Circular Dichroism and $^1$H Nuclear Magnetic Resonance Studies on the Solution and Membrane Structures of GAP-43 Calmodulin-binding Domain*

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Growth-associated protein-43 (GAP-43) is believed to be palmitoylated near the N terminus and the modification is assumed to be involved in the membrane anchoring of the protein. However, GAP-43 isolated from bovine brain is not palmitoylated as shown by mass spectrometric analysis, but still retains the ability to bind phospholipids, suggesting that other parts of the molecule are involved in the interaction. Upon addition of acidic phospholipids, purified GAP-43 showed a conformational change from random coil to α-helix as indicated by a change in CD spectra. A synthetic peptide corresponding to the calmodulin-binding domain showed a similar conformational change from random coil to α-helix in the presence of various acidic phospholipids. These results suggest that the calmodulin-binding domain of GAP-43 is directly involved in the GAP-43-membrane interaction and undergoes a conformational change upon binding to phospholipid membranes. After phosphorylation by protein kinase C, the phospholipid-induced conformational changes were no longer observed. Structural characteristics of the calmodulin-binding domain peptide in aqueous and hydrophobic solvents were further studied in detail by two-dimensional $^1$H nuclear magnetic resonance. The results obtained suggest that the domain assumes a nascent α-helical structure in aqueous solution, which is stabilized under hydrophobic environments.

Although the protein has been believed to be a specific in vivo substrate of protein kinase C (PKC), recent studies on the in vivo phosphorylation sites revealed that the protein is a substrate of so-called proline-directed protein kinases as well (3, 4). Since casein kinase II has also been shown to phosphorylate GAP-43 in vitro (5, 6), physiological functions of GAP-43 such as sequestering of calmodulin (7) and signal transduction through the binding to $G_{o}$ (8) may be regulated through various protein kinases in a very complex manner.

GAP-43 is found associated with membrane fractions or with so-called membrane-cytoskeleton fractions (9). Since the protein is very hydrophilic and lacks any apparent membrane-binding domain (10), the interaction of the protein with membranes is thought to be effected by palmitoylation at two cysteine residues near the N terminus (11, 12). However, our recent mass spectrometric study on the in vivo post-translational modifications of GAP-43 revealed that the protein isolated from the membrane fractions of adult brain lacks the palmitoyl group (3), suggesting that part(s) of the molecule other than the palmitoyl moiety is involved in the interaction of the protein with the membrane. In fact, the involvement of the calmodulin-binding domain, which is at the same time the phosphorylation domain by PKC, in the membrane association has been suggested (13, 14).

We have previously shown that the PKC phosphorylation and calmodulin-binding domain of myristoylated alanine-rich protein kinase C substrate, another in vivo major substrate of PKC belonging to the same family of acidic hydrophilic membrane-associated proteins (15), is directly involved in the interaction of the protein with membrane phospholipids (16). Furthermore, the phosphorylation of the domain regulates the reversible membrane association of myristoylated alanine-rich protein kinase C substrate (14, 16). Thus, the calmodulin-binding domain of basic amphiphilic α-helical nature seems to serve as a phosphorylation- and calmodulin-dependent membrane binding motif as well (16, 17). A similar polybasic motif found in the Src family proteins has been implicated in the membrane association of these proteins (18, 19).

In the present study, structural characteristics of the calmodulin-binding domain of GAP-43 were studied by circular dichroism (CD) spectroscopy and two-dimensional $^1$H nuclear magnetic resonance (NMR). Purified non-palmitoylated GAP-43 and a synthetic peptide corresponding to the domain showed similar conformational changes from random coil to α-helix upon binding to acidic phospholipids. The calmodulin-binding domain of GAP-43 seems to assume a "nascent" α-helical structure in aqueous solution, which is further stabilized under hydrophobic environments.

