Direct Involvement of Protein Myristoylation in Myristoylated Alanine-rich C Kinase Substrate (MARCKS)-Calmodulin Interaction*

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MARCKS, a major in vivo substrate of protein kinase C, interacts with plasma membranes in a phosphorylation-, myristoylation-, and calmodulin-dependent manner. Although we have previously observed that myristoylated and non-myristoylated MARCKS proteins behave differently during calmodulin-agarose chromatography, the role of protein myristoylation in the MARCKS-calmodulin interaction remained to be elucidated. Here we demonstrate that the myristoyl moiety together with the N-terminal protein domain is directly involved in the MARCKS-calmodulin interaction. Both myristoylated and non-myristoylated recombinant MARCKS bound to calmodulin-agarose at low ionic strengths, but only the former retained the affinity at high ionic strengths. A quantitative analysis obtained with dansyl (5-dimethylaminonaphthalene-1-sulfonyl)-calmodulin showed that myristoylated MARCKS has an affinity higher than the non-myristoylated protein. Furthermore, a synthetic peptide based on the N-terminal sequence was found to bind calmodulin only when it was myristoylated. Only the N-terminal peptide but not the canonical calmodulin-binding domain showed the ionic strength-independent calmodulin binding. A mutation study suggested that the importance of the positive charge in the N-terminal protein domain in the binding.

Since the discovery of a covalent-bound myristoyl group in the catalytic subunit of CAMP-dependent protein kinase (1), many proteins have been found to be modified not only with myristoyl group but also with a variety of fatty acids (2–4). Interestingly, the vast majority of acylated proteins are either proteins involved in cellular signaling or those of viral origin. Protein acylation is often essential for the proper functioning of these proteins (5), although in the mechanism by which the modification exerts the effect is largely unknown. Of various kinds of acylation, myristoylation has been implied in the reversible membrane association due to its intermediate hydrophobicity (3, 6). Studies from our own and other laboratories (7, 8) have established that such a mechanism is, in fact, operative in the phosphorylation-dependent interaction of MARCKS1 (myristoylated alanine-rich C kinase substrate) with membranes. In the case of recoverin, the binding of Ca2+ induces a drastic conformational change of the protein, and the myristoyl group hidden inside the protein will protrude from the protein and can interact with membrane (9). The modification has also been shown to affect the protein stability of CAMP-dependent protein kinase (10). However, the involvement of the protein myristoylation in protein-protein interaction has never been clearly demonstrated, although the issue has been the subject of extensive studies (11–13). Recently, we have shown that the modification is directly involved in the interaction of a brain-specific protein kinase C substrate, CAP-23/NAP-22, with calmodulin (14, 15). Furthermore, the phenomena is observed in the binding HIV-1 Nef with calmodulin (16). The acyl chain interacts with specifically with the hydrophobic pocket of calmodulin. Since the basic domain adjacent to the myristoyl group was found to be important for the CAP-23/NAP-22-calmodulin and HIV-1 Nef-calmodulin interaction, the interplay of the myristoyl group and the basic domain seems to function in a manner analogous to that found in the myristoylation-mediated protein-membrane interaction. MARCKS, a major in vivo substrate of protein kinase C as well as of proline-directed protein kinases such as mitogen-activated protein kinase (17), contains a highly conserved N-terminal domain with an N-terminal myristoyl group and a basic phosphorylation domain in the middle of the molecule that is at the same time the binding sites for calmodulin (18, 19). The phosphorylation of the latter domain results in the disruption of the calmodulin binding of the domain (20, 21). We have previously shown that MARCKS binds to phospholipid membrane through both the N-terminal myristoyl moiety and the phosphorylation domain of basic amphiphilic nature (7). Protein kinase C-dependent phosphorylation introduces negative charges into the positively charged effector domain, consequently neutralizing its interaction with acidic phospholipids and promoting its release from the membrane (7, 8). We have also discovered a demyristoylase activity of MARCKS and shown that the modification is dynamically regulated (22, 23). During the course of these studies, we found that the presence of non-myristoylated pool of MARCKS (24). Interestingly, the non-myristoylated MARCKS protein behaved differently from

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The abbreviations used as: MARCKS, myristoylated alanine-rich C kinase substrate; NMT, N-myristoyltransferase; non-myr MARCKS, non-myristoylated MARCKS; myr MARCKS, myristoylated MARCKS; MRP, MARCKS-related protein; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; HIV-1, human immunodeficiency virus, type 1; HPLC, high-performance liquid chromatography.

