Identification of Phosphopeptides by MALDI Q-TOF MS in Positive and Negative Ion Modes after Methyl Esterification*

Chong-Feng Xu‡, Yun Lu‡, Jinghong Ma§, Moosa Mohammadi§, and Thomas A. Neubert‡§†

We have developed an efficient, sensitive, and specific method for the detection of phosphopeptides present in peptide mixtures by MALDI Q-TOF mass spectrometry. Use of the MALDI Q-TOF enables selection of phosphopeptides and characterization by CID of the phosphopeptides performed on the same sample spot. However, this type of experiment has been limited by low ionization efficiency of phosphopeptides in positive ion mode while selecting precursor ions of phosphopeptides. Our method entails neutralizing negative charges on acidic groups of nonphosphorylated peptides by methyl esterification before mass spectrometry in positive and negative ion modes. Methyl esterification significantly increases the relative signal intensity generated by phosphopeptides in negative ion mode compared with positive ion mode and greatly increases selectivity for phosphopeptides by suppressing the signal intensity generated by acidic peptides in negative ion mode. We used the method to identify 12 phosphopeptides containing 22 phosphorylation sites from low femtomolar amounts of a tryptic digest of a model phosphopeptide. We also identified 10 phosphopeptides containing five phosphorylation sites from an in-gel tryptic digest of 100 fmol of an in vitro autophosphorylated fibroblast growth factor receptor kinase domain and an additional phosphopeptide containing another phosphorylation site when 500 fmol of the digest was examined. The results demonstrate that the method is a fast, robust, and sensitive means of characterizing phosphopeptides present in low abundance mixtures of phosphorylated and nonphosphorylated peptides. Molecular & Cellular Proteomics 4:809–818, 2005.

Reversible phosphorylation of serine, threonine, and tyrosine residues is one of the most common and important regulatory modifications of proteins and often is a key event in cellular signal transduction (1, 2). In recent years, mass spectrometry has become a key technology for characterization of protein phosphorylation and phosphoproteome analysis. Two complementary ionization techniques, MALDI and ESI, in combination with a variety of mass analyzers, have been used to identify phosphopeptides and determine the phosphorylated amino acids on the peptides (3–16). In most cases, characterization of phosphopeptides by MS requires selection of phosphorylated peptides from complex peptide mixtures resulting from proteolysis of phosphorylated proteins followed by MS/MS to confirm the phosphorylation and to identify the phosphorylated amino acid residues on phosphopeptides containing more than a single serine, threonine, or tyrosine residue. Despite many recent advances in methodology, identification of phosphorylation sites on proteins remains a difficult challenge (17).

High sensitivity, resolution, and mass accuracy make Q-TOF MS a powerful tool for the characterization of phosphopeptides. The MALDI Q-TOF allows for increased efficiency and sample throughput because identification of phosphopeptides by MS and characterization by CID MS/MS can be performed on a single sample spot (18). However, a limitation of this type of experiment is the low ionization efficiency of phosphopeptides in positive ion mode, resulting in low sensitivity of phosphopeptide detection and consumption of a large portion of the sample during the search for phosphorylated precursor peptides before MS/MS can be performed (18). Phosphorylated precursor ions can be detected by comparing MALDI spectra of a single sample taken in positive and negative ion modes, with phosphopeptides demonstrating greater relative ion intensities in negative ion mode (19, 20). However, this approach suffers from poor specificity for phosphopeptides because of the high background of nonphosphorylated acidic peptides in negative ion mode caused by the ability of carboxylate groups on glutamate or aspartate residues to develop negative charges in a manner similar to that of phosphate groups. In this article, we show that removal of these acidic groups by methyl esterification (21, 22) can greatly diminish the ion intensity of these acidic nonphosphorylated peptides in negative ion mode and therefore greatly increase the selectivity of the method for phosphopeptides in peptide mixtures. We used the method to identify 12 phosphopeptides containing 22 phosphorylation sites from low femtomolar amounts of a tryptic digest of a model phospho-
protein, β-casein, and its minor contaminant α-s-casein.

