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Presence of Conserved Domains in the C-Terminus of MARCKS, a Major *In Vivo* Substrate of Protein Kinase C: Application of Ion Trap Mass Spectrometry to the Elucidation of Protein Structures[1]

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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 analyzed MARCKS purified from bovine brain using capillary liquid chromatography/electrospray mass spectrometry and found that the protein structure differed significantly from that deduced from cDNA sequences [Taniguchi, H., Manenti, S., Suzuki, M., and Titani, K. (1994) *J. Biol. Chem.* 269, 18299-18302]. Moreover, the alignment of the protein from various species showed a lack of any conserved sequences in the C-terminal half of the molecule. This prompted us to reexamine the C-terminal amino-acid sequence of bovine MARCKS. The purified protein was digested with lysyl endoprotease, and the obtained C-terminal peptide was further digested with either *Staphylococcus* V8 protease or NTCB. The small peptides thus obtained were analyzed by liquid chromatography/electrospray/tandem mass spectrometry. This combined with gas-phase Edman sequencing allowed us to determine the C-terminal primary structure. The sequence obtained differed significantly from that reported previously, and the comparison with other species revealed the presence of a novel conserved domain in the C-terminal region of MARCKS.

Key words: ion trap, MARCKS, mass spectrometry, protein kinase C, protein structure.

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 shown to play 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 (*3*). One of the most striking characteristics of the protein is its translocation from the membrane to cytosolic fractions upon extracellular stimulation. 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 crosstalk 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 has 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 (*7, 9*). We have already shown that liquid chromatography/mass spectrometry (LC/MS) in which capillary reversed-phase high performance liquid chromatography is connected on-line to the electrospray interface of a quadrupole mass spectrometer is a powerful technique for elucidating posttranslational modifications of proteins such as phosphorylation and acylation (*10-12*). In fact, many hitherto unnoticed *in vivo* phosphorylation sites were revealed in various proteins. In addition to the various protein modifications, our mass spectrometric analysis (*11*) indicated that the protein structure of bovine MARCKS differed significantly from the primary sequence deduced from the bovine cDNA sequences (*13, 14*). The differences between the sequences were notable in the C-terminal region of the protein, where the comparison of MARCKS proteins from various sources showed a lack of any significant homology (*1*).

In view of the importance of the protein in cell biology as well as in neurophysiology, it is essential to know the correct protein structure to elucidate the structure-func-

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tion relationship of the protein. In the present study, we have purified MARCKS from bovine brains, and studied the amino acid sequence by mass spectrometry. 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 fully utilized to determine the sequence. The sequence thus obtained differed significantly from that reported previously, and the presence of a hitherto unnoticed conserved domain near the C-terminus was revealed. The C-terminal domain of MARCKS, therefore, seems to represent another functionally-independent domain.

MATERIALS AND METHODS

Materials—MARCKS was purified from membrane fractions of bovine brain as described previously (*15*). 2-Nitroso-5-thiocyanobenzoic acid (NTCB) was obtained from Sigma. 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 M urea at 37°C for 15 h (*11*). The reaction was stopped by adding 1% final concentration of trifluoroacetic acid.

Glu-C Endoprotease Digestion of C-Terminal Peptide—The Lys-C endoprotease peptides were separated by reversed-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. Interchain disulfide bonds formed by oxidation during treatments were cleaved by incubating with excess amounts of dithiothreitol before the mass analysis.

Cleavage with NTCB—Purified protein (10 μg) was dissolved in a solution containing 100 mM Tris-HCl (pH 8.0), 2.5 mM dithiothreitol, and 2 M urea. Then 100 μg of NTCB was added to the mixture which was incubated at 37°C for 20 min. The pH of the reaction mixture was then adjusted to 9.0 with NaOH and the incubation was continued for 6 h. The reaction was stopped by adding 3% final concentration of trifluoroacetic acid. The reaction mixture was directly injected to the LC/MS apparatus as described below.

