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The binding of myristoylated N-terminal nonapeptide from neuron-specific protein CAP-23/NAP-22 to calmodulin does not induce the globular structure observed for the calmodulin–nonmyristoylated peptide complex

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Abstract

CAP-23/NAP-22, a neuron-specific protein kinase C substrate, is N^{α}-myristoylated and interacts with calmodulin (CaM) in the presence of Ca^{2+} ions. Takasaki et al. (1999, *J Biol Chem 274*:11848–11853) have recently found that the myristoylated N-terminal nonapeptide of CAP-23/NAP-22 (mC/N9) binds to Ca^{2+}-bound CaM (Ca^{2+}/CaM). In the present study, small-angle X-ray scattering was used to investigate structural changes of Ca^{2+}/CaM induced by its binding to mC/N9 in solution. The binding of one mC/N9 molecule induced an insignificant structural change in Ca^{2+}/CaM. The 1:1 complex appeared to retain the extended conformation much like that of Ca^{2+}/CaM in isolation. However, it could be seen that the binding of two mC/N9 molecules induced a drastic structural change in Ca^{2+}/CaM, followed by a slight structural change by the binding of more than two but less than four mC/N9 molecules. Under the saturated condition (the molar ratio of 1:4), the radius of gyration (R_g) for the Ca^{2+}/CaM-mC/N9 complex was 19.8 ± 0.3 Å. This value was significantly smaller than that of Ca^{2+}/CaM (21.9 ± 0.3 Å), which adopted a dumbbell structure and was conversely 2–3 Å larger than those of the complexes of Ca^{2+}/CaM with the nonmyristoylated target peptides of myosin light chain kinase or CaM kinase II, which adopted a compact globular structure. The pair distance distribution function had no shoulder peak at around 40 Å, which was mainly due to the dumbbell structure. These results suggest that Ca^{2+}/CaM interacts with N^{α}-myristoylated CAP-23/NAP-22 differently than it does with other nonmyristoylated target proteins. The N-terminal amino acid sequence alignment of CAP-23/NAP-22 and other myristoylated proteins suggests that the protein myristoylation plays important roles not only in the binding of CAP-23/NAP-22 to Ca^{2+}/CaM, but also in the protein–protein interactions related to other myristoylated proteins.

Keywords: calmodulin; CAP-23/NAP-22; myristoylation; protein–protein interaction; small-angle X-ray scattering

The neuron-specific protein CAP-23/NAP-22 was first characterized in chicken brain as a 23 kDa cortical cytoskeleton-associated protein (Widmer & Caroni, 1990), and a rat homologue was later characterized as a 22 kDa neuron-specific acidic protein (Maekawa et al., 1993). The physiological function of the protein has yet to be determined, but its involvement in synaptogenesis and neuronal plasticity has been suggested (Caroni et al., 1997). CAP-23/NAP-22 is related to other neuron-specific acidic proteins such as GAP-43 and myristoylated alanine-rich protein kinase C substrate (MARCKS) (Widmer & Caroni, 1990; Blackshear, 1993; Maekawa et al., 1993) because it is also a prominent substrate of protein kinase C (PKC).

Recently, we have demonstrated that CAP-23/NAP-22 isolated from rat brain is N^{α}-myristoylated, and this modification is involved in its interaction with CaM in the presence of Ca^{2+} (Takasaki et al., 1999). The involvement of the protein myristoylation in protein–protein interactions had been implied in various studies

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Abbreviations: CaM, calmodulin; Ca^{2+}/CaM, Ca^{2+}-bound calmodulin; d_{max}, maximal pair distance; HSQC, heteronuclear single quantum coherence; M13, a peptide based on the calmodulin-binding domain of myosin light chain kinase; MARCKS, myristoylated alanine-rich protein kinase C substrate; mC/N9, myristoylated nonapeptide synthesized based on the N-terminal sequence of CAP-23/NAP-22; MLCK, myosin light chain kinase; myr, myristoylated; N^{α}, α-amino; PKC, protein kinase C; $P(r)$, pair distance distribution function; R_g, radius of gyration; SAXS, small-angle X-ray scattering; TFP, trifluoperazine, 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10H-phenothiazine; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

(Chow et al., 1987; Kawamura et al., 1994; Senin et al., 1995), but it had never been clearly demonstrated. Our study was the first direct demonstration of the involvement of the myristoylation in protein–protein interactions. The CaM-binding site was also narrowed down to the myristoyl moiety together with the N-terminal basic domain of nine amino acid residues, GGKLSKKKK. Phosphorylation of a single serine residue in the N-terminal domain by PKC abolished the binding of CAP-23/NAP-22 to Ca^{2+}/CaM. Phosphorylation of CAP-23/NAP-22 by PKC was also demonstrated to be myristoylation-dependent. These results strongly suggested the importance of myristoylation in protein–protein interactions.