EXPERIMENTAL PROCEDURES

Materials—GAP-43 was purified from membrane fractions of bovine brain after detergent extraction as described (3). PKC was purified from...
bovine brain as described previously (20). A peptide (QAS- 
FRGHIIRKLIGEKEK) corresponding to the calmodulin-binding
domain of GAP-43, named GAP peptide, was synthesized using con-
ventional t-butoxycarbonyl chemistry using an ABI 430A peptide
synthesizer (Applied Biosystems), and purified over a C18 reversed-
phase high-performance liquid chromatography (HPLC) column.
Phospholipids (Avanti Polar Lipids) were suspended in 5 mM
phosphate buffer (pH 7.5) and sonicated in a sonicator (Branson
Sonifier 250) for 30 min. After centrifugation in a tabletop centri
guge for 20 min, the supernatant was used as unilamellar liposomes.

**Analysis of Phosphorylated GAP-43 and GAP Peptide—** Phos-
phorylation of the intact GAP-43 and GAP peptide by PKC was car-
ried out in 25 mM Tris-HCl buffer (pH 7.5) containing 10 mM
MgCl₂, 100 μM CaCl₂, 80 μg/ml phosphatidyserine (PS), 8 μg/ml dioleoyl glycerol, and 1 mM ATP at 35 °C for 90 min. The reaction was stopped by adding 0.1% final concentration of trifluoroacetic acid. The extent of the phos-
phorylation was analyzed by mass spectrometry as described previously (3, 21). The phosphorylated GAP peptide was purified by reversed-phase HPLC using a Vydac C18 column (218TP52, 0.46 × 25 cm). The phosphorylated protein was concentrated by ultra-
filtration using a Centricon 10 cartridge (Amicon).

**Mass Spectrometric Analysis—** GAP-43 purified from the mem-
brane fractions of bovine brain by the detergent extraction method (3, 20) was digested with L-1-lysylamido-2-phenylethyl chloromethyl ketone-
treated trypsin (Whartinongton), and the peptides were separated over a reversed-phase HPLC column as described previously (3, 21). Fractions containing the N-terminal peptide were combined and dried with a Speed-Vac concentrator. The peptide was dissolved in 5 ml of 50%
acetonitrile containing 0.1% trifluoroacetic acid. Electrospray tandem mass spectra were recorded in a PE Sciex API-III mass spectrometer in tandem mass mode. A singly-charged precursor ion was selected with Q1 for collision-induced decomposition with argon in Q2. Fragment ion spectra were obtained by scanning Q3 of the triple quadrupole system.

**Circular Dichroism (CD) Spectrometry—** CD spectra were recorded at 25 °C in a JASCO J-720 CD spectropolarimeter using a 0.1-cm cell. The concentration of the peptide was 20 μM in 5 mM phosphate buffer (pH 7.3) unless otherwise indicated. The contents of α-helix, β-sheet, and β-turn structures were estimated from the CD spectra on an IRIS Indigo X524 workstation (Silicon Graphics, Inc.) using a CONTIN pro-
gram (22) modified by Dr. F. Ariasaka, Tokyo Institute of Technology.

**H NMR Spectrometric Analysis—** 1H NMR spectra were recorded on a Bruker DMX-500 spectrometer operating at 500 MHz. Chemical shifts were measured relative to the methyl resonance of 4,4-dimethyl-4-
silapentane-1-sulfonate, used as an internal reference. GAP peptide (5 mM) was dissolved in H₂O/D₂O (9/1, v/v), D₂O (99.98%), H₂O/D₂O/tri-
fluoroethanol (TFE)–d₂ (5/4/1), or D₂O/TFFE–d₂ (6/4). The pH of the samples was 4.0 (direct meter reading). The sequence-specific assign-
ment of resonances was obtained from two-dimensional TOCSY (24, 25), NOESY (23), DQF-COSY with phase cycling (26) or with pulsed field
gradient (27, 28), and TQF-COSY with pulsed field gradient (27, 28) spectra, which were acquired at 25 °C in the phase-sensitive mode using the time proportional phase increment technique. Various mixing times were used for NOESY (200, 100, 50, 25, and 10 ms) and TOCSY (75, 50, and 25 ms).

