Identification by Electrospray Ionization Mass Spectrometry of the Sites of Tyrosine Phosphorylation Induced in Activated Jurkat T Cells on the Protein Tyrosine Kinase ZAP-70*

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Julian D. Watts‡, Michael Affolter‡, Danielle L. Krebs§, Ronald L. Wanger†, Lawrence E. Samelson†, and Ruedi Aebersold||

From the Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3 and the ¶Cell Biology and Metabolism Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892

We have developed a rapid and sensitive two capillary-column chromatography and mass spectrometry-based method for the determination of protein phosphorylation sites following recovery of individual phosphopeptides from two-dimensional phosphopeptide maps. With a standard phosphopeptide, we demonstrate detection sensitivity of at least 250 fmol for this system. We applied this technique to the analysis of in vitro protein tyrosine phosphorylation induced on the T cell-specific protein tyrosine kinase ZAP-70 in the absence and presence of p56^ck. We show that ZAP-70 has a primary autophosphorylation site at Tyr-292, with a secondary site at Tyr-126. We also show additional phosphorylation at Tyr-69, Tyr-175, Tyr-492, and Tyr-493 upon the addition of the protein tyrosine kinase, p56^ck. By comparative two-dimensional phosphopeptide mapping, we show that ZAP-70 isolated from Jurkat T cells also autophosphorylates at Tyr-292 and Tyr-126. Similar analysis of 32P-labeled Jurkat cells stimulated with anti-T cell receptor antibodies reveals Tyr-492 and Tyr-493 as the principal sites of T cell antigen receptor-induced tyrosine phosphorylation, with additional phosphorylation at the Tyr-292, but not the Tyr-126 autophosphorylation site. The high degree of phosphorylation and sensitivity of this technology should greatly facilitate the direct biochemical determination of inducible protein phosphorylation events, an experimental strategy that until now has been both time consuming and difficult.

In recent years, it has become clear that almost all cellular responses to external stimuli, hormonal, chemical, or otherwise, are to a large part regulated on an intracellular level by reversible protein modification. Of particular importance is the role of protein phosphorylation/dephosphorylation events, which are carried out by protein kinases and phosphatases, respectively. It is now known that many of the critical early phosphorylation events that occur within a cell following stimulation are performed by protein tyrosine kinases (PTK).1*

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† Should be considered as joint first author.

‡ Participant in the Co-operative Biochemistry Program at Simon Fraser University, Burnaby, BC.

|| Recipient of a Medical Research Council (Canada) scholarship. To whom correspondence should be addressed: Dept. of Molecular Biotechnology, University of Washington, PJ-20, Seattle, WA 98195. Tel.: 206-685-4235; Fax: 206-685-6932.

1 The abbreviations used are: PTK, protein tyrosine kinase; TCR, T cell antigen receptor; TAM, tyrosine-based activation motif; pTyr, phosphotyrosine; 2D, two-dimensional; MS, mass spectrometry; IMAC, immobilized metal affinity chromatography; HPLC, high performance liquid chromatography; ESI, electrospray ionization; TIC, total ion current; PDTGR, platelet-derived growth factor receptor; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; pC12-beads: phospho-TCR conjugated agarose beads; PMSF, phenylmethylsulfonyl fluoride.
its association with the phosphorylated form of TCRζ (27, 29-32, 35) and CD3ε subunits (31, 32, 35). Recent studies have shown that ZAP-70, like p56^c^ and p59^ζ^, is vital to the induction of a full TCR-mediated response, including the induction of tyrosine phosphorylation events, through its interaction with the phosphorylated forms of TCR subunits. These studies include the analysis of immunodeficient individuals lacking ZAP-70 expression (36-38), or in cells in which the binding of ZAP-70 to phosphorylated TCRζ is blocked. However, since little is currently known about the regulation of ZAP-70 activity and the nature of its potential substrates, the mechanism by which it plays such a critical role in TCR-mediated signaling remains unclear. Since it is now clear that many protein kinases are regulated via reversible tyrosine phosphorylation (for example, all members of the src family), the determination of the TCR-induced sites of tyrosine phosphorylation on ZAP-70 should be an important first step in answering the above questions.

Current techniques for the analysis of protein phosphorylation rely largely on two-dimensional (2D) phosphopeptide mapping of isolated proteins which have been phosphorylated either in vitro kinase assays, or in vivo following metabolic labeling of cells with radiolabeled phosphate. However, due to the very small quantities of most of the phosphoproteins of interest present in living cells and the generally low stoichiometry of protein phosphorylation, direct biochemical determination of phosphorylation sites inducible in vivo is not presently a viable experimental approach. In order to circumvent these limitations, we combined and adapted a number of current techniques in column chromatography and electrospray ionization mass spectrometry (ESI-MS). We took advantage of the affinity of phosphopeptides for immobilized Fe^{III} ions (39) by linking microbore immobilized metal affinity chromatography (IMAC) and high performance liquid chromatography (HPLC) columns in series, with on-line detection of eluted peptides by ESI-MS. With this system, we demonstrated detection of a synthetic phosphopeptide with sensitivity of at least 250 fmol of injected peptide. By using recombinantly expressed and isolated ZAP-70 and p56^ζ^, we were able to recover tryptic phosphopeptides from 2D peptide maps and determine the sites of ZAP-70 phosphorylation and those induced on ZAP-70 by 56^ζ^ in vitro. The generation of tryptic phosphopeptide maps of ZAP-70 isolated from stimulated Jurkat T cells following metabolic labeling, and their comparison with our in vitro generated data thus allowed us to determine the sites of tyrosine phosphorylation induced on ZAP-70 following TCR engagement on the basis of the co-migration of in vitro and in vivo derived phosphopeptides.