CaM is a small (16.7 kDa) calcium-binding protein involved in a wide range of cellular Ca^{2+}-dependent signaling pathways through various enzymes, including protein kinases, protein phosphatases, nitric oxide synthase, inositol triphosphate kinase, nicotinamide adenine dinucleotide kinase, and cyclic nucleotide phosphodiesterase (Crivici & Ikura, 1995). The Ca^{2+}/CaM molecule adopts an "elongated" structure that comprises two globular domains connected by a highly flexible linker (Seaton et al., 1985; Kretsinger et al., 1986; Persechini & Kretsinger, 1988; Heidorn et al., 1989; Barbato et al., 1992; Finn et al., 1995; Spoel et al., 1996). The binding of Ca^{2+}/CaM to the target-peptide induces a compact globular structure caused by bending of the domain linker (Ikura et al., 1992; Meador et al., 1992, 1993). The target peptides form an α-helix in the complexes in a basic amphiphilic nature. In contrast, the basic N^{α}-myristoylated peptide, myr-GGKLSKKKK, of CAP-23/NAP-22 (mC/N9) clearly has different properties from known CaM-binding peptides. The most striking feature is that the binding of mC/N9 to CaM is dependent on the presence of the myristoyl moiety, whose general function so far has been assumed to be the membrane targeting of myristoylated proteins (Takasaki et al., 1999). Additionally, the interaction between mC/N9 and CaM has several other unique features: (1) mC/N9 does not resemble any canonical CaM-binding domain in the amino acid sequence; (2) mC/N9 is likely to adopt a nonhelical conformation even in the complex with CaM; and (3) the interaction is also controlled by phosphorylation of the peptide. All these features suggest the novelty of the interaction. Furthermore, because protein myristoylation has been implicated in the regulation of various signal transduction proteins (Resh, 1996; Towler et al., 1988), and because, in addition to signal transduction proteins, there are many other potential myristoylated proteins whose myristoylations can be predicted from their amino acid sequences, there is a possibility that myristoylation dependent protein–protein interaction plays important roles in some of these cases. Therefore, a more precise examination of the molecular mechanism for its interaction is an important subject to investigate.

In this paper, we used small-angle X-ray scattering (SAXS), NMR spectroscopy, and gel shift assay to investigate the structural changes that occur in Ca^{2+}/CaM by its binding to mC/N9, namely the structural basis of CAP-23/NAP-22 binding to Ca^{2+}/CaM. This study provides insights into the role of the protein myristoylation that is essentially required for CAP-23/NAP-22–Ca^{2+}/CaM interaction.

Results

SAXS

Figure 1 shows three representative Guinier plots for Ca^{2+}/CaM in isolation and Ca^{2+}/CaM in the presence of mC/N9 at a protein concentration of 9.0 mg/mL. Figure 2 shows the R_g values as a function of the molar ratio of the Ca^{2+}/CaM–mC/N9 complex at 9.0 mg/mL. The binding of mC/N9 to Ca^{2+}/CaM induced an insignificant structural change in Ca^{2+}/CaM at a molar ratio of 1:1. On the other hand, a drastic decrease in the R_g value upon the mC/N9 binding to Ca^{2+}/CaM was observed at a molar ratio of 1:2, followed by a slight structural change that terminates at a molar ratio of 1:4. The R_g values are shown as a function of protein concentrations in Figure 3. The R_g values of the Ca^{2+}/CaM–mC/N9 1:4 complex and Ca^{2+}/CaM at a zero concentration are given in Table 1. For comparison, Table 1 also contains the R_g values for Ca^{2+}/CaM obtained from other sources and the Ca^{2+}/CaM–trifluoperazine (TFP) complex. The R_g value for Ca^{2+}/CaM (21.9 ± 0.3 Å) is comparable with that reported previously (21.5 ± 0.3 Å) (Matsushima et al., 1989). On the other hand, the R_g value for the Ca^{2+}/CaM–mC/N9 1:4 complex (19.8 ± 0.3 Å) is inconsistent with those of Ca^{2+}/CaM by itself (21.9 ± 0.3 Å) or the Ca^{2+}/CaM–known CaM binding peptide (e.g., M13; a peptide based on the CaM-binding domain of myosin light chain kinase) complex (16.4 ± 0.2 Å). It shows a middle value between the latter two values.

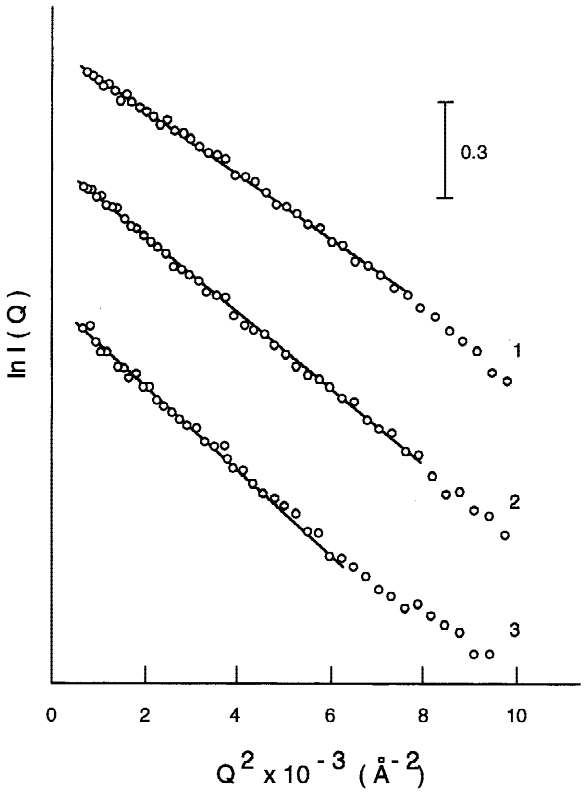

Fig. 1. Three representative Guinier plots for Ca^{2+}/CaM–mC/N9 complex and Ca^{2+}/CaM in isolation at a CaM concentration of 9.0 mg/mL. (1) Ca^{2+}/CaM–mC/N9 complex (Ca^{2+}/CaM:mC/N9=1:4); (2) Ca^{2+}/CaM–mC/N9 complex (Ca^{2+}/CaM:mC/N9=1:2); (3) Ca^{2+}/CaM without mC/N9.