**Characterization of GAP-43 Isolated from Bovine Brain—** GAP-43 purified from membrane fractions of bovine brain was digested with trypsin and the resulting peptide mixture was analyzed by the liquid chromatography-electrospray mass spectrometry (3, 21). One peptide with a mass of 796.3 Da has been tentatively assigned as the N-terminal peptide in the previous study (3). The N-terminal peptide should give a the-
oritical mass of 796.3 Da, when the N-terminal acetylation is assumed. The reversed-phase column fractions containing the peptide were combined, and the sample was analyzed by the electrospray tandem mass spectrometry (3). In this technique, a precursor ion corresponding to the peptide is selected with the first quadrupole mass analyzer, and the fragments ob-
tained by the collision with argon are analyzed using the second
mass analyzer. Most of the cleavage occur randomly in the middle of the peptide bonds, resulting in the formation of “ladder” mixtures (33). As shown in Fig. 1, the tandem mass spectrum of the peptide clearly indicated that the N-terminal is acetylated, and that the two Cys residues, which have been as-
scribed to be palmitoylated (11), are not palmitoylated at all, but form an intramolecular disulfide bond. GAP-43 has only two Cys residues, and no other modification has been detected by mass spectrometric analysis (3). Since the mass spectromet-
ic analysis of the whole protein showed a single peak with a minor peak corresponding to a phosphorylated species (3), we con-
cluded that the GAP-43 protein as isolated does not contain palmitoylated species in significant amounts.
To confirm that the non-palmitoylated GAP-43 still has the ability to bind to phospholipid membranes, the binding was studied by sedimentation analysis. When GAP-43 was mixed with multilamellar liposomes made of a mixture of PC (80%) and PS (20%), which mimics the compositions of biomembranes, at a physiological ionic strength, most of GAP-43 (89%) was found associated with the lipids (Fig. 2a). No binding was observed with pure PC liposomes (data not shown). The non-palmitoylated GAP-43, therefore, still retains the ability to bind to phospholipid membranes containing physiologically relevant combinations of lipids. A synthetic peptide corresponding to the calmodulin-binding domain of GAP-43 showed a similar binding to the phospholipid membranes. With increasing concentrations of vesicles containing PC (80%) and PS (20%), the amounts of peptide remained in the supernatant were determined by densitometry and plotted against lipid concentration (●). A similar analysis was done with pure PC liposomes (▲).

Conformational Change of GAP-43 Induced by Phospholipid

Binding—To get more insights into the mode of the GAP-43-phospholipid interactions, effects of phospholipids on the conformation of GAP-43 were studied by CD spectroscopy. Purified GAP-43 in aqueous buffer showed a CD spectrum with a single large negative peak at around 200 nm, and a typical CD spectrum of random coil with a single large negative peak below 200 nm was observed when vesicles containing pure PC was used (Fig. 4a), suggesting that the protein has a random structure. The addition of an acidic phospholipid, phosphatidylglycerol (PG), caused a small but significant change in the CD spectrum, which was dependent on the concentration of PG added. There was a decrease in the intensity and a shift of the largest negative peak around 200 nm with a concomitant increase in the negative ellipticity between 210 and 230 nm. The characteristics of the change can be more clearly observed in the difference spectrum (Fig. 4b). A broad negative peak between 210 and 230 nm with a positive peak below 200 nm suggests that a part of the protein molecule underwent a conformational change from random coil to α-helix.

