MARCKS, a major protein kinase C substrate, assumes non-helical conformations both in solution and in complex with Ca^{2+}-calmodulin

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Abstract MARCKS, a major cellular substrate for protein kinase C, plays important roles in various cellular functions and its functions are regulated by calmodulin. We have studied the conformational properties of recombinant human MARCKS in solution and in complex with calmodulin. Circular dichroism (CD) spectra showed a high content of random coil in physiological solution. When MARCKS or MARCKS-derived calmodulin-binding peptide was complexed with Ca^{2+}-calmodulin, little change was observed in the CD spectra, suggesting that MARCKS binds with calmodulin in a non-helical conformation, which is unique among the calmodulin-binding proteins.

Key words: Myristoylated alanine-rich C kinase substrate; Protein kinase C; Calmodulin; Protein structure

1. Introduction

Myristoylated alanine-rich C kinase substrate (MARCKS) is a cellular substrate for protein kinase C as well as for proline-directed protein kinases such as mitogen-associated protein (MAP) kinase [1–3]. Its precise cellular function remains unclear, but a recent gene disruption study has indicated that MARCKS is essential for the normal development of the central nervous system [4]. MARCKS contains two highly conserved domains: an N-terminal domain that is modified by myristoylation and a basic effector domain in the middle of the molecule. The latter serves as the calmodulin- and actin-binding site [5–7]. Phosphorylation by protein kinase C at four serine residues within the effector domain abolishes both the calmodulin and actin binding [5]. We have also demonstrated that MARCKS binds to phospholipid membranes through the N-terminal myristoyl moiety and the basic effector domain [8]. Phosphorylation of the latter introduces negative charges, consequently neutralizing its interaction with acidic phospholipids and facilitating its release from the membrane [8]. Since all the effector molecules compete for the same binding site, the physiological functions of MARCKS are regulated in a very complex manner that involves various components of the signal transduction pathways.

Although rotary shadowing experiments have demonstrated that MARCKS has an elongated, rod-shaped overall structure [5], no detailed structural information of the protein was reported. In the present study, we expressed and purified both myristoylated and non-myristoylated recombinant human MARCKS and studied their conformational characteristics by circular dichroism (CD) spectroscopy. CD spectra suggested that MARCKS did not have a distinct structure in solution regardless of the presence of myristoylation. Furthermore, both MARCKS and a peptide derived from the effector domain bound to calmodulin in non-helical conformations. Thus, MARCKS represents a novel class of calmodulin-binding proteins, which has a mode of interaction with calmodulin considerably different from the known calmodulin-binding proteins.

2. Materials and methods

2.1. Materials

Dansyl-calmodulin and calmodulin agarose were obtained from Sigma, while tryptone and yeast extracts were from Difco Ltd. Ampicillin, kanamycin, and isopropyl-1-thio-β-galactopyranoside (IPTG) were from Wako Pure Chemical Industries, Ltd. Bovine calmodulin was either purchased from Wako Pure Chemical Industries or purified from bovine brain [9]. Calmodulin concentration was determined from UV absorption using A_{276nm} (1%, 1 cm) = 1.8. A 25-residue peptide corresponding to the basic effector domain of MARCKS (KKKKKRFSFKKSFKLSGFSFKKNKK) was synthesized with a standard t-Boc chemistry using an Applied Biosystems peptide synthesizer 430A. A 20-residue peptide corresponding to the calmodulin-binding domain of human endothelial nitric oxide synthase (eNOS) was synthesized as described previously [10,11]. The peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) using a C18 column (Waters μBondasphere 5 μ C18-300Å, 1.9×15 cm). The peptides were judged to be of greater than 95% purity by analytical HPLC and electrospray mass spectroscopy. Peptide concentration was determined by quantitative amino acid analysis. The *Escherichia coli* strain BL21(DE3)pLysS was obtained from Stratagene. The plasmid pBB131NMT was a gift from Jeffrey I. Gordon (Washington University).