The fibroblast growth factor receptors (FGFRs) are a family of tyrosine kinase receptors that play critical roles in human skeletal development. Gain of function mutations in the tyrosine kinase domain of FGFRs are responsible for a number of human skeletal disorders. The degree of clinical severity associated with the mutations correlates with the level of constitutive kinase activity in these mutants (23–25). A method for rapidly comparing phosphorylation sites on various mutants of FGFRs and then correlating phosphorylation status with receptor activity would be very useful for understanding the molecular basis for receptor gain of function and potentially facilitate development of therapeutic interventions. We used our method to characterize phosphopeptides on the in vitro phosphorylated kinase domain of the N549H mutant of the FGFR2, which is responsible for the severe craniosynostosis disorder known as Crouzon syndrome (26). After in vitro phosphorylation by incubation with ATP, isolation by SDS-PAGE, and in-gel tryptic digestion, we identified 10 phosphopeptides containing five phosphorylation sites from 100 fmol of the mutant kinase domain and an additional phosphopeptide containing another phosphorylation site when 500 fmol of the digest was examined. These identified tyrosine phosphorylation sites correspond to those previously found to be phosphorylated on the kinase domain of FGFR1 (27), demonstrating that our method can be used to rapidly characterize phosphorylation sites on low levels of receptors or other proteins such as can be obtained in biological experiments.

EXPERIMENTAL PROCEDURES

Materials—2,6-Dihydroxyacetophenone (DHAP), α-cyano-4-hydroxycinnamic acid (CHCA), dihydroxybenzoic acid (DHB), ammonium hydrogen citrate, and acetyl chloride were purchased from Sigma-Aldrich (St. Louis, MO). Ammonium bicarbonate, TFA, [Glu1]-Fibrinopeptide B, BSA, β-casein (from bovine milk, purity >90% by electrophoresis), and the monophosphopeptide’ was purchased from Promega Co. (Madison, WI). Sequencing grade modified trypsin (from bovine pancreas) and tetraphosphopeptide from the kinase domain of FGFR1 (27) were purchased from Sigma Chemical Co. HPLC grade water, acetonitrile, and methanol were purchased from Fisher Scientific (Hanover Park, IL). Coomassie Blue R-250 and HCl regent. When more than 250 pmol of peptides were methylated, the volume was 200 μl of methanolic HCl. Methyl esterification was allowed to proceed for 2–3 h at room temperature. Solvent was removed by lyophilization, and the resulting samples were desiccated in 30% acetonitrile in 0.2% TFA.

Preparation of Matrix for MALDI Q-TOF Mass Spectrometry—A 50 mm solution of the DHAP matrix was prepared by dissolving 15.2 mg of 2,6-dihydroxyacetophenone in 1 ml water/methanol (10:90, v/v) followed by the addition of 100 mm ammonium hydrogen citrate in water at a ratio of 1:1 (v/v). CHCA matrix was prepared by dissolving 2 mg of α-cyano-4-hydroxycinnamic acid in 1 ml of water/acetonitrile (50:50, v/v) containing 0.1% TFA.

MALDI Q-TOF Mass Spectrometry—Sample and matrix were mixed at a ratio of 1:1 (v/v), and 1.0 μl of this mixture was spotted onto the MALDI sample stage. Positive and negative ion MALDI Q-TOF mass spectra were acquired with a Micromass Q-TOF Ultima MALDI mass spectrometer (Waters, MA). The instrument was operated in V mode with a mass resolution of ~10,000, which enabled the discrimination of carboxymethyl (+14 atomic mass units) and asparagine/glutamine methylation (+15 atomic mass units). Laser pulses were generated by a nitrogen laser (337 nm) with laser energy of 350 μJ per pulse. Mass spectra were acquired and processed by Masslynx 4.0 software (Micromass Ltd., Manchester, United Kingdom). A total of 200–800 laser shots were averaged per mass spectrum, the background was subtracted, and the spectrum was smoothed using a mass window appropriate for the significant peak widths. Known peptide masses were used as internal mass standards.

