a major biotinylated protein with an electrophoretic mobility that correlated with the predicted molecular weight of the HPTP- β fusion protein (44 kDa for the phosphatase and 12.5 kDa for the affinity tag). Total cell lysates from *E. coli* induced for HPTP- β expression (lanes 1, 3) or from noninduced cells (lanes 2, 4) were separated by SDS-PAGE. Proteins were detected by staining (lanes 1, 2) or, after transfer to nitrocellulose, by affinity detection using avidin (lanes 3, 4). The inducibly biotinylated protein band (lane 3) comigrated with the major induced protein band around 57 kDa, detected by protein staining (lane 1), indicating that expression of a biotinylated protein with the expected electrophoretic mobility had been induced. A major second biotinylated protein band around 22.5 kDa was constitutively expressed (lanes 3, 4). This band is most likely the major biotinylated protein of 22.5 kDa that has been observed previously in the *E. coli* strain used in these experiments (Cress et al., 1993).

Characterization of inducibly expressed phosphatase activity

To determine whether the major induced biotinylated protein band of approximately 57 kDa contained phosphatase activity, the biotinylated proteins in crude *E. coli* cell lysate were purified using monomeric avidin bound to rigid methacrylate beads.

Fig. 1. Induced expression of biotinylated protein. Proteins in lysates from *E. coli* induced (lanes 1, 3) or noninduced (lanes 2, 4) for HPTP- β expression were separated by 12.5% SDS-PAGE and stained with Coomassie blue (lanes 1, 2) or transferred to nitrocellulose for identification of biotinylated protein bands with streptavidin-alkaline phosphatase, nitroblue tetrazolium/bromochloroindolyl phosphate (lanes 3, 4). Annotated arrows on the right indicate the electrophoretic mobilities of standard proteins with the respective molecular weights.

Because the goal of this work was the development of a micro ER based on immobilized phosphatases, the enzymatic activity was assessed with the immobilized protein. The induction of phosphatase activity associated with biotinylated proteins is shown in Figure 2. A synthetic peptide modeled after residues 151–198 of the B-cell receptor-associated protein Ig β (average

mass = 7,125.75 Da) was tyrosine phosphorylated with the purified kinase p56^{lck} (Watts et al., 1992) using [γ -³²P]ATP and purified by reversed-phase HPLC. Thirty picomoles of the phosphopeptide, as determined by Cerenkov counting, was used as the substrate for proteins purified with 1 μ L slurry (50:50 v/v resin/buffer) of avidin resin either from a lysate of noninduced cells or from cells induced for HPTP- β expression. The degree of dephosphorylation achieved by the biotinylated proteins purified from induced bacteria reached a maximum of 90% in 10 min, whereas biotinylated proteins purified from noninduced bacteria showed no dephosphorylation activity with this substrate (Fig. 2).

We further determined that the phosphatase activity correlated with the slower migrating biotinylated protein in Figure 1 (lane 3). Precipitated proteins were desorbed from the avidin resin with an excess of biotin, and the smaller molecular weight biotinylated proteins were separated by ultrafiltration. Both the concentrate and the filtrate were assayed for phosphatase activity. Phosphatase activity was exclusively associated with the higher molecular weight protein (data not shown). Collectively, these results indicate that induction of the HPTP- β fusion construct in *E. coli* resulted in the expression of a biotinylated protein of approximately 57 kDa, with tyrosine phosphatase activity that could be precipitated by avidin resin. Furthermore, these experiments demonstrate that expressed biotinylated HPTP- β remained active even when immobilized on a resin. Subsequent experiments showed that the immobilized enzyme remained active for up to 8–12 weeks when stored at 4 °C and for several hours when stored at room temperature. The activity of the enzyme was pH dependent, with the optimum pH range between 7.5 and 8.0 (Harder et al., 1994).

Fig. 2. Induced phosphatase activity. Graph showing the degree of dephosphorylation versus time of 30 pmol of ³²P-labeled Ig- β peptide with SoftLink Soft Release avidin resin-purified biotinylated proteins from cell lysates from bacteria induced to express HPTP- β (solid squares) and lysates from noninduced bacteria (crosses). Points are the averages of duplicate assays.

Construction and characterization of the HPTP- β activated micro ERs

We next investigated whether an HPTP- β -modified fused silica capillary possessed sufficient phosphatase activity to warrant its use as an on-line micro ER for capillary electrophoresis. To construct the ER, the inner surface of a fused silica capillary (50 mm i.d., 360 μ m o.d.) was modified in a sequential manner with 3-aminopropyltriethoxysilane, an amino-reactive biotin derivative, avidin, and the expressed biotinylated HPTP- β . The amount of HPTP- β immobilized onto the capillary was in the same proportion as the amount of immobilized avidin that we have previously determined to be on the order of 0.12 pmol/cm of capillary (Amankwa & Kuhr, 1992). Consequently, the amount of HPTP- β immobilized was estimated to be about 0.12 pmol/cm of capillary assuming a 1:1 interaction between avidin and the biotinylated HPTP- β . To assay the phosphatase activity of the ER, a phosphopeptide solution was perfused through the modified capillary and the effluent was collected and analyzed by CE. The sample used was a 78-pmol/ μ L solution of a synthetic phosphotyrosyl peptide (PDGF-R with sequence IMRDSNY_pISKGST) corresponding to residues 851–863 of the platelet-derived growth factor receptor B chain. The sample was perfused through the ER at a flow rate of 77 nL/min. The elec-

tropherograms shown in Figure 3 document increasing substrate dephosphorylation with increasing residence time of the phosphopeptide in the ER. The residence time was adjusted by the length of the capillary. Aliquots of 1.5 pmol of the collected reaction mixture after residence time of 0 min (Fig. 3A), 5.2 min (Fig. 3B), 7.8 min (Fig. 3C), and 13.0 min (Fig. 3D) were analyzed by CE. The unmarked peaks were due to impurities in the peptide sample, and the peak marked (S) was due to salts in the sample. Integration of the peak areas corresponding to the substrate (designated "phospho") and product (designated "dephospho") indicated that 7.1%, 11.3%, and 21.0% of the substrate were dephosphorylated at a residence time in the reactor of 5.2 min, 7.8 min, and 13.0 min, respectively. Thus, the phosphatase activity of the HPTP- β -modified fused silica capillary micro reactor was determined to be 23.8 ± 2.0 pmol/cm of the flow rate used.