Figure 4 shows the pair distance distribution function ($p(r)$) for Ca^{2+}/CaM in isolation and Ca^{2+}/CaM in the presence of mC/N9 at a molar ratio of 1:4. The $p(r)$ for Ca^{2+}/CaM in isolation has a peak at around 20 Å (principally representing the interatomic distances within each domain of Ca^{2+}/CaM) and a shoulder at around

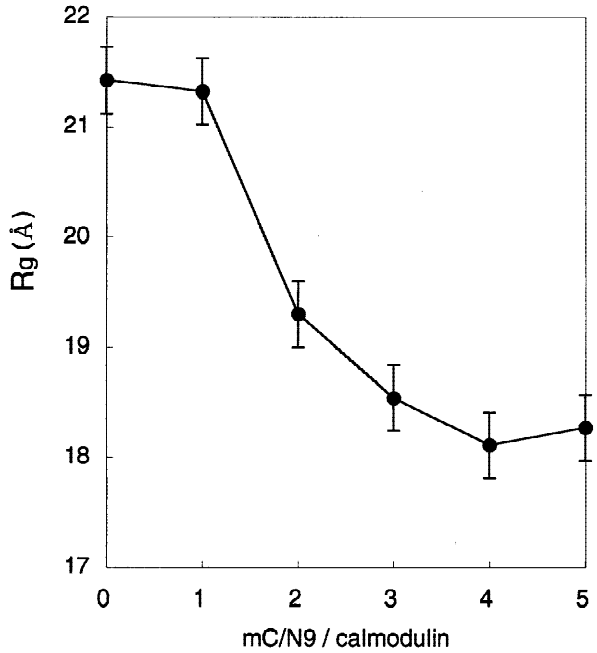

Fig. 2. The radius of gyration R_g as a function of the molar ratio of mC/N9 to Ca^{2+}/CaM at a CaM concentration of 9.0 mg/mL.

40 Å (mainly representing the interdomain distances) (Seaton et al., 1985; Matsushima et al., 1989). In contrast, in the $p(r)$ for the Ca^{2+}/CaM–mC/N9 complex, a peak appears not at 20 Å but at around 27 Å, and the shoulder peak at around 40 Å disappears. Moreover, the maximal pair distance (d_{max}) of the Ca^{2+}/CaM–mC/N9 complex is about 10 Å smaller than that of Ca^{2+}/CaM in isolation. Additionally, the $p(r)$ of the Ca^{2+}/CaM–mC/N9 complex is symmetrical, being similar to that of the Ca^{2+}/CaM–N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) complex. The determination of the three-dimensional structure indicated that the Ca^{2+}/CaM–TFP and –M13 complexes are in a compact globular form. Thus, the present data suggest that the Ca^{2+}/CaM complex with mC/N9 adopts a unique and larger globular structure that is not similar to those of the other known Ca^{2+}/CaM complexes.

Table 1. *Radius of gyration R_g and maximum dimension d_{max} for Ca^{2+}/CaM and its complexes*

	R_g (Å)	d_{max} (Å)	Reference
Ca^{2+}/CaM[a]	21.9 ± 0.3	62	This study
Ca^{2+}/CaM–mC/N9[a]	19.8 ± 0.3	50	This study
Ca^{2+}/CaM[a]	21.5 ± 0.3	69	Matsushima et al. (1989)
Ca^{2+}/CaM–M13[a]	16.4 ± 0.2	49	Heidorn et al. (1989)
Ca^{2+}/CaM–W-7[a]	17.6 ± 0.3	47	Osawa et al. (1999)

[a]Values at zero protein concentration obtained by SAXS experiment.

NMR spectroscopy

We have studied the interaction between mC/N9 and Ca^{2+}/CaM using ^{15}N labeled CaM and two-dimensional ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) NMR spectroscopy. The NMR spectra for Ca^{2+}/CaM in the absence and presence of mC/N9 are shown in Figure 5. The assignments were taken by reference to Ikura et al. (1990). When mC/N9 was added, shifts of some peaks were observed in the ^{1}H-^{15}N HSQC NMR spectra of Ca^{2+}/CaM. Some drastic shifts of the peak were observed with the addition of 2 molar equivalents of mC/N9, followed by slight shifts observed in the presence of 4 molar equivalents of mC/N9 (Fig. 5B). The fine structural analyses by NMR are now in progress, and the results will be shown elsewhere.

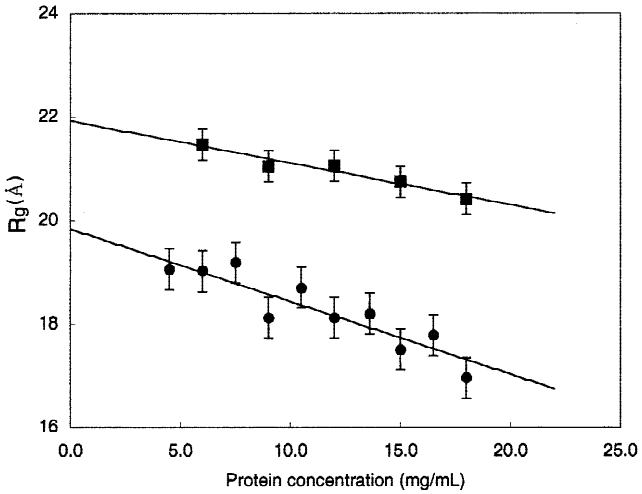

Fig. 3. The radius of gyration R_g for the Ca^{2+}/CaM–mC/N9 complex (Ca^{2+}/CaM:mC/N9=1:4) and Ca^{2+}/CaM as a function of the CaM concentration. ○, Ca^{2+}/CaM–mC/N9 complex; □, Ca^{2+}/CaM.

