Detection and Sequencing of Phosphopeptides Affinity Bound to Immobilized Metal Ion Beads by Matrix-Assisted Laser Desorption/ Ionization Mass Spectrometry

Wei Zhou*
Laboratory of Structural Biology, National Institute of Environmental Health Sciences/National Institutes of Health, Research Triangle Park, North Carolina, USA

B. Alex Merrick
Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences/National Institutes of Health, Research Triangle Park, North Carolina, USA

Morteza G. Khaledi
Department of Chemistry, North Carolina State University, Raleigh, North Carolina, USA

Kenneth B. Tomer
Laboratory of Structural Biology, National Institute of Environmental Health Sciences/National Institutes of Health, Research Triangle Park, North Carolina, USA

Consecutive enzymatic reactions of analytes which are affinity bound to immobilized metal ion beads with subsequent direct analysis of the products by matrix-assisted laser desorption/ ionization mass spectrometry have been used for detecting phosphorylation sites. The usefulness of this method was demonstrated by analyzing two commercially available phosphoproteins, β-casein and α-casein, as well as one phosphopeptide from a kinase reaction mixture. Agarose loaded with either Fe^{3+} or Ga^{3+} was used to isolate phosphopeptides from the protein digest. Results from using either metal ion were complementary. Less overall suppression effect was achieved when Ga^{3+}-loaded agarose was used to isolate phosphopeptides. The selectivity for monophosphorylated peptides, however, was better with Fe^{3+}-loaded agarose. This technique is easy to use and has the ability to analyze extremely complicated phosphopeptide mixtures. Moreover, it eliminates the need for prior high-performance liquid chromatography separation or radiolabeling, thus greatly simplifying the sample preparation. (J Am Soc Mass Spectrom 2000, 11, 273-282) © 2000 American Society for Mass Spectrometry

The reversible phosphorylation/dephosphorylation of proteins, mainly on serine, threonine, and tyrosine residues, is probably the most common and important regulatory modification of proteins. Phosphorylation is involved in the regulation of gene expression and protein synthesis which controls cell growth, division, or differentiation [1–4]. In order to better understand the molecular basis of these regulatory mechanisms, it is necessary to identify these phosphorylation sites.

Identification of protein phosphorylation sites usually entails a combination of techniques even when the sequence of a protein is known. Conventional strategies usually involve isolation of radiolabeled [^{32}P] target protein [5–8], phosphoamino acid analysis to determine the modified amino acid, tryptic mapping using thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) separation schemes, and automated Edman degradation to determine the amino acid sequence of candidate peptides with the appearance of ^{32}P at a specific cycle [9]. In vitro kinase reactions using peptide substrates homologous to various regions of the target protein, site-directed mutagenesis, isoelectric focusing, and use of anti-ser/thr/tyr antibodies are complementary methods which can be integrated into the preceding strategy for site-specific localization of phosphorylation in a target protein [10–13]. However, conventional approaches using ^{32}P may

Address reprint requests to Dr. Kenneth B. Tomer, National Institute of Environmental Health Sciences, PO Box 12233 MD F0-03, Research Triangle Park, NC 27709, E-mail: tomer@niehs.nih.gov
* Also at Department of Chemistry, North Carolina State University, Raleigh, NC 27695-8204

1044-0305/00/$20.00
PII S1044-0305(00)00100-8

Received June 28, 1999
Revised December 14, 1999
Accepted December 14, 1999

not be sufficiently sensitive because many phosphoproteins in cell signaling are in low abundance, the amount of biological sample is often limited, and the process of radiolabel incorporation into protein under cellular conditions is generally inefficient. These factors underscore the need for continued development of methods for enrichment and increasingly sensitive detection of phosphopeptides to find the precise site of phosphorylation.

Mass spectrometry has proven to be very useful in mapping posttranslational modifications of proteins, such as phosphorylation and glycosylation [14, 15]. The analysis is rapid and does not require radiolabeling. Both matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) and electrospray ionization mass spectrometry (ESI/MS) have been used to determine the phosphorylation sites on proteins [16–23]. In the mass spectrometry analysis of protein digests, however, components are not always detected due either to suppression effects or to low ionization efficiency [16]. This is particularly problematic when phosphopeptides are present, because phosphorylated peptides usually exhibit low response to mass spectrometry in positive ion mode due to the negative charge of a phosphate group. Negative charge interference in detection becomes even worse when multiple phosphate groups exist in the peptide.

To reduce suppression effects and enhance the detectability of these components, the digests can be subjected to HPLC separation. The HPLC eluent can be either directly analyzed using ESI/MS/MS to locate the phosphorylation sites or HPLC fractions can be collected and subjected individually to MALDI/MS analysis. After phosphatase treatment, phosphopeptide identification by MALDI can be facilitated by the 80 Da difference in the MALDI spectrum between the observed mass of the peptide and that calculated on the basis of the nonphosphorylated sequence [14]. MALDI-based peptide sequencing can also be achieved by post source decay (PSD), which produces predicted fragmentation along the peptide backbone [18]. The HPLC separation, however, is laborious, and incomplete separation can sometimes make data analysis difficult. Moreover, the loss of sample during the HPLC separation makes the analysis of cellular phosphoproteins present at very low concentrations more difficult.

The use of immobilized metal ion affinity chromatography (IMAC) to isolate phosphopeptides from mixture has been shown to be useful in both MALDI/MS and ESI/MS structure studies of phosphoproteins. Immobilized metal ions, such as Fe^{3+} and Al^{3+} bind with high specificity to phosphoproteins and peptides [24–26]. Recently, Tempst demonstrated that Ga^{3+} has a better selectivity for the phosphopeptides [25]. By selective isolation of phosphopeptides using metal ion affinity media before mass spectrometry analysis, suppression effects or low net average charge due to the presence of the doubly negatively charged phosphate group combined with the positively charged amino groups can be greatly reduced. This technique has been used successfully with both on-line and off-line coupling to mass spectrometry analysis [28–33]. However, elution of phosphopeptides from the metal ion column before mass spectrometry analysis could result in loss of sample. We have previously shown that it is not necessary to elute affinity bound analytes, including phosphopeptides, from affinity media prior to mass spectrometry analysis. For example, we observed that affinity-bound phosphoprotein and peptide, such as human apotransferrin and phosphokemptide, could be analyzed directly from Fe^{3+}-loaded sepharose using MALDI/MS [34]. We also found that consecutive enzymatic reactions could be carried out on affinity-bound analytes, including those immobilized on Ni^{2+} ion media. Subsequent direct analysis of the products by MALDI/MS could further accurately define the amino acid sequence of the bound peptides [35].