RESULTS

Identification of Phosphorylation Sites on β-Casein—β-Casein is a model phosphoprotein used to develop and test methods to characterize phosphopeptides. Fig. 1 shows positive and negative mode MALDI Q-TOF mass spectra of 140 fmol of a tryptic digest of β-casein before and after methylation. As shown in Fig. 1, a and b, and previously by us and others (19, 20), the phosphopeptides (indicated by asterisks) have higher relative abundance in the negative ion MALDI spectrum compared with the positive ion spectrum. However, the relative difference between the positive and negative ion intensities of the phosphopeptides is subtle and difficult to observe in the case of the monophosphorylated peptides of m/z 1949.94 from (α-casein) and 2059.81 (FQpSEEQQT-EDELODQK, calculated monoisotopic MW = 2060.82) from β-casein (Fig 1b). In addition to the tetraphosphopeptide

1 The abbreviations used are: FGFR, fibroblast growth factor receptor; DHAP, 2,6-dihydroxyacetophenone; DHB, dihydroxybenzoic acid; CHCA, α-cyano-4-hydroxycinnamic acid.

2 S. R. Weinberger, personal communication.
FIG. 1. MALDI Q-TOF MS of methylated and nonmethylated β-casein tryptic digest in positive and negative ion modes. 
a, spectrum of 140 fmol of β-casein digest acquired in positive ion mode; 
b, spectrum of 140 fmol of β-casein digest acquired in negative ion mode; 
c, spectrum of 140 fmol of methylated β-casein digest acquired in positive ion mode; 
d, spectrum of 140 fmol of methylated β-casein digest acquired in negative ion mode; 
e, spectrum of 7 fmol of methylated β-casein digest acquired in positive ion mode; 
f, spectrum of 7 fmol of methylated β-casein digest acquired in negative ion mode. 
Predominant phosphopeptides species are labeled (*), undermethylated (one or more carboxylate groups not methylated) are labeled (−), and methyl ester side products of deamidated Gln or Asn residues are labeled (+). Each spectrum is the sum of 740 laser shots. Base peak (maximum) ion counts are shown in the upper right corner of each spectrum. The 12 phosphopeptides identified in d, containing five phosphorylation sites from β-casein and 17 phosphorylation sites from the minor contaminants α-s1 and α-s2-casein, are listed in Table I.
Phosphopeptide Methylation and Detection by MALDI Q-TOF MS