Since many of the important phosphoprotein components of cell signaling pathways have now been identified, the availability of expressed forms of these proteins means that such an approach for the determination of the in vitro phosphorylation sites on such molecules should be a more rapid approach than by mutagenic analysis of all potential phosphorylation sites (ZAP-70 itself contains over 30 tyrosine residues). Additionally, by its very nature, direct biochemical determination of phosphorylation sites is more reliable than indirect analysis via mutagenesis. The availability of rapid, sensitive, and direct approaches for the determination of inducible phosphorylation sites on ZAP-70 and other proteins of interest in cell signaling pathways should thus greatly facilitate investigation of the specific roles played by these modifications via site-directed mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents—**All laboratory chemicals were from Fisher Scientific and of appropriate purity, unless otherwise stated. Solvents for 2D peptide mapping were from British Drug House and were of HPLC grade or better, trifluoroacetic acid was from Applied Biosystems. 18-methylmethylsulfanyl fluoride (PMSF) from Boehringer Mannheim; β-mercaptoethanol from Millipore; 32P-labeled reagents from ICN Biomedica; ATP, polyvinylpyrrolidone-40, protein G-Sepharose, β-methylaspartic acid, soybean trypsin inhibitor, and aprotinin from Sigma; sodium orthovanadate was obtained from British Drug House; dithiothreitol from Calbiochem; sequencing grade trypsin from Promega; cellulose thin layer chromatography plates and x-ray film from Kodak.

Synthetic peptides were generously provided by Dr. I. Clark-Lewis (Biomedical Research Centre, University of British Columbia). Phospho-ζ-beads were prepared essentially as described elsewhere (29) and were generously provided by Dr. R. Orchanetz (Department of Microbiology, University of British Columbia). The monoclonal antibody (mAb) OKT3 was prepared as described elsewhere (40), and 9E10 mAbs were prepared as ascites fluid.

**MicroIMAC Column Chromatography—**A 50-µm inner diameter × 380-µm outer diameter × 30-cm-long fused silica capillary (Polymicro Tech. Inc., Phoenix, AZ) was inserted 5–10 mm into a 250-µm-inner diameter × 1.0-mm outer diameter piece of Teflon tubing (Mandel Scientific Company Ltd., Guelph, Ontario), which was connected with standard fittings to a 1-mL syringe filled with 100 µL of a 50% slurry of chelating Sepharose Fast Flow (Pharmacia Biotech Inc.) in 20% ethanol. Since the gel bed diameter was larger than the outlet capillary inner diameter, no terminating frit had to be used at the outlet of the microIMAC column. Approximately 3 cm of the Teflon tubing was filled manually with chelating Sepharose beads as monitored under a stereo microscope (final column volume, 1.5 mL). After disconnection from the syringe, the Teflon tubing was cut 5–10 mm above the packed beds and a 5-cm long piece of fused silica capillary (50-µm inner diameter × 380-µm outer diameter) was inserted to close the open end of the column.

Fig. 1 shows schematics of the instrumentation described below. The assembled microIMAC column was connected to a Rheodyne (Cotati, CA) 8125 injector equipped with a 5-µL sample loop and washed with water delivered by a Harvard apparatus (South Natick, MA) syringe pump at a flow rate of 5 µL/min for 10 min. The column was activated with five injections of 5 µL of 50 mM FeCl₃ solution at 1-min intervals, which was washed for another 5 min with water. The water was similarly replaced with 0.1 M acetic acid, and the column washed with the same for at least 10 min at a flow rate of 5 µL/min. Prior to the initial use, the microIMAC column was washed by injecting 5 µL of 0.1% ammonium acetate, pH 8, containing 50 mM Na₂HPO₄ (elution buffer). Fig. 1, B, and C, shows an enlargement of the Rheodyne 7000 switching valve used to control delivery of samples eluted from the IMAC column to the HPLC-ESI-MS system. Samples were normally loaded onto the IMAC column in volumes of less than 5 µL, and the column was washed to waste (through the sample loop) for 10 min in 0.1 M acetic acid, pH 3 (Fig. 1B). Bound phosphopeptides were then eluted by injecting 5 µL of elution buffer, the IMAC eluate eluate filling the 7-µL sample loop (Fig. 1B) prior to delivery on-line to the HPLC-ESI-MS system (see Fig. 1C). The exact timing for the valve switching was determined using a 32P-labeled phosphopeptide and collecting 1-µL fractions following the injection of 5 µL of elution buffer onto the microIMAC column. 80–90% of the radioactivity was reproducibly recovered in fractions 1–6, thus for the above column, dimensions and flow rates, a time delay of 96 ± 8 µL was used to allow eluted phosphopeptide(s) to fill the 7-µL loop prior to switching the valve to apply the phosphopeptide to the HPLC column (Fig. 1C). Any remaining sample bound to the microIMAC column was washed out by injecting another 5 µL of elution buffer onto the column and washing to waste, while the HPLC gradient was developed. By placing the solvent back from acetic acid into water and then to water/methanol (4:1 v/v), the microIMAC column could be stored at 4°C for 2–3 weeks without a reduction in performance. For re-use, the microIMAC column was preconditioned as described above for a new column, except that a 10-min wash with elution buffer was performed prior to the column activation.

**Narrow Bore HPLC Column Chromatography—**The instrumentation used and HPLC-ESI-MS analyses performed were a modification of

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2 R. L. Wange, N. Isakov, T. R. Burke, Jr., A. Otaka, P. P. Roller, J. D. Watts, R. Aebersold, and L. E. Samelson, submitted for publication.
those described elsewhere. A Fig. 1A gives a schematic summary of the instrumentation used in these studies.

A 320-μm inner diameter × 15-cm long C18 reversed phase HPLC column (Micro-Tech Scientific, Sunnyvale, CA) was connected to port 4 of a Rhodyne 7000 valve, and by a fused silica capillary (50-μm inner diameter × 150-μm outer diameter) to the electrospray probe tip of a PE Sciex (Thornhill, Ontario) APIII triple quadrupole mass spectrometer. Valve port 5 was connected with PEEK-tubing (0.005-inch inner diameter) column (Micro-Tech Scientific, Sunnyvale, CA) was connected to port 4 of the system are given under "Experimental Procedures." Expanded views of the switching valve from Panel A (*) used to link the microIMAC column to the HPLC system are shown. B, valve position for IMAC column loading, washing, and subsequent elution of bound phosphopeptides into the sample loop. C, valve position for loading contents of the sample loop onto the HPLC system and subsequent development of HPLC column.

for the capillary column.

For analyses which required the microIMAC column to precede the HPLC column, the microIMAC outlet capillary was connected to port 1 of the switching valve with port 2 as exit to the waste, with a 7-μl sample loop installed between ports 3 and 6 (see Fig. 1, B and C). In the IMAC column load position of the switching valve, ports 1-6, 2-5, and 4-5 were connected, allowing the eluate from the syringe pump-driven low pressure microIMAC column to pass through the sample loop to waste (Fig. 1B), while the HPLC column remained connected to the high pressure pump system. Switching the valve (port connection 3-4 and 5-6) thus allowed the contents of the sample loop to be applied to the HPLC column, while the microIMAC system maintained its flow through the port 1-2 connection (Fig. 1C). The valve was switched back to its starting configuration after 5 min to reduce void volumes and time delays on the HPLC system.