A second type of HPTP- β activated micro reactor was constructed for coupling to an LC column. This was done by packing HPTP- β -activated methacrylate resin into an 8-cm long 0.25-mm i.d. teflon tubing as described in the Materials and methods and in Figure 8. Samples were introduced onto the reactor via a Rheodyne injection valve into a flowing stream of reaction buffer (50 mM ammonium formate, pH 7.8, 1 mM DTT) being pumped at a rate of 2 μ L/min by a syringe pump.

Fig. 3. Activity of an HPTP- β ER. CE electropherograms showing the separation of substrate (A) and products (B, C, D) after 6.2 nmol of phosphorylated peptide PDGF-R was perfused through the HPTP- β -modified fused silica capillary and manually collected. The residence time of the sample in the reactor was adjusted by the capillary length. **A:** Substrate peptide PDGF-R. **B:** Product from a 20-cm capillary corresponding to a residence time of 5.2 min. **C:** Product from a 30-cm capillary (residence time 7.8 min). **D:** Product from a 50-cm capillary (residence time 13.0 min). CE separation electrolyte was 50 mM ammonium formate containing 1 mM DTT, pH 7.8. Separation capillary was 70 cm (62 cm to detector) 50- μ m i.d. $\times$ 360- μ m-o.d. Samples (3 pmol each) were injected for 2 s by vacuum (15 inches of Hg) suction. Peak corresponding to the substrate phosphopeptide is labeled "phospho" and peak corresponding to the dephosphorylated product is labeled "dephospho." The peak labeled (S) was due to salts in the samples. Detection was by UV absorbance at 214 nm.

At this flow rate, the residence time of the sample on the reactor was 16.5 min and the entire sample was recovered from the reactor in about 20 min. The activity of this reactor was determined to be about 31.8 pmol/min at this flow rate.

Construction and operation of the ER/CE/MS and ER/LC/MS systems

Because the goal of this work was to develop an integrated micro ER/CE/ESI/MS or ER/LC/ESI/MS system, the reactivities of these reactors were tested while coupled on-line to either a CE separation capillary or an LC column and an ESI/MS detector. In the ER/CE/ESI/MS configuration, the ER products were introduced directly onto the CE/MS system for separation and detection. Coupling of the ER to the separation capillary was achieved as described in the Materials and methods, and a schematic of the entire instrument setup is shown in Figure 4. This system was used to dephosphorylate aliquots from a 2.4-mg/mL solution of the synthetic phosphopeptide PDGF-R. Three picomoles of the phosphopeptide were incubated for 6.5 min in

the ER, following which the reaction products were separated by CE and analyzed by ESI/MS. The results of such an analysis are shown in Figure 5. The total ion current displays all the products eluting from the separation capillary detected by the ESI/MS within an m/z range of 500–1,700. The peak designated "phospho" corresponded to the residual of the phosphopeptide substrate, and the peak designated "dephospho" corresponded to the dephosphorylated product. This assignment was confirmed by the mass spectra of the respective peaks, which are shown as inserts in Figure 5. The apparent mass difference of 40 Da (m/z 776.5 versus m/z 736.5) of the doubly charged ($M + 2H$)²⁺ peptide ions corresponds to a net mass difference of 80 Da between the singly charged ($M + H$)⁺ peptide ions. This mass difference is indicative of a loss of a phosphate group (HPO_3). A comparison of the ion currents for the phospho and dephospho forms indicates that about 50% (1.5 pmol) of the phosphopeptide substrate injected onto the ER was dephosphorylated under the conditions used, assuming comparable ESI/MS response for the phospho and dephospho peptide (Di Luca et al., 1992).

Fig. 4. Instrument setup for ER/CE/MS. A schematic diagram showing the instrument setup used for on-line ER/CE/ESI/MS. ER: Enzyme reactor consisting of a 2.5-cm length of HPTP- β -modified fused silica capillary. CE, separation capillary; TS, teflon sleeve; AR, anode reservoir; CE-HVPS, CE high-voltage power supply; ESI-HVPS, ESI high-voltage power supply; VT, Valco tee; SL, sheath liquid; SG, sheath gas; N₂, nitrogen gas. Details of the interface between the ER and the CE are shown in the inset.

Fig. 5. On-line ER/CE/MS. Mass spectral data for on-line ER/CE/MS of peptide PGDF-R showing the TIC electropherogram for $m/z = 500$ – $1,700$ and mass spectra for detected peaks. Peak corresponding to the phosphopeptide substrate is labeled “phospho” and peak corresponding to the dephosphorylated product is labeled “dephospho.” Mass spectra for these peaks are shown as inserts. The ER was 2.5 cm of HPTP- β -modified fused silica capillary and the CE separation capillary was 138 cm long. Three picomoles of phosphorylated PGDF-R were injected electrokinetically (20 s at +26 kV) onto the ER and allowed to incubate for 6.5 min before initiation of CE separation.

We further evaluated this technique by applying it to the identification of a tyrosine-phosphorylated peptide in a peptide mixture. Tryptic digestion of the peptide PDGF-R (0.4 mg/mL) resulted in three products designated, T1, T2, and T3, with the corresponding sequence, IMR, DSNYPISK, and GST, respectively. An aliquot of 51.5 fmol of this digest was analyzed by CE or on-line ER/CE and detected by on-column UV absorbance detection. Sample application was accomplished by hydrostatic pressure injection. The electropherograms for these analyses, shown in Figure 6A and B, respectively, demonstrate distinct pattern differences. Peak 7 (at RT 10.5 min) in Figure 6A was absent in Figure 6B, whereas a new peak (indicated with the arrow, RT 7.5) appeared as a result of incubation in the ER. It appeared from these observations that peak 7 (Fig. 6A) represented the phosphorylated peptide fragment in the tryptic mixture, whereas the new peak in Figure 6B contained the dephospho product. The slight difference in the elution times of corresponding peaks unaltered by the ER was the result of the smaller electroosmotic flow, caused by the coupling of the ER to the CE separation capillary.