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the myristoylated counterpart during purification procedures. While the latter bound tightly to calmodulin-agarose column in the presence of Ca\(^{2+}\), the former was eluted from the column with high ionic strength even in the presence of Ca\(^{2+}\). 

In this study, we have produced recombinant non-myristoylated (non-myr) and myristoylated (myr) MARCKS proteins and studied their binding properties to calmodulin in detail. Non-myr MARCKS was dissociated from calmodulin with high ionic strength in the presence of Ca\(^{2+}\), whereas myr MARCKS bound tightly even at high ionic strength. Furthermore, we found that a myristoylated peptide based on the N-terminal MARCKS sequence bound to calmodulin only when it was myristoylated. The results obtained suggested that the N-terminal myristoylated domain is directly involved in the MARCKS-calmodulin interaction.

EXPERIMENTAL PROCEDURES

Materials—Dansyl-calmodulin and calmodulin-agarose were obtained from Sigma. Source 15Q ion exchange column was from Pharmacia Corp. Tryptone and yeast extracts were from Difco, while ampicillin, kanamycin, and isopropyl-1-thio-β-galactopyranoside were from Wako Pure Chemical Industries. A 25-amino acid peptide corresponding to the phosphorylation domain of MARCKS (KKKKKRFSFKKNKK) was synthesized with a standard synthesizer using standard Fluka fluorenylmethoxycarbonyl chemistry, were purchased from Research Genetics. The peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) using a C18 column (Waters, μBondapak 5 μ, C18—300Å, 1.9 × 15 cm). They were judged to be greater than 95% purity by analytical HPLC and electrospray mass spectrometry (14, 25). Peptide concentration was determined by quantitative amino acid analysis. The Esccherichia coli strain BL21(DE3)pLyS8 was obtained from Stratagene. The plasmid pBB131NMT was a gift from Dr. J. Gordon (Washington University).

Expression and Purification of Non-myr MARCKS and Myr MARCKS—The E. coli strain BL21(DE3)pLyS8 was transformed with the plasmid pET3d containing the human MARCKS gene cloned by PCR based on the published sequence (26). For the construction, expression and purification of non-myr MARCKS, the published procedure was followed exactly as described previously (27). The cells containing the plasmid were selected with 100 μg/ml ampicillin. A frozen stock of transformed colonies was used to inoculate LB media containing 100 μg/ml ampicillin, and the cells were grown overnight at 37 °C until use. We have previously observed that non-myr MARCKS purified from soluble fractions of bovine brain does not bind to the calmodulin column as efficiently as the myr MARCKS (24). The non-myr MARCKS can be eluted with high ionic strength even in the presence of Ca\(^{2+}\), whereas myr MARCKS is still bound to the column and can be eluted only in the absence of Ca\(^{2+}\).

RESULTS

Binding of Myr and Non-myr MARCKS to Calmodulin—We have previously observed that non-myr MARCKS purified from soluble fractions of bovine brain does not bind to the calmodulin column as efficiently as the myr MARCKS (24). The non-myr MARCKS can be eluted with high ionic strength even in the presence of Ca\(^{2+}\), whereas myr MARCKS is still bound to the column and can be eluted only in the absence of Ca\(^{2+}\). These observations suggest that the N-terminal myristoylation of MARCKS modulates the interaction of MARCKS with calmodulin, which is effected by the calmodulin-binding domain in the middle of the MARCKS molecule (20, 24). To elucidate the underlying mechanism, we have produced recombinant human non-myr MARCKS and myr MARCKS proteins and studied the binding of the two forms to calmodulin in detail.