Mass Spectrometric and Data Analyses—ESI-MS analyses were performed on a PE Sciex APIII triple quadrupole mass spectrometer, equipped with a pneumatically assisted ESI source (ion spray). The mass spectrometer scanned repetitively over a mass to charge ratio (m/z) range of 300–2000, using a step of 0.5 unit, a dwell time of 1.0 ms (single scan duration = 3.4 s), and a 80 V orifice potential.

Computer software provided with the mass spectrometer permits mass spectra to be displayed for any observed peak of ion detection events, allowed background subtraction, as well as extraction of a defined input mass or mass range from the data set. A deconvolution algorithm was used to calculate peptide masses based on computer matching of observed signals with the predicted m/z values for the various possible charge states of the same peptide, and to perform theoretical fragmentation of any input protein sequence, listing all possible fragments along with their predicted charged mass values (M + H+).

Two-dimensional Phosphopeptide Mapping—Following SDS-polyacrylamide gel electrophoresis (PAGE), phosphoproteins were either digested in situ following Coomassie Blue staining, destaining, and partial vacuum drying of excised gel slices, or immobilized on a membrane following transfer to nitrocellulose and blocking in 1% polyvinylpyrrolidone-40, 100 mg acetic acid for 1 h at 37 °C (41). Proteolytic digestion was overnight at 37 °C in 150–200 μl of 1% ammonium bicarbonate, pH 8.3, with 1 μg of trypsin added to each sample. Eluted phosphopeptides were recovered in the supernatant, Cerenkov counted in a Packard 2200CA Tri-Carb liquid scintillation counter to quantitate peptide recovery, and dried under vacuum. Samples were then resuspended in a minimal volume of water and spotted onto (20 × 10 cm) cellulose TLC plates. These were electrophoresed in the first dimension (20 cm) in water/acetic acid/pyridine (89:10:1, v/v/v) at 10 °C and 1000 V for 110 min, dried in air, and developed in the second dimension (10 cm) in water/pyridine/butan-1-ol/ acetic acid (34:30:30:6, v/v/v). The plates were extensively dried in air, and phosphopeptides subsequently visualized by autoradiography.

Phosphopeptides required for further analysis were recovered from the cellulose TLC plate by carefully removing the cellulose matrix containing the phosphopeptide spot of interest from the plastic support with a surgical blade. The matrix was resuspended in 150–200 μl of water/acetonitrile (4:1, v/v) and samples placed in a water bath sonicator for ~15 min to break up the cellulose. Following centrifugation, phosphopeptide supernatants were removed and the recovery quantitated by Cerenkov counting the supernatants and residual cellulose pellets. These counts were also used to estimate the quantity of each...
phosphopeptide present prior to IMAC-HPLC-ESI-MS analyses. Samples were reduced to a minimal volume in a Speedvac concentrator to facilitate easy loading onto the microMAC column.

Cloning and Expression of ZAP-70 in a Baculovirus System—The generation of the ZAP-70-containing baculovirus transfer vector will be described briefly. A full length cDNA of the human ZAP-70 gene was inserted into the baculovirus transfer vector, pVL1393 (Invitrogen, San Diego, CA). The ZAP-70 cDNA also had an epitope tag with the optimum time for ZAP-70 expression being thus determined with the positive clones, designated pVL1393-ZAPmyc-6, was used to generate plasmid for baculovirus expression by Invitrogen.

Baculovirus-infected High Five cells (performed by Invitrogen) were then screened for c-Myc-tagged ZAP-70 expression by immunoblotting, with the optimum time for ZAP-70 expression being thus determined as 48 h post-infection (not shown).

In Vitro Phosphorylation of Baculovirus-expressed ZAP-70—High Five cells (1-ml pellet) expressing the c-Myc-tagged ZAP-70 described above were lysed in 2 ml of ice-cold 50 mM Tris, pH 7.5, 25 mM β-mercaptoethanol, 5 mM EDTA, 2 mM EGTA, 2 mM PMSF, 0.2 mM NaVO₃, 0.2 mM dithiothreitol, 10 mM β-mercaptoethanol, 10 mM MgCl₂, 20 μg/ml leupeptin, 50 μM aprotinin. Insoluble matter was removed by centrifugation at 200,000 × g for 20 min at 4 °C. The supernatant was recovered, glycerol added to 50% (v/v), and the pellet P-40 to 1 ml. Lysates were stored thus in 100-μl aliquots at −80 °C until required.

For analytical phosphopeptide mapping, 20 μl of lysate was precipitated with 1.5 μl of 9E10 mAb in PBS buffer and 20 μl of lysate were each precipitated with 1 μl 9E10 mAb ascites fluid. For preparative phosphopeptide mapping (for subsequent IMAC-HPLC-ESI-MS), four aliquots of 50 μl of lysate were each precipitated with 10 μl of 9E10 mAb. Samples were diluted to 500 μl in ice-cold 25 mM Tris, pH 7.5, 10% glycerol, 5 mM NaF, 2 mM EDTA, 0.1% Nonidet P-40, 1 mM NaVO₃, 1 mM Na,MoO₃, 1 mM PMSF prior to the addition of the 9E10. Precipitation was at 4°C for 2 h, followed by the addition of 30 μl of a 50% slurry of protein G-Sepharose for 1 h at 4°C. Precipitates were washed 4 × in 1 ml of 1% Nonidet P-40, 50 mM NaCl, 2 mM EDTA, 0.5% Triton X-100 prior to in vitro kinase assays.

ZAP-70 in vitro phosphorylation assays were performed by the addition of 20 μl of 50 μM Tris, pH 7.5, 20 mM MgCl₂, 0.1% Nonidet P-40, 250 μM ATP, with [γ-32P]ATP added to either 880 pmol/pmol (for preparative 2D peptide mapping) or 8800 pmol/pmol (for analytical 2D peptide mapping). When required, recombinantly expressed and purified 56 kDa (43) was also added in three 5-μl aliquots at 20-min intervals. Phosphorylation reactions were performed for 1 h at 30 °C with frequent mixing. Reactions were terminated with the addition of SDS-PAGE sample loading buffer, boiled for 5 min and run on 10% SDS gels. 2D phosphopeptide mapping was performed in situ in the (wet) gel as described above.