2.2. Expression and purification of non-myristoylated MARCKS (non-myr MARCKS) and myristoylated MARCKS (myr MARCKS)

Both non-myr MARCKS and myr MARCKS were expressed in *E. coli*, and purified to homogeneity following the published procedures [12], the details of which will be published elsewhere (Matsubara et al., in preparation). The expression and purification of non-myr MARCKS were carried out as described previously [12]. For expression of myr MARCKS, the *E. coli* strain BL21(DE3)pLysS was co-transformed with the plasmid pBB131NMT, which contains the gene encoding yeast NMT [13]. Bacterial culturing was performed similarly except that 25 μg/ml kanamycin was added to the media. Coexpression of MARCKS and NMT was induced by addition of 0.4 mM IPTG to the log-phase culture. Myr MARCKS was purified from the heat-stable protein fractions by calmodulin-agarose chromatography [14]. The fractions containing myr MARCKS were pooled, concentrated/buffer exchanged with 20 mM Tris-HCl buffer (pH 7.5) with Centricon 10 and kept frozen at −80°C until use. Electrospray mass spectroscopic analysis of the recombinant non-myr MARCKS and myr MARCKS proteins indicated that the mass difference of the two proteins was 210 Da, which corresponded very well to protein myristoylation. Protein concentration was determined by quantitative

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amino acid analysis or by densitometric analysis of the Coomassie blue-stained SDS gels.

2.3. CD spectroscopy

CD spectra were measured in a Jasco J-720 CD spectropolarimeter as described previously [10,11]. Spectra were recorded over a 190–260 nm range using a 1-mm quartz cuvette. Temperature was controlled at 25°C using a water bath. Protein concentration was adjusted to 1 μM in 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl and 0.5 mM $CaCl_2$. For CD spectra of the calmodulin-MARCKS protein complex, equimolar concentrations (1 μM) of calmodulin and MARCKS were mixed in the same buffer. For calmodulin-peptide complexes, the concentrations of both components were increased to 10 μM. Results are expressed as the mean residue molar ellipticity, [θ]. The contents of secondary structures were calculated according to Greenfield and Fasman [15].

2.4. Fluorescence measurements

Binding of MARCKS or MARCKS peptide to dansyl-calmodulin was analyzed in a Jasco FP-777 spectrofluorometer using a 11-cm quartz cuvette as described previously [11]. With the excitation wavelength set at 340 nm, emission spectra of dansyl-calmodulin in the presence or absence of MARCKS or MARCKS peptide were recorded in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM $CaCl_2$ and indicated concentrations of NaCl. Binding of both MARCKS and MARCKS peptide to calmodulin was monitored by recording the fluorescence emission at 490 nm. Dissociation constants of calmodulin-MARCKS complexes were determined by a direct fit of the data to the mass equation using a non-linear least squares method [11].

2.5. Binding of MARCKS peptide to calmodulin-agarose gel

Binding of MARCKS peptide to calmodulin was also determined by calmodulin-agarose assay [10] in 20 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM $CaCl_2$ and indicated concentrations of NaCl. After sedimenting the agarose by centrifugation, the supernatant (unbound fraction) was removed. The calmodulin-agarose was washed three times with the same buffer, and the bound peptide was eluted with the SDS sample buffer containing 1% SDS. Both unbound and bound fractions were analyzed by SDS gel electrophoresis.

3. Results

3.1. Conformational properties of myr and non-myr MARCKS

To study the conformational properties of MARCKS, myr MARCKS and non-myr MARCKS were produced in *E. coli*. Coexpression of human MARCKS with or without the yeast *N*-myristoyltransferase expression vector in *E. coli* yielded myr MARCKS and non-myr MARCKS, respectively. The proteins were purified to homogeneity by calmodulin-agarose chromatography (Fig. 1a). The recombinant myr MARCKS migrated in a 10% polyacrylamide gel with an apparent molecular mass of 80 kDa, whereas the recombinant non-myr MARCKS showed an apparent molecular mass of 68 kDa. The migrations correspond very well to those of the native myr and non-myr MARCKS proteins purified from bovine brain [16,17]. Since the difference in mass caused by protein myristoylation is only 210 Da, the shift in migration of about 12 kDa in SDS gel may suggest a conformational change caused by the myristoyl moiety.