First, the effects of ionic strength on the binding were assessed by dansyl-agarose assay as described under “Experimental Procedures.” Both myr and non-myr MARCKS were mixed with calmodulin-agarose, and the bound protein was analyzed by SDS-gel electrophoresis. The extent of the binding of non-myr MARCKS to calmodulin-agarose gradually decreased with increasing concentrations of NaCl, whereas that of myr MARCKS was almost unaffected (Fig. 1). Even in the presence of 1 M NaCl, myr MARCKS did not lose its affinity to calmodulin-agarose, while the binding of non-myr MARCKS to calmodulin was almost abolished. When agarose beads were instead of calmodulin-agarose as a control, neither myr nor non-myr MARCKS showed significant binding to the agarose.

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demonstrating that we were dealing with specific interactions with calmodulin (data not shown). These results suggest that the myristoylation indeed affects the binding of MARCKS to calmodulin, and myr and non-myr MARCKS have different calmodulin-binding modes, especially in terms of the sensitivity to ionic strength.

Next, fluorescence change of dansyl-calmodulin upon binding of target protein/peptides (30–32) was used to analyze the binding quantitatively. The addition of 150 nM myr or non-myr MARCKS to 50 nM dansyl-calmodulin in a buffer containing 0.1 M NaCl induced similar shifts in the maxima of fluorescence emission spectra of dansyl-calmodulin from 510 to 490 nm and about 2-fold increases in the intensity, suggesting that both forms bound tightly to calmodulin (Fig. 2a). Although the two emission spectra obtained with myr and non-myr MARCKS were very similar, there was a clear difference in the peak maxima, and the difference was observed repeatedly. This phenomenon was definitely observed when both the concentration of dansyl-calmodulin and that of MARCKS were increased (data not shown). This may reflect a difference in the conformation of the protein that may expose a new binding site. To assess the former possibility, a myristoylated
peptide based on the N-terminal sequence (myr-GAQLSKTAAK) was synthesized, and the binding of the peptide to dansyl-calmodulin was examined. As shown in Fig. 3, the addition of the myristoylated peptide to dansyl-calmodulin at 0.1 mM NaCl caused a drastic increase in the intensity and a shift of the peak maximum of the emission spectra similar to those observed with the myr and non-myr MARCKS proteins (see Fig. 2). On the other hand, no significant change in the fluorescence spectra was observed when a non-myristoylated N-terminal peptide (GAQFSKTAAGK) was added to calmodulin (Fig. 3). Furthermore, the addition of myristic acid alone or that of the mixture of myristic acid and non-myristoylated peptide to dansyl-calmodulin did not affect the fluorescence spectra significantly (data not shown). These results not only indicate that the myristoyl moiety is directly involved in the peptide-calmodulin interaction but also suggest that the N-terminal peptide part is also important for the interaction. The dissociation constant was calculated from the titration data to be 2.0 μM, a value that was lower than that obtained for the basic effector domain (3.8 μM), but similar to those observed with low-affinity calmodulin-binding proteins such as GAP-43 (33). Interestingly, the binding of the myristoylated N-terminal peptide to calmodulin was not affected by ionic strength. The dissociation constant obtained at 0.5 mM NaCl was 1.5 μM, which was almost identical to that observed at 0.1 mM NaCl.