Mass Spectrometry—LC/MS analysis of intact proteins was carried out using an electrospray ionization/quadrupole mass spectrometer (PE Sciex API-III) as described previously (*10*). LC/MS and LC/MS/MS analyses of protein digests were done with an electrospray ionization/ion trap mass spectrometer (Finnigan-MAT LCQ). A Monitor C18 column (2.1×150 mm) was eluted with a linear gradient of H_2O-acetonitrile in the presence of 0.02% trifluoroacetic acid and 0.1% acetic acid at a flow rate of 40 μl/min. The ion source voltage was set at 4.5 kV, and N_2 gas was used as the nebulizer gas. The temperature of the heated capillary was set at 200°C. The LC/MS/MS analysis was carried out either 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, or in a preset mode, where the m/z values of the ions to be analyzed were preset. The isolation width of the precursor ions was set either at 2 (for the first run) or at 10 a.m.u. (during the second run), while the relative collision energy was set at 35%.

Other Analytical Methods—Protein concentration was determined by Coomassie Blue binding (BioRad). Gas-phase Edman degradation was carried out with 470A protein sequencer (Applied Biosystems).

RESULTS

Electrospray Mass Spectrometry of Purified Protein—When MARCKS purified from membrane fractions of bovine brain was subjected directly to the LC/MS analysis, one major peak together with a few minor species was observed (Fig. 1). We have previously shown that the minor peaks corresponded to *in vivo* phosphorylated species with mass differences of 80 Da (*15*). The molecular mass observed for the major species was 31,748 Da, which differed significantly from the value calculated from the deduced amino acid sequence, 31,971 Da (*15*). Our previous LC/MS analysis on the lysyl endoprotease digests, which covered more than 95% of the whole sequence, indicated that the difference lies in the C-terminal peptide (residues 269-332 in the corrected protein sequence[3]) (*11*). When a few corrections that have been found in our previous study are taken into account, the theoretical mass of the C-terminal peptide would be 5,917.4 Da, which is slightly but significantly larger than the observed mass, $5,910.2 \pm 1.5$ Da (data not shown). Since only one peptide corresponding to the C-terminal peptide was observed, and since there was only one major protein species observed in the mass spectrum of the intact protein, except for a few phosphorylated species, the presence of isoforms that differ in the C-terminal region could be dismissed.

Sequencing Analysis of C-Terminal Peptide—To elucidate the cause of the mass difference, we first performed an Edman sequencing analysis of the C-terminal peptide isolated by reversed-phase chromatography as described under "MATERIALS AND METHODS." The gas-phase se-

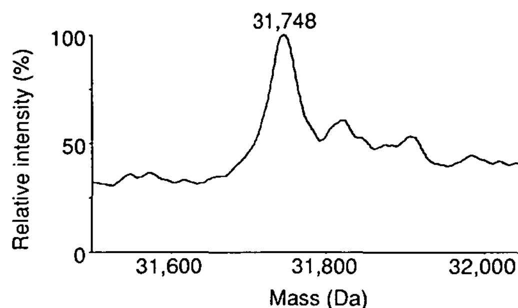

Fig. 1. **Deconvoluted electrospray mass spectrum of bovine MARCKS.** MARCKS purified from the membrane fractions of bovine brain was analyzed by the LC/MS method as described under "MATERIALS AND METHODS."

[3] Residue numbering for the protein sequence corresponds to the bovine protein sequence deduced from cDNA sequence (*13, 14*) except that the initial methionine is omitted and the corrections described in our previous (*11*) and present studies are incorporated.

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quencing gave a sequence, Ala-Glu-Glu-Ala-Gly-Val-Ser-Ala-Ala-Gly-Xaa-Glu-Ala-Pro-Ser-Ala-Ala-Gly-Pro-Gly-Val-Pro-Pro-Glu-Gln-Glu-Ala-Ala-Pro-Ala, where Xaa was an unidentified amino acid. In addition to the lack of the 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 (see Fig. 6).