### Table I

<table>
<thead>
<tr>
<th>Protein and seq. no.</th>
<th>Sequence</th>
<th>MW (without methylation)</th>
<th>MW-H (after methylation)</th>
<th>Phosphorylation sites</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Casein</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33–48</td>
<td>FQSEEQQOTEDELQDK</td>
<td>2060.82</td>
<td>2157.82</td>
<td>Ser-35</td>
</tr>
<tr>
<td>30–48</td>
<td>IEKFQSEEQQOTEDELQDK</td>
<td>2431.04</td>
<td>2542.07</td>
<td>Ser-35</td>
</tr>
<tr>
<td>2–25</td>
<td>ELEELNVPGVEISELpSpSpSSEEISTR</td>
<td>2965.16</td>
<td>3076.24</td>
<td>Ser-15, Ser-17, Ser-18, Ser-19</td>
</tr>
<tr>
<td>1–25</td>
<td>RELEELNVPGVEISELpSpSpSSEEISTR</td>
<td>3121.26</td>
<td>3232.35</td>
<td>Ser-15, Ser-17, Ser-18, Ser-19</td>
</tr>
<tr>
<td><strong>α-s1-Casein</strong></td>
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<td></td>
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</tr>
<tr>
<td>106–119</td>
<td>VPQLEIPNpSAEER</td>
<td>1659.79</td>
<td>1714.84</td>
<td>Ser-115</td>
</tr>
<tr>
<td>43–58</td>
<td>DlVS_STEDQAMEDIK</td>
<td>1926.68</td>
<td>2023.79</td>
<td>Ser-46, Ser-48</td>
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<tr>
<td>104–119</td>
<td>YKVQLEIPNpSAEER</td>
<td>1950.95</td>
<td>2006.00</td>
<td>Ser-115</td>
</tr>
<tr>
<td><strong>α-s2-Casein</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>138–149</td>
<td>TVDMEpSTEVFTK</td>
<td>1465.60</td>
<td>1520.62</td>
<td>Ser-143</td>
</tr>
<tr>
<td>138–150</td>
<td>TVDMEpSTEVFTKK</td>
<td>1593.69</td>
<td>1648.72</td>
<td>Ser-143</td>
</tr>
<tr>
<td>46–70</td>
<td>NANESEPQpSipSpSSEEpSAEVATEEVK</td>
<td>3007.02</td>
<td>3132.10</td>
<td>Ser-56, Ser-57, Ser-58, Ser-61</td>
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<tr>
<td>1–24</td>
<td>KNTMEHVpSipSpSpSEESHpSQETQYQEK</td>
<td>3131.19</td>
<td>3214.27</td>
<td>Ser-8, Ser-9, Ser-10, Ser-16</td>
</tr>
</tbody>
</table>

Phosphopeptides detected in a methylated tryptic digest of 140 fmol of β-casein

MW (without methylation) is the calculated molecular weight of the phosphopeptides before methylation; MW-H (after methylation) is the observed m/z of the singly charged methylated phosphopeptides measured in the negative ion spectra shown in Figs. 1 and 2.

**Identification of Phosphorylation Sites in a Mixture of BSA and β-Casein Tryptic Digests**—To demonstrate that the method can be used to study more complex peptide mixtures, a similar analysis was carried out on a tryptic digest of a mixture of 140 fmol of BSA and 140 fmol of β-casein (Fig. 2). The presence of the BSA peptides increased the complexity of the spectra of the unmethylated peptides in both positive and negative modes (Fig. 2, a and b) and the methylated peptides in positive ion mode (Fig 2c). However, only phosphopeptides produced peaks of significant ion intensity in the negative ion spectra of the methylated peptide digest (Fig. 2d). In Fig. 2f, three phosphopeptides (including one missed cleavage peptide) containing all five phosphorylation sites from β-casein and four of the α-casein phosphopeptides can be observed in negative ion mode spectra of 14 fmol of the methylated peptide mixture (containing less than 2 fmol of the α-casein contaminants). These results indicate that our method is capable of identifying small amounts of phosphopeptides directly from simple protein mixtures without the need for further purification or enrichment.