To obtain further information on the protein structures, CD spectra of myr and non-myr MARCKS were measured (Fig. 1b). The CD spectrum of myr MARCKS and that of non-myr MARCKS taken under physiological conditions showed single negative peaks at around 200 nm, together with small negative peaks at 220–230 nm, suggesting that the recombinant proteins have a high content of random coil structure with a small amount of α-helix. The contents of secondary structures were calculated to be 5% (α-helix), 28% (β structure), and 67% (random coil) for myr MARCKS. The content of α-helix (9%) and that of β structure (33%) are slightly higher in non-myr MARCKS with a corresponding decrease in random coil content (58%). We have observed a similar CD spectrum with native MARCKS purified from bovine brain [14]. These results suggest that MARCKS has a so-called natively unfolded structure [18]. Although myr and non-myr MARCKS showed CD spectra with similar characteristics, the intensity of the negative peak at around 220–230 nm is smaller in myr MARCKS (Fig. 1b). This suggests that the presence of a myristoyl moiety may affect part of the three-dimensional structure of MARCKS.

3.2. Conformation of MARCKS in calmodulin complex

Although MARCKS has been previously shown to bind to calmodulin tightly through the basic effector domain [7,12], no structural study on MARCKS-calmodulin complex has been reported. The addition of 150 nM recombinant myr MARCKS to 50 nM dansyl-calmodulin induced a shift in the fluorescence emission maximum of dansyl calmodulin from 510 to 490 nm as well as a 2-fold increase in the intensity at 490 nm (Fig. 2a), suggesting that the recombinant protein

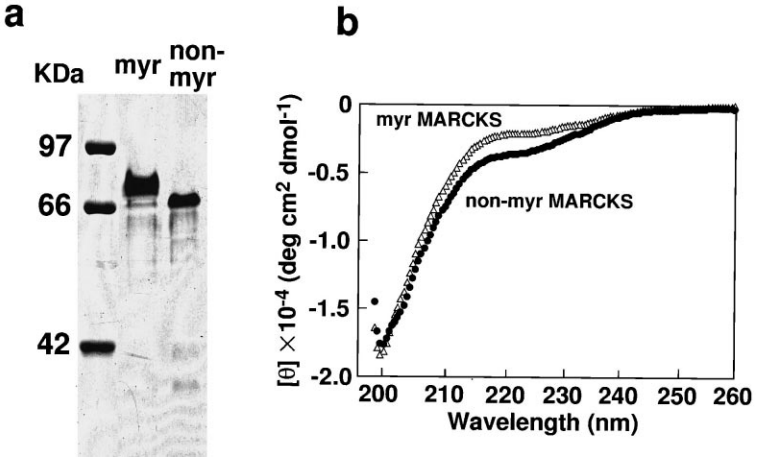

Fig. 1. SDS gel electrophoresis and CD spectra of human recombinant myr and non-myr MARCKS. a: Coomassie blue-stained SDS-polyacrylamide gel (10%) of myr and non-myr MARCKS. b: CD spectra of 1 μM myr (△) and non-myr (●) MARCKS in 20 mM HCl buffer (pH 7.5) containing 0.1 M NaCl and 0.5 mM $CaCl_2$.