LC/MS and LC/MS/MS Analysis of NTCB Fragments—Because of the lack of suitable amino acids for further proteolytic cleavage, the purified MARCKS protein was subjected to chemical cleavage at Cys with NTCB. The peptide mixture thus obtained was directly injected into the LC/MS apparatus, and tandem mass spectra were obtained simultaneously. Four peptides including two large N-terminal fragments and two small C-terminal fragments were observed. While the former did not yield any interpretable tandem mass spectra, we were able to obtain the tandem mass spectra of two short peptides derived from the C-terminal region (Fig. 3). Both the molecular masses of the peptides and the partial sequence obtained with the tandem mass spectra confirmed that the two peptides were derived from the C-terminus. The last 21 amino acids near the C-terminus do not differ in either the cDNA sequence or the protein sequence (see Fig. 2).

LC/MS and LC/MS/MS Analysis of V8 Digests of C-Terminal Peptide—To obtain sequence information on the middle part of the C-terminal peptide K28, the peptide was further subjected to partial digestion with V8 protease. The obtained peptide mixture was directly injected into the LC/MS apparatus. Several peptides derived from peptide K28 together with other peptides derived from contaminating peptides in the fraction were observed (Fig. 4a). Only peptide E8 showed a mass that was smaller than the theoretical value by 112 Da, while the masses of other peptides corresponded very well to the theoretical ones based on the revised sequence (Table I). This narrowed down the sequence difference to the N-terminal half of peptide E8.

The tandem mass spectrum of the peptide E8 obtained in the data-dependent mode during the LC/MS run is shown in Fig. 4b. Certain b series (b_{10} to b_{15}) and y series ions (y_2 to y_6) confirmed the sequence in the C-terminal half of the peptide. Furthermore, y_8 to y_{13} ions gave a partial sequence, Ala-Pro-Ala-Ser-Ser, and a part of the sequence was confirmed also with b series ions (b_3 to b_8). The mass difference between b_8 and b_{10} and that between y_6 and y_8 corresponded to one Cys plus one Ala. Since the presence of a Cys was confirmed by the NTCB cleavage as described above, these results together gave the sequence Ala-Pro-Ala-Ser-Ser-Ala-Cys-Ala-Ala-Pro-Ser-Gln-Glu. Although no sequence ions for the N-terminal part of the peptide

