Phosphatidylserine Liposomes Can Be Tethered by Caldesmon to Actin Filaments

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ABSTRACT Rotary shadowing electron microscopy revealed that attachment of caldesmon to phosphatidylserine (PS) liposomes was mainly through its C-terminal end. To determine the PS-binding sites of caldesmon, we have made use of synthetic peptides covering the two C-terminal calmodulin binding sites and a recombinant fragment corresponding to the N-terminal end of the C-terminal domain that contains an amphipathic helix. Interactions of these peptides with the PS liposomes were studied by nondenaturing gel electrophoresis and fluorescence spectroscopy. The results showed that both calmodulin-binding sites of caldesmon were able to interact with PS. The affinity (K_d) of PS for these sites was in the range of 1.8–14.3 $\times$ 10^{-5} M, compared to 0.69 $\times$ 10^{-5} M for the whole caldesmon molecule. Fragments located outside of calmodulin-binding sites bound PS weakly (3.85 $\times$ 10^{-4} M) and thus may contain a second class of lipid-binding sites. Binding of PS induced conformational changes in regions other than the C-terminal PS-binding sites, as evidenced by the changes in the susceptibility to proteolytic cleavages. Most significantly, the presence of caldesmon greatly increased binding of PS to F-actin, suggesting that caldesmon may tether PS liposomes to actin filaments. These results raise the possibility that caldesmon-lipid interactions could play a functionally important role in the assembly of contractile filaments near the membranes.

INTRODUCTION

Caldesmon (CaD) is a smooth muscle and nonmuscle thin filament component that interacts with contractile proteins including actin, myosin, and tropomyosin, and, in the presence of Ca^{2+}, with calmodulin (CaM) (Bryan and Wang, 1993; Dabrowska, 1994; Marston and Huber, 1996; Marston and Redwood, 1991; Matsumura and Yamashiro, 1993; Sobue and Sellers, 1991). It is generally believed that CaD takes part in the regulation of actomyosin interactions and that it may be involved in the organization of contractile filaments (Walsh, 1990), as well as assembly (Galazkiewicz et al., 1985, 1989) and stabilization of microfilaments (Dabrowska et al., 1996; Ishikawa et al., 1989a,b). The role of CaD in cell motility has been implicated by its participation in the secretion of catecholamine from adrenal chromaffin cells (Burgoyne et al., 1986), receptor capping in splenic T-lymphocytes (Mizushima et al., 1987) and T-lymphoma cells (Walker et al., 1989), changes in the shape and structure of cells during mitosis (Yamashiro et al., 1990, 1991), and cell attachment and spreading on substratum (Surgucheva and Bryan, 1995; Warren et al., 1994). In some cells CaD is localized in the subplasmalemmal region: in patches at the internal periphery of adrenal chromaffin cells (Burgoyne et al., 1986), directly beneath the caps of Con-A receptor (Mizushima et al., 1987; Walker et al., 1989), and in the ruffle regions of fibroblast membranes (Bretscher and Lynch, 1985). Such localization as well as binding to cytosolic granules during secretion (Burgoyne et al., 1986) suggest its direct interaction with phospholipid-containing membranes. Studies on the interaction of CaD with liposomes composed of various phospholipids, by the use of native gel electrophoresis, light scattering, and fluorescence spectroscopy, revealed complex formation with anionic phospholipids, particularly with phosphatidylserine (PS) (Czurylo et al., 1993; Vorotnikov et al., 1992; Vorotnikov and Gusev, 1990). The interaction of CaD with PS was reversed either by Ca^{2+}/CaM (Czurylo et al., 1993; Vorotnikov et al., 1992) or by phosphorylation of CaD catalyzed by protein kinase C (PKC) (Vorotnikov et al., 1992).

On the basis of sequence similarity to phospholipid-binding sites of other proteins, one or more of the following segments were proposed to be the PS-binding sites of CaD: 446–459, 655–668, and 718–731 (in analogy to neuromodulin and neuroregulin; see Vorotnikov et al., 1992), or 633–639 and 701–711 (in analogy to gelsolin, gCap39, cofilin, and profilin; see Bogatcheva et al., 1994). (All amino acid position numbers used in this work refer to the chicken gizzard CaD sequence according to Bryan et al., 1989.) The latter predictions are in good agreement with the assignment of residues 626–710 as the major PS-binding site on the basis of light scattering measurements of PS suspensions in the presence of CaD peptides (Bogatcheva et al., 1994). Analyses of the CaD sequence using the algorithm of Eisenberg et al. (1984) indicated that residues 649–662 form an amphipathic α-helix that may be involved in interactions with PS, as suggested by Bogatcheva and

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Gusev (1995). All of these results and predictions agree well with the observation that CaD phosphorylation by PKC (Vorotnikov et al., 1992) and its binding to Ca^{2+}/CaM (Czurylo et al., 1993; Vorotnikov et al., 1992) interfere with PS binding, because the major sites recognized by PKC as well as CaM are also located in the C-terminal region of CaD (Marston et al., 1994; Mezgueldi et al., 1994; Vorotnikov et al., 1994; Zhan et al., 1991).

