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*

(Received for publication, June 24, 1992)

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A major in vitro substrate of Ca\textsuperscript{2+}-phospholipid-dependent protein kinase (myristoylated alanine-rich C-kinase substrate (MARCKS)) has been purified to apparent homogeneity from the particulate as well as from the cytoplasmic fractions of calf brain using a calmodulin affinity column. The two preparations were characterized and compared with various biochemical and biophysical techniques. Although they behave similarly in various chromatographic procedures during purification, their elution positions from the gel filtration column are markedly different. Stokes radii of 85 and 45 Å were measured for the cytoplasmic and membrane MARCKS, respectively. Once purified, however, they show a similar small Stokes radius (45 Å), suggesting the dissociation of a component or a drastic conformational change in the cytoplasmic preparation during purification. The electrrospray mass spectroscopic analysis of the two preparations revealed the existence of at least three major subpopulations with molecular mass differences of 80 daltons, which suggests the presence of protein phosphorylated in different degrees. The cytoplasmic preparation contains more phosphorylated species compared with the membrane preparation, whereas the calculated molecular weight of each peak was indistinguishable between the two preparations. Correspondingly, when the two preparations were phosphorylated by purified protein kinase C in vitro, more phosphate groups were transferred to the membrane preparation (4 mol/mol) than to the cytoplasmic preparation (2.9 mol/mol). A significant difference was also observed in the inhibition of calmodulin of the phosphorylation reaction. On the other hand, the circular dichroism of the two preparations showed similar spectra rich in random coil with little contribution of α-helix (~10%), suggesting that there is not a significant difference in the overall conformation. These results clearly established that the two preparations are the same protein coded by a single gene but they differ in their degree of phosphorylation, and that the difference observed in their Stokes radius is due to the presence of an unidentified factor that is removed from the cytoplasmic MARCKS during purification.

* This work was supported by Grants NF 31-252 30.88 and NF 31-32 188.91 of the Swiss National Fonds. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Protein kinase C (PKC)\textsuperscript{1} is a key enzyme of the cellular signal transduction pathway, involved in various processes such as cell differentiation, cell division, and tumor promotion (1). Most of the characterized cellular substrates for this enzyme are either integral membrane proteins or associated with the membrane (2). In the last few years, a number of studies have focused on a major in vivo substrate (also called MARCKS for myristoylated alanine-rich C kinase substrate) of PKC. This molecule was well characterized in rat and bovine brains and in macrophages, although its distribution is not limited to these tissues (3, 4).

The cDNA cloning of MARCKS from different species revealed several important features of the protein (5–9). Its calculated molecular mass (~31 kDa) is different from the apparent molecular mass obtained in gel electrophoresis (65–85 kDa). Overall the protein is very acidic and rich in alanine and glutamic acid. Four potential sites of phosphorylation by PKC are localized in a 28-amino acid very basic region in the middle of the sequence (amino acids 151–178 in the bovine sequence) (10), which has also been recently characterized as a calmodulin (11, 12) and an actin filament (13) binding site. This domain and the myristoylation consensus sequence localized at the N terminus of the molecule are highly conserved between species. The latter covalent modification of the MARCKS protein has been described in vivo (14, 15) and seems to be necessary for association with the membrane, but not for phosphorylation by PKC (16).

One interesting property of MARCKS is its stimulation-dependent translocation from the plasma membrane to the cytosolic compartment when it is phosphorylated by PKC (17, 18). Aderem and co-workers (19) recently found that this process is reversible and that progressive dephosphorylation of the protein leads to its reassociation with the membrane. The translocation between these two cellular compartments is also one of the characteristics of PKC. Although the physiological function of MARCKS and the meaning of its translocation is at present not clear, the immunocytochemical localization of the protein in focal contacts (where PKC is also present) in macrophages (20) and the recent report that MARCKS is an actin filament cross-linking protein (13) may implicate that it is involved in cytoskeleton membrane interaction processes.

In order to elucidate the function of MARCKS, it is of interest to compare the protein that is present in the two different cellular compartments. The purification of the pro-
tein from the soluble fractions of bovine brain has been reported by several groups (3, 21), and a MARCKS-like protein has also been isolated from the particulate fraction of bovine brains (22, 23). The relationship between them is not clear. Furthermore, all the purification procedures described so far involve one or more denaturing steps. In order to study the function of MARCKS in vitro we decided to develop a new purification procedure that avoids such denaturing steps and is applicable to the purification of MARCKS from the cytoplasmic as well as from the particulate fractions. The characterization of the two preparations thus obtained showed that they are identical proteins containing a mixture of phosphorylated species, with more phosphorylated species being found in the cytoplasmic fraction. The presence of a factor in the cytoplasmic MARCKS fraction that forms a complex with MARCKS and is removed during the purification procedures is also suggested.

