Identification of the Calmodulin-binding Domain of Neuron-specific Protein Kinase C Substrate Protein CAP-22/NAP-22

DIRECT INVOLVEMENT OF PROTEIN MYRISTOYLATION IN CALMODULIN-TARGET PROTEIN INTERACTION*

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Various proteins in the signal transduction pathways as well as those of viral origin have been shown to be myristoylated. Although the modification is often essential for the proper functioning of the modified protein, the mechanism by which the modification exerts its effects is still largely unknown. Brain-specific protein kinase C substrate, CAP-23/NAP-22, which is involved in the synaptogenesis and neuronal plasticity, binds calmodulin, but the protein lacks any canonical calmodulin-binding domain. In the present report, we show that CAP-23/NAP-22 isolated from rat brain is myristoylated and that the modification is directly involved in its interaction with calmodulin. Myristoylated and non-myristoylated recombinant proteins were produced in Escherichia coli, and their calmodulin-binding properties were examined. Only the former bound to calmodulin. Synthetic peptides based on the N-terminal sequence showed similar binding properties to calmodulin, only when they were myristoylated. The calmodulin-binding site narrowed down to the myristoyl moiety together with a nine-amino acid N-terminal basic domain. Phosphorylation of a single serine residue in the N-terminal domain (Ser5) by protein kinase C abolished the binding. Furthermore, phosphorylation of CAP-23/NAP-22 by protein kinase C was also found myristoylation-dependent, suggesting the importance of myristoylation in protein-protein interactions.

Since the finding of protein myristoylation in the catalytic subunit of cAMP-dependent protein kinase (1), various proteins in the signal transduction pathways as well as those of viral origin have been shown to be fatty-acylated (2). The modification is often essential for the proper functioning of the modified protein; the transforming activity of p60src from Rous sarcoma virus, for example, is dependent on its myristoylation (3). However, the mechanism by which the modification exerts its effects is still largely unknown (2, 4).

It is generally assumed that the hydrophobic acyl groups such as myristoyl and palmitoyl groups are often involved in the protein-membrane interactions. Due to its intermediate hydrophobicity, myristoylation has been implied in the reversible membrane association (5, 6). Studies from our own and other laboratories have established that such a mechanism, in fact, is operative in the phosphorylation-dependent interaction of myristoylated alanine-rich protein kinase C substrate (MARCKS)1 with membranes (7, 8). 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 membranes (9).

However, not all the myristoylated proteins are associated with membranes. The first protein that has been found myristoylated, cAMP-dependent protein kinase, is not a membrane protein. In the case of calcineurin, the second protein found myristoylated, the myristoylation seems to not affect the association of the protein with membranes, and both myristoylated and non-myristoylated proteins are found in membrane and soluble fractions (10, 11). Interestingly, the modification has been shown to affect the protein stability of both proteins (10, 12). This suggests that the myristoyl moiety interacts with the protein part in a manner analogous to that found in recoverin. The third possible function of the protein myristoylation, namely the involvement of the modification in protein-protein interaction, has been the subject of various studies (10, 13–15).

To our knowledge, however, the last possibility has never been clearly demonstrated.

Brain-specific protein CAP-23/NAP-22 has been characterized first in chicken brain as a cortical cytoskeleton-associated protein of 23 kDa (16), and its rat homologue was later characterized as a neuron-specific acidic protein of 22 kDa (17). It is related to another neuron-specific acidic protein called GAP-43, a major neuronal growth cone-associated protein (16, 17). Although the physiological function of the protein has yet to be determined, the involvement in the synaptogenesis and neuronal plasticity has been suggested (18). Like the two related proteins, GAP-43 and MARCKS (19), CAP-23/NAP-22 is a prominent substrate of protein kinase C (PKC), and the phosphorylation by PKC is regulated by the binding to calmodulin (20). However, unlike GAP-43 and MARCKS, which have a typical calmodulin-binding domain of basic amphiphilic nature, CAP-23/NAP-22 lacks any canonical calmodulin-binding domain (20).

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The abbreviations used are: MARCKS, myristoylated alanine-rich protein kinase C substrate; PKC, protein kinase C; CD, circular dichroism; PCR, polymerase chain reaction; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
In the present report, we show that NAP-22/CAP-23 isolated from rat brain is myristoylated and that the modification is directly involved in its interaction with calmodulin. The calmodulin-binding site of the protein was narrowed down to the myristoyl moiety together with a nine-amino acid N-terminal basic domain. The acyl chain seems to interact with the hydrophobic pocket of calmodulin, which is usually occupied by the hydrophobic amino acids of target proteins. Phosphorylation of CAP-23/NAP-22 by PKC was also found to be myristoylation-dependent.