The purpose of this work was to use synthetic peptides to test the postulated location of regions in CaD that are involved in PS binding, and to elucidate the relationship between the PS-binding and the CaM-binding sites. We would also like to know whether binding of PS induces local or more extensive changes in the structure of the CaD molecule. Moreover, to determine whether CaD can mediate the interaction between actin filaments and membranes, we have investigated the interaction of F-actin with PS liposome-bound CaD by cosedimentation and electron microscopy. Our data showed that the peptide stretch encompassing the two CaM-binding sites in the C-terminal region of CaD contains the major elements responsible for lipid binding, and that CaD enhances PS binding to F-actin, most likely by tethering liposomes to the actin filament.

MATERIALS AND METHODS

Protein and peptide preparation

Chicken gizzard CaD was prepared according to the method of Bretscher (1984). Peptides corresponding to Gly^{651}-Ser^{667} (GS17C), Val^{685}-Gly^{713} (VG29C), and Met^{658}-Gly^{713} (MG56C) of chicken gizzard CaD, with a cysteine residue added to the C-terminus of each of them, were synthesized and purified as described by Zhuang et al. (1995). Recombinant fragment Glu^{413}-Lys^{579} (EK167) was overexpressed in *Escherichia coli* and purified on a CM-cellulose (Sigma) column at pH 5, using a linear gradient (30–500 mM) of NaCl. The positions of these peptides are marked in Fig. 1. Rabbit skeletal muscle actin was prepared as described by Spudich and Watt (1971). The purity of CaD, its fragment (EK167), and actin was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The concentrations of actin, CaD, and the synthetic peptides were determined spectroscopically, using extinction coefficients of ϵ_{290} = 26,400 cm^{-1} M^{-1} for G-actin (Houk and Ue, 1974; M_r for actin 42,000), ϵ_{280} = 29,400 cm^{-1} M^{-1} for CaD (Graceffa et al., 1988; M_r for CaD 89,000), ϵ_{280} = 5600 cm^{-1} M^{-1} for GS17C and VG29C, and ϵ_{280} = 11,200 cm^{-1} M^{-1} for MG56C (Zhuang et al., 1995). The concentration of EK167 polypeptide was determined according to the method of Lowry et al. (1951), using bovine serum albumin (Sigma) as a standard.

Preparation of PS liposomes

Liposomes were prepared according to the method of Szoka and Papahadjoboulos (1980). Briefly, dried powder of PS (Sigma) was suspended in a buffer containing either 40 mM Tris, 20 mM sodium acetate (pH 7.4) and 2 mM EDTA (buffer A; for electron microscopy, chymotryptic digestion, nondenaturing gel electrophoresis, and actin cosedimentation experiments), or 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 1 mM NaN_3, and 1 mM 2-mercaptoethanol (buffer B; for fluorescence measurements). Dispersion of PS was facilitated by sonication in an ice bath with an Ultrasonic Disintegrator (model UD-11; Techpan, Warsaw, Poland) working at maximum output, with 30-s pulses for 15 min under N_2 to prevent oxidation. The concentration of PS was determined by phosphate assay (Rouser et al., 1970).

Electron microscopy

PS liposomes in buffer A (see above) were first centrifuged at 180,000 × *g* for 30 min (Sorvall RC M100 ultracentrifuge); the supernatant was used for electron microscopic studies. The liposome solution was diluted 300-fold into a solution containing 30% glycerol, 100 mM ammonium acetate (pH 7.2), and when present, CaD (1 μg/ml, or ~11 nM) and/or monoclonal