**EXPERIMENTAL PROCEDURES**

**Materials**—Calmodulin-agarose gel (phosphodiesterase 3',5'-cyclic nucleotide activator-agarose), protease inhibitors (leupeptin, pepstatin, aprotinin), and the Sephacryl S-400 gel filtration obtained from Sigma. L-α-Phosphatidylinerine from bovine brain was purchased from Avanti Polar Lipids, Alabaster, AL. Diolein (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine) was obtained from Whatman, and hydroxylapatite (Bio-Gel HTP) from Bio-Rad Laboratories. Calmodulin-agarose was purchased from Pharmacia LKB Biotechnology, Inc. [γ-32P]ATP (specific activity 3000 Ci/mmmol) was obtained from Amer sham Corp. All other reagents used were of the highest grade commercially available.

**Purification of MARCKS from the Crude Cytoplasmic Fraction**—The purification of MARCKS from the cytoplasmic fractions (cMARCKS) was performed as described (3), until the last reversed phase column step, with the following modifications. The calf brains (from five animals) obtained from local slaughterhouse were homogenized in the presence of 0.25 M sucrose. After centrifugation at 27,000 g x 45 min, the supernatant was used as the crude cytoplasmic fraction for further purification, and the pellet was frozen and kept at -70 °C until used for the purification of MARCKS from the crude membrane fractions (see below). DEAE-Sephacel chromatography was replaced by a TSK DEAE 5PW column (10 mm x 25 cm) that had been equilibrated with 40 mM Tris-HCl (pH 7.3), 1 mM EDTA, 10% ethylene glycol, and 1 mM DTT and was further purified to apparent homogeneity by the phosphatidylserine affinity column as described (24).

**Comparison of Two MARCKS Forms**

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**Electrospray Mass Spectrometry Analysis**—Approximately 7.5 µg (~0.2 nmol) of the protein from the purified fractions were applied on a C18 reversed phase HPLC column (2.1 x 5 mm; Vydac 214TP5250, The Separations Group, Hesperia, CA), and the column was eluted with a linear gradient of 0-95% acetonitrile in the presence of 0.1% trifluoroacetic acid for 20 min at a flow rate of 0.25 ml/min. The peak fractions were collected, dried under vacuum, and immediately redissolved in 6 µl of 50% acetonitrile containing 0.1% acetic acid. Method A was used for the mass analysis of the samples using a VG BioQ mass spectrometer (VG Biotech Ltd., Altrincham, United Kingdom), an instrument that consists essentially of an electrostatic spray ion source operating at atmospheric pressure followed by a quadrupole mass analyzer with a mass range of 4000. Protein solutions (6 µl) were introduced into the ion source at a flow rate of 3 µl/min. Calibration was performed using the multiply charged ions from a separate introduction of horse heart myoglobin (16,905.0 Da).

**Phosphorylation by Protein Kinase C and Inhibition with Calmodulin**—The standard phosphorylation assay contained 20 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 50 µM CaCl2, 80 µM phosphorylation inhibi tor, 8 µg/ml diestatin, 1 ng of the purified PKC, and 75 ng of pure MARCKS in a total volume of 25 µl. The reaction was initiated by the addition of 250 µM [γ-32P]ATP and was performed at 30 °C for the indicated times. For the inhibition experiments the indicated amounts of calmodulin and 2 mM CaCl2 were included. The reaction was stopped by the addition of SDS-sample buffer, and SDS gel electrophoresis was performed according to Laemmli (25). The gels were dried, and autoradiography was performed at -70 °C with Kodak X-Omat film. Quantification of these autoradiograms was done either by densitometry of the film or by excision of the bands from the gel and analysis of the radiolabel by Cerenkov radiation counting.

**Other Analytical Methods**—20 µl of the purified cMARCKS (15 µg/ml) was lyophilized, and the protein was resuspended in 30 µl of the sample buffer. Gels were stained either with Coomassie Blue or by silver staining. Protein determination was performed by the method of Bradford (26) or by densitometry of the bands obtained after silver staining of the gel electrophoresis. Circular dichroism (CD) measurements were carried out at 25 °C in an AVIV 60 spectropolarimeter with a 0.1-cm cell. The results were expressed in terms of mean residue molar ellipticity.