In this study, a method that minimizes suppression effects and enables direct analysis of phosphopeptides from the protein digest without any HPLC separation is demonstrated. Agarose loaded with either Fe^{3+} or Ga^{3+} is used to isolate phosphopeptides from the protein digest. The affinity-bound phosphopeptides are treated with phosphatase, and the number of phosphorylation sites are determined from 80 Da (or multiples of 80 Da) mass shifts. Carboxypeptidase Y treatment of the affinity-bound phosphopeptides then is used to cleave the amino acids from the C-terminus, with subsequent direct analysis of the enzymatic products by MALDI/MS to locate the phosphorylation sites on the bound phosphopeptides. We have applied this method to the analysis of a peptide and two commercially available proteins, β-casein and α-casein. Results from using either Fe^{3+} or Ga^{3+} metal ions are complementary. Ga^{3+}-loaded agarose shows less overall suppression effect and the ability to isolate phosphopeptides with multiple phosphate groups. However, the selectivity for monophosphorylated peptides is better using Fe^{3+}-loaded agarose. High sensitivity, absence of the need for radiolabeling or HPLC separation, ease of use, and the ability to analyze extremely complicated phosphopeptide mixtures make this method attractive.

Materials and Methods

Chemicals and Reagents

Ni-NTA (nickel-bound nitrilotriacetic, a quadradentate metal chelator) agarose was purchased from QIAGEN (Chatsworth, CA). Hi-trap chelating IDA (iminodiacetic, a tridentate metal chelator) sepharose was obtained from Pharmacia Biotech (Piscataway, NJ). The β-casein, α-casein, and carboxypeptidase Y were obtained from Sigma (St. Louis, MO). Calf intestinal alkaline phosphatase and buffer sets were obtained from Gibco BRL Products (Grand Island, NY). Immobilized trypsin was from PerSeptive Biosystems (Framingham, MA). Ferric chloride was from Allied Chemical (Morristown, NJ).

The α-cyano-4-hydroxycinnamic acid, gallium (III) chloride, and other reagents were purchased from Aldrich Chemical (Milwaukee, WI). Compact reaction columns and filters were made by Amersham Pharmacia Biotech (Piscataway, NJ).

Methods

Tryptic digest. Phosphoproteins were digested by immobilized trypsin to avoid trypsin autodigestion products. A 10 μL aliquot of immobilized trypsin slurry was washed with 50 μL of 50:50 acetonitrile:0.1 M ammonium bicarbonate (3×), followed by 50 μL of 5:95 acetonitrile:0.1 M ammonium bicarbonate (3×) to activate the enzyme. A 40 μL (1 μg/μL) aliquot of protein and 50 μL 100 mM ammonium bicarbonate digestion buffer (pH = 7.8) were added to the treated immobilized trypsin. The mixture was incubated at 37 °C for 4 h on a rotator.

IMAC column. IMAC columns were prepared by adding 30 μL 50% slurry of Ni-NTA resin into a compact reaction column. The column was washed with 30 μL 100 mM EDTA (3×) to remove any bound Ni^{2+} metal ions. The column was then washed sequentially with 30 μL water (3×), 30 μL 0.1 M acetic acid (3×), and 30 μL 60 mM $GaCl_3$ or 100 mM $FeCl_3$ (3×) to load the column with metal ions. The column was then washed with 30 μL water (3×) followed by 30 μL 0.1 M acetic acid (3×) to remove any unbound metal ions. Binding of phosphopeptides to the metal chelator was achieved by loading the protein digest (2 μg protein digest dissolved in 50 μL 0.1 M acetic acid) onto the IMAC column and incubated at 37 °C for 30 min on a rotator. The column was washed with 50 μL water (3×) and 50 μL 0.1 M acetic acid (3×) to remove less tightly bound analytes. A 0.5 μL aliquot of the resin was placed directly onto the MALDI target followed by a 0.5 μL aliquot of the matrix solution. The sample was then allowed to dry at room temperature before loading into the mass spectrometer. In some experiments, a 0.5 μL aliquot of the resin, a 0.5 μL aliquot of the matrix solution, and a 0.5 μL aliquot of 25 mM ammonium citrate were placed onto MALDI target and allowed to dry at room temperature. Ammonium citrate has been reported to be able to enhance the detection of phosphopeptides in MALDI/MS analysis [21].

On-gel carboxypeptidase Y digest. 40 μL of digestion buffer (50 mM sodium citrate, pH = 6.0) was added to the phosphopeptides bound to Ga-NTA beads. Carboxypeptidase Y solution was then added (enzyme to peptide ratio is 1:1 by weight). The mixture was incubated at 37 °C on a rotator. The digestion course was monitored by removing a small aliquot of the reaction mixture at a specific time. The mixture was centrifuged and the supernatant was discarded. The resin was then washed three times with 50 μL of 0.1 M acetic acid followed by MALDI/MS analysis.

ON-GEL DEPHOSPHORYLATION. A small amount (5 μL) of phosphopeptide affinity bound to immobilized metal ion agarose was mixed with 1 unit of calf intestine alkaline phosphatase, 1 μL digestion buffer, and 2 μL water. The mixture was incubated at 37 °C on a rotator. The dephosphorylation course was monitored by removing a 0.5 μL aliquot of the reaction mixture (both beads and supernatant) at specific times and analyzing the resulting products by MALDI/MS.

Kinase assay. A serine phosphopeptide was generated enzymatically by an in vitro kinase reaction. The synthetic peptide, RDFYHSKRRLI (*m/z* 1489.7) was synthesized commercially (Sigma-Genosys Biotechnologies, The Woodlands, TX) at 95% purity. The carboxy terminus was amidated. Cdk2-cyclin kinase complexes were used as the enzyme source and were isolated by immunoprecipitation from HT1080 human fibrosarcoma cells [34]. Briefly, cells were lysed with an immunoprecipitation buffer (50 mM Tris pH 8, 130 mM NaCl, 20 mM NaF, 1 mM EDTA, 1% NP-40 with protease and phosphatase inhibitors). Whole cell lysates were created by progressive shearing with 19 and 23 gauge needles to fragment DNA. Cell debris was pelleted by centrifugation at 12,000 × g for 20 min at 4 °C. The protein concentration of the lysate was determined, and 200 μg of protein was incubated with anti-cdk2 (M-2 antibody; Santa Cruz Biotech., Santa Cruz, CA). Immune complexes were collected by rotation with protein-G agarose beads reacted with anti-rabbit IgG. The beads were washed four times with immunoprecipitation buffer and twice with kinase buffer (50 mM HEPES pH 7.6, 10 mM $MgCl_2$ and 1 mM dithiothreitol). Each immunoprecipitate received 15 μL of kinase buffer also containing 1 mM NaF, 10 mM β-glycerylphosphate, 200 μM ATP, and 0.5 mM of synthetic peptide as a substrate. Reactions were conducted at 30 °C for 30 min. Supernatants from five kinase reactions were pooled and frozen on dry ice until mass spectrometry analysis.

