The C-terminal Conserved Domain of MARCKS Is Phosphorylated in Vivo by Proline-directed Protein Kinase

APPLICATION OF ION TRAP MASS SPECTROMETRY TO THE DETERMINATION OF PROTEIN PHOSPHORYLATION SITES*

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MARCKS, the major protein kinase C substrate in various cells and tissues, binds to calmodulin, acidic membrane phospholipids, and actin filaments, and these interactions are regulated by protein phosphorylation. We have previously shown that MARCKS purified from bovine brain is phosphorylated not only by protein kinase C but also by so-called proline-directed protein kinases in the well conserved N-terminal half of the molecule (Taniguchi, H., Manenti, S., Suzuki, M., and Titani, K. (1994) J. Biol. Chem. 269, 18299–18302). Although the presence of other phosphorylation sites in the C-terminal peptide was also noticed, the ambiguity in the C-terminal domain of the bovine protein hampered a more detailed analysis. In the present study, we analyzed MARCKS purified from rat brain by electrospray ionization/ion trap mass spectrometry. The results obtained revealed two additional novel phosphorylation sites in the C-terminal region. Both phosphorylation sites (Ser291 and Ser299) are immediately followed by proline, suggesting that these sites are also phosphorylated by the proline-directed protein kinase(s). Since Ser299 is within the C-terminal domain, which is well conserved among various species, the function of the domain, whatever it is, seems to be controlled by phosphorylation.

MARCKS, a major in vivo substrate of protein kinase C (PKC), has been characterized in various tissues and cells (for reviews, see Refs. 1 and 2). Although its physiological function has yet to be elucidated, MARCKS has been implicated in playing an important role in brain function. It is enriched in the nerve terminals, and its expression is developmentally regulated. Furthermore, a recent gene knockout study showed that its deficiency in mice leads to abnormal brain development and perinatal death (1). One of the most striking characteristics of the protein is its translocation from the membrane to cytosolic fractions upon extracellular stimuli. A highly conserved basic amphiphilic phosphorylation domain in the middle of the molecule, which contains all the four PKC phosphorylation sites, serves at the same time as the phospholipid-binding site (4). Since calmodulin shares the same binding site, and since the PKC-dependent phosphorylation abolishes the binding of both calmodulin and acidic phospholipids, MARCKS may play a key role as one of the cross-talk points in the signal transduction pathways (4, 5). The ability of the same domain to sequester phosphatidylinositol-derived signaling molecules led to a suggestion that the MARCKS protein may control the activity of phospholipase C (6).

Recent development in mass spectrometry made it possible to determine the molecular mass of large proteins with high precision and resolution (7, 8). The technique is now widely used to analyze various aspects of proteins including primary and tertiary structures (9). We have already shown that the liquid chromatography/mass spectrometry (LC/MS), in which capillary reverse-phase high performance liquid chromatography is connected on-line to the electrospray interface of a quadrupole mass spectrometer, is a powerful technique in elucidating post-translational modifications of proteins such as phosphorylation and acylation (10–12). In fact, many hitherto unnoticed in vivo phosphorylation sites were revealed in various proteins.

Our mass spectrometric analysis on the in vivo phosphorylation sites of MARCKS showed that it is phosphorylated also at sites other than those by PKC (11). All the phosphorylation sites found are followed by proline, suggesting that MARCKS is a physiological substrate of proline-directed protein kinase such as microtubule-associated protein kinase and Cdk5. These phosphorylation sites are distributed only in the N-terminal half of the molecule, where the homology among various species is high, suggesting that the functions of the domains in the N-terminal regions are regulated by protein phosphorylation. It should be stressed that PKC is not the only kinase which phosphorylates MARCKS, although MARCKS has been regarded as a specific marker of PKC activation (1, 2). In a preliminary study, we found that bovine MARCKS contained additional phosphorylation sites in its large C-terminal peptide. However, the ambiguity of the sequence and the lack of residues suitable for proteolytic digestion in the bovine protein hampered a more detailed analysis. In the present study, we used MARCKS protein purified from rat brains, and studied the in vivo protein phosphorylation sites. The capability of an electrospray ionization/ion trap mass spectrometer to perform on-line LC/MS and tandem mass spectrometry (LC/MS/MS) analyses was utilized to determine these sites. The two additional in vivo phosphorylation sites thus identified, Ser291 and Ser299, are followed by proline, suggesting that they are also phosphorylated by proline-directed protein kinase(s). Since the
**EXPERIMENTAL PROCEDURES**

**Materials**—MARCKS was purified from membrane fractions of bovine brain as described previously (13). The same procedure was applied to purify MARCKS from membrane fractions of rat brain, except that all buffers contained 0.5 mM NaF and 125 mM β-glycerophosphate as phosphatase inhibitors. Microcystin-LR (Calbiochem, 1 μM) was also included in the homogenization buffer. All other chemicals and biochemicals used were of analytical grade.