To confirm whether the interpretation of the UV absorbance data shown in Figure 6 was correct, the experiment was repeated with the exception that eluting peaks were detected by on-line ESI/MS. A 13.9-pmol sample was injected electrokinetically to compensate for the lower sensitivity of the MS compared to UV absorbance detection. Figure 7A shows the TIC of peptides detected in the ER/CE/MS experiment. Figure 7B and Figure 7C compare the mass spectra of peaks E and G, respectively, generated by CE/MS and ER/CE/MS. The CE/MS and ER/CE/MS spectra of peak G, which was diminished by the ER indicate masses at $m/z = 454.0$ and $m/z = 906.5$ (Fig. 7C) correspond-

ing to $(M + 2H)^{2+}$ and $(M + H)^+$ ions of the tryptic phosphopeptide T2, respectively. The mass spectra of peak E, which was enhanced by the ER, were different (Fig. 7B) with distinct signals at $m/z = 826.5$ and $m/z = 413.5$ corresponding to a peptide with $m/z = 826.5$ only present in the sample generated by ER/CE/MS. This measured mass corresponded well with the predicted $m/z = 826.5$ for the dephosphorylated peptide T2. Fragment T2 was therefore conclusively identified as a phosphopeptide in the peptide mixture. In the experiment shown in Figure 7, complete dephosphorylation was not achieved because a large amount (13.9 pmol) of substrate was injected onto the ER. The degree of dephosphorylation could be increased by increasing the residence time of the phosphopeptide in the reactor. The longer elution times observed in Figure 7 compared to Figure 6 for the same peptides was due to the increase in the length of the separation capillary. A longer capillary was required to make the CE/MS connection.

For analysis by ER/LC/ESI/MS, a 5.6- μ L packed resin ER was coupled to the LC/MS system as described in the Materials and methods and shown schematically in Figure 8. We have used this setup to identify the 13-amino acid residue phosphotyrosyl peptide in a mixture of several peptides obtained from tryptic digestion of carboxymethylated α -lactalbumin (average mass = 14,651.40 Da). A 1- μ L aliquot of the sample containing 13.6 pmol of the digested protein was added with 2.6 pmol of phosphopeptide PDGF-R and introduced via valve 1 (V1) onto the ER at a flow rate of 2 μ L/min with the reaction buffer. The effluent from the ER was collected in the sample loop of valve 2 (V2) and applied to the HPLC column for LC/MS analysis. A second aliquot of the sample was injected directly onto the LC column via V2 and analyzed by LC/MS. The results of

Fig. 6. On-line ER/CE. CE electropherograms for on-line ER/CE analysis of a 51.5-fmol tryptic digest of peptide PDGF-R. The ER was a 2.5-cm length of HPTP- β -modified fused silica capillary. **A:** CE electropherogram of the sample. **B:** Electropherogram obtained after on-line ER/CE was performed. Samples were injected for 2 s by vacuum (15 in. of Hg) suction. During on-line ER/CE, the sample was allowed to incubate in the ER for 6.5 min before initiation of CE separation (time = 0.0 min). Other conditions were as given in Figure 3. Peptides were detected by UV absorbance measurement on the capillary. Peak annotations are by numbers. The peak indicated with the arrow (Fig. 6B) is the dephosphorylated product of peak 7 (Fig. 6A).

these analyses are shown in Figure 9. Figure 9A shows the TIC chromatogram of the components eluting from the LC column and detected by ESI/MS within the m/z range of 500–2,000 when only LC/MS analysis was performed on the sample and Figure 9B shows the TIC chromatogram when ER/LC/MS was performed. The dark arrows indicate the phosphopeptide PDGF-R, whereas the open arrows indicate the retention time for the dephosphor-

ylated form of PDGF-R. The mass spectra of these species are shown as inserts in Figure 9A and B, respectively. A comparison of Figure 9A and B shows that the peak corresponding to phospho-PDGF-R in the data for the LC/MS analysis (Fig. 9A) disappeared as a result of exposure to the phosphatase while a new peak corresponding to the dephospho-PDGF-R appeared (Fig. 9B). Based on the specificity of the enzyme used and the mass difference of 80 Da between the $(M + H)^+$ ions of phospho and dephospho species, we were able to easily identify the phosphotyrosyl peptide in the mixture of several peptides. The shift in the retention times of the two late-eluting peaks (indicated by **) between the LC/MS and the ER/LC/MS analyses was likely due to problems with the LC gradient during the ER/LC/MS analysis. Although the detection limit of the techniques described here was not evaluated, it is expected from the magnitude of the ion currents obtained for the data in Figure 9 that detection sensitivities at 1–5 pmol or lower (by ER/CE/MS) is possible by these techniques.

Discussion

The work presented in this report describes the construction and use of an integrated micro ER/separation/MS system for the rapid, sensitive, and conclusive identification of the sites of protein tyrosine phosphorylation without the use of radioisotopes. The system is based on the specific hydrolysis of phosphate ester bonds in phosphotyrosine side chains mediated by HPTP- β immobilized in a micro ER, the separation of reaction products by CE or LC, and analysis of the separated products by ESI/MS. Although the mass, electrophoretic mobility, and polarity of most peptides in a typical peptide mixture are unaltered by the ER, tyrosine-phosphorylated peptides passing through the ER are characterized by an increase in electrophoretic mobility, a decrease in chromatographic retention, as well as a decrease in mass.

Knowledge of the mass of the dephosphorylated peptide is normally sufficient to locate a peptide within a protein sequence, provided that the entire protein sequence is known and that the protein is fragmented with a proteolytic enzyme of defined specificity. Application of the system to peptide mixtures by proteolysis of a tyrosine-phosphorylated protein therefore locates the phosphorylated peptide and possibly the site of tyrosine-phosphorylation within the amino acid sequence of proteins.