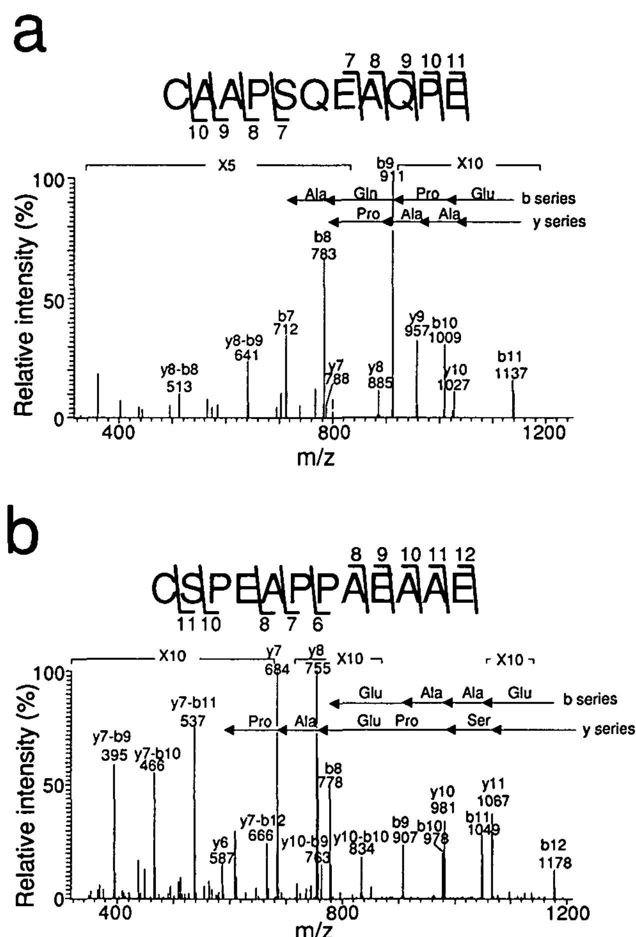

Fig. 3. **Electrospray tandem mass spectra of two C-terminal NTCB Fragments obtained with ion trap mass spectrometer.** MARCKS purified from bovine brain was treated with NTCB to cleave it at Cys residues, and the resulting peptide mixture was subjected to the LC/MS and LC/MS/MS analysis. The tandem mass spectra were obtained "on the fly" in the data-dependent mode, in which the base peaks were automatically subjected to the tandem mass spectrometry. (a) Tandem mass spectrum of C3 peptide, (b) tandem mass spectrum of C4 peptide. b_n and y_n ions, formed by the cleavage of the peptide bond of the *n*th amino acid from the C terminus or N terminus, refer to the C-terminal and N-terminal fragments, respectively (*23*).

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                                 **                                  *
cDNA            PEEAAVAPEKPPARRGAKAVEEPSKAEEKAEEAGVSAAGAAGCEAPSAAGPGCPRAGG-   298
PCR products    PEEAAVAPEKPPASEEAKAVEEPSKAEEKAEEAGVSAAG---CEAPSAAGPGVPPEQEA
peptide         PEEAAVAPEKPPASEEAKAVEEPSKAEEKAEEAGVSAAG---CEAPSAAGPGVPPEQEA
protein         PEEAAVAPEKPPASEEAKAVEEPSKAEEKAEEAGVSAAG---CEAPSAAGPGVPPEQEA   295

                  *     *
cDNA            APREEAAPPRASSACSAPSQEAQPECSPEAPPAEAAE 335
PCR products    APAEEAAPPRASSACAAPSQEAQPECS
peptide         APA
protein         APAEEAAAAPASSACAAPSQEAQPECSPEAPPAEAAE 332
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Fig. 2. **Comparison of the C-terminal sequence of MARCKS deduced from cDNA and that determined by the Edman sequencing.** Part of the C-terminal sequence of bovine MARCKS deduced from the cDNA sequences (*13, 14, 22*) and that determined by the DNA sequencing of a PCR product (*21*) were compared with the sequence obtained by the direct Edman sequencing of the C-terminal peptide K28 and the final primary structure determined in the present study. Arg residues that are replaced with either neutral or acidic amino acids in the final protein structure are marked with asterisks. Residue numbering for the cDNA sequence includes the initial methionine.

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were obtained, the difference between the mass of the whole peptide and that of y_{13} ion, and the mass of b_3 can be explained by three consecutive Ala residues. Although other combinations of amino acids such as Arg and Gly or Val and Asn would yield the same mass difference of 213, the comparison with MARCKS proteins from other species favors the presence of the three Ala residues. The total mass of the peptide Ala-Ala-Ala-Ala-Pro-Ala-Ser-Ser-Ala-Cys-Ala-Ala-Pro-Ser-Gln-Glu would give a monoisotopic mass of 1,402.6 Da (MH^+), an excellent agreement with the observed mass (1,402.7 Da). The theoretical mass of the C-terminal peptide K28 based on the determined sequence is 5,913.3 Da, in good agreement with the observed mass (5,910.2 Da). Furthermore, the corrected sequence would give a molecular mass of 31,744.0 Da for the intact protein, which is within the experimental error of the mass observed (31,748 Da). The presence of deamidation at four Asn residues can account for the difference of 4 Da (*11*). In all, these results suggest that the protein sequence determined in the present study represents the correct sequence of the protein.