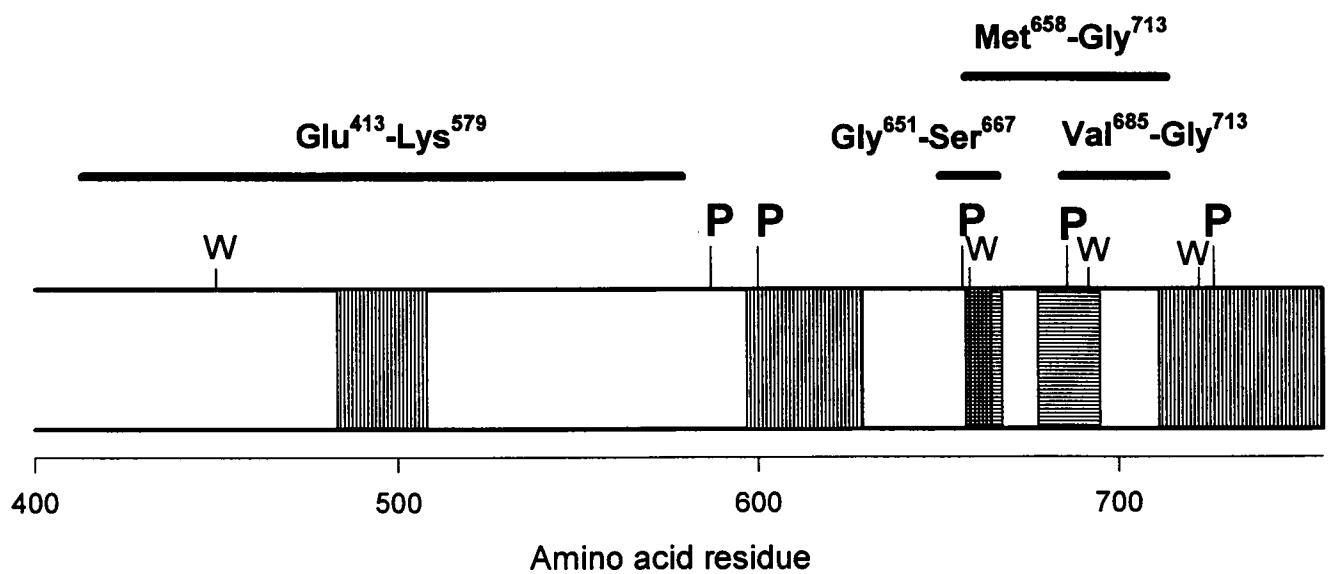


FIGURE 1 Domain mapping of the C-terminal region of CaD. The CaM-binding (*shaded with horizontal lines*) and actin-binding sites (*shaded with vertical lines*) in the C-terminal region (from residue 400 to 756) are shown here, along with the synthetic peptides and fragment used in this study marked as references. Also included are the positions of Trp residues (denoted by W) and Ser residues that are phosphorylated by PKC (denoted by P) (according to Vorotnikov et al., 1994) in this region.

anti-CaD antibody (1 μg/ml), which recognizes an epitope in the N-terminal region of CaD (Lin et al., 1991; Mabuchi et al., 1993). After ~20 min at room temperature, the liposomes with or without proteins were adsorbed onto freshly cleaved mica sheets by floating the sheets on a drop of the liposome solution. Liposomes and proteins on the mica sheets were then processed for electron microscopic visualization by rotary shadowing (Mabuchi, 1991; Mabuchi and Wang, 1991). Specimens were observed with a Philips 300 electron microscope at 60 kV.

The mixtures of F-actin (~1 μM) and PS liposomes (120 μM) in the presence and absence of CaD (0.22 μM) were incubated for 20 min at room temperature, negatively stained with 1% uranyl acetate, and examined by electron microscopic imaging on Formvar-carbon coated grids.

Digestion of CaD with α-chymotrypsin in the presence of PS liposomes

Changes in CaD conformation upon binding of PS liposomes were probed by proteolytic cleavages with α-chymotrypsin. CaD (2.2 μM) in buffer A with or without PS liposomes (125 μM) prepared in the same buffer was preincubated for 30 min at 4°C. Digestion was carried out at a chymotrypsin:CaD weight ratio of 1:500 at 30°C. At various time intervals, aliquots were withdrawn, and 0.1 mM phenylmethylsulfonyl fluoride was added to stop the reaction; samples were then boiled for 4 min with a sample buffer prepared according to the method of Laemmli (1970) and subjected to SDS-PAGE. Gels were stained with Coomassie Brilliant Blue and analyzed by laser scanning densitometry with a Personal Densitometer (Molecular Dynamics).