The described system has several advantages over present techniques for determining sites of protein tyrosine phosphorylation. First, the system is sensitive. We show that the ER can function reproducibly with low picomole amounts of substrate (picograms of phosphopeptide) and that the reaction products can be separated and detected at that level. Second, the system eliminates the need for radioisotopic protein labeling. This feature not only eliminates a considerable health hazard to the biochemist and reduces cost, but, significantly, permits the analysis of tyrosine-phosphorylated proteins isolated from sources that are incompatible with high-level isotopic labeling. In addition, constitutively phosphorylated proteins that show poor radioisotopic incorporation in pulse-labeling experiments become accessible for analysis. Third, the integration of the enzymatic reaction, product separation, and mass analysis in an on-line fashion dramatically reduces problems, such as sample loss and contamination, that normally plague the analysis of very low amounts of sample. Fourth, the integration on-line of the different sub-

Fig. 7. Identification of the phosphopeptide in a mixture of peptides by ER/CE/MS. A tryptic digest of peptide PDGF-R (13.9 pmol) was subjected to CE/MS and ER/CE/MS. The ER was 2.5 cm of HPTP- β -modified fused silica capillary. The sample was incubated on the ER for 6.5 min before initiation of CE separation. CE and MS conditions were as described above. **A:** Mass spectra data (TIC, mass range 400–1,800) of ER/CE/MS. **B:** Mass spectra of elution window for peak E from samples subjected to CE/MS and ER/CE/MS, respectively. **C:** Mass spectra of elution window for peak G from samples subjected to CE/MS and CE/ER/MS, respectively.

Fig. 8. Instrument setup for ER/LC/MS. A schematic diagram showing the instrument setup used for on-line ER/LC/ESI/MS. ER: Enzyme reactor consisting of an 8-cm length of 254- μ m-i.d. teflon tubing packed with HPTP- β -modified methacrylate resin. V1 and V2 were Rheodyne (model 8125) valves equipped with 5- μ L and 20- μ L sample loops, respectively. A 320- μ m-i.d. $\times$ 15-cm-long C_{18} reversed-phase HPLC column was connected from the outlet port of V2 to the ESI interface of the MS. Details of the ESI interface were as described in Figure 4.

Fig. 9. Identification of the phosphopeptide in a mixture of peptides by ER/LC/MS. Tryptic digest of carboxymethylated α -lactalbumin (13.6 pmol) was spiked with 2.6 pmol of peptide PDGF-R and subjected to LC/MS and ER/LC/MS. Enzyme reaction, LC, and MS conditions were as described above. **A:** Mass spectra data (TIC, mass range 500–2,000) of LC/MS. **B:** Mass spectra data (TIC, mass range 500–2,000) of ER/LC/MS. Dark arrows indicate the retention time for the phosphorylated species of peptide PDGF-R, and open arrows indicate the retention time of the dephospho-PDGF-R. Mass spectra of these species are shown as insets.

systems lends itself to automation of the whole system, and fifth, the specificity of the enzyme as well as the high accuracy and sensitivity provided by the MS enhance the confidence in data generated by this technique.

The configurations of instruments described in this system represent only a few of several possible arrangements. The ER described here can be equally interfaced with other MS techniques such as the one based on matrix-assisted laser desorption ionization and fast atom bombardment ionization. Although matrix-assisted laser desorption ionization MS is simpler, more affordable, and more sensitive than ESI/MS (Liao et al., 1994), the ease with which the ESI/MS can be interfaced with CE or capillary LC and the additional dimension provided by product separation prior to mass analysis support the described system configuration. An obvious limitation of the use of CE to separate reaction products is low concentration detection limit (10^{-5} M) and the small sample volumes (nL) required. This limitation can be overcome by performing product separation by micro-column chromatography instead of CE as shown here, or by using on-column sample concentration in the capillary as done in capillary isotachopheresis (Thompson et al., 1993).

HPTP- β , the enzyme used in this study, is a highly active single-domain phosphatase of relatively wide substrate specific-

ity (Wang & Pallen, 1992; Harder et al., 1994). The enzyme was expressed in *E. coli* as a fusion protein with an attached biotin purification tag (Cronan, 1990; Cress et al., 1993) that could be used to purify the enzyme and to produce the ER by simply perfusing a crude lysate of HPTP- β -expressing *E. coli* cells through an avidin-modified matrix. Each HPTP- β molecule immobilized in the ER contained one biotin group that was attached away from the catalytic phosphatase domain. This circumvented the reduction or total ablation of the catalytic activity, which is a common problem with chemical biotinylation of proteins.

Bacterial overexpression of the enzyme also provides an abundant source for the enzyme, which is of relatively low abundance in genetically unaltered cells. Finally, the addition of a significant polypeptide segment to the catalytic domain localized the enzyme further away from the capillary surface, thus making it possible for the enzyme to interact more freely with the substrate. As we have shown with ERs of different specificity, the ER offered the advantage that the reactor could be used on-line under low pressure flow conditions (Amankwa & Kuhr, 1992). The relatively large surface area-to-volume ratio within these reactors promotes adequate interaction between the immobilized enzyme and the substrate, thus accelerating the catalysis in dilute samples. This was confirmed by the observation that enzyme

reactions that normally take several hours to complete were performed within minutes in the ER. It also appeared that the stability of the enzyme was enhanced by immobilization. It was therefore possible to use the same ER several times in an on-line fashion.

Although current techniques involving collision-induced elimination of the phosphate group and mass analysis of the fragmentation products by MS provide an alternative nonradioisotopic method for the determination of phosphorylation sites (Covey et al., 1991; Huddleston et al., 1993), the approach described in this report provides enhanced sensitivity, specificity of fragmentation provided by the enzyme reaction, requires a simple, relatively inexpensive MS, and can conceptually be extended to the analysis of different types of modifications.

The scope of this approach is limited by the availability of enzymes that specifically remove posttranslational modifications. Because essentially all biochemical reactions are enzyme mediated, it is reasonable to assume that, for most types of reversible protein modifications, there is an enzyme with the specificity to remove that group. It is therefore expected that the scope and application of this technique will be significantly broadened by the development of a large array of ERs, each of which is specific for removal of one type of posttranslational modification. In fact, the use of a specific ER will be a significant advantage over tandem MS methods in cases in which collision-induced fragmentation produces isobaric fragments, a situation that is frequently encountered in the analysis of carbohydrates.