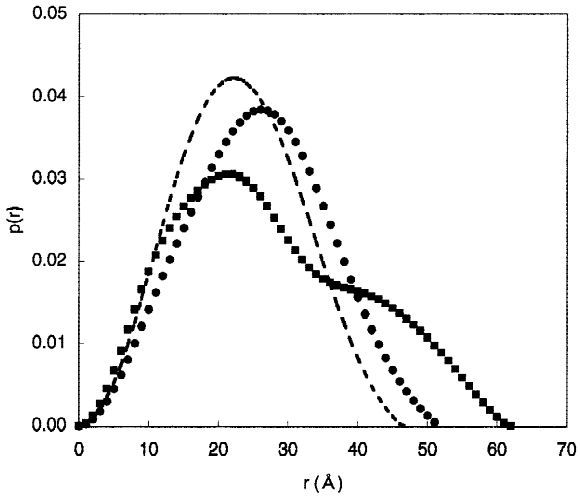

Fig. 4. Pair distance distribution function $p(r)$ for the Ca^{2+}/CaM–mC/N9 complex (Ca^{2+}/CaM:mC/N9=1:4), Ca^{2+}/CaM, and W-7 (Osawa et al., 1999). ○, Ca^{2+}/CaM–mC/N9 complex; □, Ca^{2+}/CaM; dashed line, Ca^{2+}/CaM–W-7 complex.