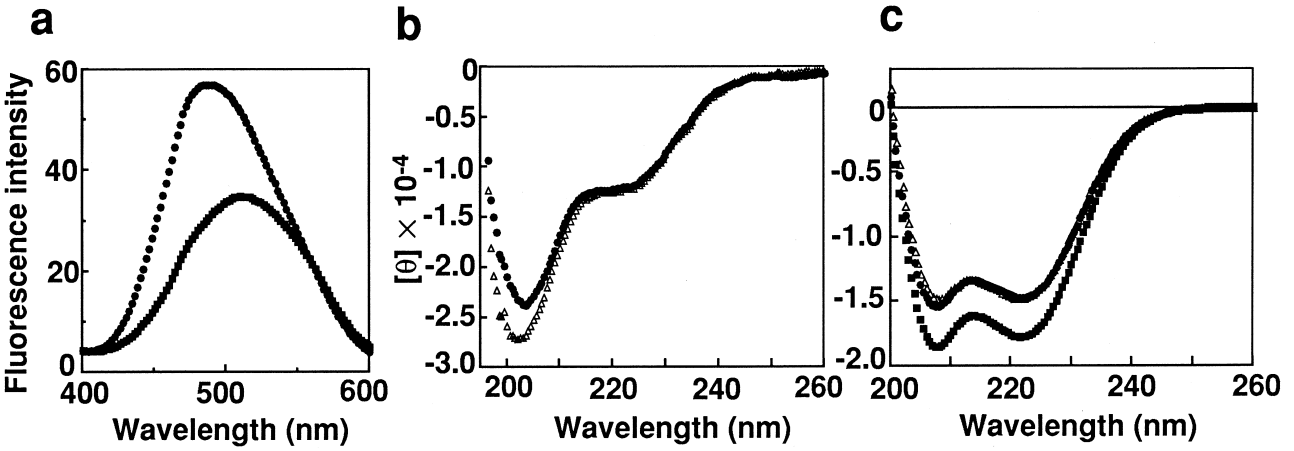

Fig. 2. Binding of myr MARCKS to calmodulin. a: Fluorescence spectra of 50 nM dansyl-calmodulin in the presence (●) and absence of 150 nM myr MARCKS (■). The excitation wavelength was set at 340 nm. b: CD spectrum of calmodulin-myr MARCKS complex (●), the individual CD spectra obtained with calmodulin alone and myr MARCKS alone were mathematically added (△). c: CD spectra of calmodulin alone (●), calmodulin-MARCKS peptide complex (△), and calmodulin-eNOS peptide complex (■).

retained the ability to bind to calmodulin. The dissociation constant determined by the direct fit of titration data to mass equation was 4.5 ± 0.4 nM ($n = 3$), a value comparable to those reported for native and recombinant protein preparations [7,12]. Non-myr MARCKS has a slightly reduced affinity to MARCKS (data not shown), as has been reported previously for the native protein [16].

Next, we investigated the secondary structures of MARCKS in complex with calmodulin by CD spectroscopy. CD spectra were measured with myr MARCKS mixed with calmodulin at a 1:1 ratio in the presence of Ca^{2+} or with the individual component alone. The CD spectra obtained with MARCKS alone or with calmodulin alone were mathematically added and the obtained spectrum was compared with the CD spectrum of the mixture (Fig. 2b). Although the depth of the large negative peak at 202 nm showed a difference, the broad peak around 220–230 nm, an indicator of the α-helical content, did not differ significantly. This implies that the full-length myr MARCKS binds to calmodulin in a non-helical structure. This is rather unusual for a calmodulin-binding protein; an increase in the α-helical content is observed upon the binding of a target protein to calmodulin. Similar results were obtained with non-myr MARCKS (data not shown).

Since the calmodulin-binding site of MARCKS has been shown to reside in the basic effector domain in the middle of the molecule, we also studied the conformational characteristics of the domain in calmodulin complex. When the CD spectrum of the equimolar complex of the peptide and calmodulin was compared to the sum of those of the individual components, no significant difference was observed, indicating that the α-helical content of the peptide did not change significantly upon binding with calmodulin. As a comparison, the calmodulin-binding domain of eNOS, which binds to calmodulin in a typical α-helical conformation [10], was used in a similar experiment. The CD spectrum of the calmodulin-eNOS calmodulin-binding domain peptide complex showed an increased negative mean residue ellipticity at 208 and 222 nm (Fig. 2c). This increase in the α-helical content is due to the formation of an α-helical structure of the peptide upon calmodulin-peptide complex formation as has been reported for various calmodulin-binding peptides [19]. It is generally assumed that Ca^{2+}-calmodulin itself does not gain secondary structure when bound to a target peptide, since only the central helix of calmodulin bends to accommodate the peptide bound [19]. Therefore, the changes in the α-helical contents can be attributed mainly to those of the target peptides. These results established that the calmodulin-binding domain of MARCKS binds to calmodulin in an extended conformation rather than in an α-helical structure, which is unique among the calmodulin-binding proteins.

4. Discussion

In the present study, we studied the structural characteristics of recombinant human MARCKS in solution and in

Table 1
Protein sequence resembling the calmodulin-binding domain of MARCKS

Protein	Sequence	Basic amino acids (%)	Hydrophobic amino acids (%)	Binding mode with calmodulin	Reference
MARCKS (human)	KKKKKRFSFKKSFKLSGFSFKKNKK	52	24	non-helical	this study
F52/MRP (mouse)	KKKKFSFKKPFKLSGLSFKRNRK	50	29	non-helical	[29]
hDGKζ (human)[a]	KKKKRASFKRKSSKKGPEE	53	11	?	[31]
α-Adducin (human)	KKKKKFRTPSFLKKSKKKSDS	52	19	?	[32,33]
β-Adducin (human	KKKKKFRTPSFLKKSKKEKVES	52	22	?	[33]
35H (rat)	KKKKKFRTPSFLKKNKKKSDS	52	19	?	[34]
Ral-A (human)[b]	SKEKNGKKKRKSLAKRIR	56	17	non-helical	[30]
eNOS	RKKTFKEVANAVKISASLMG	25	40	helical	[11]
MLCK[c]	KRRWKKNFIAVSAANRFKKISSSGAL	31	42	helical	[19]