The development and characterization of such a comprehensive system for identification of sites of posttranslational modifications in proteins is currently being investigated and its application would significantly enhance diverse areas in biochemical research.

Materials and methods

Materials and instrumentation

The recombinant protein expression vector (PinPoint Xa-2™ and accessories, cat. no. V4201) were purchased from Promega Corporation (Madison, Wisconsin). The synthetic phosphopeptides PDGF-R, corresponding to the human PDGF-R B chain (residues 851–863) and Igβ, corresponding to the murine B-cell receptor associated protein Igβ (residues 151–198) were synthesized and generously donated by Dr. Ian Clark-Lewis (Biomedical Research Centre, Vancouver, British Columbia, Canada). NHS-LC-biotin was obtained from Pierce (Rockford, Illinois). All other chemicals and reagents were acquired either from Sigma, BDH, or Fisher, and the highest available grade was used. CE separations were performed on a Quanta 4000 CE instrument (Waters, Bedford, Massachusetts). MS analyses were performed on a prototype single quadrupole MS with an attached ESI interface (PE-Sciex, Thornhill, Ontario, Canada). Fused silica capillaries were purchased from Polymicro Technologies (Phoenix, Arizona). A Rheodyne injection valve (no. 8125) (Cotati, California) and Harvard apparatus syringe pump (South Natick, Massachusetts) were used. A 320-μm i.d. × 15-cm-long C₁₈ reversed-phase HPLC column (Micro-Tech Scientific, Sunnyvale, California) was used. The HPLC instrument used in this work was a Michrom ultrafast microprotein analyzer (BioResources Inc., Pleasanton, California).

Buffers for protein isolation and purification

Buffer A was 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM 2-mercaptoethanol. Buffer B was the same as buffer A except that it also contained soybean trypsin inhibitor (0.6 μg/mL), leupeptin (0.5 μg/mL), phenylmethylsulfonylfluoride (1 mg/mL), and pepstatin (0.7 μg/mL).

Expression of in vivo biotinylated HPTP-β

The expression of the biotinylated HPTP-β was performed according to the procedure previously described for the expression of pET-HPTP-β (Harder et al., 1994). Plasmid pHPTP-β was digested with *Hin* dIII and *Bgl* II and the HPTP-β cDNA fragment was then subcloned into *Hin* dIII- and *Bgl* II-digested PinPoint Xa-2™ plasmid, to give PinPoint-HPTP-β. This plasmid was then transformed into competent XL1-β blue bacteria (Stratagene, La Jolla, California). This strain contains an integrated copy of the T7 RNA polymerase gene and is under the control of the IPTG-inducible *lac* UV5 promoter (Wang & Pallen, 1992). A 20-mL overnight culture of PinPoint-HPTP-β grown in 100 μg/mL ampicillin/Luria broth was added to 1 L of the same media and grown with shaking to an OD of 0.7 (λ = 600 nm) at 37 °C in the absence of IPTG. Recombinant fusion protein production was simulated by the addition of IPTG to a final concentration of 1 mM. After 4–5 h of induced growth, the bacterial cells were pelleted by centrifugation at 3,500 rpm for 15 min at 4 °C (Beckman J-6B). The cells (2 mL) were then washed in buffer A, sedimented, and then resuspended in 5 mL of buffer B. A total of 770 μL of 10 mg/mL lysozyme was added and the mixture incubated on ice for 15 min. Later, 3 cycles of alternating freeze-thaws in liquid nitrogen were performed to ensure cell lysis, then 55 μL of 1 M MgCl₂ and 50 μL of 10 mg/mL DNase I were added to the lysate. The resulting mixture was incubated at 25 °C for 20 min, 100 μL of 500 mM EDTA and 600 μL of 10% Triton X-100 were then added, mixed, and reincubated for a further 30 min at 25 °C. Finally, the resulting mixture was subjected to ultracentrifugation at 19,000 rpm at 4 °C for 1 h. The supernatant (8 mL total) was carefully separated, aliquoted into 500-μL fractions, frozen in liquid nitrogen, and stored at –80 °C until ready for use. In order to check for the efficiency of the expression, a crude cell lysate from non-HPTP-β-induced bacteria was also similarly prepared.

SDS-PAGE analysis of the crude cell lysate

The crude cell lysate was analyzed by SDS-PAGE. Gel electrophoresis and blotting were performed using a Mini-Protein II apparatus (Bio-Rad, Richmond, California). Two microliters of the crude cell lysate were added to 38 μL of SDS-PAGE sample buffer, then boiled for 5 min in a water bath, and then centrifuged (Eppendorf centrifuge, model no. 5415 C) for 2 min at 14,000 rpm. Twenty microliters of the resulting supernatant were then loaded onto 12.5% SDS-PAGE gels (0.75 mm) and electrophoresis performed as described by the manufacturer. The gels were run in duplicates. Following electrophoresis, one gel was stained with Coomassie blue for location of the major protein bands. Protein bands in the unstained gel were transferred onto nitrocellulose as done for Western blotting. Later the membrane was incubated for 1 h at room temperature with a 2%

solution of bovine serum albumin in $1 \times$ TBS saline. The membrane was subsequently rinsed three times for 5 min each with $1 \times$ TBST, after which a 30-min incubation of the membrane in $3 \mu\text{L}$ of streptavidin-alkaline phosphatase (Promega) in 15 mL of $1 \times$ TBST was performed. Subsequently, the membrane was washed three times for 5 min each with TBST, rinsed briefly with distilled water, and then incubated in a freshly prepared solution (containing nitroblue tetrazolium and bromochloroindolyl phosphate) with slight agitation until dark purple bands appeared. A similar SDS-PAGE analysis was performed using a crude cell lysate obtained from the noninduced bacteria.