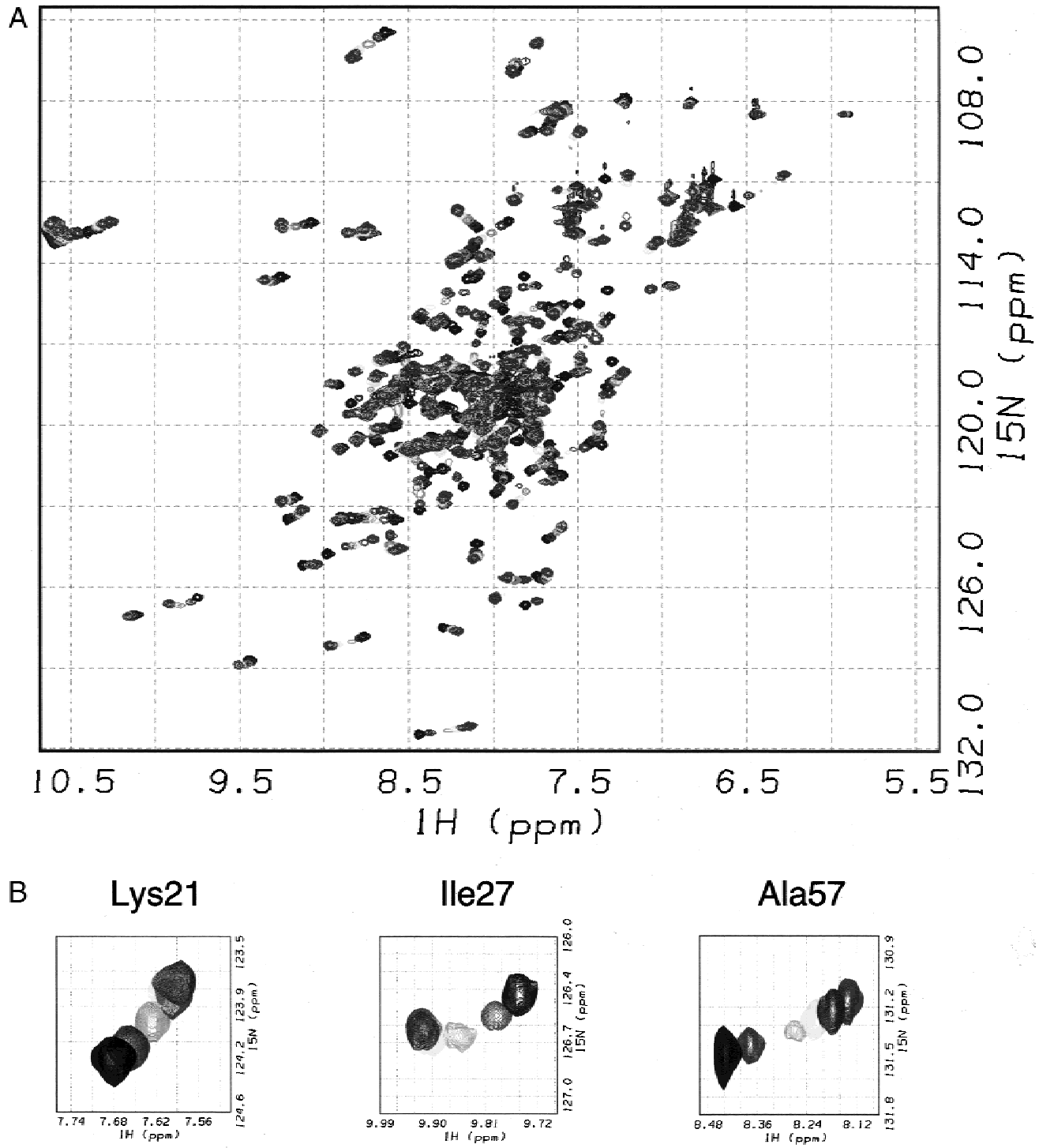

Fig. 5. Titration of Ca^{2+}/CaM with mC/N9 as studied by CaM labeled uniformly with ^{15}N and ^{1}H-^{15}N HSQC NMR spectroscopy. The sample contained 0.5 mM CaM, 120 mM NaCl, 2.5 mM $CaCl_2$, and 50 mM deuterated TrisHCl (pH 7.5) in 90% H_2O and 10% D_2O. The resonance assignments were taken by reference to Ikura et al. (1990). (**A**) The whole region and (**B**) three well isolated regions are indicated. The tentative assignments (Lys21, Ile27, Ala57) were obtained referring to Ikura et al. (1990). The spectra of Ca^{2+}/CaM in the presence of 0, 1, 2, 3, 4, and 5 molar equivalents of mC/N9 are shown in black, magenta, blue, yellow, brown, and red, respectively.

Nondenaturing urea-PAGE

We used a gel band shift assay to assess the interaction between Ca^{2+}/CaM and mC/N9. At increasing ratios of mC/N9 to Ca^{2+}/CaM, a slight band shift due to the formation of a complex between mC/N9 and Ca^{2+}/CaM can be seen (Fig. 6). It was found that the band shift lasted until 2 molar equivalents of mC/N9 were added. No changes occurred when more than 2 molar equivalents

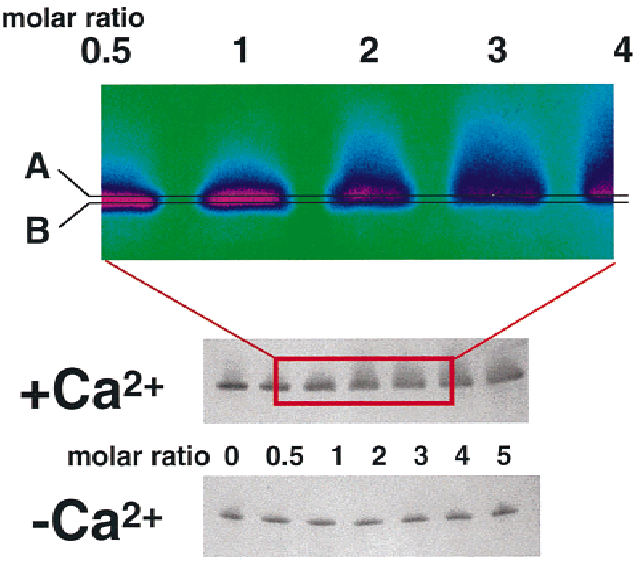

Fig. 6. Nondenaturing urea-PAGE of the Ca^{2+}/CaM–mC/N9 complexes. The ratio of mC/N9 to CaM is indicated as a molar ratio. Because mobilities of the bands in the presence of Ca^{2+} were small, an enlarged and enhanced gel image is shown simultaneously on which two lines are drawn corresponding to the centers of the bands; Line A for the bands of Ca^{2+}/CaM in the presence of more than 2 molar equivalents of mC/N9, and line B for the band of Ca^{2+}/CaM in the presence of 0.5 molar equivalents of mC/N9 whose position was almost the same as the center of the Ca^{2+}/CaM band in the absence of mC/N9. The center of the band of Ca^{2+}/CaM in the presence of 1 molar equivalent of mC/N9 was located between line A and B. Note that, without Ca^{2+}, no band shift was observed.

of mC/N9 were added. These results suggest that two mC/N9 molecules bind to one CaM molecule.

Discussion

Structure of the Ca^{2+}/CaM–mC/N9 complex

Very interestingly, the SAXS analysis indicated that the binding of one mC/N9 molecule induced an insignificant structural change in Ca^{2+}/CaM. The 1:1 complex appeared to retain the extended conformation much like that of Ca^{2+}/CaM in isolation. In fact, a recent report describes an NMR structure for a complex between a peptide from Ca^{2+} pump and CaM which differs from that of the MLCK peptide complex in that it does not assume a collapsed globular structure (Elshorst et al., 1999). It cannot be assumed that the mC/N9 and Ca^{2+} pump peptides bind to CaM in the same manner because mC/N9 is unlikely to form a helical structure (Takasaki et al., 1999) differently from the Ca^{2+} pump that takes a helical structure in solution. The detailed manner of the binding of mC/N9 to CaM appears to be different from that of the Ca^{2+} pump.

Furthermore, the binding of two mC/N9 molecules induced a drastic structural change in Ca^{2+}/CaM that could be detected not only by SAXS and NMR but also by gel mobility assay, followed by a slight structural change with four mC/N9 molecules that caused small changes of the R_g value of SAXS, small shifts of peaks in the ^{1}H-^{15}N HSQC NMR spectra, and no band shift on nondenaturing urea-PAGE. It was shown by the SAXS analyses that the overall shape changed from an elongated structure to a globular structure in solution, but the final conformation appeared to be different from the known compact globular structures of CaM induced by the binding of TFP/W-7 (Cook et al., 1994; Osawa et al., 1999) or skeletal muscle myosin light chain kinase (MLCK) (Ikura et al., 1992). An example where not one but two peptides bound to Ca^{2+}/CaM has been also reported (Yuan & Vogel, 1998).