MALDI/MS. Mass spectra were acquired on a Voyager-RP (PerSeptive Biosystems, Framingham, MA) equipped with a nitrogen laser (λ = 337 nm). The accelerating voltage used was 30 kV. All spectra were recorded in the positive linear mode except spectra from the carboxypeptidase digest experiments which were recorded in the negative ion mode. A saturated solution of recrystallized α-cyano-4-hydroxycinnamic acid in ethanol:water:formic acid (45:45:10), prepared fresh daily, was used as the MALDI matrix. Samples were prepared by mixing equal volumes of analyte and matrix solution on the target and letting them dry. When ammonium citrate additive was used, an equal volume of ammonium salt solution was also mixed with the analyte and matrix solutions. Mass calibration was performed by using angiotensin I [$(M + H)^+$ = 1297.51] and insulin (bovine) [$(M + H)^+$ = 5734.59] as external standards.

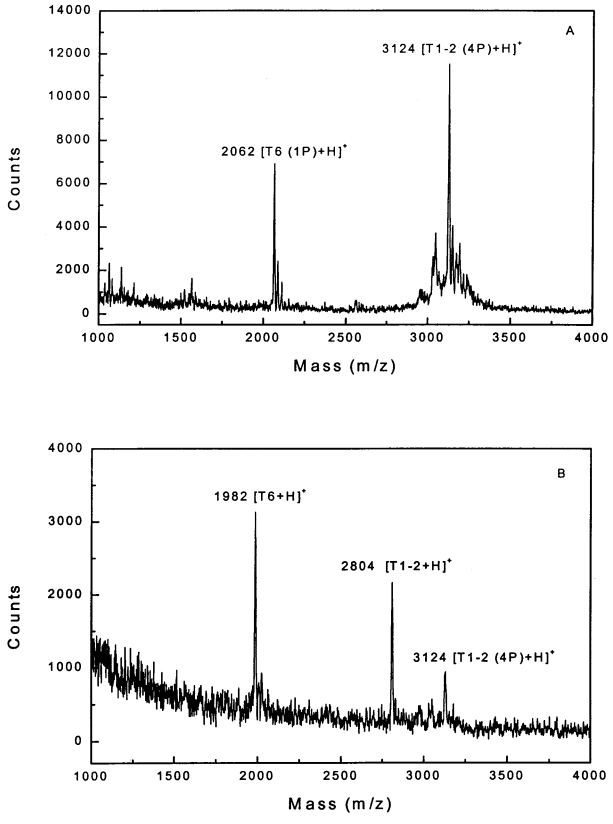

Figure 1. MALDI/TOF mass spectra of (**A**) Ga^{3+}-NTA bound phosphopeptides from β-casein tryptic digest and (**B**) the same peptides after the phosphatase treatment (both beads and supernatant). T6 (1P): FQpSEEQQQTEDELQDK; T1-2 (4P): RELEENVPGEIVEpSLpSpSpSEESITR.

Results and Discussion

Selective Detection of Phosphorylation Sites in β-Casein

Bovine β-casein was chosen as a model compound because it is a commercially available phosphoprotein and has five well-characterized phosphorylation sites at serine residues [37, 38]. Tryptic digests of this protein were loaded onto Ga^{3+}-NTA beads. After intensive washing to remove any unbound digest, the Ga^{3+}-NTA beads were analyzed directly by MALDI/MS. Two major peaks, *m/z* 2062 and 3124, were observed in the MALDI spectrum, shown in Figure 1A. A number of smaller signals corresponding to sodium adducts of the major peaks and a peak of *m/z* 3044 were also observed. The still affinity-bound peptides were then treated by phosphatase. The dephosphorylation course was monitored by taking a small aliquot (0.5 μL) of the reaction mixture at specific time points for direct MALDI/MS analysis. The reaction was complete within 1 h. Figure 1B shows that the peak at *m/z* 2062 shifted −80 Da to *m/z* 1982 indicating there was only one phosphorylation site on this peptide. Based on the known protein sequence, T6, FQSEEQQQTEDELQDK, with a calculated $[M + H]^+$ at 1982, fits the data. Because there is only one serine in this peptide, the monophosphorylated peptide can be unambiguously assigned as T6 (1P), FQpSEEQQQTEDELQDK. The peak at *m/z* 3124 shifted −320 Da to *m/z* 2804, indicating there were four phosphorylation sites on this peptide, while the *m/z* 3044 peak was no longer seen, indicating that it, too, shifted to *m/z* 2804 and had three phosphorylation sites. Based on the known protein sequence, T1-2, RELEENVPGEIVESLSSSEESITR, with a calculated $[M + H]^+$ of 2804, fits these data.

Because there were five serines, but only four phosphorylated residues on this peptide, the still affinity-bound phosphopeptides were treated with carboxypeptidase Y with subsequent direct analysis of the reaction products by MALDI/MS to locate the phosphorylation sites. Figure 2 shows the time course study of the carboxypeptidase Y reaction of T1-2 (4P). A MALDI mass spectrum of the resulting mixtures yielded the molecular weight information corresponding to each fragment affinity bound to Ga^{3+}-NTA beads. The mass difference between consecutive peaks can be directly related to the mass of one or more amino acid residues from the C-terminus of T1-2 (4P). After overnight treatment with carboxypeptidase Y, the still affinity-bound phosphopeptides were treated with phosphatase, Figure 2D shows that there were −320 Da shifts for both carboxypeptidase Y digest products. The molecular weights of these two peptides matched the molecular weights of RELEENVPGEIVESLSSSEES and RELEENVPGEIVESLSSSEE. These results demonstrate that the carboxypeptidase Y could cleave through the first serine of T1-2 from the C-terminal. However, the enzyme could not cleave further, indicating that glutamic acid may interact with the gallium ions [35]. The interaction is apparent sufficiently strong that it prevented the enzymatic reaction from going further. Both resulting fragments still had four phosphorylation sites, indicating that the first serine from the C-terminus was not phosphorylated. The tetraphosphorylated T1-2 peptide is, therefore, assigned as RELEENVPGEIVEpSLpSpSpSEESITR. Peaks could still be observed with a mass 80 Da lower than the tetraphosphorylated peptides, indicating that the nonphosphorylated serine in the triphosphorylated peptide was located within the SLSSS sequence. From the MALDI/MS spectra, it also can be seen that these two peptides, especially T1-2, had a strong tendency to form multiple sodium adducts. This may have been due to the multiple phosphate groups and negatively charged amino acids on this peptide.