**EXPERIMENTAL PROCEDURES**

**Preparation of Recombinant Proteins**—The complete gene of 683 base pairs including tag regions for NcoI and XhoI restriction sites at both ends was synthesized based on the rat cDNA sequence (17), except that Ser2 in the deduced sequence was replaced with a Gly and that nucleotide sequence was rewritten using the bacterial codon usage. The double-stranded DNA was divided into 12 regions that overlap each other, and the DNA fragments that extended from 18 to 60 nucleotides were synthesized. They were annealed, ligated, and used as template for PCR. The synthetic gene thus obtained was incorporated between the NcoI-XhoI sites of pET14b expression vector. This approach allowed a high expression of the protein in Escherichia coli (86 mg/liter of culture medium). For the myristoylated protein, a pBB131 vector containing yeast N-myristoyltransferase cDNA was co-transformed (21). Both proteins were purified from the heat-stable protein fractions by successive column chromatography on Phenyl-Sepharose and Resource RPC (Amersham Pharmacia Biotech).

**Myristoylation of Peptides**—Peptides synthesized by standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry (Research Genetics, Huntsville, AL) were myristoylated by recombinant yeast N-myristoyltransferase in vitro as described (22). The myristoylated peptides were purified over a reversed-phase column (Vydac 218TP, 2.1 × 150 mm) using a linear gradient of H2O-acetonitrile in the presence of 0.1% trifluoroacetic acid.

**Phosphorylation by PKC**—The synthetic peptides and recombinant proteins were phosphorylated by PKC purified from bovine brain as has been described previously (23). The phosphorylated peptides were purified by reversed-phase column chromatography. For the mass spectrometric studies, the phosphorylation reaction mixtures were directly analyzed by liquid chromatography/mass spectrometry (24).

**Mass Spectrometry**—CAP-23/NAP-22 protein purified from rat brain (24) was digested with lysyl endoprotease, and the resulting peptide mixture was subjected to the collision-induced dissociation (Fig. 1a) as described (24). The myristoylated peptides were identified by reversed-phase column chromatography. The peptide isolated was subjected to the collision-induced dissociation as described under "Experimental Procedures." m/z and yn ions denote the N-terminal and C-terminal fragments, respectively. b, comparison of the N-terminal domain of CAP-23/NAP-22 from various species. Ser2 that is mistakenly read in the rat sequence is underlined. The initial Met that is cut off before myristoylation is omitted. Sequences were from Ref. 17 (a), accession number P80723 (b), accession number 1939063 (c), and Ref. 16 (d).

**RESULTS**

**N-terminal Myristoylation of Rat CAP-23/NAP-22**—First we have characterized the N terminus of CAP-23/NAP-22 purified from rat brain. The purified protein was digested with lysyl endoprotease, and the resulting peptide mixture was subjected to the liquid chromatography/electrospray mass spectrometric analysis. Most of the peptides were assigned by comparing the observed masses with those calculated from the amino acid sequence deduced from the rat cDNA sequence (17), which established the authenticity of the protein. One peptide eluted from the reversed-phase column near the end of the gradient was subjected to the collision-induced dissociation (Fig. 1a). The results obtained demonstrated clearly that the N-terminal Gly is modified with a myristoyl group. Interestingly, the third amino acid is clearly identified as Gly and not a Ser found in the deduced sequence (17). Comparison of the amino acid sequence shows that the third Gly together with other amino acids in the N-terminal region is well conserved among CAP-23/NAP-22 proteins from various species (Fig. 1b), suggesting that the original rat cDNA sequence contains an error at the N terminus.

**Characterization of Recombinant Proteins**—To study the effects of myristoylation on the interaction of CAP-23/NAP-22 with calmodulin, two recombinant proteins, i.e. non-myristoylated and myristoylated proteins, were produced in Escherichia coli as described under "Experimental Procedures." The latter was obtained by co-transforming the bacteria with a plasmid containing yeast N-myristoyltransferase gene (21). The authenticity of the two proteins thus produced was established by mass spectrometry. The mass of the non-myristoylated protein was determined to be 21,629.2 ± 2.9 Da (theoretical mass is 21,629.1 Da for the non-myristoylated protein with the initial Met removed), while that of the myristoylated protein was 21,839.5 ± 2.0 Da (theoretical mass is 21,839.5 Da). These results indicate that the two proteins start with the second Gly residue in the reduced sequence and differ only in the N-terminal myristoylation.