To study the role of the N-terminal protein moiety in the myristoylated peptide-calmodulin interaction, a series of myristoylated peptides were synthesized, and their binding characteristics to calmodulin were analyzed by fluorescence measurements. Since both hydrophobic and basic amino acids play important roles in the calmodulin-target-protein interactions (34, 35), we have replaced either the Phe^4 or Lys^8 in the original peptide with various amino acids (Fig. 4). When Phe^4 was replaced with an Ala or an Asp, no significant change in the fluorescence spectrum was observed, suggesting that the hydrophobic amino acid is important for the calmodulin interaction. Interestingly, when the same residue was replaced with a Lys, an increase in intensity and a shift of the peak maximum of the emission spectra similar to those observed with the original sequence were observed. The dissociation constant obtained at 0.1 mM NaCl was 1.4 μM, which was almost identical to that of the wild type peptide. When Lys^8 was replaced with an Asp, no detectable change in the fluorescence spectrum was observed. Therefore, both hydrophobic and basic residues are involved in the interaction of the myristoylated peptides with calmodulin, and the presence of positively charges seems to be important. Taken together, these results suggest that the myristoyl moiety, together with the N-terminal protein domain, constitutes the ionic strength-insensitive second calmodulin-binding site in myr MARCKS.

FIG. 3. Fluorescence spectra of dansyl-calmodulin in the presence of N-terminal myristoylated peptides. Fluorescence spectra of 200 nM dansyl-calmodulin in the presence of 0.5 mM CaCl_2 (solid line), plus 4 μM myristoylated peptide (dashed line), plus 4 μM non-myristoylated peptide (dashed line) were obtained as described under “Experimental Procedures.”

FIG. 4. Binding of mutant myristoylated peptides to dansyl-calmodulin. Fluorescence spectra of dansyl-calmodulin were obtained in the presence of 0.5 mM CaCl_2 (—), plus each 1 μM of myr-GAQLSKTAAK (—–), myr-GAQKSKTAAK (—–), or myr-GAQLSSTAAK (—–).
binding site with low affinity affected the overall affinity of myr MARCKS, we can assume that the N-terminal domain and the effector domain bind to calmodulin cooperatively. The elucidation of the detailed mechanism would require the determination of the three-dimensional structure. However, it should be noted in this context that protein domains other than canonical calmodulin-binding domains in other calmodulin-target proteins have been shown to be involved in the calmodulin interaction (44). The canonical calmodulin-binding domain, therefore, is clearly not the sole interaction site of the calmodulin-target proteins with calmodulin.

We have previously demonstrated the presence of demyristoylation activity in the cytoplasmic fraction of synaptosomes (22, 23). We also found that calmodulin inhibited the demyristoylation reaction in a Ca\(^{2+}\)-independent manner. This observation was rather puzzling at that time, since the N-terminal domain where the cleavage occurs and the calmodulin-binding domain are about 150 amino acids apart, and MARCKS has been shown to assume an elongated overall structure (31, 45–47). The direct interaction of the N-terminal domain with calmodulin as demonstrated in the present study solved the puzzle easily; the direct interaction of calmodulin with the basic effector domain are functionally interdependent. The demyristoylation of MARCKS is regulated by calmodulin, and since the demyristoylation affects in turn the MARCKS-calmodulin interaction, the N-terminal myristoyl moiety and the basic effector domain are functionally interdependent.

Schleiff et al. (48) have reported the lack of effects by protein myristoylation on the properties of MARCKS-related protein (MRP or also called F52) in solution. Using myr MRP and non-myrm MRP, they have investigated the secondary structure of MRP, phosphorylation by protein kinase C, binding to calmodulin, and the inhibitory effect of divalent cations. According to their results, none of these properties were significantly altered by myristoylation. They have pointed out, however, that myristoylation might modulate the mechanism of recognition of MRP by calmodulin and consequently alter the kinetics of complex formation. They have recently demonstrated that the myristoylated N-terminal domains of MRP interact with calmodulin in the complex, whereas the non-myristoylated N-terminal domains do not (49). We have repeated the calmodulin binding experiments with MRP and observed a difference in sensitivity to ionic strength similar to that observed with MARCKS. Therefore, it is reasonable to assume that the myristoyl moiety of MRP also interacts with calmodulin. Thus, the myristoylated N-terminal domains of MARCKS family proteins play important roles both in their interaction with membrane phospholipids and in the protein-protein interaction with calmodulin.

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