Alignment of the C-Terminal Sequences of MARCKS from Various Species—The overall sequencing strategy is shown in Fig. 5. The protein sequence obtained in the present study differs significantly from the cDNA sequence but agrees very well with the sequence based on the PCR product, except for a few residues (Fig. 2). It is notable that five Arg residues present in the cDNA sequence are all replaced either with neutral or with acidic residues, making the C-terminal region highly acidic. The incorporation of

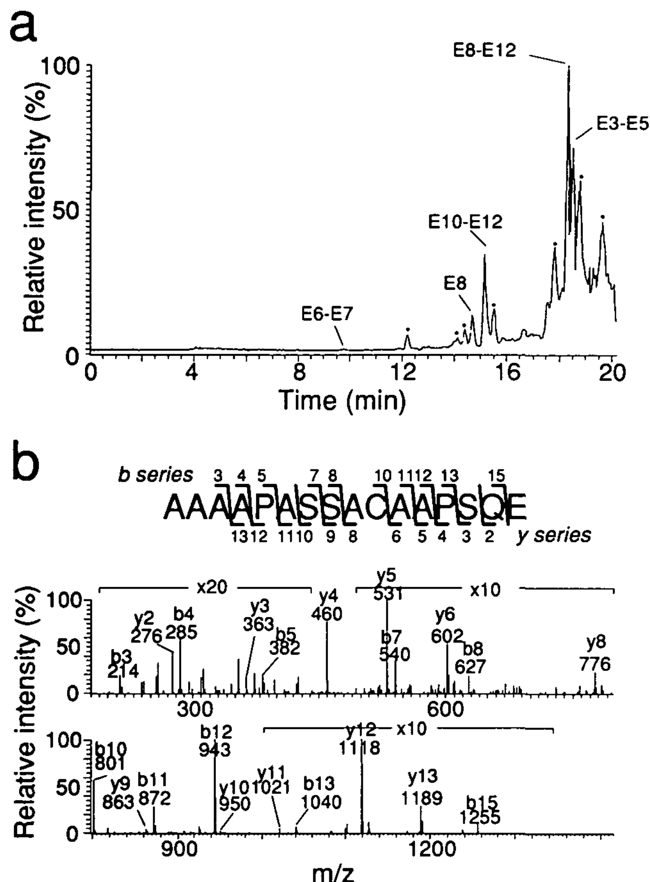

Fig. 4. **LC/MS and LC/MS/MS analyses of the V8 protease digests of C-terminal peptide K28.** The isolated C-terminal peptide was further digested with V8 protease and subjected to the LC/MS and LC/MS/MS analyses as described under "MATERIALS AND METHODS." (a) Total ion chromatogram of the LC/MS analysis. Peptides assigned are indicated. Peptides derived from contaminating peptides in the peptide K28-containing fractions used are marked with dots. (b) Tandem mass spectrum of E8 peptide, in which the protein sequence and that deduced from cDNA differ.

TABLE I. **Assignment of MARCKS C terminal peptides.**

Peptide	Residues[a]	Mass of MH^+ (Da)	
		Observed	Calculated
E3-E5	272-294	2,052.8	2,053.2
E6-E7	295-300	587.0	587.3[b]
E8	301-316	1,402.7	1,514.7 (1,402.6)[a,b]
E8-E12	301-332	2,981.5	3,093.3 (2,982.2)[a]
E10-E12	321-332	1,171.6	1,171.5[b]

[a]Based on the corrected sequence. [b]Monoisotopic mass. The other values are average mass.

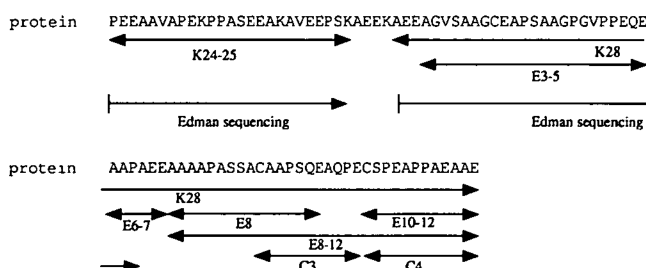

Fig. 5. **Sequencing strategy of the C-terminal structure of MARCKS.** Peptides identified from the masses and partial sequencing by tandem mass analysis are indicated by arrows. Amino acid sequences determined by the Edman sequencing are also indicated.