Nondenaturing gel electrophoresis

Electrophoresis of the peptide-PS liposome complexes under nondenaturing conditions was performed on agarose-polyacrylamide gels essentially as described by Cohen et al. (1986), except that 0.135% *N,N'*-methylene-bis-acrylamide and 3% acrylamide were used. Synthetic peptides (1 mg/ml) in buffer A were mixed with a suspension of PS vesicles (10–300 μg; total volume 85 μl) and incubated for 20 min at room temperature. The samples were subjected to electrophoresis in both directions (toward cathode or anode) at 4°C. Gels were stained with Coomassie Brilliant Blue for peptides or Sudan Black B for liposomes (Prat et al., 1969). The amount of PS-bound fragment was calculated after laser scanning densitometry as a difference between the control PS-free sample of peptide and the unbound peptide.

Fluorescence measurements

The effect of PS liposomes on the tryptophan fluorescence of CaD and its fragments was determined in buffer B with a Spex 1680 spectrofluorimeter. The excitation wavelength was 295 nm. All spectra were corrected for light scattering, solvent background, and dilution. Relative quantum yield increase ($\Delta Q/Q_0$) was calculated as a ratio of the quantum yield increase in tryptophan emission of CaD (or its fragments) at a given PS concentration to the quantum yield of CaD (or fragments) alone. The fitting of experimental results with theoretical curves and calculation of the binding parameters were carried out on a personal computer with the program Marqit (kindly provided by Dr. A. Kasprzak, Centre National de la Recherche Scientifique, Montpellier).

Actin cosedimentation experiments

Actin cosedimentation assays were performed in buffer A. Before the experiments CaD and liposome suspension were first clarified by ultracentrifugation at 180,000 × *g* for 30 min (Sorvall RC M100 ultracentrifuge). Samples containing PS liposomes (150 μM) and/or CaD (0.8 μM) were preincubated at room temperature for 30 min. Actin (4 μM) was then added and the mixture was incubated for another 30 min at room temperature. Samples were ultracentrifuged as above, and the protein amounts in the pellet and supernatant were quantified after SDS-PAGE (Laemmli, 1970) by laser scanning densitometry. The amount of PS remaining in the supernatant was determined as described by Rouser et al. (1970).

RESULTS

Interaction of CaD with PS liposomes

Liposomes visualized by heavy metal shadowing appeared as round, smooth-surfaced objects with diameters varying from a few nanometers to ~0.1 μm (Fig. 2 *A*). When mixed with anti-CaD IgGs, the surface of some liposomes became more irregular, suggesting that the IgG molecule is incorporated into the liposomes (Fig. 2 *B*). Complexes made up of CaD and liposomes exhibited images with multiple projections (Fig. 2 *C*), indicating that only one end of the CaD molecule interacts with the liposomes. Images corresponding to linear aggregates of CaD were also observed occasionally (Fig. 2 *C*, *asterisks*). The subsequent addition of monoclonal anti-CaD antibodies reactive to the N-terminal region of CaD resulted in images containing many triangular IgGs tethered to the liposomes via CaD molecules (Fig. 2 *D*, *arrowheads*), indicating that it is the C-terminal region of CaD that binds to the PS liposomes. However, there were also some IgG molecules closely bound to the liposomes (Fig. 2 *D*, *arrows*); it is not clear whether these IgGs are associated with the liposomes via the N-terminus of CaD or directly incorporated into the liposomes, as seen in Fig. 2 *B*. It should be noted, however, that the background concentration of free IgG in Fig. 2 *D* was much lower than that in Fig. 2 *B*, because the majority of the antibodies were bound to CaD, and therefore, direct incorporation of IgG to the liposomes should occur much less frequently.

Identification of the PS binding site(s) of CaD

Chymotryptic digestion

By comparing the patterns of chymotryptic digestion of CaD in the presence and absence of liposomes, regions of the molecule affected by phospholipid can be readily identified (Fig. 3). Whereas the major cleavage site (between Trp^{450} and Asp^{451}) was not affected by the presence of liposomes, as evidenced by the unchanged peak height of the Asp^{451}-Pro^{756} peptide (a 40-kDa actin- and CaM-binding fragment), there were clear changes in the susceptibility of chymotryptic cleavage sites in the C-terminal region, which produced several shorter fragments (23 kDa, 18 kDa, and 16 kDa). (Apparent molecular masses are according to Szpacenko and Dabrowska, 1986.) Peptide analyses of the gel bands indicated that the peptide bond between Tyr^{625} and Thr^{626} and the nearby sites are affected by PS binding.