Interaction of Calmodulin-binding Domain Peptide with Phospholipids—The CD spectrum of the GAP peptide showed again a typical CD spectrum of random coil with a single large negative peak at around 198 nm (Fig. 4c). The partition constant of peptide binding to PC liposomes (0.98 ± 0.01 M) was incubated with increasing amounts of the same liposomes, and the peptide remained in the supernatant was analyzed by SDS-gel electrophoresis. Concentrations of liposomes were 0, 0.1, 0.5, 1.0, 3.0, 5.0, and 9.6 mg/ml. A similar study with the whole GAP-43 protein was not feasible due to the amounts of samples needed and its tendency to aggregate at high concentrations.
with GAP peptide, and CD spectra were recorded to study the phospholipid specificity. Although neutral phospholipids such as PC did not affect the spectrum significantly, all acidic phospholipids examined caused similar changes in the CD spectra (Fig. 5). Although the final spectral change observed at saturating concentrations were very similar regardless of the phospholipid used (data not shown), the affinities of various acidic phospholipids varied appreciably, suggesting that the binding of the peptide to the phospholipids involves not only ionic but also specific structural interactions. Among various acidic phospholipids, phosphatidic acid showed the highest affinity to GAP peptide (20 \( \mu \text{M} \)).

\[ \text{CD spectra of GAP-43 and those of GAP peptide.} \]

**Effects of TFE on the Conformation of GAP Peptide**—To study the structural characteristics of the calmodulin-binding domain of GAP-43 under hydrophobic environments in detail, effects of TFE, a membrane mimicking reagent, on the conformation of GAP peptide were studied. As shown in Fig. 6, the addition of TFE caused a concentration dependent induction of a CD spectrum typical for \( \alpha \)-helix with two negative peaks at 222 and 208 nm, a cross-over near 200 nm, and a maximum near 192 nm (35). The \( \alpha \)-helical content reached almost 100% in 30% TFE, and the \( \alpha \)-helical content of GAP peptide (20 \( \mu \text{M} \)) was increased about two-thirds of the N-terminal side of the peptide, assumes a regular \( \alpha \)-helix (38, 39) as shown in Fig. 8 (a, inset). The \( \alpha \)-helix (38, 39) is characterized by two negative peaks at 222 and 208 nm, a cross-over near 200 nm, and a maximum near 192 nm (35). The \( \alpha \)-helical content reached almost 100% in 40% TFE (Fig. 6, inset). Therefore the secondary structure of GAP peptide was further analyzed by two-dimensional \( ^1 \text{H} \) NMR. To reduce the amide exchange rate and simultaneously increase the correlation time of the peptide, most of the spectra were obtained at acidic pH (Fig. 7a). The assignment of the NMR resonances was obtained by following standard procedures for two-dimensional \( ^1 \text{H} \) NMR of proteins (36). Fig. 8a summarizes the short and medium range NOE cross-peaks observed with GAP peptide. A substantial number of \( \alpha \beta (i, i+3) \) and \( \alpha \text{N}(i, i+3) \) NOE cross-peaks were observed with GAP peptide in 50% H\(_2\)O, 10% D\(_2\)O, 40% TFE-d\(_4\) solution, suggesting that GAP peptide assumes an \( \alpha \)-helical structure in the presence of TFE. Furthermore, comparison of the chemical shifts of the \( \alpha \) protons observed with GAP peptide to those obtained with peptides in random coil (37) shows upfield shifts characteristic for \( \alpha \)-helix (38, 39) as shown in Fig. 8b. Altogether, these results established that GAP peptide, especially about two-thirds of the N-terminal side of the peptide, assumes a regular \( \alpha \)-helix in the presence of TFE.

**Conformation of GAP Peptide in Aqueous Solution**—In the absence of TFE, many cross-peaks in the NMR spectra could not be assigned due to resonance overlaps. However, it was possible to assign several residues from their unique spin systems. Since Ala\(^2\), Ile\(^8\), Thr\(^9\), and Leu\(^13\) occurs only once in the peptide, their well resolved methyl group signals in higher magnetic field region were used to assign peaks belonging to these residues. Interestingly, the chemical shifts of all the as-
The overlapped signals of cross-peaks between typical shifts between those observed with typical random coil (36, 37) are plotted (b). Negative values correspond to higher magnetic field shifts.