**RESULTS**

**Purification of MARCKS Protein from Cytoplasmic and Particulate Fractions**—From 1.3 kg of calf brain, about 500 µg of purified protein were routinely obtained from the particulate fractions, whereas 100–150 µg were obtained from the cytoplasmic fractions. The difference in the yield recovery is mainly due to the second HPLC DEAE step chromatography included in the purification of cMARCKS, which can be omitted from the mMARCKS purification without affecting the purity of the final preparation. We could not determine the recovery of MARCKS because its phosphorylation by PKC in the initial steps was strongly inhibited, especially with cMARCKS. cMARCKS was first observed as one of the minor phosphoproteins in the crude homogenate and in the DE52 fractions. MARCKS was observed as a major phospho-
the second step of the cMARCKS purification, we could not remove the inhibition. The only significant difference observed is the elution positions from the gel filtration column. As shown in Fig. 1, cMARCKS was eluted much earlier than mMARCKS. The Stokes radii, determined using standard proteins, are 85 and 45 Å for the cMARCKS and mMARCKS, respectively. Since the only difference before the gel filtration step in the two purification procedures is the presence of Triton X-100 during the first DE52 chromatography step, we purified cMARCKS in the presence of the detergent during the initial steps. Under these conditions, no significant change in the elution position of cMARCKS in the gel filtration was observed. The difference observed is, therefore, not due to the effect of the detergent but due to the different cellular compartments of the starting material. Interestingly, when the purified cMARCKS preparation was subjected to the same gel filtration column under the same conditions, cMARCKS was eluted at a similar position to that of mMARCKS (indicated by the arrowhead in Fig. 1). Either cMARCKS contains an unidentified factor that is removed, or its conformation changes drastically during the last steps of the purification.

**Graphs:**

The two MARCKS preparations behave similarly during the various chromatographic procedures. The only significant difference observed is the elution positions from the gel filtration column. As shown in Fig. 1, cMARCKS was eluted much earlier than mMARCKS. The Stokes radii, determined using standard proteins, are 85 and 45 Å for the cMARCKS and mMARCKS, respectively. Since the only difference before the gel filtration step in the two purification procedures is the presence of Triton X-100 during the first DE52 chromatography step, we purified cMARCKS in the presence of the detergent during the initial steps. Under these conditions, no significant change in the elution position of cMARCKS in the gel filtration was observed. The difference observed is, therefore, not due to the effect of the detergent but due to the different cellular compartments of the starting material. Interestingly, when the purified cMARCKS preparation was subjected to the same gel filtration column under the same conditions, cMARCKS was eluted at a similar position to that of mMARCKS (indicated by the arrowhead in Fig. 1). Either cMARCKS contains an unidentified factor that is removed, or its conformation changes drastically during the last steps of the purification.

**Graphs:**

**Fig. 2.** SDS-polyacrylamide gel electrophoresis of the two MARCKS preparations. The MARCKS-containing fractions from gel filtration step (a) and from calmodulin affinity column (b) of the mMARCKS preparation and those from the second HPLC DEAE step (c) and the calmodulin column (d) of the cMARCKS preparation are shown. After phosphorylation by PKC, these fractions were submitted to 12.5% gel electrophoresis, and stained with Coomassie Blue. An autoradiogram of these gels was then performed (e–h). The positions of molecular weight markers are indicated.

**Fig. 3.** Electrospray mass spectra of cMARCKS (a) and mMARCKS (b). The charge number of the main peak is indicated.

**Comparison of Two MARCKS Forms**

In order to establish the relationship between the two MARCKS preparations obtained from the different cellular compartments, the purified preparations were analyzed by electrospray mass spectrometry as described under “Experimental Procedures.” Typical electrospray mass spectrometry spectra with multiply charged species obtained with both preparations are presented in Fig. 3. a and b, for cMARCKS and mMARCKS, respectively. When a part of the spectra was expanded (Fig. 4, a and b), three major peaks (indicated as A–C) were clearly distinguished in both spectra, although the third peak (C) having the highest molecular weight appears only as a shoulder in the cMARCKS (a) spectrum. Species with higher molecular weight than the three major peaks are also present, although they are not well resolved, and their amount is not significant. These high molecular weight peaks may arise from the cation-bound proteins. Average masses were calculated from the multiply charged species, and the
values obtained for the three peaks were 31,750 ± 3, 31,825 ± 6, and 31,885 ± 10 Da for the cMARCKS and 31,750 ± 2, 31,827 ± 3, and 31,905 ± 2 Da for the mMARCKS. The molecular masses of the three peaks are therefore identical for the two MARCKS preparations within the experimental error. The differences in the mass observed between the three peaks (~80 Da) correspond exactly to the mass difference expected for the phosphorylation of a protein. The isolated MARCKS preparations, therefore, seem to contain multiply phosphorylated species. Although the purified mMARCKS (Fig. 4b) contains peak A (31,750 Da) as the major component, the purified cMARCKS (Fig. 4a) contains B (31,825 Da) as its main species, with a small amount of C (31,895-31,905 Da) in both cases. These results were reproduced in three separate experiments.