**Effects of Myristoylation on the Calmodulin Interaction**—The interaction of the recombinant proteins with calmodulin was analyzed by binding to calmodulin-agarose beads. Myristoylated CAP-23/NAP-22 and non-myristoylated-CAP-23/NAP-22 were mixed with calmodulin beads in the presence of Ca2+, and the bound proteins were eluted from the beads with EGTA. As shown in Fig. 2a, the myristoylated protein bound to the cal-

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**FIG. 1. Myristoylation and N-terminal sequence of rat CAP-23/NAP-22.**

- **a, tandem mass spectrum of the N-terminal peptide of CAP-23/NAP-22 purified from rat brain.** Purified protein was digested with lysyl endoprotease, and the N-terminal peptide was isolated by reversed-phase column chromatography. The peptide is shown in Fig. 2a.
- **b, comparison of the N-terminal domain of CAP-23/NAP-22 from various species.** Sequences were from Ref. 17 (a), accession number P80723 (b), accession number 1939063 (c), and Ref. 16 (d).

**FIG. 2.**

- **a, tandem mass spectrum of the N-terminal peptide of CAP-23/NAP-22.** The myristoylated protein bound to the calmodulin-agarose beads. Myristoylated and non-myristoylated proteins, were produced in Escherichia coli as described under "Experimental Procedures." The latter was obtained by co-transforming the bacteria with a plasmid containing yeast N-myristoyltransferase gene (21). The authenticity of the two proteins thus produced was established by mass spectrometry. The mass of the non-myristoylated protein was determined to be 21,629.2 ± 2.9 Da (theoretical mass is 21,629.1 Da for the non-myristoylated protein with the initial Met removed), while that of the myristoylated protein was 21,839.5 ± 2.0 Da (theoretical mass is 21,839.5 Da). These results indicate that the two proteins start with the second Gly residue in the reduced sequence and differ only in the N-terminal myristoylation.
modulin agarose, and most of the bound protein was eluted with the Ca\(^{2+}\)-free buffer. Therefore, the binding of CAP-23/NAP-22 to calmodulin is Ca\(^{2+}\)-dependent, as has been shown with the CAP-23/NAP-22 protein purified from rat brain (20). These results also suggest that the recombinant protein is fully functional. In contrast, the non-myristoylated protein did not bind to the calmodulin beads to a significant extent under the same conditions. There seems to be an absolute requirement of N-terminal myristoylation in the CAP-23/NAP-22-calmodulin interaction.

**Binding of N-terminal Peptides to Calmodulin**—To elucidate the mechanisms underlying the myristoylation-dependent calmodulin binding, peptides were synthesized based on the N-terminal sequence and myristoylated using a purified recombinant N-myristoyltransferase in vitro. Their binding to calmodulin was assessed using calmodulin-agarose as above. Surprisingly, not only the longest peptide synthesized containing the N-terminal 18 residues (GGKLSKKKGGYGVNDEKA) but also the shortest peptide of nine amino acids (GGKLSK) showed a similar Ca\(^{2+}\)-dependent binding to calmodulin (Fig. 2b). Corresponding non-myristoylated peptides did not show any significant binding to calmodulin.

The myristoylated peptide can fully mimic the behavior of the intact protein, suggesting that the myristoylated N-terminal domain is solely responsible for the binding of CAP-23/NAP-22 to calmodulin.

A more quantitative analysis was carried out by measuring the fluorescence change of dansyl-calmodulin upon binding of the target peptide (26). While the addition of the myristoylated N-terminal peptide caused a drastic increase in the intensity and a shift of the peak maximum of the emission spectra, little, if any, change was observed with the addition of the non-myristoylated peptide (Fig. 2c). This is in good agreement with the direct binding results studied with calmodulin-agarose, and suggests that the binding of the myristoylated peptide is associated with a conformational change of calmodulin. Using the fluorescence change, the dissociation constant of the short peptide, myr-GGKLSKKK, was determined from the titration data to be 3.0 nM, which is similar to that observed with the intact myristoylated protein (20). These values suggest that the binding of the myristoylated peptide to calmodulin is among the highest of calmodulin-binding proteins (28). Although calmodulin has been known to bind to phospholipids through hydrophobic interactions, the affinities toward the membrane lipids are very low (29). Furthermore, the addition of myristic acid to calmodulin did not affect the calmodulin fluorescence (data not shown). These results suggest that the binding of CAP-23/NAP-22 to calmodulin involves specific interactions rather than simple hydrophobic interactions between calmodulin and the myristoyl moiety.