Effects of Phosphorylation on GAP-43-membrane Interaction—To examine effects of PKC-dependent phosphorylation of GAP-43 on the GAP-43-membrane interaction, GAP-43 and GAP peptide were phosphorylated by PKC and purified as described under “Experimental Procedures.” The basic domain of GAP-43 contains only one phosphorylatable residue (Ser41) and the phosphorylation of the protein prevents its binding to calmodulin (7). Phosphorylated GAP-43 and phosphorylated GAP peptide showed CD spectra similar to those of non-phosphorylated species. When acidic phospholipids such as PG was added, no significant change in the CD spectra was observed with both the phosphorylated protein and the peptide (data not shown), suggesting that the phosphorylation by PKC reduced their affinities to the phospholipids. Phosphorylation of GAP peptide has already been shown to affect the interaction (14). It is easily conceivable that the incorporation of negative charges reduces the net positive charge of the basic domain, which should affect the electrostatic interaction involved in the binding.

Although the CD spectrum of the phosphorylated peptide was very similar to that of the non-phosphorylated peptide in aqueous solution, those obtained in the presence of TFE differed significantly. Even in the presence of 50% TFE, the phosphorylated peptide showed an intermediate state between the random and α-helical structures (Fig. 9), similar to that observed with the non-phosphorylated peptide in 20% TFE (Fig. 6). These results suggest that phosphorylation of the Ser41, which is located near the N terminus of the peptide, not only changes the net charge of the peptide, but also impairs significantly the ability of the peptide to form the α-helical structure.

Effects of Calmodulin on GAP-43-membrane Interaction—Since the same domain shows the ability to bind both acidic phospholipids and calmodulin, it is of interest to examine the effects of calmodulin on the GAP-43-membrane interactions. As shown in Fig. 10, the addition of calmodulin to the membrane-bound GAP-43 reversed the binding, and a part of the protein was found in the supernatant. In the presence of 0.15 M KCl, the amounts of GAP-43 remained in the supernatants were similar regardless of Ca2+ (40). However, at a low ionic strength, where GAP-43 shows higher affinity to apo-calmodulin than to Ca2+-calmodulin (40), the amounts of GAP-43 remained in the supernatants was very similar to that of the non-phosphorylated peptide in 20% TFE (Fig. 6). These results suggest that phosphorylation of the Ser41, which is located near the N terminus of the peptide, not only changes the net charge of the peptide, but also impairs significantly the ability of the peptide to form the α-helical structure.

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Fig. 9. Effects of TFE on CD spectra of phosphorylated GAP peptide. GAP peptide was phosphorylated by PKC, and purified over an HPLC reversed-phase column as described under “Experimental Procedures.” CD spectra of phosphorylated GAP peptide (20 μM) were measured in the absence (○) or presence (●) of 50% TFE.

Fig. 10. Effects of calmodulin on GAP-43-phospholipid interaction. GAP-43 (1 μM) was mixed with PS liposomes (1 mM), and incubated at 25 °C for 30 min in the presence of 0.5 mM Ca²⁺ or 2 mM EGTA. The solutions were divided, and calmodulin (CaM) (35 μM) was added to samples indicated. The protein remained in supernatant after centrifugation was analyzed as described in the legend to Fig. 2. The experiments were carried out in 5 mM phosphate buffer (pH 7.3) containing 150 mM KCl (a) or in 20 mM phosphate buffer containing no additional salt (b). The relative amounts of GAP-43 remained in supernatants were determined by densitometry to be 4.7% (+PS), 11.4% (+PS + Ca²⁺), 60.9% (+PS + calmodulin), and 61.1% (+PS + calmodulin + Ca²⁺) at 150 mM KCl. The corresponding values obtained at low ionic strength were 3.9% (+PS), 8.9% (+PS + Ca²⁺), 35.1% (+PS + calmodulin), and 19.1% (+PS + calmodulin + Ca²⁺).