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MARCKS C-terminal
                         **** **  ** **            *   * ***  **  **
bovine    AGCEAPSAAGPGVPPEQEAAPA--EEAAAAPASSACAAPSQEAQPECSPEAPPAEAAE
human     AACEAPSAAGPGAPPEQEAAPA--EEPAAAAASSACAAPSQEAQPECSPEAPPAEAAE
rat       TADDAPSAAG----PEQEA-PAATDEPAASAAPSAS--P--EPQPECSPEAPPAPVAE
mouse     TAGDASSAAG----PEQEA-PAATDEAAASAAPAAS--P--EPQPECSPEAPPAPTAE
chick     AGAAATSEAGSG---EQEAAPA--EEPAAAR---------QEAPSESSPEG-PAEPAE

NAP22          PAPAAPAAEP-QAEAPVASSEQSVAVKE
(rat)
F52                                            EPSTPSGPESGPTPASAEQNE
(mouse)
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Fig. 6. **Sequence alignment of MARCKS proteins from various species together with homologous sequences found in F52 and NAP-22.** Part of the C-terminal sequence of bovine MARCKS determined in the present study was aligned with those reported for other species. Two domains where high homologies were found are boxed. Identical amino acids are marked with asterisks. Homologous sequences found in the C termini of MARCKS-related proteins, F52 and NAP-22, are also shown. The positions where identical amino acids are found in one of the MARCKS sequences are underlined.

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the corrections gave clearly better homology among MARCKS proteins from various species, and the alignment of the C-terminal sequences of MARCKS from various species revealed two conserved domains in the region (Fig. 6). One is the C-terminal domain of 17 amino acids, and the other is a domain near the C-terminus consisting of 12 (13 in rat and mouse) amino acids. Both domains consist mainly of Ala, Glu, and Pro, and the presence of a characteristic motif of two Pro residues with a spacing of varying length Pro-$(Xaa)_n$-Pro is notable.

DISCUSSION

MARCKS has a few peculiar characteristics in its sequence. The protein lacks any large hydrophobic amino acids and is very acidic (*1*). Only the domain in the middle of the molecule that serves as the calmodulin-, PKC-, and acidic phospholipid-binding domain is highly-basic and amphiphilic. The previous alignment of the MARCKS proteins from various species indicated that the N-terminal half including the PKC phosphorylation domain is well conserved among MARCKS from various species, while the C-terminal half is less well conserved. In the former, three conserved domains have been identified: the N-terminal myristoyl moiety, the MH2 (MARCKS homology domain 2), and the calmodulin-binding, phosphorylation domain (*2, 16*). On the contrary, 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 particular motifs at the particular positions seems to be not necessary for the function of this part of the molecule. This has been noticed previously with GAP-43, another PKC substrate protein and a relative of MARCKS (*17*). In both proteins, amino acid compositions are well conserved, but extensive sequence conservation is lacking.

Our new sequence data, however, showed homology in the region near the C-terminus that was not apparent in the previous alignment (*1*). Thereby, we identified two conserved domains in the region. One is the C-terminal domain consisting of 17 amino acids. In this domain 14 amino acids are identical in human, bovine, mouse, and rat, and 9 amino acids are conserved also in chicken, suggesting that this domain may constitute another functionally-independent domain. Interestingly, the C-terminal domain is also conserved in F52, a short homologue of MARCKS which shares all the N-terminal conserved regions with MARCKS (Fig. 6) (*18*). It should also be noted that the Ser residue together with its neighboring Pro that is well conserved among various species is the target of an unknown proline-directed kinase (*19*). This suggests that the physiological function of the domain, whatever it is, may well be regulated by the protein phosphorylation at the site.