Determination of the activity of the expressed biotinylated HPTP- β

In order to determine the phosphatase activity of the metabolically biotinylated HPTP- β , a small amount ($500 \mu\text{L}$) of the crude cell lysate was purified via affinity sorption with a $50\text{-}\mu\text{L}$ (1:1 resin:buffer) slurry of affinity resin (SoftLink Soft Release avidin resin, Promega). Purification was performed in a batchwise manner by gently shaking the mixture of the lysate and the resin overnight at 4°C . The resin was later sedimented by centrifugation at 6,000 rpm for 5 min at 4°C and then rinsed three times with 1 mL each of buffer A. The final sediment was stored in an equal volume of buffer A at 4°C until use. The phosphatase activity of the avidin resin-immobilized biotinylated HPTP- β was assessed using the synthetic peptide Ig β , which was phosphorylated *in vitro* with recombinant baculovirus expressed and purified p56^{lck} (Watts et al., 1992) in the presence of [γ - ^{32}P]ATP (100 dpm/pmol) following the protocol described by Gold et al. (1994). Four microliters of a solution of the phosphopeptide (containing 30 pmol) in buffer A were added to $1 \mu\text{L}$ of the slurry of the biotinylated HPTP- β avidin resin in $20 \mu\text{L}$ of buffer A and then thoroughly mixed. Twenty microliters of the supernatant were pipetted off at a predetermined time and placed on a $2 \times 2\text{-cm}$ piece of a Whatmann chromatography paper P81 (cat. no. 3698 915, Whatmann Laboratory Division, Maldstone, England). The paper was allowed to dry for 5 min and then washed four times for 15 min each with 100 mL of 1% phosphoric acid. The residual ^{32}P radioactivity on the paper was later measured in a model 2200CA liquid scintillation counter (Canberra-Packard). A similar analysis was performed using avidin resin-purified proteins from noninduced bacterial cell lysate.

Construction of the HPTP- β -activated ERs

The technique used for the construction of the fused silica capillary ER for on-line ER/CE/MS analysis is similar to the procedure that we described previously for a trypsin micro ER (Amankwa & Kuhr, 1992). Simply, the inner surface of a fused silica capillary was modified with 3-aminopropyltriethoxysilane. This treatment provided free amino groups on the capillary surface to which biotin was coupled by the formation of an amide bond. This reaction was performed by slow perfusion of the capillary with 5 mg/mL solution of NHS-LC- β biotin in 50 mM NaHCO_3 (pH 8.6). Subsequent treatment (overnight) at 4°C of the biotin-modified surface with a solution of avidin (10 mg/mL) in 50 mM sodium phosphate (pH 7.2) yielded an avidin-modified capillary. The final step in the ER construction was performed by slowly perfusing (by gravity flow) the avidin-modified capillary with $300 \mu\text{L}$ of HPTP- β -induced bacterial cell lysate over-

night at 4°C . Finally, the capillary was rinsed with about 5–10 column volumes of buffer A and stored at 4°C until ready to use.

For on-line ER/LC/MS analysis, the ER was constructed in a slightly different manner. First, avidin-modified methacrylate resin was used to purify biotinylated proteins from a crude bacterial cell lysate as described earlier in the text. The resin was then packed into 10-cm-long, $254\text{-}\mu\text{m}$ -i.d. teflon tubing until an 8-cm-long plug of resin was obtained. The packing was done by suction of a 50% resin:buffer (v/v) slurry of the resin. A 10-mL plastic syringe (Becton Dickinson and Company, Franklin Lakes, New Jersey) was used to suck the resin into the teflon tubing. Later, a 5-cm-long piece of $50\text{-}\mu\text{m}$ -i.d. $\times$ $360\text{-}\mu\text{m}$ -o.d. fused silica capillary was inserted about 1 cm into one end of the teflon tubing. Another 50-cm-long piece of the same fused silica capillary was inserted at the other end of the packed tubing to secure the resin in the tubing. Because the average particle size of the resin was between 40 and $90 \mu\text{m}$, the inserted fused silica capillaries served both as frit as well as solvent transfer lines. The reactor was then stored at 4°C until ready to use.

Test for phosphatase activity of the ER

The phosphatase activity of the ER was tested both off-line and on-line. In the off-line mode, the synthetic phosphopeptide PDGF-R in 50 mM ammonium formate and 1 mM DTT (pH 7.8) was used as substrate. An aliquot of the substrate containing 6.2 nmol was perfused by gravity through an HPTP- β -modified capillary of 20 cm length at a constant flow rate of 77 nL/min as previously described (Amankwa & Kuhr, 1992). This experiment was repeated using capillaries of 30 cm and 50 cm length, respectively. In each case, the flow rate was maintained at 77 nL/min . Consequently, the parameter that was varied was the amount of time the sample stayed in contact with the enzyme. The effluent from each reactor was collected separately and analyzed by CE with an on-column UV absorbance detector set at 214 nm.

Construction and operation of the coupled ER/CE/MS and ER/LC/MS systems

An integrated system consisting of an ER connected on-line with a CE instrument, which in turn was coupled to an ESI/MS detector, was constructed by connecting an HPTP- β -modified fused silica capillary of 2.5 cm length butt-to-butt at the injection end of the CE separation capillary. The connection was formed using a 1-cm piece of teflon tubing ($254 \mu\text{m}$ i.d.; Mandel Scientific Co. Ltd., Guelph, Ontario) as a sleeve. The $360\text{-}\mu\text{m}$ o.d. fused silica capillary used in this work fits tightly into the sleeve to give a leak-free joint. The joining ends of the reactor and the separation capillaries were both cleanly cut in order to minimize dead volume at the joint. To ensure the maintenance of the activity of the ER, good separation efficiency during electrophoresis, as well as a low background noise in the MS data, a 50 mM solution of ammonium formate containing 1 mM DTT, pH 7.8, was used as the separation buffer. During operation, an aliquot of the sample (20 nL) was introduced onto the ER. In experiments involving CE or on-line ER/CE, sample injection was accomplished by vacuum (15 inches of Hg) suction, whereas electrokinetic injection was used when on-line ER/CE/MS was performed. The sample was then allowed to incubate for a predetermined time inside the ER at ambient temperature.