**Digestion with Lys-C Endoprotease**—Purified protein (30 μg) was digested with 3 μg of *Achromobacter* lysyl endoprotease in 100 mM Tris-HCl (pH 8.9) in the presence of 2 mM urea at 37 °C for 18 h. After stopping the reaction by adding 1% trifluoroacetic acid, the peptide mixtures were directly analyzed by LC/MS and LC/MS/MS.

**Asp-N Endoprotease Digestion of C-terminal Peptide**—The lysyl endoprotease peptides were separated by reverse-phase high performance liquid chromatography (Vydac 218TP52 column, 2.1 × 150 mm) using a linear gradient of H₂O-acetonitrile in the presence of 0.1% trifluoroacetic acid. Fractions containing the C-terminal peptide were combined and dried in a SpeedVac concentrator. The peptide was dissolved with 25 μl of 50 mM sodium phosphate buffer (pH 8.0) and incubated with 0.2 μg of endoprotease Asp-N (Takara, Kusatsu, Japan) at 37 °C for 18 h. The resulting peptide mixture was directly analyzed by LC/MS and LC/MS/MS.

**Glu-C Endoprotease Digestion of C-terminal Peptide**—The Asp-N endoprotease peptides were separated by reverse-phase high performance liquid chromatography as above. The C-terminal peptide obtained was dissolved in 25 μl of 50 mM ammonium bicarbonate, and incubated with 1 μg of Glu-C endoprotease (*Staphylococcus* V8 protease, Boehringer Mannheim) at 25 °C for 14 h. After stopping the reaction by adding 0.1% final concentration of trifluoroacetic acid, the resulting peptide mixture was directly analyzed by LC/MS and LC/MS/MS. The inter-chain disulfide bond formed by oxidation during the treatments was cleaved by incubating with excess amounts of dithiothreitol before the mass analysis.

**Mass Spectrometry**—LC/MS analysis was carried out with an electrospray ionization/quadruple mass spectrometer (PE Sciex API-III) as described previously (10, 11). LC/MS and LC/MS/MS analyses were done with an electrospray ionization/ion trap mass spectrometer (Finnigan-MAT LCQ). A linear gradient of solvents at a flow rate of 200 μl/min produced with a conventional high performance liquid chromatography apparatus (ThermoQuest P3000) was split with a tee. A Monitor C18 column (1 × 150 mm, packed with 5-μm, 100-Å pore material) was connected to one outlet as a separation column. Another monitor C18 column (2.1 × 150 mm) was connected to the other outlet as a balancing column to make 1:4 split ratio. The elution was done with a linear gradient of H₂O-acetonitrile in the presence of 0.02% trifluoroacetic acid and 0.1% acetic acid. The ion source voltage was set at 4.5 kV, and N₂ gas was used as a nebulizer gas. The temperature of the heated capillary was set at 200 °C. The LC/MS/MS analysis was carried out in a data-dependent mode, in which the largest peak in each single scan obtained in the LC/MS mode was automatically subjected to the collision-induced dissociation. The isolation width of the precursor ions was set either at 2 (for the first run) or at 10 atomic mass units for the phosphopeptide analysis during the second run, while the relative collision energy was 35%.

**Other Analytical Methods**—Protein concentration was determined by Coomassie Blue binding (Bio-Rad). The gas phase Edman degradation was carried out with a 470A protein sequencer (Applied Biosystems).