**Identification of the Calmodulin-binding Domain of NAP-22**—To elucidate the amino acid residues involved in the binding, the myristoylated peptide (myr-GGKLSKKK) was partially digested with lysyl endopeptidase. After stopping the reaction by boiling, the obtained peptide mixture was either directly injected to the liquid chromatography/mass spectrometry apparatus (total), or treated with calmodulin-agarose. The fraction not bound to the agarose (unbound), or that bound and eluted from calmodulin-agarose with EGTA (bound) was analyzed by mass spectrometry as described under "Experimental Procedures." Original electrospray mass spectra were deconvoluted.
phobic part of calmodulin, but also involves specific interactions. In this context, it is of interest to note that the replacement of the second Gly with a Ser, which was found in the original deduced sequence, resulted in the reduced binding affinity by 1 order of magnitude (data not shown). These results again support our view that the peptide moiety plays an important role in the interaction, and that specific structural requirements exist to achieve a high affinity binding of nonglobular range.

N-terminal Peptide Assumes Non-helical Structure in Calmodulin Complex—To understand the structural basis of CAP-23/NAP-22 binding to calmodulin, CD spectra of the myristoylated N-terminal domain peptide and those of calmodulin complex were measured. It has been shown that peptides derived from calmodulin-binding domain of target proteins assume random structures in solution, but form a-helical structures in calmodulin complex. Since the overall secondary structures of calmodulin, and hence its a-helical content, do not change appreciably, changes in CD spectra associated with complex formation are mainly attributed to the change in the target proteins (30). CD spectrum of the equimolar complex of the N-terminal peptide and calmodulin was compared with the mathematical sum of those of the individual components. As shown in Fig. 4, there was practically no change upon the complex formation. The peptide alone in solution showed a CD spectrum with a single negative peak below 200 nm, which is characteristic for a random coil (data not shown). These results suggest that the conformation of the myristoylated peptide does not change upon calmodulin binding, and the peptide assumes a non-helical conformation in the complex.

Effects of Phosphorylation on Calmodulin Binding—Phosphorylation of the intact protein by PKC has been shown to abolish its interaction with calmodulin (20). We have examined whether the N-terminal peptide (myr-GGKLSKKKK) can be phosphorylated by PKC, and if it can, whether the phosphorylation affects the binding of the myristoylated N-terminal peptide with calmodulin. Both myristoylated and non-myristoylated N-terminal peptides were stoichiometrically phosphorylated by PKC as shown by mass spectrometry (Fig. 5a). Since the peptides contained only one phosphorylatable residue, we concluded that Ser5 is the phosphorylation site by PKC. Phosphorylation by PKC abolished the binding of the peptide to calmodulin completely, as is the case with the intact myristoylated protein (Fig. 5b). From these results, we conclude that the myristoylated N-terminal basic domain is the sole calmodulin-binding and PKC phosphorylation domain of CAP-23/NAP-22, and that the interaction with calmodulin is regulated by the phosphorylation of a single Ser in the domain.

Effects of Myristoylation on PKC-dependent Phosphorylation—Since the N-terminal myristoylated domain is phosphorylated by PKC, it is of interest to examine whether the phosphorylation is affected by myristoylation. Both myristoylated and non-myristoylated CAP-23/NAP-22 were incubated with purified PKC, and the resulting mixtures were analyzed by mass spectrometry. As shown in Fig. 6, both proteins were stoichiometrically phosphorylated, but there was a clear difference in the time course. The myristoylated protein was almost completely phosphorylated after 10-min incubation, while only 60% of the non-myristoylated protein was phosphorylated at that time. Therefore, the myristoylation seems to facilitate the interaction of CAP-23/NAP-22 with PKC.

DISCUSSION

In the present report, we could narrow down the calmodulin-binding domain of CAP-23/NAP-22 to the myristoyl moiety together with the nine-amino acid N-terminal domain (myristoyl-GGKLSKKKK). Even a shorter peptide (myristoyl-GGKL) binds to calmodulin albeit weakly. Addition of each basic Lys increases the affinity to calmodulin incrementally. Since a myristoylated peptide consisting of the myristoyl moiety and the first 18 N-terminal amino acids showed an affinity similar to that of the nine-amino acid peptide (data not shown), the first 9 amino acids should suffice for calmodulin binding. As is the case for the typical calmodulin-binding motif (31), the interaction between the basic residues of the binding protein and the acidic residues of calmodulin seems to play an important role in the binding. However, a more striking result is the fact that the binding of the intact CAP-23/NAP-22 protein as well as that of the N-terminal peptide is dependent on the presence of the myristoyl moiety.