In Vitro Phosphorylation of Jurkat ZAP-70—Jurkat cells were maintained in RPMI 1640, supplemented to 10% with fetal calf serum, 290 mM l-glutamine, and 25 mM β-mercaptoethanol. Cells were spun down and resuspended at 1 x 10⁷/ml in ice-cold 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM NaF, 2 mM EDTA, 0.5% Triton X-100, 1 mM NaVO₃, 1 mM Na,MoO₃, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 5 μg/ml aprotinin. Insoluble matter was removed by centrifugation, and supernatants were precipitated with non-phosphorylated or phosphorylated TCRC beads as described above (the non-phosphorylated TCRC beads were added only to the stimulated, 5 μl-labeled sample). Precipitates were subsequently washed four times each with 1 ml of the above lysis buffer, boiled for 5 min following the addition of 100 μl 1 x SDS-PAGE sample buffer, and run on 10% SDS gels. 2D phosphopeptide mapping was performed as described above following transfer of the gel-separated proteins to nitrocellulose.

RESULTS

We have previously described a method for the recovery of phosphopeptides from 2D phosphopeptide maps and their analysis by microbore HPLC with on-line detection by electrospray ionization mass spectrometry (HPLC-ESI-MS). In those studies, we were analyzing phosphorylated synthetic peptides and thus had an abundant supply of material. We found that the lower sensitivity limit for this system was about 10–20 pmol of injected peptide due to background signal interference, most likely originating from the cellulose TLC plate-coating material or the solvent system used during the chromatography dimension.

To develop a more suitable system for the analysis of phosphopeptide maps derived from phosphoproteins isolated by immunoprecipitation from cultured cell lines, an improvement in sensitivity was required.

Earlier studies had shown that phosphopeptides have an affinity for immobilized Fe³⁺ ions (39). However, attempts to couple Fe³⁺ IMAC columns with MS detection resulted in the simultaneous detection of all phosphopeptide species eluted from the column, with poor detection sensitivity (10–20 pmol lower limit) (44). Also in these studies, the optimal conditions for the elution of phosphopeptides from the IMAC column and their detection by ESI-MS are not compatible, leading to a lower detection sensitivity due to the need to compromise. Under these conditions, the IMAC column also rapidly de novo, making its frequent replacement necessary. We overcame these limitations by linking an HPLC column in series following a microIMAC column. This allowed both the removal of proteins by the background signal resulting from the IMAC elution conditions and the detection of multiple phosphopeptides. The schematic in Fig. 1A shows the instrumentation used in these studies. The flow to the IMAC column was delivered by a mechanical syringe drive to provide a low pressure flow rate and to facilitate the frequent changes of buffers required for delivery to the IMAC column. Following loading and extensive washing of the IMAC column, the phosphopeptide was eluted with 0.1% ammonium acetate, pH 8, 50 mM Na₂HPO₄, and a switching valve was used to inject the sample into the HPLC system (Fig. 1, B and C). Following a 10-min isocratic wash, a 15-min acetoniitrile gradient was used to elute bound material from the HPLC column. Sample detection was by on-line ESI-MS.

The IMAC-HPLC-ESI-MS system was calibrated by loading different amounts of a synthetic phosphopeptide based on the platelet-derived growth factor receptor (PDGFR) (residues 851–863) (45). Fig. 2A shows the extracted total ion current (TIC) in the 500–2000 m/z range observed in the time window where the peptide eluted (~15 min) following a 25-μl initial loading (~400 pg). The data analysis software scales the data set to the largest peak observed, the peak intensities in this case being relative to the large injection peak caused by the phosphate salt in the IMAC elution buffer (not shown). Fig. 2B shows the m/z spectrum observed within the indicated peak

4 T. R. Isakov, R. L. Wange, and L. E. Samelson, manuscript in preparation.

5 M. Affolter, unpublished observations.
Peak intensities are expressed as a percentage of the largest signal peak of the data analysis, was performed five times and averaged for each data set to smooth out variations in resulting ion counts at time 0.

The doubly charged species (\(M + 2H^+\)) was a dominant peak in the 12-22 min time window in two 250-fmol loadings, and in one of two 125-fmol loadings (data not shown). Thus the IMAC-HPLC-ESI-MS system currently has an effective lower sensitivity limit of \(~125\) fmol for a phosphopeptide of unknown mass. For a peptide of known mass (where the data set can be extracted for the predicted \(m/z\) values), we could easily detect 50 fmol of injected PDGFR peptide signal above background mass detection levels (Fig. 2C and data not shown).

We then compared the IMAC-HPLC-ESI-MS system with the HPLC-ESI-MS system alone to test whether it was capable of improving the detection of phosphopeptides recovered from 2D phosphopeptide maps. For this we used a phosphopeptide derived from the in vitro phosphorylation of ZAP-70 by \(p56^{dc}\) recovered from a 2D phosphopeptide map (described below). The mass spectrum for the indicated peak is shown in the inset, with the singly (\(M + H^+ = 1247.0\)) and doubly (\((M + 2H^+) = 624.5\)) charged species being observed. It is clear that in the HPLC-ESI-MS system, any peptide eluting \(~22\) min post-injection would be difficult to find due to the characteristic "hump" of observed contaminants resulting from the 2D mapping procedure. Fig. 3B shows the significant improvement in the nonspecific mass background for an estimated 5.0 pmol loading of the same peptide upon inclusion of the IMAC column in chromatography system. The inset again gives the mass spectrum observed within the indicated peak. As can be seen from Fig. 3B, the signal peak for a 5-pmol loading is well resolved above the noise levels (signal to noise \(~15:1\)), thus the detection sensitivity of phosphopeptides eluted from TLC plates appears comparable to that observed with the PDGFR standard peptide. Thus the IMAC-HPLC-ESI-MS was clearly suitable for the analysis of the low picomole quantities of phosphopeptide recoverable from 2D phosphopeptide maps.
A single phosphopeptide resulting from the in vitro phosphorylation of expressed ZAP-70 by expressed p56\textsuperscript{ck} was used to estimate quoted pmol values from the known mental Procedures was analyzed on both systems. Cerenkov counting of sample onto a previously described HPLC-ESI-MS system (Molter, M., Watts, J. D., Krebs, D. L., and Aebersold, R. (1994) Anal. Biochem., in press). The solid line represents the TIC plotted against time (minutes) given as a percentage relative to the largest signal peak. The broken line gives the acetonitrile concentration delivered by the pump system to elute bound material from the HPLC column. The inset gives the complete \( m/z \) spectrum for the indicated peak following background subtraction. Peak intensities (RI) are given as a percentage, relative to the largest signal peak. The singly charged \((M + H^+ = 1247.0)\) and doubly charged \((M + 2H^+ = 624.5)\) phosphopeptide species are indicated. B, an estimated 5.0-pmol loading of sample onto the IMAC-HPLC-ESI-MS system. Data analysis and representation is as described for A and shows the significant improvement in the signal to noise ratio for the peak of interest.