The globular structures of Ca^{2+}/CaM in complexes with its target molecules, such as M13 or TFP, are stabilized by extensive van der Waals interactions in addition to electrostatic interactions, where the target peptide forming an α-helix binds to both the CaM domains simultaneously (Ikura et al., 1992). In contrast, in the case of W-7, which is an analogue of TFP and has a binding affinity with Ca^{2+}/CaM about 10^3 times lower than those of the target peptides, a similar globular structure to that of Ca^{2+}/CaM is induced by the main interaction of W-7 with the hydrophobic pocket of the two CaM domains, although the relative orientation of the two domains is not always fixed, and the time-averaged shape remains globular and compact (Osawa et al., 1999). In addition, it has been suggested that bridging of both the domains by a polypeptide chain of the target molecule is not necessary for the formation of the globular structure, and that the known globular structures are not necessarily required for the binding of the target molecules to Ca^{2+}/CaM.

These data suggest new insights for a structural aspect of CaM recognition. The differences in the R_g values and the $p(r)$ functions between the Ca^{2+}/CaM–mC/N9 complex and the Ca^{2+}/CaM–known CaM binding peptide (such as M13) complex may suggest the presence of a novel class of "myristoylated" CaM-binding proteins including CAP-23/NAP-22. Furthermore, considering the nonhelical conformation of mC/N9 in solution, we propose that the CaM binding domain of CAP-23/NAP-22, which has a basic-amphiphilic nature including the myristoyl moiety, should be a novel candidate for a structural motif of CaM recognition. At any rate, the elucidation of their three-dimensional structures should provide more precise information about the residues of these proteins involved in their binding to CaM, and the NMR analyses are now in progress.

mC/N9 and other nonmyristoylated targets: Features of the primary and secondary structure, and structural and functional implications of the myristoyl moiety

In the previous study, the CaM-binding domain of CAP-23/NAP-22 was identified as being located in the N-terminal region, and the interaction between Ca^{2+}/CaM and CAP-23/NAP-22 was assumed to be a novel type of protein–protein interaction (Takasaki et al., 1999). Unlike other CaM target proteins, CAP-23/NAP-22 lacked any canonical CaM-binding motif of a basic amphiphilic nature, suggesting that the myristoyl moiety of the protein plays a direct role in the protein–protein interaction.

In the case of M13 (the CaM-binding domain of MLCK), the amphiphilic nature of the peptide required for its binding to CaM is induced by the α-helical conformation. The CaM-binding domain of CAP-23/NAP-22 adopts a nonhelical conformation in the Ca^{2+}/CaM-complex (Takasaki et al., 1999). The N-terminal domain of CAP-23/NAP-22 contains one hydrophobic residue (Leu4) in addition to five basic residues (Lys3, Lys6, Lys7, Lys8, and Lys9). In this domain, one hydrophobic acyl group (N-terminal myristoyl moiety) is followed by one basic residue (Lys3) and then one hydrophobic residue (Leu4). This is actually reminiscent of the canonical CaM-binding motif, in which positively charged hy-

drophilic and hydrophobic residues appear alternately (Blumenthal et al., 1985; O'Neil & DeGrado, 1990). If the acyl group is substituted for a large hydrophobic residue, such as Trp or Leu found in the canonical CaM-binding motif, the overall structural characteristics seem to be very similar to each other. The distance between the myristoyl moiety and Leu4 is comparable to that between the two critical hydrophobic residues found in M13 (Fig. 7; shown in red). Although the chemical structure of TFP/W-7 clearly differs from that of mC/N9 or M13, it also contains hydrophobic groups (Fig. 7; shown in red) as well as positively charged groups (Fig. 7; shown in blue), and these groups cause the amphiphilic nature of the molecule. All these observations together seem to emphasize the importance of the amphiphilic nature required for the binding of CaM binding molecules, of not only proteins but also bioactive small molecules, to Ca^{2+}/CaM.

Phosphorylation of Ser5 (Fig. 7; shown in yellow) in the N-terminal region of CAP-23/NAP-22 by PKC abolished the binding of CAP-23/NAP-22 to Ca^{2+}/CaM (Takasaki et al., 1999). This was assumed to be caused by the introduction of an oppositely-charged group into the middle of the basic residues essential in making the ionic contact with the negatively-charged CaM.

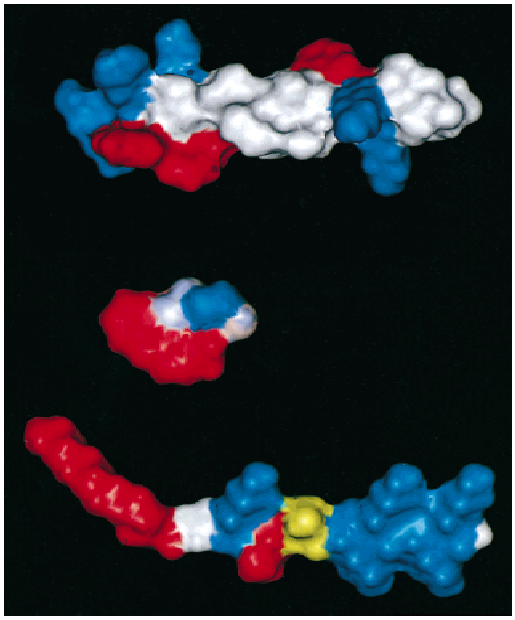

Fig. 7. A comparison among the canonical CaM-binding peptide, TFP, and the myristoylated mC/N9. Space-filling model of the M13 peptide derived from skeletal muscle MLCK in a helical conformation (top); the hydrophobic amino acid residues that play important roles in the CaM interaction are shown in red, and the positively charged amino acid residues are shown in blue. TFP (middle); the hydrophobic aromatic group is shown in red, and the positively charged group is shown in blue. The myristoylated N-terminal peptide of CAP-23/NAP-22 (myr-GGKLSKKKK) in an elongated structure (bottom); the myristoyl moiety and Leu4 are shown in red; the positively charged amino acid residues are shown in blue, and one phosphorylatable amino acid residue, Ser5, is shown in yellow. All of these molecules include the basic amphiphilic natures (basic group, blue; hydrophobic group, red) in them. The models were constructed and rendered on an IRIS Indigo 2 workstation (SGI) using Insight II (Molecular Simulations, Burlington, Massachusetts) and SYBYL/BASE software (Tripos, Inc., St. Louis, Missouri).