The second conserved domain revealed near the C-terminus in the present study resembles the C-terminal domain in terms of amino acid composition and the presence of a similar Pro-$(Xaa)_n$-Pro motif. This latter feature is less well conserved, however, and the lack of the Ser-Pro motif that is the recognition sequence of the proline-directed protein kinase is notable. Interestingly, the domain is also conserved in NAP-22, a brain-specific acidic protein kinase C substrate protein belonging to the MARCKS family of proteins (Fig. 6) (*20*). In NAP-22, this part of the sequence constitutes a part of the C-terminal domain. This domain, therefore, may constitute a functional domain that is common to the two proteins. It should be also noted that the regions adjacent to the domain are well conserved among the MARCKS species from bovine, human, rat, and mouse. Only in the chick sequence are the regions less well conserved. This may be due to the diversity of the protein, although it is also possible that the published cDNA sequence is not correct. When one considers that 29 of the 334 amino acids in the originally reported bovine sequence have been corrected by subsequent studies including the present study (*11, 13, 21*), a detailed structural analysis on the chick protein might yield a new insight into the structure of MARCKS.

Finally, the present study demonstrates the advantage of using the ion trap mass spectrometer in the determination of protein structures. Both LC/MS and LC/MS/MS data can be obtained in a single chromatographic run, which facilitates the identification of peptides, especially those showing masses which are unaccountable from the theoretical values based on the known sequence. The MS/MS data obtained "on the fly" are good enough to read partial amino acid sequences. Especially with proteins such as MARCKS, whose sequencing at the DNA level is troublesome due to its high GC content, it is often necessary to "correct" primary structure at protein level. The automatic LC/MS and LC/MS/MS data collection feature of the ion trap instrument offers a unique solution for a rapid screening, confirmation, and correction of doubtful protein sequences.

REFERENCES

1. Blackshear, P.J. (1993) The MARCKS family of cellular protein kinase C substrates. *J. Biol. Chem.* **268**, 1501-1504
2. Aderem, A. (1992) The MARCKS brothers: a family of protein kinase C substrates. *Cell* **71**, 713-716
3. Stumpo, D.J., Bock, C.B., Tuttle, J.S., and Blackshear, P.J. (1995) MARCKS deficiency in mice leads to abnormal brain development and perinatal death. *Proc. Natl. Acad. Sci. USA* **92**, 944-948
4. Taniguchi, H. and Manenti, S. (1993) Interaction of myristoylated alanine-rich protein kinase C substrate(MARCKS) with membrane phospholipids. *J. Biol. Chem.* **268**, 9960-9963
5. Kim, J., Blackshear, P.J., Johnson, J.D., and McLaughlin, S. (1994) Phosphorylation reverses the membrane association of peptides that correspond to the basic domains of MARCKS and neuromodulin. *Biophys. J.* **67**, 227-237
6. Glaser, M., Wanaski, S., Buser, C.A., Boguslavsky, V., Rashidzada, W., Morris, A., Rebecchi, M., Scarlata, S.F., Runnels, L.W., Prestwich, G.D., Chen, J., Aderem, A., Ahn, J., and McLaughlin, S. (1996) Myristoylated alanine-rich C kinase substrate (MARCKS) produces reversible inhibition of phospholipase C by sequestering phosphatidylinositol 4,5-bisphosphate in lateral domains. *J. Biol. Chem.* **271**, 26187-26193
7. Roepstorff, P. (1997) Mass spectrometry in protein studies from genome to function. *Curr. Opin. Biotechnol.* **8**, 6-13
8. Biemann, K. (1992) Mass spectrometry of peptides and proteins. *Annu. Rev. Biochem.* **61**, 977-1010
9. Taniguchi, H. (1996) Capillary LC/MS analysis of posttranslational and posttranscriptional modifications of proteins and nucleic acids. *J. Mass Spectrom. Soc. Jpn.* **44**, 443-457
10. Taniguchi, H., Suzuki, M., Manenti, S., and Titani, K. (1994) A mass spectrometric study on the in vivo posttranslational modification of GAP-43. *J. Biol. Chem.* **269**, 22481-22484
11. Taniguchi, H., Manenti, S., Suzuki, M., and Titani, K. (1994) Myristoylated alanine-rich C kinase substrate (MARCKS), a major protein kinase C substrate, is an in vivo substrate of proline-directed protein kinase(s). A mass spectroscopic analysis