We also probed the Ga^{3+}-NTA bound phosphopeptides with aminopeptidase, but no cleavage was observed. This may be due either to the affinity media binding the metal ion required for the activity of enzyme or the steric hindrance of the cleavage sites by affinity media. The same results were also observed when aminopeptidase was used to cleave the Ni-NTA bound recombinant proteins [33].

Carboxypeptidase Y can rapidly hydrolyze most

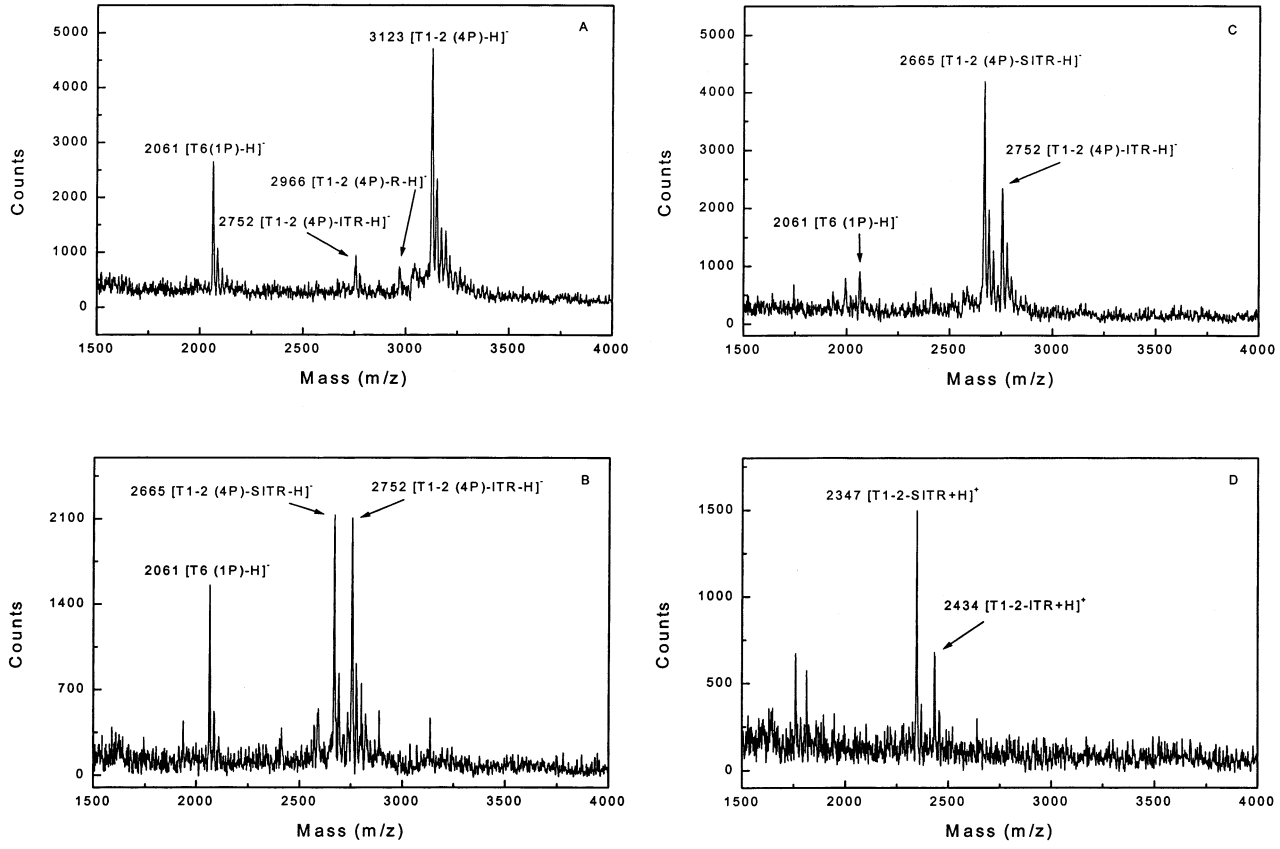

Figure 2. MALDI/TOF mass spectra of (**A–C**) Ga^{3+}-NTA bound phosphopeptides from β-casein tryptic digest after carboxypeptidase Y digest for (**A**) 25 min, (**B**) 4 h, and (**C**) overnight. (**D**) Peptides after the phosphatase treatment of the Ga^{3+}-NTA bound phosphopeptides from β-casein tryptic digest after carboxypeptidase Y digest overnight (both beads and supernatant). T1-2 (4P): RELEEN-VPGEIVEpSLpSpSpSEESITR. Spectra **A–C** were recorded in the negative mode.

amino acids from the C-terminus. The release of G and D, however, is considerably retarded. The digest rate for phosphopeptide T6 is extremely slow because of the presence of D as the second residue from the C-terminus.

Selective Detection of Phosphopeptides in α-Casein

The same strategy was applied to the other commercially available phosphoprotein studied here, α-casein, another milk-derived protein possessing multiple phosphorylation sites. Carr [17] reported the presence of different variants in the commercially available α-casein by mass spectrometry. He found that this protein contains at least two variants that have low sequence homology. 12 out of 18 predicted phosphorylation sites were observed using precursor scans. The method they used could not detect and sequence all the phosphopeptides present in the protein digest, especially those phosphopeptides with multiple phosphorylation sites. They proposed that this was due either to suppression effects or to the fact the peptides observed in the negative ion mode may not always produce analytically useful signals in the positive ion mode required for sequencing.

In the present study, agarose loaded with Ga^{3+} was used to isolate phosphopeptides from the protein tryptic digest. Direct analysis by MALDI/MS of the phosphopeptides affinity bound to the Ga^{3+}-loaded agarose showed that two ions corresponding to phosphopeptides that had been observed in the β-casein tryptic digest analysis in addition to the expected phosphopeptides from these two α-casein variants (shown in Figure 3A). There were also a few unidentified peaks that may be either from unknown variants of the protein or nonphosphorylated peptides that co-purified. The still affinity-bound phosphopeptides were treated with phosphatase. As shown in Figure 3B and Table 1, the *m/z* of most of the resulting peaks matched the predicted tryptic peptides. These results show that using Ga^{3+}-loaded agarose allows the detection of phosphopeptides with multiple phosphate groups. Because of the complexity of the sample, carboxypeptidase Y digest was not performed on the analytes to further locate the phosphorylation sites. Sequence information of mixtures of bound phosphopeptides may also be