samples before and after digestion consistently gave a recovery of 80–90% of the phosphopeptides into solution (data not shown), suggesting that most, if not all major phosphorylation sites are represented in Fig. 4, B and C. Phosphoamino acid analyses of each spot confirmed all as containing exclusively pTyr (data not shown). Spot G was analyzed by IMAC-HPLC-ESI-MS as described above, the data set being shown in Fig. 3B. The singly charged ion \((M + H^+ = 1247.0)\) and doubly charged ion \((M + 2H^+ = 624.5)\) correspond to a phosphopeptide mass of 1246.0, which matches the predicted mass of 1166.6 for a partial tryptic fragment of ZAP-70 corresponding to residues 176–186 (KLYSGAQTDGK) plus an additional 80 mass units due to the phosphate ester group.

The remaining spots (A–F) marked in Fig. 4C were analyzed in the same way, and these data are summarized in Table I. These analyses revealed that two of the autophosphorylation spots (A and B) corresponded to autophosphorylation at the same residue (Tyr-292) being derived from partial digestion at

**Fig. 3.** Comparison of HPLC-ESI-MS and IMAC-HPLC-ESI-MS. A single phosphopeptide resulting from the in vitro phosphorylation of expressed ZAP-70 by expressed p56\textsuperscript{ck} following trypsin digestion and elution from a 2D phosphopeptide map, as described under “Experimental Procedures” was analyzed on both systems. Cerenkov counting of sample onto a previously described HPLC-ESI-MS system (Molter, M., Watts, J. D., Krebs, D. L., and Aebersold, R. (1994) Anal. Biochem., in press). The solid line represents the TIC plotted against time (minutes) given as a percentage relative to the largest signal peak. The broken line gives the acetonitrile concentration delivered by the pump system to elute bound material from the HPLC column. The inset gives the complete \( m/z \) spectrum for the indicated peak following background subtraction. Peak intensities (RI) are given as a percentage, relative to the largest signal peak. The singly charged \((M + H^+ = 1247.0)\) and doubly charged \((M + 2H^+ = 624.5)\) phosphopeptide species are indicated. B, an estimated 5.0-pmol loading of sample onto the IMAC-HPLC-ESI-MS system. Data analysis and representation is as described for A and shows the significant improvement in the signal to noise ratio for the peak of interest.

**Fig. 4.** 2D tryptic phosphopeptide mapping of in vitro auto-phosphorylated and p56\textsuperscript{ck}-phosphorylated ZAP-70. A, expressed ZAP-70 was immunoprecipitated from cell lysate by means of an affinity tag recognized by the 9E10 mAb. Immune complex kinase assays were performed in the absence (lane 1) or presence (lane 2) of p56\textsuperscript{ck}. Phosphoproteins were separated by SDS-PAGE on 10% gels and visualized by autoradiography of the (wet) gel. The migrations of molecular mass standard proteins (kdA) along with that of ZAP-70 and p56\textsuperscript{ck} are indicated. ZAP-70 bands were excised from the gel and digested in situ with trypsin. Eluted peptides were run on 2D peptide maps and phosphopeptides visualized by autoradiography. 2D tryptic phosphopeptide map of B, autophosphorylated ZAP-70 (A, lane 1) and C, ZAP-70 phosphorylated with p56\textsuperscript{ck} (from A, lane 2) are shown. The orientation of the positive and negative electrodes during electrophoresis is indicated along with the sample origin (O). Exposure times were 1.5 h at room temperature for Panel A and 20 and 4 h at –80 °C with an intensifying screen for Panels B and C, respectively.
Phosphorylation Sites on ZAP-70 Determined by MS

Table I

Sequence of ZAP-70-derived phosphopeptides inferred from mass spectrometric measurements

Spots A to G (from Fig. 4C) were analyzed by IMAC-HPLC-ESI-MS as described under “Experimental Procedures.”

| Spot | Observed phosphopeptide mass | Calculated peptide mass | ZAP-70 residue nos. | Inferred peptide sequence |
|------|-------------------------------|-------------------------|---------------------|--------------------------|
| A    | 1727.99 ± 0.01                | 1647.78                 | 284–298             | (R) IDTNSDGpYTPEPAR (I)  |
| B    | 1883.24 ± 0.36                | 1803.88                 | 238–298             | (R) RIDTNSDGpYTPEPAR (I)  |
| C    | 651                         | 551.28                  | 125–128             | (R) DpYVR (Q)              |
| D    | 1272.24 ± 1.06                | 1191.63                 | 64–75               | (Q) LIGNTPYAIAGGK (A)      |
| E    | 1361.49 ± 0.71                | 1301.6                  | 485–496             | (K) ALGADDSpYpYTAR (S)     |
| F    | 1461.99 ± 0.70                | 1301.6                  | 485–496             | (K) ALGADDSpYpYTAR (S)     |
| G    | 1246.49 ± 0.70                | 1166.6                  | 176–186             | (K) KLpYSQAQTGDGK (F)      |

*a* Observed masses are the deconvoluted phosphopeptide masses, and are given ± the standard deviation values calculated by the program. The observed peptide mass must be 80 mass units greater than the calculated mass to allow for the addition of a single phosphate ester group.

*b* Calculated peptide masses for a theoretical tryptic digest of the human ZAP-70 protein, corresponding to the nonphosphorylated peptide masses.

*c* ZAP-70 residue nos. and inferred peptide sequences thus determined are given.

*d* Spot C gives only a singly charged species at an m/z = 632, being too small to observe additional charged species. Since only one charge state was detected, this assignment and matching with the indicated ZAP-70 fragment was done manually.