Functional implications of the myristoyl moiety of other myristoylated proteins

Myristoylation is one of the often identified post-translational modifications of proteins. It was first identified in the catalytic subunit of cAMP-dependent protein kinase (Carr et al., 1982). The hydrophobic acyl group is often involved in protein-membrane interactions. Due to its intermediate hydrophobicity, the myristoyl moiety plays an important role in reversible membrane associations of myristoylated proteins including the phosphorylation-dependent membrane interaction of MARCKS with membranes (Taniguchi & Manenti, 1993; Kim et al., 1994). The binding of Ca^{2+} to some proteins, such as recoverin (Ames et al., 1997) and flagellar calcium-binding protein (Godsel & Engman, 1999), induces drastic conformational changes of these proteins, so that the myristoyl group hidden inside the proteins becomes exposed and can interact with membranes. Interestingly, the presence of only the myristoyl group is insufficient for stable membrane anchoring of myristoylated proteins. With the myristoyl moiety, the cooperation of the basic residue cluster often found near the acyl group makes stable membrane anchoring possible (Resh, 1994; McLaughlin & Aderem, 1995). However, even if the cooperative effects of the basic residue cluster are taken into account, in the case of large proteins, the myristoyl group is presumed not to have sufficient ability to anchor the myristoylated proteins on membranes. The comparison of myristoylated proteins of various molecular sizes (Table S1, see Supplementary material in the Electronic Appendix) suggests that not all the myristoyl groups are sufficient by themselves for the anchoring, and one might predict the presence of other functions of myristoylation.

The alignment of the N-terminal amino acid sequence (10 residues) in many myristoylated proteins including CAP-23/NAP-22 is given in Table S1 (see Supplementary material in the Electronic Appendix). Like CAP-23/NAP-22, many myristoylated proteins also have basic residues following the myristoyl moiety. Furthermore, some of them also have phosphorylatable residue(s) in the N-terminal domain. Thus, it is reasonably presumed that some myristoylated proteins, as well as CAP-23/NAP-22, bind to CaM through the N-terminal domain. These observations suggest that the signal transduction pathways and/or the catalytic functions regulated by these myristoylated proteins are directly regulated by Ca^{2+}/CaM in a myristoylation-dependent manner, and the regulations are controlled by phosphorylation.

Conclusion

The present SAXS results indicate that the binding of mC/N9, the myristoylated N-terminal nonapeptide of CAP-23/NAP-22, to Ca^{2+}/CaM induces an insignificant structural change in Ca^{2+}/CaM at a molar ratio of 1:1. On the other hand, at a molar ratio of 1:2, the binding of mC/N9 to Ca^{2+}/CaM induces a drastic structural change in Ca^{2+}/CaM, as suggested from the inconsistency of the R_g values for the Ca^{2+}/CaM–mC/N9 complex compared to those for Ca^{2+}/CaM—known target molecule complexes. A slight structural change in Ca^{2+}/CaM with four mC/N9 molecules was also detected by SAXS and NMR analyses, although the biological significance of this 1:4 complex remains unclear. In spite of the difference in the final conformations, the comparison of target molecule structures has so far revealed the importance of the basic amphiphilic nature for the binding to CaM in all the cases. The present SAXS results and the N-terminal amino acid sequence

comparison of myristoylated proteins suggest the presence of a novel class of CaM-binding proteins including CAP-23/NAP-22, and raise a possibility that protein myristoylation plays direct roles not only in the binding of CAP-23/NAP-22 to CaM, but also in protein–protein interactions related to other myristoylated proteins.

Materials and methods

Sample preparation

Myristoylated CAP-23/NAP-22 peptide (myr-GGKLSKKKK: mC/N9) was purchased from Research Genetics Inc. (Huntsville, Alabama). Rat CaM was expressed in *Escherichia coli* and purified to homogeneity as previously described (Hayashi et al., 1998). For the SAXS experiments, the recombinant CaM was dissolved in Tris buffer (50 mM Tris-HCl, pH7.6) containing 120 mM NaCl and a sufficient amount (five molar equivalents relative to CaM) of $CaCl_2$. The solutions for Ca^{2+}/CaM and the Ca^{2+}/CaM–mC/N9 complex were prepared with five equivalents of Ca^{2+} ion, considering the molar ratio of Ca^{2+} ion to CaM in the Ca^{2+}/CaM crystal or solution structure; CaM:Ca^{2+} = 1:4 (Seaton et al., 1985; Kretsinger et al., 1986; Persechini & Kretsinger, 1988; Heidorn et al., 1989; Barbato et al., 1992; Finn et al., 1995; Spoel et al., 1996). The protein concentration was determined by amino acid analysis. The solutions for the Ca^{2+}/CaM–mC/N9 mixtures with molar ratios of 1:1, 1:2, 1:3, 1:4, and 1:5 were each prepared at a CaM concentration of 9.0 mg/mL. The solutions for the Ca^{2+}/CaM–mC/N9 mixtures with a molar ratio of 1:4 were prepared at CaM concentrations of 6.0, 9.0, 12.0, 15.0, and 18.0 mg/mL.