**RESULTS**

**Characterization of the C-terminal Peptide of Bovine MARCKS**—Our previous LC/MS analysis, which covered more than 95% of the whole sequence, indicated some differences between the published cDNA and protein sequences (11). Of the two lysyl endoprotease peptides which showed masses different from the theoretical ones based on the cDNA sequence, one peptide corresponding to residues 241–258 (K24–25) was completely sequenced, and the corrected sequence gave a theoretical mass which is within experimental error of the observed mass (11). The other peptide, corresponding to the C-terminal peptide, was partially sequenced, and we could identify a few amino acid differences. When these corrections are taken into account, the theoretical mass of the peptide would be 5,917.4 Da, which is slightly but significantly larger than the observed mass, 5,910.2 ± 1.5 Da (Fig. 1). The peptide was found accompanied with a peptide of 5990.2 ± 1.3 Da, which can be a singly phosphorylated peptide. To elucidate the cause of the mass difference, we first repeated the sequencing of the peptide isolated from the Lys-C endoprotease digest as described under “Experimental Procedures.” The gas phase sequencing gave a sequence, Ala-Glu-Glu-Ala-Gly-Val-Ser-Ala-Ala-Gly-Xaa-Glu-Ala-Pro-Ser-Ala-Ala-Gly-Pro-Gly-Pro-Pro-Glu-Gln-Glu-Ala-Ala-Pro-Ala, where Xaa was an unidentified amino acid. In addition to the missing Ala-Ala-Gly sequence in the protein sequence as noticed in our previous study (11), the peptide sequence started to differ completely from the deduced sequence from the 20th cycle (Fig. 2). A part of the new sequence was found in the corresponding part of the rat, mouse, and chick sequences, suggesting that the published bovine sequence deduced from the cDNA sequence contains more errors in the C-terminal region. A sequencing study on the polymerase chain reaction products containing a part of the C-terminal region has been conducted with various species (14). The sequence obtained with bovine MARCKS in the study shows a complete agreement with our present peptide sequence. The incorporation of these corrections to the bovine sequence made a better homology among MARCKS proteins from various species (see Fig. 7). However, the C-terminal peptide based on the sequence would have a theoretical mass of 6024.4 Da, which is much larger than the observed value, 5910.2 Da. Since the removal of the C-terminal Glu would not account for the difference, the composite sequence shown in Fig. 2 should still differ from the protein sequence.

**LC/MS Analysis of Rat MARCKS**—Because of the ambiguity of the C-terminal sequence and the lack of suitable amino acid residues for further proteolytic digestion in the bovine protein, we sought MARCKS protein from other species that would not pose these problems. Rat MARCKS was chosen because of the presence of a few well positioned Asp residues in the C-terminal peptide. MARCKS was purified from rat brain in the presence of phosphatase inhibitors, and digested with lysyl endoprotease. The peptide mixtures obtained were analyzed directly by reverse-phase capillary liquid chromatography and identified by LC/MS/MS.
Phosphorylation coupled on-line to an electrospray ionization/ion trap mass spectrometer. As has been described in a previous analysis on the bovine protein (11), the peptides were identified by comparing the molecular masses observed with those predicted from the cDNA sequence. Furthermore, automatic switching to the LC/MS/MS mode was used to generate fragment ions produced by the collisionally activated dissociation. The partial sequences thus obtained were used to confirm the assignment. More than 90% of the whole sequence was confirmed. No significant difference between the theoretical and observed masses was found, suggesting that the published amino acid sequence deduced from the rat cDNA sequence is correct. In addition to the three major in vivo phosphorylation sites by proline-directed protein kinase found in the bovine protein, i.e. peptides K3, K5, and K9 (11), the C-terminal peptide K24 (residues 253–309) was also found phosphorylated (Fig. 3). Both singly and doubly phosphorylated peptides, which are larger than the parent peptide by 80 and 160 Da, respectively, were present. These results indicate that the in vivo phosphorylation sites by proline-directed protein kinase(s) are not species restricted to bovine MARCKS.

Characterization of the C-terminal Peptide of Rat MARCKS—Fractions containing the C-terminal K24 peptide from the reverse-phase column chromatography were combined and subjected to further digestion with Asp-N endoprotease to produce smaller peptides suitable for tandem mass analysis. The digest mixture was directly analyzed by LC/MS (Fig. 4A, inset). Only peptide N4 (residues 280–309) containing the C terminus was found phosphorylated (Fig. 4A). The presence of doubly phosphorylated species indicated that the peptide contained two phosphorylation sites. The two other peptides, N1 (residues 253–262) and N2–3 (residues 263–279), were not phosphorylated to a significant extent (data not shown). To identify the phosphorylation sites in the N4 peptide, the peptide was isolated by reverse-phase chromatography and subjected to further digestion with Glu-C endoprotease. The digestion yielded two peptides, DEPAASAAPASPEPKPE (E1) and CSPEAP-PAPVAE (E2–3), both of which were found singly phosphoryl-
ated, suggesting that each peptide contained one phosphorylation site (Fig. 4, B and C).