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of the post-translational modifications. *J. Biol. Chem.* **269**, 18299-18302

12. Matsubara, M., Kusubata, M., Ishiguro, K., Uchida, T., Titani, K., and Taniguchi, H. (1996) Site-specific phosphorylation of synapsin I by mitogen-activated protein kinase and Cdk5 and its effects on physiological functions. *J. Biol. Chem.* **271**, 21108-21113
13. Seykora, J.T., Ravetch, J.V., and Aderem, A. (1991) Cloning and molecular characterization of the murine macrophage "68-kDa" protein kinase C substrate and its regulation by bacterial lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* **88**, 2505-2509
14. Stumpo, D.J., Graff, J.M., Albert, K.A., Greengard, P., and Blackshear, P.J. (1989) Molecular cloning, characterization, and expression of a cDNA encoding the "80- to 87-kDa" myristoylated alanine-rich C kinase substrate: a major cellular substrate for protein kinase C. *Proc. Natl. Acad. Sci. USA* **86**, 4012-4016
15. Manenti, S., Sorokine, O., Van, D.A., and Taniguchi, H. (1992) Affinity purification and characterization of myristoylated alanine-rich protein kinase C substrate (MARCKS) from bovine brain. Comparison of the cytoplasmic and the membrane-bound forms. *J. Biol. Chem.* **267**, 22310-22315
16. Li, J. and Aderem, A. (1992) MacMARCKS, a novel member of the MARCKS family of protein kinase C substrates. *Cell* **70**, 791-801
17. LaBate, M.E. and Skene, J.H. (1989) Selective conservation of GAP-43 structure in vertebrate evolution. *Neuron* **3**, 299-310
18. Blackshear, P.J., Verghese, G.M., Johnson, J.D., Haupt, D.M., and Stumpo, D.J. (1992) Characteristics of the F52 protein, a MARCKS homologue. *J. Biol. Chem.* **267**, 13540-13546
19. Yamauchi, E., Kiyonami, R., Kanai, M., and Taniguchi, H. (1998) The C-terminal conserved domain of MARCKS is phosphorylated *in vivo* by proline-directed protein kinase. *J. Biol. Chem.* **273**, 4367-4371
20. Maekawa, S., Maekawa, M., Hattori, S., and Nakamura, S. (1993) Purification and molecular cloning of a novel acidic calmodulin binding protein from rat brain. *J. Biol. Chem.* **268**, 13703-13709
21. Herget, T., Brooks, S.F., Broad, S., and Rozengurt, E. (1992) Relationship between the major protein kinase C substrates acidic 80-kDa protein-kinase-C substrate (80K) and myristoylated alanine-rich C-kinase substrate (MARCKS). Members of a gene family or equivalent genes in different species. *Eur. J. Biochem.* **209**, 7-14
22. Stumpo, D.J., Graff, J.M., Albert, K.A., Greengard, P., and Blackshear, P.J. (1989) Nucleotide sequence of a cDNA for the bovine myristoylated alanine-rich C kinase substrate (MARCKS). *Nucleic Acids Res.* **17**, 3987-3988
23. Biemann, K. (1990) Appendix 5. Nomenclature for peptide fragment ions (positive ions). *Methods Enzymol.* **93**, 886-887

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