In each negative ion spectrum of the methylated peptides, the major phosphopeptide peaks, designated by asterisks in the figures, correspond to the peptides with complete methylation of carboxyamide groups. For example, in Figs. 1, d and f, and 2, d and f, the peak at m/z 2157 corresponds to FQpS_{m} _E_{m} _EQQQT_{m} _E_{D_{m}} _ELQ_{m} _D_{m} _K, where m indicates the methyl esterified acidic or C-terminal amino acid residue, whereas 3232 corresponds to the tetraphosphorylated peptide with complete methylation of carboxyamide groups. Minor peaks, designated by (−), correspond to phosphopeptides with incomplete methyl esterification of acidic groups. Some peptides, indicated by (+) in Figs. 1–3, undergo deamidation followed by methyl esterification on side chains of asparagine and/or glutamine, resulting in a mass increase of 15 Da be-
FIG. 2. MALDI Q-TOF MS of methylated and nonmethylated mixtures of \( \beta \)-casein and BSA tryptic digests in positive and negative ion modes. a, spectrum of 140 fmol of nonmethylated digest mixture acquired in positive ion mode; b, spectrum of 140 fmol of nonmethylated digest mixture acquired in negative ion mode; c, spectrum of 140 fmol of methylated digest mixture acquired in positive ion mode; d, spectrum of 14 fmol of methylated digest mixture acquired in negative ion mode; e, spectrum of 14 fmol of methylated digest mixture acquired in positive ion mode; and f, spectrum of 14 fmol of methylated digest mixture acquired in negative ion mode. c-f, signal intensity of \( m/z \) above 2500 is increased 10-fold. Predominant phosphopeptides species are labeled (*), undermethylated (one or more carboxyl groups not methylated) are labeled (\( \ldots \)), and methyl ester side products of deamidated Gln or Asn residues are labeled (\( \dagger \)). The labeled phosphopeptides are listed in Table I.
cause of substitution of methyl-oxide (-OCH₃) for the amine group (-NH₂) (29). Consistent with the results of He et al. (29), we have found that this side reaction is unavoidable even when using dehydrated methanol and extensively drying the peptides, given that the reaction must proceed for a minimum of 2–3 h to ensure nearly complete methyl esterification of acidic groups (21). We evaluated the methylation of Glu₁-Fibrinopeptide B peptide (sequence EGVNDNEEGFFSAR) and a tryptic digest of \(/H₉₂₅₁/s-casein tryptic digest in a time course experiment. Methyl esterification of acidic groups of most peptides was more than 95% complete after 2 h without side product (amine) methylation in 60 and 80% of peptides containing 2 to 4 and mono Gln/Asn, respectively. However, those peptides with multiple acidic amino acids and Gln/Asn, such as the tetraphosphopeptide in \(/H₉₂₅₂/s-casein and the penta-phosphopeptide in \(/H₉₂₅₁/s₁-casein, required 3 h to achieve complete methylation of acidic groups on 95% of the peptides.

Identification of FGFR2 Autophosphorylation Sites—We used the method to identify phosphorylation sites on the N549H mutant FGF receptor kinase domain after incubation with ATP to induce autophosphorylation. The minimum amount of autophosphorylated kinase domain (2 pmol, 76 ng) required to visualize by Coomassie Blue staining was subjected to SDS-PAGE. After electrophoresis, the corresponding gel band (37 kDa) was excised for in-gel digestion with trypsin, and half of the resulting tryptic peptides were methyl esterified as described under “Experimental Procedures.” The results of the analysis of 10% of the methylated peptides (100 fmol before electrophoresis and digestion) by MALDI Q-TOF in positive and negative modes are shown in Fig. 3 and Table II.

Fig. 3b shows 10 phosphopeptides containing four tyrosine and one serine phosphorylation sites (Table II). All of the major peaks in the spectrum acquired in negative ion mode (Fig. 3b) are caused by FGFR2 phosphopeptides with the exception of the peak of m/z 1342.58. Tandem mass spectra in positive ion mode of the phosphopeptides were acquired from the same spot on the MALDI sample plate to confirm the identity of the phosphopeptides, and in most cases to determine the phosphorylated amino acids. The sequences of these phosphopeptides are shown in Table II. Fig. 3a (inset) shows the MS/MS spectrum for the precursor ion of m/z 1487.63 sequence mDINNImDpYpYKmK. This peptide is derived from the activation loop of the kinase domain. Autophosphorylation of both corresponding tyrosine residues in FGFR1 is required for up-regulation of kinase activity (27). The MS/MS spectrum supports phosphorylation on the two tyrosine residues. The immonium ion of phosphotyrosine is observed at m/z 216.04, peaks at m/z 1407.76 and 1327.72 indicate the loss of one and two HPO₃ from the molecular ion (MH⁺ = 1487.63), respectively, and the y₄ to y₇ fragment ions are clearly seen. Interpretation of the previous spectrum was straightforward.
Phosphopeptide Methylation and Detection by MALDI Q-TOF MS