The tandem mass spectra of the two peptides were obtained by LC/MS/MS analysis (Fig. 5). Although the N-terminal peptide, E1, contained three phosphorylatable residues (serines), the difference between $y_6$ and $y_7$ ions that corresponded to a phosphoserine clearly identified the third Ser in the peptide (Ser291) as the phosphorylation site (Fig. 5A). On the other hand, the difference between $y_8$ and $y_9$ and that between $y_{12}$ and $y_{13}$ corresponded to values expected for a normal Ser. The lack of phosphorylation at the other two Ser residues was also confirmed by $b_8$, $b_{10}$, and $b_{11}$ ions. From these results we concluded that Ser291 is the sole phosphorylation site in the peptide. The C-terminal peptide, E2–3, contained only one phosphorylatable residue, Ser299. The tandem mass spectrum of the peptide confirmed the lack of phosphorylation between the Ser residue and the C terminus (Fig. 5B). These results established that the two Ser residues within the Ser-Pro motif (Ser 291 and Ser299) are the in vivo phosphorylation sites in the C-terminal domain.

**Characterization of the N-terminal Peptide**—Although MARCKS is believed to be myristoylated at its N terminus, the modification has never been demonstrated directly. As has been reported previously (11), the LC/MS analysis showed the presence of a peptide with a mass corresponding to the myristoylated N-terminal peptide K1. An MS/MS spectrum of the N-terminal peptide was obtained in the LC/MS/MS mode (Fig. 6). The data not only confirmed the N-terminal sequence of the protein, but also indicated clearly the Nα-amino group of the N-terminal glycine is modified by a group corresponding to a myristoyl group.

**DISCUSSION**

In a previous study we reported that MARCKS is a physiological substrate of proline-directed protein kinases (11). These phosphorylation sites were all contained in the half of the molecule N-terminal to the PKC-phosphorylation domain, where most of the sequence is well conserved. Here we added two novel sites to the list of the MARCKS phosphorylation sites. Considering the small size of the protein, the presence of altogether more than 10 in vivo phosphorylation sites is

**Fig. 5.** Tandem mass spectra of the C-terminal peptides, E1 and E2–3. The doubly charged ion of the phosphorylated E1 peptide (A, $m/z$ 916.4) and the singly charged ion of the phosphorylated E2–3 peptide (B, $m/z$ 1247.0) were subjected to the collision-induced dissociation in the ion trap mass analyzer. Fragment ions observed are indicated above and below the peptide sequence, respectively. The two phosphorylated Ser residues are underlined.

**Fig. 6.** Tandem mass spectrum of the N-terminal peptide. The singly charged ion of the N-terminal peptide ($m/z$ 847.5) was subjected to the fragmentation in the ion trap mass analyzer. Fragment ions observed are indicated above and below the peptide sequence.

**Fig. 7.** Alignment of the C terminus of MARCKS proteins from various species. The Ser residue found phosphorylated is highlighted together with the following proline. Note that the phosphorylation site identified in the present study together with the surrounding amino acids is well conserved among various species (boxed).
rather surprising, and MARCKS can be considered to be hyperphosphorylated.

MARCKS has interesting characteristics in its sequence. The whole protein lacks large hydrophobic amino acids and is very acidic except for the N-terminal myristoylation site and the highly basic amphiphilic domain which serves as the calmodulin-, PKC-, and acidic phospholipid-binding domain (15–17). As a whole, the N-terminal half including the PKC phosphorylation domain is well conserved among MARCKS from various species, while the C-terminal one is less conserved. The latter is rich in a few amino acids such as Ala, Pro, and Glu. Although the presence of many repeats consisting of these characteristic residues is noted, the presence of exact motifs at the exact positions seems to be not necessary for the function of the part of the molecule. This has been noticed previously with GAP-43, another PKC substrate protein and a relative of MARCKS (18). In both proteins, amino acid compositions are well conserved, but extensive sequence conservation is lacking. However, the C-terminal end consisting of about 15 amino acids is well conserved among MARCKS proteins from various species (Fig. 7). Thirteen amino acids are identical in human, bovine, mouse, and rat, and 8 amino acids are conserved also in chicken, suggesting that this region might constitute another functionally independent domain. It should be noted that one of the phosphorylated Ser found in the C-terminal domain is well conserved together with the surrounding residues, suggesting that the physiological function of the domain, whatever it is, is controlled by the protein phosphorylation at the site.

Interestingly, the C-terminal domain is not conserved in F52, a short homologue of MARCKS that shares all the N-terminal half including the PKC phosphoamino acids. As will be described elsewhere, the tandem spectrum of the N-terminal myristoylated peptide obtained with the ion trap mass analyzer was almost indistinguishable from that obtained in the low energy collision-induced dissociation in a triple-stage quadrupole instrument. A detailed study on the tandem mass analysis of various peptides would provide the nature of the fragmentation mechanisms of the collision-induced dissociation by resonance excitation performed in the ion trap mass analyzer, which should, in turn, give more clues in analyzing the sequence information of peptides.

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