[a]Human diacylglycerol kinase ζ. [b]Ras-related GTP-binding protein. [c]Skeletal muscle myosin light chain kinase.

complex with calmodulin. The CD spectroscopic studies suggested that MARCKS has a high content of random coil structure with a small amount of helical structure in solution. This corresponds very well to the results obtained by physicochemical and rotary shadowing electron microscopic studies, suggesting that MARCKS has an elongated rod-shaped structure [5,14,20]. Thus, unlike typical globular proteins, MARCKS is energetically minimized as unfolded structure. Recently, this type of protein has been called 'natively unfolded protein' by Weinreb and coworkers [18]. Several natively unfolded proteins contain many structural proteins in brain, such as MAP2 [21], tau [22] and non-β-amyloid component of Alzheimer's disease (NACP) [23]. Weinreb and coworkers have suggested that their random coil structure may be important to facilitate protein-protein interaction. Since MARCKS also binds to calmodulin, actin and phospholipid membranes, the natively unfolded structure of MARCKS might be critical for the rapid interactions with the target macromolecules. A recent report, which demonstrated that the association of MARCKS effector domain peptide with membranes is extremely rapid, supports this idea [24].

We further demonstrated that the binding of both full-length MARCKS and the effector-binding peptide to calmodulin has no observable effect on the overall α-helical structure of the protein. These results suggest that MARCKS binds to calmodulin in a non-helical conformation in contrast to the other typical calmodulin-binding proteins [19]. Therefore, MARCKS represents a novel class of calmodulin-binding motif, which has a mode of interaction with calmodulin considerably different from the known calmodulin-binding domains. An extended structure, which is observed as a random structure in the CD studies, does not necessary mean that the domain assumes a flexible random structure. A recent electron paramagnetic resonance spectroscopic study has indicated that the MARCKS peptide assumes a less flexible structure in the calmodulin complex compared to a peptide derived from GAP-43 [25]. The latter contains the so-called IQ motif that shows a Ca^{2+}-independent binding to calmodulin, whose mode of interaction with calmodulin is completely different from that of the typical calmodulin-binding proteins [26]. Interestingly, the calmodulin-binding domain of MARCKS contains more basic amino acid residues (52%) but fewer hydrophobic amino acids (24%) compared to the typical calmodulin-binding domains [27,28]. The interaction of the MARCKS calmodulin-binding domain with calmodulin was disrupted by increasing the ionic strength (data not shown), suggesting that the ionic interaction between the basic amino acids in the calmodulin-binding domain and the acidic residues of calmodulin plays an important role in the interaction. This is in contrast to the interaction mode of calmodulin with other typical calmodulin target proteins, which involves mainly hydrophobic interactions [19]. The calmodulin-binding domain of MARCKS, therefore, constitutes a novel class of calmodulin-binding proteins in terms of structure.

A search in the protein database revealed other calmodulin-binding proteins with characteristics similar to those of MARCKS (Table 1). It is apparent that there are sequence homologies among these proteins. Compared to the conventional calmodulin-binding domains found in eNOS or in myosin light chain kinase, they are rich in basic amino acid residues (often more than 50%) and contain fewer hydrophobic amino acid residues. Recent reports on F52 (also called MARCKS-related protein, MRP) and Ras-related GTP-binding protein (Ral-A) have shown that these proteins bind to calmodulin in a non-helical conformation [29,30]. Interestingly, they share not only the structural features but also several biochemical characteristics. These include predicted or proven protein kinase C phosphorylation sites and predicted or proven binding domains of calmodulin, F-actin, and plasma membranes found in adducin isoforms and in diacylglycerol kinase [31–34]. This suggests that the protein sequence motifs common to these proteins may function as a crosstalk point in various signal transduction pathways.

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