The observed mass for spot E indicates the addition of only a single phosphate ester group. Since the peptide has 2 tyrosine residues, we were unable to determine which was phosphorylated, or what the ratio of the two possible peptide species was.

Arg-282/Arg-283, a tryptic artifact we had previously observed while mapping phosphorylation sites in TCRζ. To confirm the assignments indicated in Table I, based on mass analysis alone, peptides were synthesized corresponding to regions of ZAP-70 containing Tyr-126, Tyr-292, and Tyr-492-Tyr-493, along with surrounding tryptic cleavage sites. These were phosphorylated in vitro with p56k, digested with trypsin and analyzed on 2D phosphopeptide maps. By mixing these peptides, following tryptic digestion, with the corresponding peptide eluted from a 2D map of ZAP-70 phosphorylated in vitro with p56k (Fig. 4C) and re-running the 2D analyses, the appearance in each case of a single spot for both A and C confirmed the assignments indicated in Table I (not shown). In the case of spots E and F, a partial phosphorylation of a synthetic peptide encompassing residues 482–498 of ZAP-70 was performed. Following tryptic digestion, the peptides apparently corresponding to the singly (spot E) and doubly (spot F) phosphorylated form were then separated on a 2D map and eluted prior to mixing with the ZAP-70-derived phosphopeptides from Fig. 4C and re-analysis. Once again, the appearance in both cases of single spots for both E and F confirmed the assignments indicated in Table I (data not shown).

We next investigated the autophosphorylation of ZAP-70 isolated from Jurkat T cells to test whether it would produce the same autophosphorylation pattern as the expressed ZAP-70. We used the affinity of ZAP-70 for the phosphorylated form of TCRζ as a means of isolating ZAP-70 from the cell extract. We phosphorylated a synthetic TCRζ peptide corresponding to the entire cytoplasmic domain (residues 52–164) in vitro with p56k and coupled this to agarose beads (pζ beads). We have previously determined that these beads are capable of precipitating ZAP-70 in Jurkat cells. The ZAP-70 band from Fig. 5A, lane 1, was cleaved with trypsin on the nitrocellulose membrane, and eluted phosphopeptides were analyzed by 2D phosphopeptide mapping (Fig. 5B). By comparison with Fig. 4B, it is clear that the ZAP-70 precipitated from Jurkat cells by binding to pζ beads phosphorylates in the same manner as the baculovirus-expressed ZAP-70 precipitated through a C-terminal affinity tag. Scintillation counting of the membrane before and after trypsin digestion showed a ~90% recovery of counts into solution (not shown), ruling out the possibility of the selective loss of a major autophosphorylation peptide to the membrane. As before, to confirm Tyr-292 as the major autophosphorylation site of Jurkat ZAP-70, spot A from Fig. 5B was eluted from the TLC plate and mixed with a synthetic peptide centered around Tyr-292 of ZAP-70, which had been phosphorylated in vitro with p56k and digested with trypsin. The observation of a single spot following 2D phosphopeptide mapping confirmed this assignment (not shown).

We next investigated sites of phosphorylation induced on ZAP-70 in Jurkat cells following stimulation with the anti-
Phosphorylation Sites on ZAP-70 Determined by MS

Fig. 6. 2D tryptic phosphopeptide mapping of ZAP-70 precipitated from stimulated 32P-labeled Jurkat cells. A, 1.6 \times 10^6 Jurkat cells were labeled with either 5 mCi (lanes 1 and 3) or 10 mCi (lane 2) of [32P]orthophosphate for 1.5 h in phosphate-free medium at 37 °C. Two samples were stimulated with OKT3 for 2 min at 37 °C prior to cell lysis as indicated. Cell lysates were then precipitated with either phosphorylated \(\zeta\)-beads (p\(\zeta\)) or non-phosphorylated \(\zeta\)-beads (non-p\(\zeta\)) as indicated prior to SDS-PAGE. Phosphoproteins were visualized by autoradiography following transfer to nitrocellulose. The migration of molecular mass standard proteins are indicated (kDa) along with the 70-kDa phosphoprotein (p70) subjected to further analysis. B, 2D tryptic phosphopeptide map of the 70-kDa band from A, lane 2. The orientation of the positive and negative electrodes during electrophoresis are indicated along with the sample origin (O). Peptide spots A, E, and F were eluted from the cellulose matrix, along with the spots labeled x and y. Spots A, E, and F were mixed with equal quantities (measured by counts/min) of their respective spots derived from the in vitro phosphorylation of ZAP-70 with p56\(\zeta\)k (Fig. 4C) with spots x and y being similarly mixed with spot G. These samples were then re-analyzed on 2D phosphopeptide maps. Co-migration of in vitro and in vitro produced C, spot A; D, spots F; and E, spot E was observed, while in F, spots y and G did not co-migrate. The orientation of the positive and negative electrodes during electrophoresis are indicated along with the sample origin (O). Exposure times were 1 and 40 h for Panels A and B, respectively, and 24 h for Panels C–F; all at -80 °C with an intensifying screen.

CD3ε mAb, OKT3. Jurkat cells were metabolically labeled with 32P-labeled phosphate, and ZAP-70 again precipitated with \(\zeta\)-beads. Immunoblotting of such precipitates with anti-pTyr antibodies (4G10) had shown that the pTyr content of ZAP-70 was undetectable in unstimulated cells and was maximal at -2 min post-OKT3 stimulation (data not shown). Following cell labeling, stimulation and precipitation with \(\zeta\)-beads, phosphoproteins were visualized by autoradiography following SDS-PAGE and transfer to nitrocellulose. As shown in Fig. 6A, upon OKT3 stimulation, there is an increase in the phosphorylation of a 70-kDa protein (lane 2) which is not apparent when stimulated cells were precipitated with the non-phosphorylated TC- \(\zeta\)-beads (lane 3).