The theoretical molecular mass calculated from the protein sequence deduced from the published cDNA cloning (6, 7) is 31,971 Da, when the existence of a myristic acid covalently bound to the N terminus glycine is taken into account. This value does not correspond to the molecular mass obtained for the three major peaks. Since a MARCKS protein with a free N terminus and glycine as the first amino acid, i.e., with the initial methionine being cut out, would have a molecular mass of 31,761 Da, which is close to the mass observed for the A component, an aliquot of the cMARCKS preparation used for the mass analysis was subjected to Edman sequencing. No free N terminus was detected as has been previously described by others (6). The N terminus is therefore blocked, probably myristoylated, implicating that the discrepancy between the measured mass and the calculated one is due to other reasons, such as a difference in the primary sequence between the isolated proteins and that deduced from the cDNA sequence.

Phosphorylation by PKC and Its Inhibition by Calmodulin—The time course of phosphorylation of MARCKS by PKC in vitro is shown in Fig. 5. A plateau was reached within 30 min with both preparations. Although the reactions proceeded in a similar way, a significant difference was observed in the maximal level of phosphorylation. Although about 4 mol of phosphate/mol of protein were incorporated into mMARCKS, a maximal incorporation was observed at about 3 mol of phosphate/mol of protein with cMARCKS. This difference was repeatedly observed using different preparations of both cMARCKS and mMARCKS. The cMARCKS preparation seems to have almost one phosphorylation site less than the mMARCKS preparation.

Since MARCKS is known to bind calmodulin in the presence of calcium and the phosphorylation domain by PKC has typical characteristics of the well characterized calmodulin-binding domain (11, 12), we studied the effect of calmodulin on the PKC-catalyzed phosphorylation of the two MARCKS preparations. As shown in Fig. 6, the phosphorylation reaction after 5 min of incubation was inhibited progressively with the increasing amount of calmodulin. Inhibition was more prominent when cMARCKS was used as a substrate; almost complete inhibition was observed with 10 μg of calmodulin in the incubation medium, whereas only 50% inhibition was observed with mMARCKS at the same concentration. This difference may be due to the presence of multiple phosphorylation sites (3-4 sites) with different sensitivities to the calmodulin inhibition or simply to the difference of affinity of the two MARCKS preparations to calmodulin.

Circular Dichroism of MARCKS Protein—In order to elucidate the secondary structure of purified MARCKS protein, CD spectra were measured with both preparations. Similar spectra were obtained with the protein from both origins (Fig. 7). The large negative peak observed at 198 nm, together with a small negative peak between 220 and 230 nm, suggests a high content of random coil structure with a small amount of α-helix (~10%). There is no significant difference in the overall conformation of the two MARCKS preparations.

DISCUSSION

In the present work, we described a purification method that can be adapted for the purification of MARCKS protein...
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The large Stokes radius compared with a small molecular mass of 31 kDa suggests a highly elongated structure (3). Indeed, in a recent publication, Hartwig et al. (13) demonstrated by rotary shadowing electron microscopy that MARCKS is a rod-shaped molecule, although this was done with a preparation obtained after reversed phase column chromatography, a procedure that may denature the protein. In any case, the elucidation of the factor that causes the observed difference between the two preparations and the change observed during the purification may facilitate the understanding of the physiological function of MARCKS.

The electron mass spectrometry analysis of the purified protein showed that our two purified fractions contain three major subpopulations of different molecular masses. The molecular masses of these three components are indistinguishable between the cMARCKS and mMARCKS. Only their relative amounts are different. These species differ by a mass of 80 Da, which is exactly the value produced by protein phosphorylation. These results suggest that the two MARCKS proteins purified from different cellular compartments are actually the same protein and differ only in their phosphorylation degree. The multipotential pattern of MARCKS observed by electrofocusing in various species and tissues (4) also suggests the coexistence of various phosphorylated forms of the protein. Interestingly, the subspecies with a mass of 31,825 Da is more abundant in the cMARCKS preparation than in the mMARCKS, suggesting a higher degree of phosphorylation. This is well correlated with previous in vivo observations (17–19), which showed that the phosphorylation of MARCKS by PKC in the cells leads to its translocation from the plasma membrane to the cytoplasmic compartment.