During this time, a small hydrostatic pressure was applied at the injection end by elevating this end 15 cm relative to the detector end while it was still immersed in the separation buffer. This was done for two reasons. First, this ensured that the sample plug at the entrance of the ER was not dispersed back into the separation buffer reservoir by diffusional or convective forces. Second, by elevating the injection end, the sample plug underwent a slow hydrostatic flow (10 nL/min) inside the ER, ensuring maximum interaction between the sample and the immobilized enzyme. After this incubation time, the injection end was once again brought back to the same level as the detection end of the capillary before separation was initiated. In the on-line mode, detection of the CE-separated components was by either on-column UV absorbance measurement or by mass measurement with the ESI/MS.

The integrated system for on-line ER/LC/MS was constructed in a different manner. The teflon tubing containing the packed HPTP- β -activated resin was connected to an LC/MS system as shown in Figure 8. The inlet end of the reactor was connected to the outlet port of a Rheodyne injection valve, V1, and the outlet end of the reactor was connected to the sample loop of a second injection valve, V2. During operation, sample was injected onto the ER via the sample loop (5 μ L) of V1 into a flowing stream of reaction buffer consisting of 50 mM ammonium formate, pH 7.8, and 1 mM DTT, which was being pumped by a syringe pump at a rate of 2 μ L/min. The effluent from the reactor was directed through the 20- μ L sample loop of V2 (which was set at load position) to waste. At a predetermined time (20.5 min), V2 was switched to the injection position to direct the effluent collected inside the loop onto the LC column for separation and detection by MS. The LC gradient and MS data acquisition were also initiated at this time. A delay time of 20.5 min was necessary to allow the sample plug that was injected onto the ER to migrate through the reactor into the injection loop of the LC column. Dephosphorylation of phosphorylated peptides occurs as the sample migrates through the resin. The necessary delay time required to capture all of the sample plug in the sample loop of V2 was determined by injecting an aliquot of 125 I-labeled pentapeptide (125 I-YGGFL) onto the ER while it was disconnected from V2. Two-minute fractions of the effluent from the ER were collected and monitored for 125 I activity with an Isoflex Gamma Counter (ICN Micro-medics Systems, Huntsville, Alabama). About 95% of the starting 125 I activity was recovered in fractions 8–10. Consequently, a delay time of 20.5 min was allowed in order to capture all of the sample band in the sample loop of V2. The average time the sample spent inside the reactor was roughly 16.5 min. For reuse, the ER can be flushed with buffer A and then stored at 4 °C. During analysis by only LC/MS, the sample was injected directly via the sample loop of V2 onto the LC column.

CE

Two different lengths of capillary were used. When detection was by on-column UV absorbance monitoring, a capillary of 70 cm length was used, whereas a 140-cm length capillary was used when detection was by UV monitoring and ESI/MS. The voltage for CE separation was (+)26 kV (current = 45 μ A for the 70-cm capillary). During experiments involving on-line ER/CE/MS, an auxiliary high-voltage power supply (Spellman CZE1000R, High Voltage Electronics Corp., Plainview, New

York) was used to supply (+)5 kV to the electrospray needle in the ESI interface instead of the MS high-voltage power supply. This was necessary in order to prevent cross-talk between the electronics of the MS and the CE instrument.

Capillary HPLC chromatography

A 320- μ m-i.d. $\times$ 15-cm-long C₁₈ reversed-phase HPLC column was connected from the outlet port of V2 to the ESI interface of the MS with a 50-cm-long $\times$ 50- μ m-i.d. $\times$ 150- μ m-o.d. fused silica capillary. The HPLC gradient was performed and delivered by the Michrom ultrafast microprotein analyzer. The chromatography mobile phases were 0.05% TFA/2% acetonitrile in water (phase A) and 0.045% TFA/80% acetonitrile in water (phase B). Samples were introduced onto the column isocratically for 5 min with phase A followed by a gradient from 0% phase B to 50% phase B over another 35 min at flow rate of 5 μ L/min. A precolumn flow split was installed to reduce the flow rate from 100 μ L/min being delivered by the pumps in the HPLC chromatograph.

MS and ESI interface

The MS was a prototype single-quadrupole instrument equipped with an ESI interface (PE/Sciex, Thornhill, Ontario, Canada). For operation on-line with a CE instrument, the original ion-spray interface setup was modified according to an earlier design in order to provide both a coaxial liquid sheath as well as a coaxial gas sheath (Thibault et al., 1991). The double coaxial interface consisted of two tees (Valco) and a stainless steel needle. The back tee was used to provide the liquid sheath make-up solution necessary for good electrical conductivity for the CE separation and also for the maintenance of good electrospray. The liquid sheath was supplied by a 50- μ m-i.d. $\times$ 150-mm-o.d. fused silica capillary at a flow rate of 6.0 μ L/min and was pumped from a reservoir by an LC pump (Shimadzu, LC-10AD; Shimadzu Corp., Kyoto, Japan) via a valve (Rheodyne, no. 8125). The sheath liquid was composed of 0.2% formic acid in 50% methanol/water. The front tee was used for introduction of the nebulization sheath gas and to connect the high-voltage cable for the ionspray needle. The nebulization sheath gas was supplied via 1-mm-i.d. teflon tubing at a rate of 2 L/min. The CE capillary protruded about 0.1 mm from the ionspray needle, which in turn protruded about 0.5 mm from the concentric nebulizer sheath gas needle. A drying nitrogen curtain gas at ambient temperature was supplied at a flow rate of 1.8 L/min to the region between the ionspray ionization source and the MS orifice. The same ESI interface setup was used when LC/MS analyses were performed except that no liquid sheath was used during these analyses because the flow rate of 5 μ L/min was adequate for obtaining good electrospray ionization. Tuning and calibration of the MS was performed either with polypropylene glycol or myoglobin. Typically, the quadrupole was scanned from m/z 300 to 2,000 at 2.9 s/scan unless otherwise stated. All mass spectra data were background subtracted.