The phosphorylated 70-kDa band indicated in Fig. 6A (lane 2) was subjected to 2D phosphopeptide mapping following trypsin digestion in situ on the nitrocellulose (Fig. 6B). By comparison with Fig. 4C, it appears that the major ZAP-70 autophosphorylation, spot A (Tyr-292) is present, consistent with the reported activation of ZAP-70 following TCR engagement. However, the major induced spot is the doubly phosphorylated spot F (Tyr-492–Tyr-493), with the singly phosphorylated form of the same tryptic peptide (spot E) additionally present. Once again, Cerenkov counting of the samples before and after trypsin digestion gave phosphopeptide recoveries into solution of ~80%. Spots A, E, and F were eluted from the cellulose matrix and mixed with equal quantities (by Cerenkov counting) of the appropriate peptide spot eluted from a 2D phosphopeptide map of ZAP-70 phosphorylated in vitro with p56\(\zeta\)k (Fig. 4C). In each case, the observation of a single spot confirmed the assignments indicated in Fig. 6B (Fig. 6, C–E). It was also thought that one of the minor spots visible in Fig. 6B migrating toward the negative electrode (marked x and y) might correspond to spot G (Fig. 4C), thus these two spots were eluted and mixed with equal quantities each of spot G and re-run on 2D phosphopeptide maps. Fig. 6D shows the 2D map observed for spot g (Fig. 6B) mixed with spot G (Fig. 4C). While the spots y and G are clearly very similar in mobility, their failure to co-migrate indicates that it is unlikely that spot y corresponds to in vivo phosphorylation of ZAP-70 at Tyr-178. Similar analysis of spot x also gave a different mobility to spot G (not shown). While the identities of these minor spots (x and y) remain unknown, they may in fact not be derived from ZAP-70. As can be seen from Fig. 6A, the extensive co-precipitation of other phosphoproteins with ZAP-70 suggests that spots x and y could be derived from a ~70-kDa phosphoprotein which is co-precipitating with ZAP-70 and the \(\zeta\)-beads. Attempts to further reduce the co-precipitating phosphoproteins were unsuccessful, suggesting that they may be interacting specifically with the phospho-\(\zeta\) or ZAP-70 (data not shown).

From the comparison of in vitro and in vivo produced data, we can thus conclude that ZAP-70 has a primary autophosphorylation site at Tyr-292, with a minor in vitro autophosphorylation site at Tyr-128. Additionally, from the analysis of ZAP-70 from labeled stimulated Jurkat cells, we observe induced phosphorylation of ZAP-70 at Tyr-492 and Tyr-493, with additional phosphorylation again at Tyr-292.
Phosphorylation Sites on ZAP-70 Determined by MS

| N | SH2 | 102 | 162 | 254 | 338 | Kinase | Residue No. |
|---|-----|-----|-----|-----|-----|--------|------------|
| 69 | ↑   | 126 | ↑   | 178 | 292 | 482/493|            |
| -  | +   | -   | +   | +   | +   | +/-     | Phosphorylated residue |
| +  | +   | +   | +   | +   | +   | +/+     | in vitro autophosphorylation |
| -  | -   | +   | -   | -   | -   | +/-     | in vitro + p56<sup>ck</sup> |
|   |     |     |     |     |     |         | in vivo + OKT3 |

![Fig. 7. Location of inducible phosphorylation sites on ZAP-70.](image)

DISCUSSION

Due to growing interest into the role of reversible protein modification in the regulation of a wide range of cellular events and responses, especially in the initiation of cell proliferation, the need to devise rapid and sensitive methodologies for the determination of the sites and nature of such modifications has also grown. Of particular importance is the role played by protein phosphorylation and dephosphorylation events. Conventional approaches for the analysis of protein phosphorylation usually rely on 2D phosphopeptide mapping (46), phosphoamino acid analysis and anti-pTyr immunoblotting. While these techniques are very sensitive, they do not reveal the actual phosphorylation sites. Unfortunately, the quantities of many phosphoproteins of interest obtainable from tissue samples or cultured cells are also too small, and may be phosphorylated to too low a stoichiometry to permit direct biochemical analysis of the phosphorylation sites.

Until now, the determination of in vivo phosphorylation sites could only be achieved by making predictions of likely phosphorylation sites. These can then be investigated by expressing mutant molecules in cell lines and looking for a change in phosphorylation patterns, or by synthesizing phosphopeptides corresponding to predicted proteolytic fragments and testing for their co-migration in a 2D peptide map with those produced from some other function of the protein. Additionally, if there is a need to devise rapid and sensitive methodologies for the analysis of protein phosphorylation and dephosphorylation events, conventional approaches for the analysis of protein phosphorylation usually rely on 2D phosphopeptide mapping (46), phosphoamino acid analysis and anti-pTyr immunoblotting. While these techniques are very sensitive, they do not reveal the actual phosphorylation sites. Unfortunately, the quantities of many phosphoproteins of interest obtainable from tissue samples or cultured cells are also too small, and may be phosphorylated to too low a stoichiometry to permit direct biochemical analysis of the phosphorylation sites.

We applied this technology to the analysis of the T cell specific PTK ZAP-70, which is known to be critical in mediating TCR response and to be inducibly phosphorylated upon TCR engagement (27, 29–31, 35). The availability of expressed forms of both ZAP-70 and p56<sup>ck</sup> allowed us to directly determine the sites of ZAP-70 autophosphorylation, as well as a number of additional tyrosine phosphorylation sites induced on ZAP-70 upon addition of p56<sup>ck</sup> (Fig. 4 and Table 1). Comparative 2D phosphopeptide mapping of ZAP-70 isolated from Jurkat T cells allowed us to confirm the major autophosphorylation site of T cell ZAP-70 as Tyr-292 (Fig. 5) and identify Tyr-492 and Tyr-493 as the two principal sites of phosphorylation induced following TCR engagement (Fig. 6). The schematic shown in Fig. 7 indicates the location of all the major phosphorylation sites we observed on ZAP-70 and indicates the conditions under which they have so far been induced. Interestingly, synthetic peptides modeled on ZAP-70 around Tyr-128, Tyr-292 and Tyr-492–Tyr-493 were found to not be substrates for the expressed ZAP-70 in vitro.

The role of the tyrosine phosphorylation of ZAP-70 during T cell activation is still unclear. Mutation of Tyr-292, Tyr-492, and Tyr-493 should now permit these questions to be addressed by the overexpression of such ZAP-70 mutants in T cell lines, or their expression in cell systems lacking ZAP-70 expression. Recent studies have shown that the tyrosine phosphorylation of ZAP-70, its association with the phosphorylated forms of the TCR<sub>C</sub> and CD3<sub>e</sub> subunits and catalytic activation are interdependent (27, 29, 30, 32). The observation that p56<sup>ck</sup> can induce phosphorylation of ZAP-70 at the principal sites observed in vivo is made more interesting by the recent observation that ZAP-70 and p56<sup>ck</sup> associate in an SH2-dependent manner (48). We have also found that the two kinases can associate in vitro, and in a phosphorylation-dependent manner.