SAXS

The measurements were performed using synchrotron orbital radiation with an instrument for SAXS installed at BL-10C of Photon Factory, Tsukuba (Ueki et al., 1985). An X-ray wavelength of 1.488 Å was selected. The samples were contained in a quartz cell with a volume of 80 μL, and the temperature was maintained at 25 ± 0.1 °C by circulating water through the sample holder. The reciprocal parameter Q, equal to $4\pi \sin\theta/\lambda$, was calibrated by the observation of peaks from dried chicken collagen, in which 2θ was the scattering angle and λ was the X-ray wavelength. Scattering data were collected for 250 or 300 s at individual protein concentrations.

Two methods of data analysis were used. The first method was that of Guinier (Guinier, 1939; Glatter, 1982), which gives the R_g. The range of Q (Å^{-1}) used for Guinier plots was 0.02 to 0.09 for the Ca^{2+}/CaM–mC/N9 complex and 0.02 to 0.07 for the Ca^{2+}-saturated CaM in isolation. The second method was the calculation of $p(r)$, which is the frequency of the distances r within a macromolecule obtained by combining any volume element with any other volume element (Glatter, 1982). The $p(r)$ was calculated by a direct Fourier transformation (Glatter, 1982). Data to Q (Å^{-1}) = 0.7 were used for $p(r)$ analysis. The d_{max} was also estimated from the $p(r)$. $p(r)$ becomes zero at values of r equal to or greater than the maximum d_{max} of the particle.

NMR spectroscopy

All NMR experiments were carried out on a Bruker DMX-500 spectrometer using a 5 mm broadband, z-axis gradient-shielded probe at 298 K. Two-dimensional ^{1}H-^{15}N HSQC spectra were obtained by pulse field gradient selection (Grzesiek & Bax, 1993). The sweep width was 12 ppm in the ^{1}H dimension and 30 ppm in the ^{15}N dimension, with the ^{1}H carrier set at 500.1324 MHz and the ^{15}N carrier at 50.6814 MHz. The size of the HSQC spectra was a 1,024 × 1,024 real data matrix with eight scans for each experiment. Proton chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate as 0 ppm. Nitrogen-15 chemical shifts were referenced to liquid NH_3. NMR spectra were processed on a Silicon Graphics Indigo2 workstation using Bruker XWIN-NMR and MSI Felix 95.0 software packages. The partial and tentative assignments were obtained referring to Ikura et al. (1990).

Nondenaturing urea-PAGE

Nondenaturing urea-PAGE gel band shift assays were performed using a published procedure (Erickson-Viitanen & DeGrado, 1987). The urea was normally included in the gel to prevent the formation of nonspecific interactions.

Supplementary material in the Electronic Appendix

Table S1. N-terminal amino acid sequence alignment of human myristoylated proteins; CAP-23/NAP-22 (Takasaki et al., 1999), (2′-5′)oligoadenylate synthetase (Sarkar et al., 1999), annexin XIII (Wice & Gordon, 1992), guanylate cyclase activating protein 1 (Olshevskaya et al., 1997), NADH-ubiquinone oxidoreductase B18 subunit, NADH-cytochrome b5 reductase (Borgese et al., 1996), endothelial nitric oxide synthase (Liu & Sessa, 1994; Feron et al., 1998), acetylcholine receptor-associated 43 kD protein (Musil et al., 1988), T-lymphoma invasion and metastasis inducing protein 1, visinin like protein 1 (Spilker et al., 1997), recoverin (Ames et al., 1995), calcineurin B (Zhu et al., 1995; Klee et al., 1998), neuron-specific calcium-binding protein hippocalcin (Kobayashi et al., 1993), neurocalcin δ (Faurobert et al., 1996), calcium-binding protein P22/calcineurin homologous protein, cAMP-dependent protein kinase, α, β, and γ catalytic subunit (Carr et al., 1982), tyrosine protein kinase transforming protein src (p60-Src) (Glover et al., 1988), proto-oncogene tyrosine protein kinase src (p60-Src) (Glover et al., 1988; Rocca et al., 1997), b lymphocyte tyrosine protein kinase (Koegl et al., 1994), proto-oncogene tyrosine protein kinase Fyn (p59-Fyn) (van't Hof & Resh, 1999), tyrosine protein kinase Hck (hemopoietic cell kinase) (p59-Hck and p60-Hck) (Robbins et al., 1995), proto-oncogene tyrosine protein kinase Lck (p56-Lck) (Zlatkine et al., 1997), tyrosine protein kinase Lyn, proto-oncogene tyrosine protein kinase Yes (p61-Yes) (Koegl et al., 1994), ADP-ribosylation factor 1, 3, 4, 5, and 6 (Haun et al., 1993), human immunodeficiency virus type 1 gag polyprotein (Shoji et al., 1988), human immunodeficiency virus type 1 negative factor (Guy et al., 1987), guanine nucleotide-binding protein G_o, α subunit 1 and 2, G_i, α subunit 1 and 2, G_t, α subunit 1 and 2 (Mumby et al., 1990), MARCKS (Harlan et al., 1991), MARCKS-related protein, F52 protein (Blackshear et al., 1992) (including predicted myristoylated proteins).

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