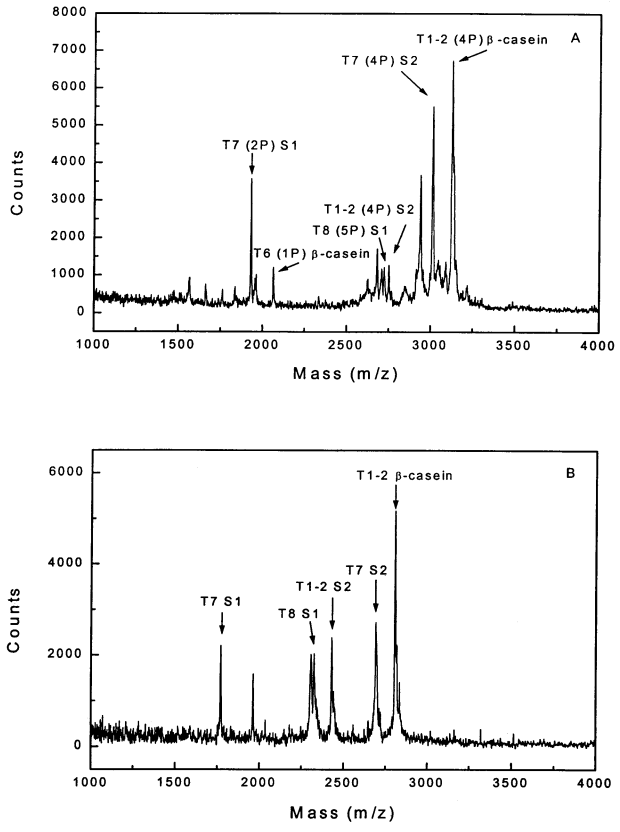

Figure 3. MALDI/TOF mass spectra of (**A**) Ga^{3+}-NTA bound phosphopeptides from the α-casein tryptic digest and (**B**) the same peptides after the phosphatase treatment (both beads and supernatant). 25 mM ammonium citrate was in the matrix.

achieved using PSD [16]. As the purpose of the present experiment was to test the usefulness of the Ga^{3+} to isolate phosphopeptides from protein tryptic digests, no attempt was made to further locate the phosphorylation sites on the phosphopeptides by PSD.

Selectivity Evaluation of Metal Ions

Tryptic digests of β-casein and α-casein were also analyzed using Fe^{3+}-loaded agarose. It is interesting to note that Fe^{3+} and Ga^{3+} exhibited different selectivities for phosphopeptides. Shown in Figure 4A, direct analysis of phosphopeptides from the β-casein tryptic digest affinity bound to Fe^{3+}-loaded agarose showed only the monophosphorylated peptides T6 (1P) and T4-6 (1P). However, T1-2 (*m/z* 2804) and T1-2 (1P) (*m/z* 2884) appeared after the treatment of the affinity-bound phosphopeptides with phosphatase, shown in Figure 4B. This implies that Fe^{3+}-loaded agarose may also bind to multiphosphorylated peptides, but the binding is sufficiently strong that the multiphosphorylated peptides are not easily dissociated from the agarose by the laser, making detection very difficult. Direct analysis of phosphopeptides from an α-casein tryptic digest affinity bound to Fe^{3+}-loaded agarose only showed the monophosphorylated peptides T14-15 (1P) and T15 (1P) from the S1 variant of α-casein. After phosphatase treatment of the digest affinity bound to Fe^{3+}-loaded agarose, the *m/z* of the resulting peaks matched the predicted tryptic digests (Figure 5).

In summary, eight tryptic phosphopeptides containing a total of 21 phosphorylated residues from the three phosphoproteins could be identified by using both Fe^{3+}- and Ga^{3+}-loaded agarose beads as shown in Table 1. Only one expected tryptic phosphopeptide, containing two phosphorylated residues, was not observed. This may be due to suppression effects or to a very low yield of this peptide from the digest.

It was noticed that Fe^{3+}-loaded IDA agarose showed less specificity for the phosphorylated peptides than Fe^{3+}-loaded NTA agarose (data not shown). Unlike NTA agarose, some unphosphorylated peptides were also co-isolated using IDA agarose. This may be due to the NTA agarose having a higher affinity to Fe^{3+}, thus

Table 1. Expected phosphopeptides in the tryptic digest of α-casein and β-casein

Tryptic fragment	Peptide sequence	Source	# of phosphorylation sites	Calculated $[M + H]^+$	Observed $[M + H]^+$	Observed $[M + H]^+$ after dephosphorylation
T15 (aa106-119)	VPQLEIVPNpSAEER	S1 variant	1	1662	1662	1582
T14-15 (aa104-119)	YKVPQLEIVPNpSAEER	S1 variant	1	1953	1954	1874
T7 (aa43-58)	DIGpSEpSTEDQAMEDIK	S1 variant	2	1929	1930	1770
T8 (aa59-79)	QMEAEpSIpSpSp SEEIVPNpSVEQK	S1 variant	5	2722	2723	2324
T1-2 (aa1-21)	KNTMEHVpSpSp SEESIIpSQETYK	S2 variant	4	2749	2750	2430
T7 (aa46-70)	NANEEEYSIGpSpSp SEEpSAEVATEEVK	S2 variant	4	3010	3011	2691
T14 (aa126-136)	EQLpSTpSEENSK	S2 variant	2	1412	[a]	[a]
T1-2 (aa1-25)	RELEELNVPGEIVEp SLpSpSpSEESITR	β-casein	4	3124	3125	2805
T4-6 (aa29-48)	KIEKFQpSEEQQQT EDELQDK	β-casein	1	2561	2558	2478
T6 (aa33-48)	FQpSEEQQQTEDELQDK	β-casein	1	2062	2064	1984

[a]Not observed with this method.

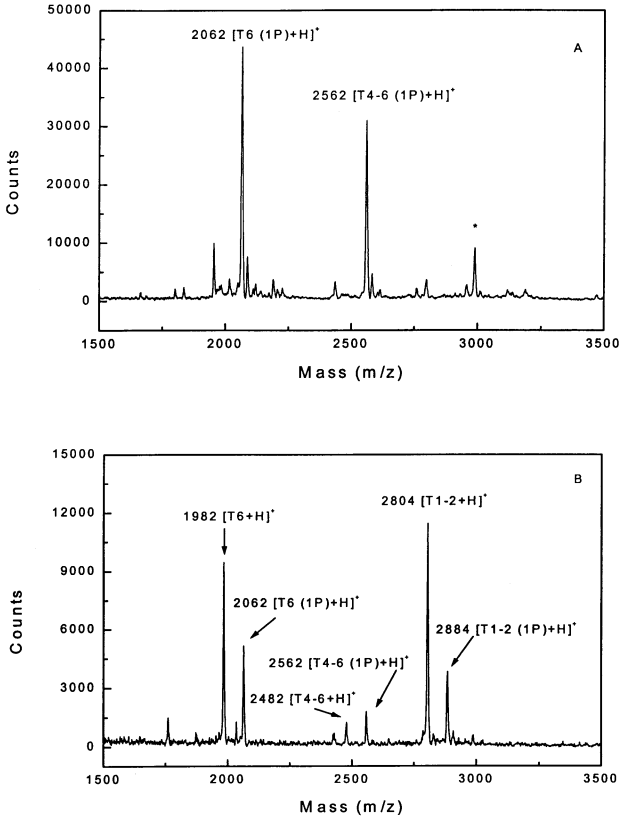

Figure 4. MALDI/TOF mass spectra of (**A**) Fe^{3+}-NTA bound phosphopeptides from the β-casein tryptic digest and (**B**) the same peptides after the phosphatase treatment (both beads and supernatant). T6 (1P): FQpSEEQQQTEDELQDK; T4-6 (1P): KIEKFQpSEEQQQTEDELQDK; T1-2 (4P): RELEENVPGEIVEpSLpSpSpSEESITR. Asterisk: unidentified peak.