**Table II**
Phosphorylation sites identified in autophosphorylated kinase domain of the N549H mutant of the FGF receptor 2

MW (without methylation) is the calculated molecular weight of the phosphopeptides before methylation, and MW-H (after methylation) is the observed m/z of the singly charged methylated phosphopeptides measured in the negative ion spectrum of 100 fmol of a tryptic digest of FGFR2 kinase domain shown in Fig 3. The peptide his tag + 459–473 (sequence GSSHHHHHHSQDPMLAGVSEYELPEDPK) was only observed by analysis of the tryptic digest of 500 fmol of the FGFR2 kinase domain. For comparison with results published previously, amino acid numbers refer to the sequence of full-length FGFR2, not the kinase domain construct used in our experiments.

<table>
<thead>
<tr>
<th>Sequence no.</th>
<th>Primary sequence</th>
<th>MW (before methylation)</th>
<th>M-H (after methylation)</th>
<th>Phosphorylation sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>650–669</td>
<td>DINVYKKWKKKKKKKK</td>
<td>1236.51</td>
<td>1405.60</td>
<td>Tyr-565/Tyr-565</td>
</tr>
<tr>
<td>580–592</td>
<td>RPPGEYDINR</td>
<td>1676.74</td>
<td>1717.74</td>
<td>Ser-587/Tyr-588</td>
</tr>
<tr>
<td>578–592</td>
<td>ARRPPGEYDINR</td>
<td>1836.68</td>
<td>1877.71</td>
<td>Tyr-586/Ser-587/Tyr-588</td>
</tr>
<tr>
<td>459–473</td>
<td>GSSHHHHHHSQDPMLAGVSEYELPEDPK</td>
<td>2926.16</td>
<td>2967.24</td>
<td>Tyr-586/Ser-587/Tyr-588</td>
</tr>
</tbody>
</table>

because both potential sites on the peptide were phosphorylated. In many cases MALDI Q-TOF MS/MS can also be used to identify phosphorylated amino acids on peptides with several potential phosphorylation sites (18). Fig. 3c shows the positive ion MS/MS spectrum of the monophosphorylated peptide RPPGEYDINR (MH+ = 1677.71). Lack of immonium ion at 216.04, the abundant b7 (831.38) and c7 (848.41) ions, as well as a minor b5 ion, and lack of c7 + 80, c7 + 80, and b5 + 80 ions indicated that the tyrosine residues were not phosphorylated or were phosphorylated at very low stoichiometry. The loss of H3PO4 from the b8 ion indicated that the serine was phosphorylated, which is also consistent with the loss of H3PO4 from c9, b10, c11, b12, and the molecular ion. We concluded that most or all of the monophosphorylated peptides are phosphorylated on serine, which has not been previously reported. For comparison, we have shown the MS/MS spectrum of both methylated and nonmethylated forms of this peptide in supplemental Fig. S1. We find no systematic advantage or disadvantage from fragmenting the singly charged methylated phosphopeptides measured in the negative ion spectrum of 100 fmol of a tryptic digest of FGFR2 kinase domain shown in Fig 3. The peptide his tag + 459–473 (sequence GSSHHHHHHSQDPMLAGVSEYELPEDPK) was only observed by analysis of the tryptic digest of 500 fmol of the FGFR2 kinase domain. For comparison with results published previously, amino acid numbers refer to the sequence of full-length FGFR2, not the kinase domain construct used in our experiments.