A closer look at the N-terminal sequence immediately shows that the domain contains one hydrophobic residue (Leu) in addition to the five basic amino acids (Lys). This is actually reminiscent of the canonical calmodulin-binding motif, a basic amphiphilic motif, in which basic hydrophilic residues and hydrophobic ones appear alternatingly (28, 32). In the calmodulin-binding domain of CAP-23/NAP-22, a hydrophobic acyl group is followed by a basic Lys residue, which is followed...
by a hydrophobic Leu residue. If one assumes that the acyl group can substitute large hydrophobic amino acids such as Trp and Leu found in the canonical binding motif, the overall structural characteristics are very similar. A canonical motif, however, would span a minimal length of 12 amino acids between the two critical hydrophobic residues (31). However, when the CAP-23/NAP-22 peptide assumes an elongated structure, the distance between the myristoyl moiety and Leu4 is comparable to that between the two critical hydrophobic amino acids found in the target proteins (Fig. 7). A CD spectrum of the myristoylated peptide-calmodulin complex, in fact, suggests that the peptide assumes an elongated structure rather than typical $\alpha$-helix. Therefore, it is reasonable to assume that the acyl chain interacts with the hydrophobic pocket in the C-terminal lobe of calmodulin, while the Leu4 interacts with that in the N-terminal lobe in a manner analogous to the other canonical calmodulin-binding motif (31). In fact, the hydrophobic caves offered by calmodulin for the hydrophobic interactions have very flexible structures and can fit a large variety of target proteins.

CAP-23/NAP-22 is not the only example of calmodulin target protein that assumes a non-helical structure in the calmodulin complex. We have recently shown that the calmodulin-binding domain of MARCKS binds to calmodulin in a non-helical elongated structure despite the classical basic amphiphilic character (27). We have also pointed out the presence of a novel class of calmodulin-binding proteins that assume non-helical structures in calmodulin complex. The calmodulin-binding domains of these proteins are characterized by the dominance of basic amino acids contrary to the canonical motif in which hydrophobic amino acids are dominant (27). It is, of course, not easy to estimate the hydrophobicity of the CAP-23/NAP-22 calmodulin-binding domain, which clearly constitutes a third class of calmodulin-binding proteins. However, the fact that the myristoyl group has only weak or intermediate hydrophobicity compared with large hydrophobic amino acids (5, 6), and the fact that the basic amino acids are important in the CAP-23/NAP-22-calmodulin interaction, may suggest a close relationship of CAP-23/NAP-22 to the non-classical class of the target proteins. The elucidation of the three-dimensional structures should yield a more insight into the structural consideration of these proteins.

Protein myristoylation has been implicated in the regulation of various signal transduction proteins (4, 5). It is obvious that the modification is essential for the membrane targeting of these proteins (4, 7). Interestingly, the presence of a myristoyl group is not enough for the stable membrane anchoring of these proteins. A basic amino acid cluster is often found near the acyl group (33), and the two domains work cooperatively in the reversible membrane binding of these proteins (34). Either protein phosphorylation or calmodulin binding regulates the reversible membrane binding. Since CAP-23/NAP-22 seems to be anchored to membrane through its N-terminal domain (35), and since the same domain binds calmodulin and PKC, the membrane association of CAP-23/NAP-22 may be regulated in a similar manner.

In contrast to the role in the membrane binding, the role of protein myristoylation in the protein-protein interaction is still largely unknown. However, the three-dimensional structure of recoverin revealed the presence of direct interaction of the acyl group with its own protein moiety (9, 36). The effects on protein stability found in cAMP-dependent protein kinase and calcineurin also imply some kind of interaction between the myristoyl group and protein parts. Furthermore, we have already shown that the N-terminal myristoylation modulates the MARCKS-calmodulin interaction (37), and the involvement of the modification in the protein-protein interactions has also
been speculated in G proteins (38). In the present report, we have also shown that the myristoylation affects the phospho-
rylation of peptides by PKC. Therefore, protein myristoylation may be also involved in the protein-protein interaction between PKC and its substrate proteins. In fact, potentiation of PKC inhibitor peptides by N-terminal myristoylation has been re-
ported (39, 40), and even the presence of a myristyl binding site in PKC has been speculated (41). Altogether, these results raise the possibility that the protein myristoylation plays direct roles in the protein-protein interaction of various proteins.

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