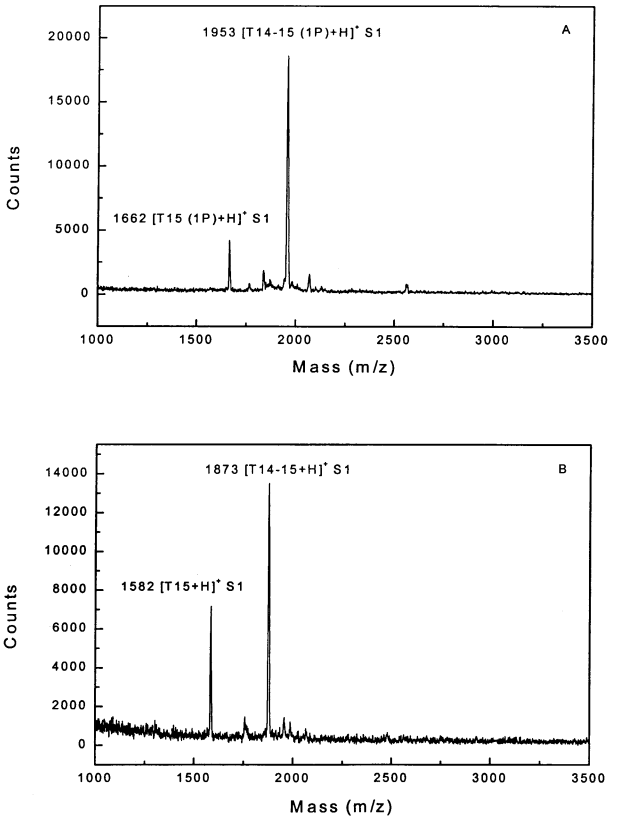

Figure 5. MALDI/TOF mass spectra of (**A**) Fe^{3+}-NTA bound phosphopeptides from the α-casein tryptic digest and (**B**) the same peptides after the phosphatase treatment (both beads and supernatant). T15 (1P): VPQLEIVPNPSAEER; T14-15 (1P): YKVPQLEIVPNPSAEER. 25 mM ammonium citrate was in the matrix.

avoiding the nonspecific adsorption of nonphosphorylated peptides onto the matrix. This result was also consistent with the published data where MALDI/MS was performed on the phosphopeptides eluted from Fe^{3+}-loaded agarose [32, 39].

Application of IMAC to Biochemical Analysis

One application of IMAC isolation of phosphopeptides is in biochemical analysis of peptide substrates from kinase reactions. Substrates from many kinase reactions are often large molecular mass proteins which can be radiolabeled using ^{32}P-ATP. The ^{32}P-labeled proteins can be separated by polyacrylamide gel electrophoresis and detected by autoradiography with high sensitivity. Although some small peptides can be separated by gel electrophoresis, the practical lower limit for effective separation of labeled peptides from unreacted ^{32}P-ATP is about 2000 Da. Peptide mapping studies to determine putative phosphorylation sites often employ 10- to 15-mer peptides which may separate poorly by electrophoresis or may have low enzyme affinity, and thus, are difficult to detect [20]. Direct MALDI/MS analysis of the reaction mixture may be unable to detect the presence of a phosphopeptide if the stoichiometry of the kinase reaction is low. HPLC also may have difficulty in separating phosphopeptides from the parent peptide. IMAC column chromatography can concentrate and separate the phosphopeptide from enzyme reactants and provide definitive evidence of phosphorylation based on mass spectrometry analysis. An early example of the direct analysis of metal ion affinity-bound peptides demonstrated femtomole level sensitivity [34].

This method was then applied to a system, RDFY-HpSKRRLI (*m/z* 1580), currently under investigation in our laboratories. The substrate showed low affinity and low phosphopeptide production under a variety of kinase conditions. Using ^{32}P, the phosphopeptide product was barely visible upon tricine gel electrophoretic separation using autoradiographic detection. The reaction mixture was first tested on Fe^{3+}-loaded agaroses. After intensively washing the sample affinity bound to Fe^{3+}-loaded agarose, we were able to observe the phosphorylated peptide. The intensity of the signal of the phosphorylated peptide increased dramatically after another intensive wash cycle on Fe^{3+}-loaded agarose (Figure 6). It is difficult to wash off the parent peptide completely because of its relatively high concentration.

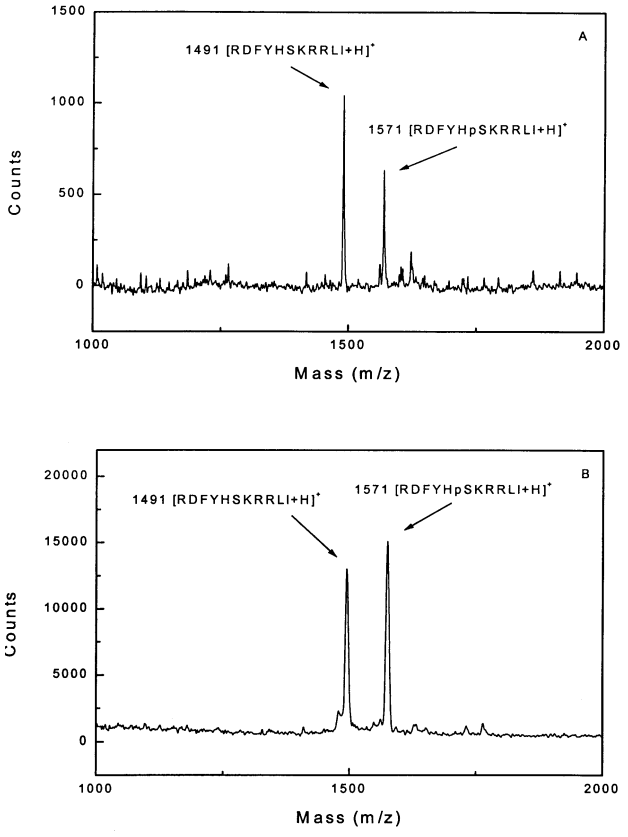

Figure 6. MALDI/TOF mass spectra of Fe^{3+}-NTA bound peptide: RDFYHpSKRRLI (**A**) before and (**B**) after intensively washing the Fe^{3+}-NTA beads with 30 μL water (3×) followed by 30 μL 0.1 M acetic acid (3×).