In summary, we demonstrated that the FGFR2 kinase domain contains a total of six phosphorylation sites, including four identified tyrosine residues and an identified serine residue as well as an additional phosphopeptide containing an undetermined phosphorylation site. This result is consistent with a native gel showing the time course of phosphorylation of the FGFR2 kinase domain, which shows nearly complete phosphorylation of five or six sites after 10 min of incubation with ATP (supplemental Fig. S4). The result is also consistent with a mass of the intact phosphoprotein as determined by MALDI-TOF MS of 37,458 Da (data not shown). This mass is 505 Da higher than the predicted average mass of the nonphosphorylated protein, 36,953 Da, suggesting that six phosphate groups modified the FGFR kinase domain at high stoichiometry. However, the mass of the intact protein alone is not definitive because of variable and uncertain stoichiometry of phosphorylation as well as the possibility of additional posttranslational modifications. Although we detected some potentially multiphosphorylated peptides that contained partially phosphorylated sites (Table II and supplemental table), we did not detect any nonphosphorylated versions of the phosphopeptides in any of our experiments.

Comparison of DHAP, DHB, and CHCA Matrices—We used DHAP as the matrix because it is a “cooler” matrix than CHCA (30) and resulted in less PSD at the relatively high fixed laser energy (350 μJ/pulse) of our MALDI Q-TOF instrument. Fig. 4 shows positive ion MALDI Q-TOF mass spectra of 500 fmol of two synthetic standard phosphopeptides from β-casein using CHCA (Fig. 4a) and DHAP (Fig. 4b) as matrix. The loss of H3PO4 is significantly greater in Fig. 4a than in 4b for both the mono- and tetraphosphorylated peptides, which decreases the ion intensity generated by the intact phosphopeptides as well as increases spectrum complexity. The increased sensitivity is also demonstrated by the clear presence of an addi-
tional peak at m/z 2967.23 in Fig. 4b but not Fig. 4a, which corresponds to a contaminant in the peptide mixture because of a missing N-terminal arginine residue. A comparison of positive and negative ion mode spectra of methyl esterified β-casein digest using CHCA and DHAP matrices is shown in supplemental Fig. S5, also demonstrating less β elimination of phosphate in the DHAP spectra. Whereas we found that use of DHB as matrix improved the ion intensity for nonmethylated phosphopeptides in both positive and negative ion modes, and inclusion of phosphoric acid improved sensitivity in negative ion mode (31), lack of improvement of the signal for methylated phosphopeptides makes DHB suboptimal for our method (data not shown).

DISCUSSION

Previous studies have shown that comparing MALDI-TOF spectra of mixtures of phosphorylated and nonphosphorylated peptides acquired in positive and negative modes can be useful for identifying the phosphopeptides based on the relatively higher ion intensities generated by the phosphorylated peptides in negative ion mode. However, a limitation of this method is its poor specificity based on the tendency of acidic peptides to exhibit behavior similar to that of phosphopeptides. We have greatly improved the selectivity of the method by methylation of carboxylate groups on the peptides with methanolic HCl (22) to suppress the contribution of these groups to formation of negative charges on the peptides in negative ion mode. We then analyze the phosphopeptides on the same sample spot by CID in the MALDI Q-TOF instrument to confirm phosphopeptide identification and locate the phosphorylated amino acid(s).

We have demonstrated the utility of the method by identifying all of the known phosphorylation sites on low femtomolar amounts of a simple protein mixture, including β-casein and its contaminant α-s-casein. We have also shown that the method can be used to rapidly monitor all previously identified phosphorylation sites on subpicomolar amounts of the medically important N549H mutant FGFR2 tyrosine kinase domain. This study is the first characterization of the autophosphorylation sites on the FGFR2. Based on a previous study of the highly homologous (more than 90% identical) FGFR1 kinase domain, we expected to find six tyrosine phosphorylation sites on the kinase domain of FGFR2. Indeed, four of our identified tyrosine phosphorylation sites correspond to those on FGFR1, and we found a fifth site on a tryptic peptide that contains tyrosine 466, which corresponds to phosphorylated tyrosine 463 on FGFR1. We did not find phosphorylation on tyrosine 733 of FGFR2, which corresponds to tyrosine 730 on FGFR1, which was shown by Mohammadi et al. (27) to be phosphorylated. However, the crystal structure of FGFR1 kinase domain shows that tyrosine 730 is poorly solvent-exposed and therefore would be expected to be a poor substrate for phosphorylation (32). We found an additional
phosphorylation on serine 587, which we believe was phosphorylated by a heterologous serine kinase during protein expression (data not shown).