The molecular masses of the three species do not correspond exactly to the mass (31,971 Da with a myristic acid bound to the N-terminal glycine) calculated from the amino acid sequences from a corrected cDNA sequence (7). Since a MARCKS protein with a free N terminus and glycine as the first amino acid has a molecular mass of 31,761 Da, which is not far from the mass observed for peak A (31,750 ± 3 Da), we first suspected the absence of the myristination in the purified MARCKS preparations. However, this is probably not the case, since the N terminus was found to be blocked. The N-terminal modifications other than myristination (such as acetylation) can also be ruled out, since such changes should be detected as a change in the mass. Another possibility to obtain smaller mass is the removal of a few amino acids from the C terminus. This can also be ruled out; the omission of the C-terminal glutamic acid and alanine will not give the observed mass. It is probable that the purified MARCKS proteins have a sequence that is slightly different from the deduced amino acid sequence. They can be different variants, and mistakes in the DNA sequencing cannot be ruled out. In fact, a part of the cDNA clone of bovine MARCKS was resequenced by another group, and 13 out of 70 amino acid positions differed from the sequence published by guest on November 14, 2017

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phosphorylated in the majority of cMARCKS molecules. Four serine residues are present within the 28-amino acid phosphorylation domain, but only three of them are in a consensus sequence for PKC phosphorylation. Recent results obtained by quantitative analysis of individual sites by microsequencing (27) indicate that only three of the four serines could be phosphorylated by PKC, although four sites of phosphorylation were found by others (28). These contradictory results may be due to different experimental conditions, and the true physiological level of phosphorylation of MARCKS by PKC remains to be established. Since the determination of the phosphorylation degree is also dependent on the estimation of the protein amount and that of the molecular weight, the absolute values obtained in the present study may not necessarily be correct, but the difference between the two MARCKS preparations clearly indicates the existence of various populations of the protein in the two fractions.

MARCKS is a calmodulin-binding protein, and the site of interaction with this effector protein also resides in the same 28-amino acid PKC phosphorylation domain (12). There are contradicting reports on the effect of calmodulin on the phosphorylation of MARCKS by PKC in vitro. Albert et al. (29) and, more recently, Zhao et al. (30) described a strong inhibition of cytoplasmic MARCKS phosphorylation by calmodulin. On the other hand, Tokumitsu et al. (23) reported that the phosphorylation of ACAMP-81, a MARCKS-like protein isolated from bovine brain membrane fractions, was not inhibited by the presence of calmodulin. This was one of the main arguments that their protein was different from MARCKS. The results described in the present work, which were obtained with the two preparations in parallel, indicate a different behavior of the two fractions. Although inhibition was observed with both preparations, the extents of this inhibition were significantly different. Almost complete inhibition (85%) was observed with cMARCKS, whereas the inhibition observed with mMARCKS was only 55%. The difference may arise from the presence of three or four phosphorylation sites, which are not necessarily equivalent with respect to inhibition by calmodulin. In this case the phosphorylation site that is already phosphorylated in cMARCKS is less sensitive to inhibition by calmodulin than the other sites. Another possibility is the difference in the affinity with calmodulin between the two MARCKS preparations. A more detailed kinetic study is necessary to elucidate the reason for the observed difference.

Circular dichroism spectra of the two MARCKS preparations showed no significant difference between them, indicating that the higher degree of phosphorylation observed in cMARCKS does not significantly affect the overall secondary structure of the protein. This seems to be in agreement with the rotary shadowing of phospho- and dephospho-MARCKS (13) showing a similar (if not identical) shape of both proteins. However, this does not exclude a local change of secondary structure within the molecule, which cannot be detected by this method. The CD spectra obtained suggest a small content of α-helix (less than 15%) and that the whole molecule consists mainly of random coil structure. The high content of proline may account for this lack of α-helix.

During the preparation of the manuscript we noticed a paper recently published by Mizutani et al. (31). They describe a partial amino acid sequencing of their MARCKS-like protein isolated from the particulate fractions of bovine brains and determine about 36% of the total sequence. All of the peptides so far identified can be found in the deduced amino acid sequence of MARCKS, and the authors conclude that their protein is identical to MARCKS, which of course confirms our results presented in this paper.

Acknowledgment—We are very grateful to Professor G. Schwarz for helpful discussion about the manuscript.

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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.

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