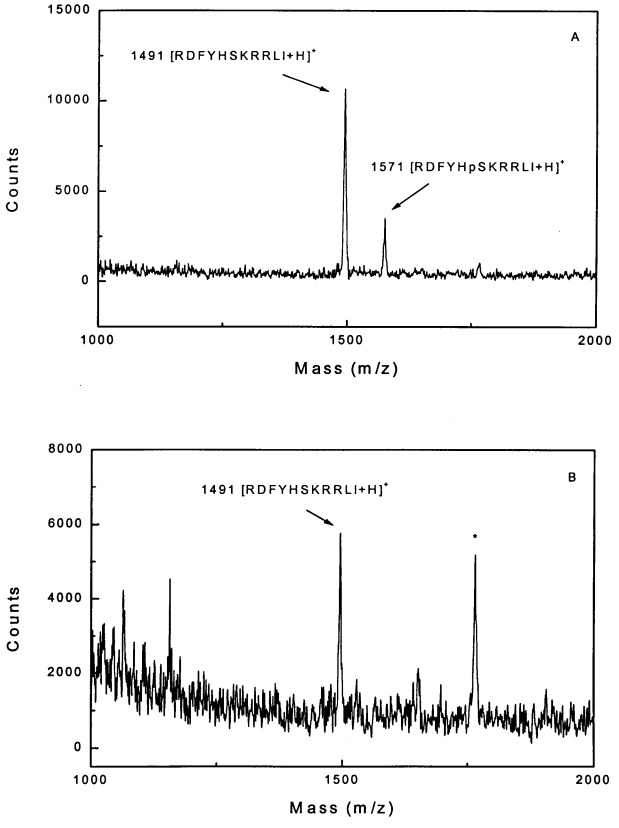

Figure 7. MALDI/TOF MS spectra of Ga^{3+}-NTA bound peptide: RDFYHpSKRRLI (**A**) before and (**B**) after intensively washing Ga^{3+}-NTA beads with 30 μL water (3×) followed by 30 μL 0.1 M acetic acid (3×). Asterisk: unidentified peak.

In order to further evaluate the selectivity difference between Fe^{3+} and Ga^{3+} affinity media, isolation of this peptide was also tested using Ga^{3+}-loaded agarose. Ga^{3+}-loaded agarose showed affinity for the monophosphopeptide as well (Figure 7). The phosphorylated peptide peak, however, was markedly reduced after a second wash cycle on Ga^{3+}-loaded agarose, suggesting that Ga^{3+} has weaker affinity for the monophosphorylated peptides than does Fe^{3+}-loaded agarose. Apparently, although both Fe^{3+} and Ga^{3+} have high specificity for phosphopeptides, the Ga^{3+} binds more weakly with multiphosphorylated peptide than Fe^{3+}, enabling the efficient dissociation and detection of phosphopeptides.

Conclusions

We have shown that the rapid identification of phosphorylation sites can be achieved by direct analysis of enzymatic digestion products affinity bound to metal ions by MALDI/MS. The number of phosphorylation sites can be easily obtained based on the molecular weight shift before and after treatment with phosphatase. The use of carboxypeptidase Y enables the determination of the exact location of the phosphorylation sites. This method eliminates HPLC separation, and thus greatly simplifies the sample preparation.

This method was successfully tested on two commercially available phosphoproteins, and was also successfully applied to the study of a low mass synthetic peptide in a kinase reaction. This method provides a rapid means to determine putative phosphorylation sites during peptide mapping. The ability to detect phosphopeptides from reaction mixtures containing a low yield of the phosphopeptide should enable the application of this method to detection of phosphorylation sites of proteins isolated from cells, where only small fraction of protein will be modified.

Both metal ion-loaded agaroses show selectivity for phosphopeptides. Although Ga^{3+}-loaded agarose has better overall selectivity for phosphopeptides, Fe^{3+}-loaded agarose shows higher selectivity for the monophosphorylated peptide. It is our recommendation that the phosphoproteins should be analyzed with both metal ions to have the greatest chance for the characterization of all the phosphorylation sites.