Although each of the phosphorylation sites on these two proteins is phosphorylated to very high stoichiometry, we also believe the method is capable of detecting phosphorylation events at low stoichiometry. We detected 16 of 18 previously reported phosphorylation sites as well as an additional site not previously reported on $\alpha$-casein that was present at less than 10% of the $\beta$-casein in the same sample. After methylation, the ratio of intensity (ion counts) of phosphorylated peptides in negative ion mode compared with positive ion mode was more than 25 greater than that of nonphosphorylated peptides (see supplemental table), further suggesting the method should efficiently detect phosphopeptides of low stoichiometry. However, it is possible for the method to fail to detect phosphorylated peptides when enzymatic digestion of a phosphoprotein fails to yield a sufficient quantity of phosphopeptides for MALDI-TOF MS analysis, when the resulting phosphopeptides are too small or too large to be efficiently detected by MALDI-TOF MS, or when a highly abundant nonphosphorylated peptide has nearly the exact same molecular weight as the phosphorylated peptide.

The identification of multiphosphorylated peptides by mass spectrometry has been especially challenging because of low ionization efficiency in positive ion mode and a high propensity for nonspecific adsorption to metallic and hydrophilic surfaces (33). For example, without methyl esterification, the tetraphosphopeptide of $\beta$-casein (containing eight acidic amino acids and four phosphates) and the pentaphosphopeptide from $\alpha$-s1-casein (amino acids 59–79, containing six acidic amino acids and five phosphates) were difficult to detect by MALDI-TOF or nanoflow LC-MS (Q-TOF) in positive ion mode at the 50 pmol level, or several pmol in negative ion mode for the $\alpha$-s1-casein pentaphosphorylated peptide (data not shown and Kim et al. (33)). However, after methyl esterification, the limit of detection by MALDI Q-TOF in negative ion mode for these peptides decreased dramatically to the low femtomolar level. The most likely explanation is that methyl esterification achieves this effect by neutralizing the carboxylate groups on the aspartate and glutamate side chains as well as the carboxyl terminus of each peptide. The phosphate groups of phosphopeptides are then the only remaining acidic groups, and only phosphopeptides ionize efficiently in negative ion mode. In this case, the ionization of phosphopeptides is less likely to be suppressed by non-phosphopeptides, so that the negative ion mode spectrum of a mixture of methyl-esterified phosphorylated and nonphosphorylated peptides is similar in ion detection efficiency to that of purified phosphopeptides. By comparing negative and positive ion spectra of methylated peptides, low abundance phosphorylated peptides can be discriminated from highly abundant nonphosphorylated peptides that produce detectable signals in negative ion mode because the nonphosphorylated peptides produce much stronger relative signals in positive ion mode.

The results demonstrate that the method is a fast, robust, and sensitive means of characterizing phosphopeptides present in low abundance mixtures of phosphorylated and nonphosphorylated peptides. An advantage of the method is that it can be used to characterize peptides phosphorylated on serine, threonine, or tyrosine residues. Another advantage is that the method also can be used with other MALDI instrumentation that can be operated in positive and negative ion modes. Because the method relies on methylating carboxyl groups on peptide mixtures before mass spectrometry, it could be used for relative quantification experiments using stable isotopic labeling of the methyl groups (29), though partial methylation of glutamine and asparagine residues would complicate analyses of such experiments. In addition, our method is an ideal complement to enrichment of phosphopeptides from complex mixtures using immobilized metal affinity chromatography IMAC (22). Such pre-enrichment would enable the method to be used in large scale analyses of the phosphoproteome.

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