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References

- Aebersold R, Watts J, Morrison HD, Bures EJ. 1991. Determination of the site of tyrosine phosphorylation at the low picomole level by solid-phase sequence analysis. *Anal Biochem* 199:51-60.
- Amankwa LN, Kuhr WG. 1992. Trypsin-modified fused silica capillary microreactor for peptide mapping by capillary zone electrophoresis. *Anal Chem* 64:1610-1613.
- Bolen JB. 1993. Nonreceptor tyrosine kinases. *Oncogene* 8(8):2025-2031.
- Carr SA, Biemann K. 1984. Identification of post-translationally modified amino acids in proteins by mass spectrometry. *Methods Enzymol* 106:29-58.
- Covey T, Shushan B, Bonner R, Schroder W, Hucho F. 1991. LC/MS, LC/MS/MS screening for the sites of post-translational modification in proteins. In: Jornvall H, Hoog JO, Gustavsson AM, eds. *Methods in protein sequence analysis*. Basel: Birkhauser Verlag. pp 249-256.
- Cress D, Shultz J, Breitlow S. 1993. A one-step nondenaturing purification method for recombinant proteins produced in *E. coli*. *Promega Notes* 42:2-7.
- Cronan JE Jr. 1990. Biotinylation of proteins in vivo. *J Biol Chem* 265:10327-10333.
- Di Luca M, De Graan PN, De Angelis L, Gispén WH, Cattabeni F. 1992. Measurement of relative amounts of phospho- and dephospho- β -50(GAP-43) peptides by fast atom bombardment-mass spectrometry. *FEBS Lett* 301:150-154.
- Fry MJ, Panayotu G, Booker GW, Waterfield MD. 1993. New insights into protein-tyrosine kinase receptor signaling complexes. *Protein Sci* 2:1785-1797.
- Gold MR, Chiu R, Ingham RJ, Saxton TM, van Oostveen I, Watts JD, Af-folter M, Aebersold R. 1994. Activation of serine phosphorylation of the p56^{lck} protein-tyrosine kinase in response to antigen receptor cross-linking in B lymphocytes. *J Immunol* 153(6):2369-2380.
- Harder KW, Owen P, Wong LKH, Aebersold R, Clark-Lewis I, Jirik F. 1994. Characterization and kinetic analysis of the intracellular domain of human tyrosine phosphatase β (HPTP β) using synthetic phosphopeptides. *Biochem J* 298:395-401.
- Hou J, McKeenhan K, Kan M, Carr SA, Huddleston MJ, Crabb JW, McKeenhan WL. 1993. Identification of tyrosines 154 and 307 in the extracellular domain and 653 and 766 in the intracellular domain as phosphorylation sites in the heparin-binding fibroblast growth factor receptor tyrosine kinase (flg). *Protein Sci* 2:86-92.
- Huddleston MJ, Annan RS, Bean MF, Carr SA. 1993. Selective detection of phosphopeptides in complex mixtures by electrospray liquid chromatography-mass spectrometry. *J Am Soc Mass Spectrom* 4(9):710-717.
- Hunter T. 1987. A thousand and one protein kinases. *Cell* 50:823-829.
- Johnson LN, Barford D. 1993. The effects of phosphorylation on the structure and function of proteins. *Annu Rev Biophys Biomol Struct* 22:199-232.
- Krueger NX, Streuli M, Saito H. 1990. Structural diversity and evolution of human receptor-like protein tyrosine phosphatases. *EMBO J* 9:3241-3252.
- Liao P, Leykam J, Andrews PC, Gage DA, Allison J. 1994. An approach to locate phosphorylation sites in a phosphoprotein: Mass mapping by combining specific enzymatic degradation with matrix-assisted laser desorption/ionization mass spectrometry. *Anal Biochem* 219:9-20.
- Nuwaysir LM, Stults JT. 1993. Electrospray ionization mass spectrometry of phosphopeptides isolated by on-line immobilized metal-ion affinity chromatography. *J Am Soc Mass Spectrom* 4:662-669.
- Palczewski K, Buczylo J, Hooser PV, Carr SA, Huddleston MJ, Crabb JW. 1992. Identification of the autophosphorylation sites in rhodopsin kinase. *J Biol Chem* 267:18991-18998.
- Pelech SL, Sanghera JS, Daya-Makin M. 1990. Protein kinase cascades in meiotic and mitotic cell cycle control. *Biochem Cell Biol* 68:1290-1330.
- Schlessinger J, Ullrich A. 1992. Growth factor signaling by tyrosine kinases. *Neuron* 9(3):383-391.
- Thibault P, Pleasance J, Laycock MV. 1991. Analysis of paralytic shellfish poisons. *J Chromatogr* 542:483-501.
- Thompson TJ, Foret F, Vouros P, Karger BL. 1993. Capillary electrophoresis/electrospray ionization mass spectrometry: Improvement of protein detection limits using on-column transient isotachophoretic sample preconcentration. *Anal Chem* 65:900-906.
- Uy R, Wold F. 1977. Post-translational covalent modification of proteins. *Science* 198:890-896.
- Wang Y, Pallen CJ. 1992. Expression and characterization of wild type, truncated and mutant forms of the intracellular region of the receptor-like protein tyrosine phosphatase HPTP β . *J Biol Chem* 267:16696-16702.
- Watts JD, Wilson GM, Ettehadieh E, Clark-Lewis I, Kubanek CA, Astell CR, Marth JD, Aebersold R. 1992. Purification and initial characterization of the lymphocyte-specific protein-tyrosyl kinase p56^{lck} from a baculovirus expression system. *J Biol Chem* 267:901-907.
- Wettenhall EH, Aebersold R, Hood LE. 1991. Solid-phase sequencing of ³²P labelled phosphopeptides. *Methods Enzymol* 201:186-199.
- Wold F. 1981. In vivo chemical modification of proteins *Annu Rev Biochem* 50:783-814.
- Wold F, Moldave K, eds. 1984. *Post-translational modification. Methods in enzymology, vol 107*. New York: Academic Press.
- Yip T, Hutchens TW. 1992. Mapping and sequence-specific identification of phosphopeptides in unfractionated protein digest mixtures by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *FEBS Lett* 308:149-153.