Additionally, since the tyrosine phosphorylation of the TCR<sub>C</sub> requires functional p56<sup>ck</sup> (31, 49), these findings taken together would support a model whereby p56<sup>ck</sup> (and/or possibly p59<sup><sub>65</sub></sup>) is required for TCR-induced phosphorylation of Tyr-492 and Tyr-493 of ZAP-70 in vivo. However, the mechanism for ZAP-70 activation still remains unresolved, but is most likely regulated via either tyrosine phosphorylation, or its interaction with the phosphorylated TCR<sub>C</sub> or p56<sup>ck</sup>, or any combination of the three. Due to the complexity of the problem, it thus seems apparent that much careful work has yet to be done. The identification of the in vivo sites of TCR-induced phosphorylation of ZAP-70 is thus an important first step in this process.

Unlike src-family kinases, the ZAP-70 autophosphorylation site does not lie within the kinase domain, instead being between the second SH2 domain and the kinase domain (see Fig. 3). By utilizing this two capillary column system, we have achieved significant improvements in detection sensitivity over previous single column approaches for the analysis of protein phosphorylation and dephosphorylation events. With a standard ESI-MS system involved a two-step chromatography system (immobilized Pe<sup>ck</sup> affinity and C<sub>18</sub> HPLC connected in series) with on-line detection by ESI-MS (Fig. 1). With a standard phosphopeptide we demonstrated sensitivity down to at least 250 fmol of injected sample (Fig. 2). By utilizing this two capillary column system, we have achieved significant improvements in detection sensitivity over previous single column strategies (44), with the added benefits of the elimination of background signals and sample separation as a direct result of the on-line connection of the two chromatography systems (see Fig. 3). The level of sensitivity demonstrated here is within the range appropriate for the analysis of peptide samples recovered from 2D phosphopeptide maps following protein labeling and isolation.

We applied this technology to the analysis of the T cell specific PTK ZAP-70, which is known to be critical in mediating TCR response and to be inducibly phosphorylated upon TCR engagement (27, 29–31, 35). The availability of expressed forms of both ZAP-70 and p56<sup>ck</sup> allowed us to directly determine the sites of ZAP-70 autophosphorylation, as well as a number of additional tyrosine phosphorylation sites induced on ZAP-70 upon addition of p56<sup>ck</sup> (Fig. 4 and Table 1). Comparative 2D phosphopeptide mapping of ZAP-70 isolated from Jurkat T cells allowed us to confirm the major autophosphorylation site of T cell ZAP-70 as Tyr-292 (Fig. 5) and identify Tyr-492 and Tyr-493 as the two principal sites of phosphorylation induced following TCR engagement (Fig. 6). The schematic shown in Fig. 7 indicates the location of all the major phosphorylation sites we observed on ZAP-70 and indicates the conditions under which they have so far been induced. Interestingly, synthetic peptides modeled on ZAP-70 around Tyr-128, Tyr-292 and Tyr-492–Tyr-493 were found to not be substrates for the expressed ZAP-70 in vitro.

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**Footnotes:**

6 J. Watts, unpublished observations.

7 D. Krebs, unpublished observations.
Phosphorylation Sites on ZAP-70 Determined by MS

1. Can you explain the basic mechanism of phosphorylation and its role in cellular signaling?

Phosphorylation is a fundamental post-translational modification that regulates the activity of many proteins in cells. It involves the transfer of a phosphate group from ATP to a specific amino acid residue (usually serine, threonine, or tyrosine) in a protein. This modification can alter the protein's conformation, interacting partners, and activity, enabling cells to respond to various stimuli. By studying the phosphorylation sites on proteins, researchers can gain insights into the complex signaling networks that underpin cellular functions.

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11. Piotrowska, B. (Biotechnology Laboratory, University of British Columbia, Research Centre: src kinases regulated via reversible phosphorylation at a C-terminal tyrosine residue, p56lck, in the same manner as do the tyrosine phosphorylation techniques for the sequencing of phosphopeptides, or of the determination of protein modifications other than phosphorylation. Thus it is reasonable to suppose that src-family kinases such as p56lck might be able to induce phosphorylation at one or more of these residues. While src-family kinases are regulated via reversible phosphorylation at a C-terminal tyrosine residue, p56lck contains no analogous sequence. It is possible that phosphorylation of Tyr-491 of p56lck, or at other sites, may play roles in its catalytic activation. They may also contribute to the ability of ZAP-70 to associate with TCR or p56lck. Once again, the identification of these phosphorylation sites should permit these hypotheses to be tested. Unfortunately, our initial attempts to characterize the role of tyrosine phosphorylation of ZAP-70 on its in vitro activity have so far been unsuccessful, but we have yet to identify a ZAP-70-specific substrate, and are unable to remove p56lck from ZAP-70 following an in vitro kinase reaction due to the strong interaction of the two.

It is clear that the determination of the mechanism by which ZAP-70 plays such a critical role in T cell activation will be difficult. However, the direct biochemical determination of in vitro sites of induced tyrosine phosphorylation of ZAP-70 will greatly facilitate these efforts. We believe that the IMAC-HPLC-ESI-MS technology will prove a valuable tool in not only addressing these questions, but also elucidating a wide range of related problems in the area of intracellular signaling. Its high sensitivity makes the technology compatible with the analysis of proteins isolated by means of immunoprecipitation and SDS-PAGE. Mass spectrometric analysis is also compatible with the analysis of serine-phosphorylated peptides, and by its nature, is also suitable for the determination of protein modifications other than phosphorylation (e.g. methylation, acetylation etc.) on the basis of the defined mass differences induced on modified peptides. In fact, the method can be directly interfaced with tandem MS techniques for the sequencing of phosphopeptides, or verification of phosphorylation sites by collision-induced fragmentation of the phosphate ester bond and the resulting elimination of 80 mass units (51-53). Finally, without direct biochemical analysis of the molecules involved in cell signaling pathways, the mechanism by which they create the desired response will remain elusive. Without understanding of such mechanisms, the development of treatment for diseases resulting from dysfunctional cell signaling pathways, based on biochemical intervention, will remain severely hindered.