References

1. Krebs, E. G. The growth of research on protein phosphorylation. *Trends Biol. Sci.* **1994,** *19*, 439–439.

2. Hunter, T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* **1995,** *80*, 225–236.
3. Posada, J.; Cooper, J. A. Molecular signal integration. Interplay between serine, threonine, and tyrosine phosphorylation. *Mol. Biol. Cell* **1992,** *3*, 583–592.
4. *Peptides and Protein Phosphorylation*; Kemp, B. E., Ed., CRC: Boca Raton, FL, 1990.
5. Yan, J. X.; Packer, N. H.; Gooley, A. A.; Williams, K. L. Protein phosphorylation: Technology for the identification of phosphoamino acids. *J. Chromatogr.* **1998,** *808*, 23–41.
6. Boyle, W. J.; Van der Geer, P.; Hunter, T. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* **1991,** *201*, 110–149.
7. Aebersold, R.; Watts, J. D.; Morrison, H. D.; Bures, E. Determination of the site of tyrosine phosphorylation at the low picomole level by automated solid-phase sequence analysis. *Anal. Biochem.* **1991,** *199*, 51–60.
8. Wettenhall, R. E. H.; Aebersold, R.; Hood, L. E. Solid-phase sequencing of ^{32}P-labeled phosphopeptides at picomole and subpicomole levels. *Methods Enzymol.* **1991,** *201*, 186–200.
9. Guy, G. R.; Philiip, R.; Tan, Y. H. Analysis of cellular phosphoproteins by two-dimensional gel electrophoresis: applications for cell signaling in normal and cancer cells. *Electrophoresis* **1994,** *15*, 417–444.
10. Zhao, J. Y.; Kuang, J.; Adlakha, R. C.; Rao, P. N. Threonine phosphorylation is associated with mitosis in hela cells. *FEBS Lett.* **1989,** *249*, 389–395.
11. Abu-Iawi, K. I.; Sultzer, B. M. Induction of serine and threonine protein phosphorylation by endotoxin-associated protein in murine resident peritoneal macrophages. *Infect. Immun.* **1995,** *63*, 498–502.
12. *Protein Phosphorylation. A Practical Approach*; Hardie, D. G., Ed.; Oxford University Press: New York, 1993.
13. Arad-Dann, H.; Beller, U.; Haimovitch, R.; Gavrieli, Y.; Ben-Sasson, S. A. Immunohistochemistry of phosphotyrosine residues: Identification of distinct intracellular patterns in epithelial and steroidogenic tissues. *J. Histochem. Cytochem.* **1993,** *41*, 513–519.
14. Annan, R. S.; Carr, S. A. The essential role of mass spectrometry in characterizing protein structure: Mapping posttranslational modifications. *J. Protein Chem.* **1997,** *16*, 391–402.
15. Qin, J.; Chait, T. Identification and characterization of posttranslational modifications of proteins by MALDI ion trap mass spectrometry. *Anal. Chem.* **1997,** *69*, 4002–4009.
16. Liao, P. C.; Leykam, J.; Andrews, P. C.; Cage, D. A.; Allison, J. 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.* **1994,** *219*, 9–20.
17. Carr, S. A.; Huddleston, M. J.; Annan, R. S. Selective detection and sequencing of phosphopeptides at the femtomole level by mass spectrometry. *Anal. Biochem.* **1996,** *239*, 180–192.
18. Annan, R. S.; Carr, S. A. Phosphopeptide analysis by matrix-assisted laser desorption time-of-flight mass spectrometry. *Anal. Chem.* **1996,** *68*, 3413–3421.
19. Immler, D.; Gremm, D.; Kirsch, D.; Spengler, B.; Presek, P.; Meyer, H. E. Identification of phosphorylated proteins from thrombin-activated human platelets isolated by two-dimensional gel electrophoresis by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) and liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). *Electrophoresis* **1998,** *19*, 1015–1023.
20. Hardie, D. G.; Campbell, D. G.; Caudwell, F. B.; Haystead, T. A. J. In *Protein Phosphorylation*; Hardie, D. G.; Ed.; IRL Press at Oxford University Press: New York, 1993; p 61.
21. Asara, J. M.; Allison, J. Enhanced detection of phosphopeptides in matrix-assisted laser desorption/ionization mass spectrometry using ammonium salts. *J. Am. Soc. Mass Spectrom.* **1999,** *10*, 35–44.
22. Neubauer, G.; Mann, M. Mapping of phosphorylation sites of gel-isolated proteins by nanoelectrospray tandem mass spectrometry: Potentials and limitations. *Anal. Chem.* **1999,** *71*, 235–242.
23. Zhang, X. L.; Herring, C. J.; Romano, P. R.; Szczepanowska, J.; Brzeska, H.; Hinnebusch, A. G.; Qin, J. Identification of phosphorylation sites in proteins separated by polyacrylamide gel electrophoresis. *Anal. Chem.* **1998,** *70*, 2050–2059.
24. Anderrsson, L.; Porath, J. Isolation of phosphoproteins by immobilized metal (Fe^{3+}) affinity chromatography. *Anal. Biochem.* **1986,** *154*, 250–254.
25. Muszynska, G.; Dobrowolska, G.; Medin, A.; Ekamn, P.; Porath, J. O. Model studies on iron (III) ion affinity chromatography II. Interaction of immobilized iron (III) ions with phosphorylated amino acids, peptides and proteins. *J. Chromatogr.* **1992,** *604*, 19–28.
26. Olcott, M. C.; Bradley, M. L.; Haley, B. E. Photoaffinity labeling of creatine kinase with 2-azido- and 8-azidoadenosine triphosphate: Identification of two peptides from the ATP-binding domain. *Biochemistry* **1994,** *33*, 11935–11941.
27. Posewitz, M.; Tempst, P. Immobilized gallium (III) affinity chromatography of phosphopeptides. *Anal. Chem.* **1999,** *71*, 2883–2892.
28. Nuwaysir, L. M.; Stults, J. T. Electrospray ionization mass spectrometry of phosphopeptides isolated by on-line immobilized metal-ion affinity chromatography. *J. Am. Soc. Mass Spectrom.* **1993,** *4*, 662–669.
29. Michel, H.; Hunt, D. F.; Shabanowitz, J.; Bennett, J. Tandem mass spectrometry reveals that three photosystem II proteins of spinach chloroplasts contain N-acetyl-O-phosphothreonine at their NH_2 termini. *J. Biol. Chem.* **1988,** *263*, 1123–1130.
30. Hanger, D. P.; Betts, J. C.; Loviny, T. L. F.; Blackstock, W. P.; Anderton, B. H. New phosphorylation sites identified in hyperphosphorylated tau (paired helical filament-tau) from Alzheimer's disease brain using nanoelectrospray mass spectrometry. *J. Neurochem.* **1998,** *71*, 2465–2476.
31. Cleverley, K. E.; Betts, J. C.; Blackstock, W. P.; Gallo, J.-M.; Anderton, B. H. Identification of novel in vitro PKA phosphorylation sites on the low and middle molecular mass neurofilament subunits by mass spectrometry. *Biochemistry* **1998,** *37*, 3917–3930.
32. Neville, D. C. A.; Rozanas, C. R.; Price, E. M.; Gruis, D. B.; Verkman, A. S.; Townsend, R. R. Evidence for phosphorylation of serine 753 in CFTR using a novel metal-ion affinity resin and matrix-assisted laser desorption mass spectrometry. *Protein Sci.* **1997,** *6*, 2436–2445.
33. Li, S.; Dass, C. Iron (III)-immobilized metal ion affinity chromatography and mass spectrometry for the purification and characterization of synthetic phosphopeptides. *Anal. Biochem.* **1999,** *270*, 9–14.
34. Papac, D. I.; Hoyes, J.; Tomer, K. B. Direct analysis of affinity-bound analytes by MALDI/TOF MS. *Anal. Chem.* **1994,** *66*, 2609–2613.
35. Qian, X. H.; Zhou, W.; Khaledi, M. G.; Tomer, K. B. Direct analysis of the products of sequential cleavages of peptides and proteins affinity-bound to immobilized metal ion beads by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem.* **1999,** *274*, 174–180.
36. Hitomi, M.; Shu, J.; Strom, D.; Hiebert, S. W.; Harter, M. L.;

Stacey, D. W. Prostaglandin A_2 blocks the activation of G_1 phase cyclin-dependent kinase without altering mitogen-activated protein kinase stimulation. *J. Biol. Chem.* **1996,** *271*, 9376–9383.
37. Dumas, B. R.; Brignon, G.; Grosclaude, F.; Mercier, J.-C. Structure primaire de la casein β bovine. *Eur. J. Biochem.* **1972,** *25*, 505–514.
38. Dumas, B. R.; Brignon, G.; Grosclaude, F.; Mercier, J.-C. Structure primaire de la casein β bovine. *Eur. J. Biochem.* **1971,** *20*, 264–268.
39. Hochuli, E.; Dobeli, H.; Schacher, A. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. *J. Chromatogr.* **1987,** *411*, 177–184.