DISCUSSION

Although GAP-43 has been reported to be palmitoylated and the acylation has been believed to be responsible for the membrane localization of the protein, our previous (3) and present studies established that GAP-43 purified from membrane fractions is not palmitoylated but still retains the ability to bind to phospholipid membranes. Since GAP-43 is a very hydrophilic protein without any clear membrane-binding domain, this seems to be puzzling. However, we have already shown that myristoylated alanine-rich protein kinase C substrate, which itself is a major PKC substrate protein and shows stimulation-dependent reversible membrane binding, interacts with phospholipid membranes through the PKC phosphorylation domain of basic amphiphilic nature (16). A similar observation has been reported with the GAP-43 calmodulin-binding domain (14). The calmodulin-binding domain of nitric oxide synthase, too, seems to serve as a membrane-binding domain (41). Although the calmodulin-binding motif has no clear conserved amino acid sequence, basic hydrophilic and hydrophobic amino acids appear alternately at certain intervals. When it assumes an α-helical structure, the two groups of amino acids segregate on opposite sides of the helices (42, 43). The calmodulin-binding domain of GAP-43, which is at the same time the phosphorylation domain by PKC, belongs to the same phosphorylation-dependent membrane binding motif.

As shown in the present study, both the intact protein and the phosphorylation domain of GAP-43 bind to phospholipid membranes and adopt α-helical structures under hydrophobic environments. The degree of change in the CD spectra of the whole protein induced by the phospholipid binding is similar to that observed with GAP peptide, suggesting that upon phospholipid binding only the phosphorylation domain undergoes a conformational change while the conformation of the rest of the molecule remains unchanged. It seems reasonable to assume that only the phosphorylation domain of GAP-43 interacts with phospholipid liposomes, since the whole protein except for the domain is very hydrophilic and acidic.

Compared with conventional CD measurements, two-dimensional 1H NMR studies gave more accurate and residue-specific information on the conformation. Most parts of the peptide, especially the N-terminal two-thirds, formed a regular α-helix in the presence of TFE, as was evidenced by the consecutive NOE connectivities and the characteristic upfield shift of α protons. The C-terminal side of the peptide, on the other hand, showed less tendency to form an α-helix. The advantage to use the NMR technique was more pronounced in the analysis of the conformation of the peptide in aqueous solution. The CD spectrum of GAP peptide in aqueous solution showed no indication of the presence of any regular structure. On the other hand, chemical shifts of most of the α protons obtained in aqueous solution showed intermediate values between those observable with an α-helix and those seen with “true” random coil. So-called random coil can be considered to be a set of many different conformations, within which rapid exchanges occur. While the CD spectroscopy does not indicate the nature of the conformations included in such sets of the conformations, the NMR studies suggest that a significant portion of the peptide molecules adopts an α-helical conformation. Such a nascent helical structure may deviate from ideal geometry, and/or the ends of the α-helix can fray (44, 45). The interaction of GAP peptide with phospholipids seems to stabilize the conformation to induce an α-helical structure. Several calmodulin-binding peptides have been reported to form such a nascent α-helical structure in aqueous solution. Such a structure is usually further stabilized either by addition of TFE (46, 47) or by binding to...
It should be noted that the shape of the CD spectrum obtained in the presence of phospholipids was not the same as that observed in TFE, suggesting that the conformation of GAP peptide bound to phospholipids is not a simple α-helix. Since the extents of the change observed upon phospholipid binding were also smaller than those observed in the presence of TFE, it is possible that only a part of the domain undergoes such a conformational change. A more detailed study of the interaction between phospholipid liposomes and the GAP-43 phosphorylation domain by NMR is now in progress.

The calmodulin-binding domain of GAP-43 interacts with membrane phospholipids, and the phosphorylation by PKC reduces the interaction. The phosphorylation seems to affect the interaction not only by reducing the positive charge of the domain but also by impairing the ability of the domain to form an α-helical nature which involves various components. The interaction of the domain with PKC and phospholipids, physiological functions of GAP-43, whatever it is, are regulated in a very complex manner which involves various components of the signal transduction pathways (Fig. 11). The domain of basic amphiphilic α-helical nature may function as one of the cross-talk points in the calcium-dependent signal transduction.

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