Changes in the digestion pattern of CaD upon binding of PS also seemed to occur in regions outside of the C-terminal domain: the amount of the 57-kDa fragment (Gln^{166}-Trp^{450}) was increased, the relative amounts of the high-molecular-mass peptides (110 kDa and 80 kDa) lacking the C-terminal

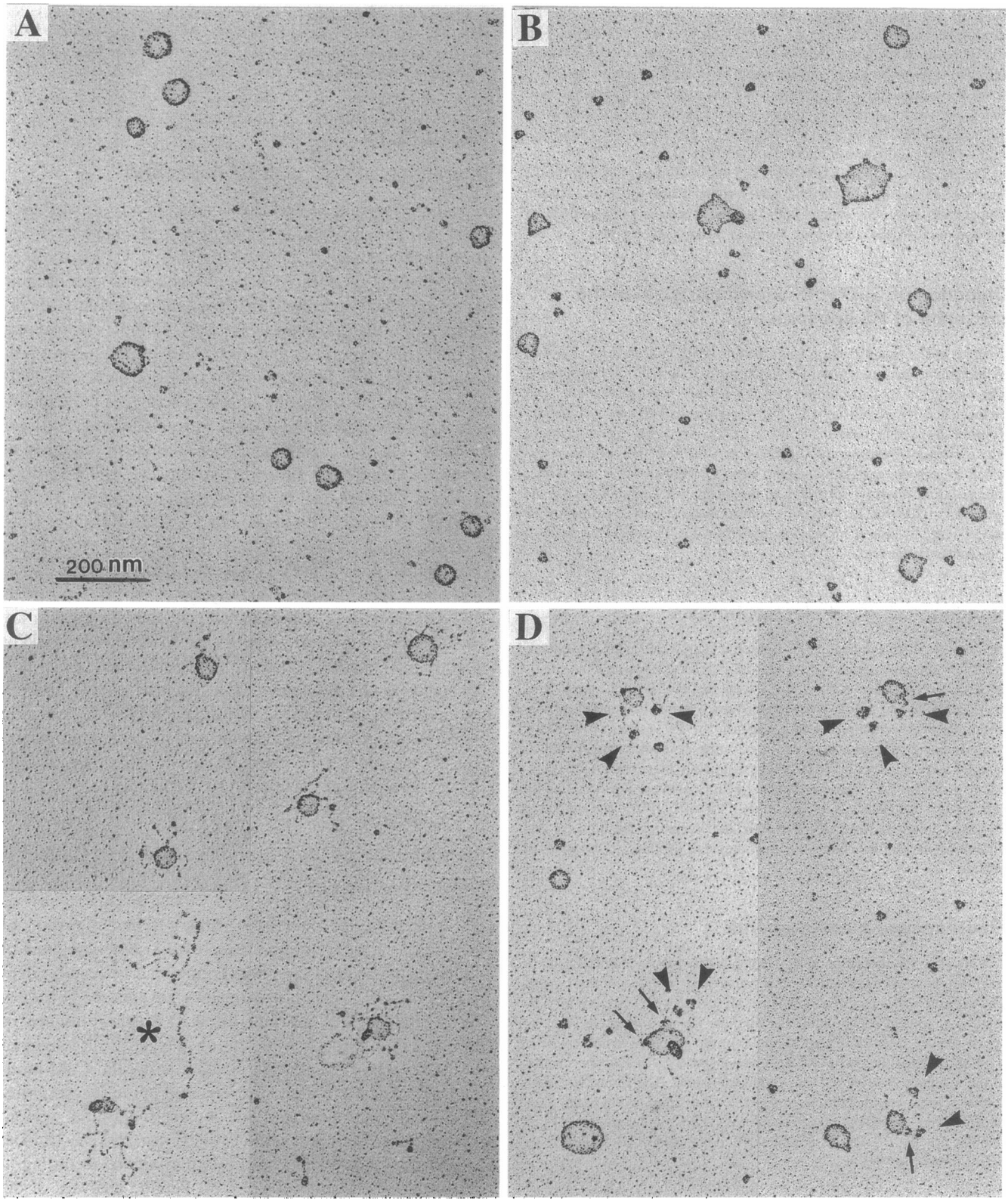


FIGURE 2 Rotary shadowed images of liposomes and complexes with CaD. (*A*) Liposomes alone. (*B*) Liposomes with incorporated IgG (anti-CaD) molecules. (*C*) Liposomes complexed with CaD. (*D*) Complexes formed between liposomes and anti-CaD antibody-labeled CaD. In all cases the same amount (1 μg/ml) of antibody was used. The background on left side of *D* has fewer free IgG molecules than in *B* because most of the antibody molecules were bound to CaD. See text for more details. Magnification: ×80,000.

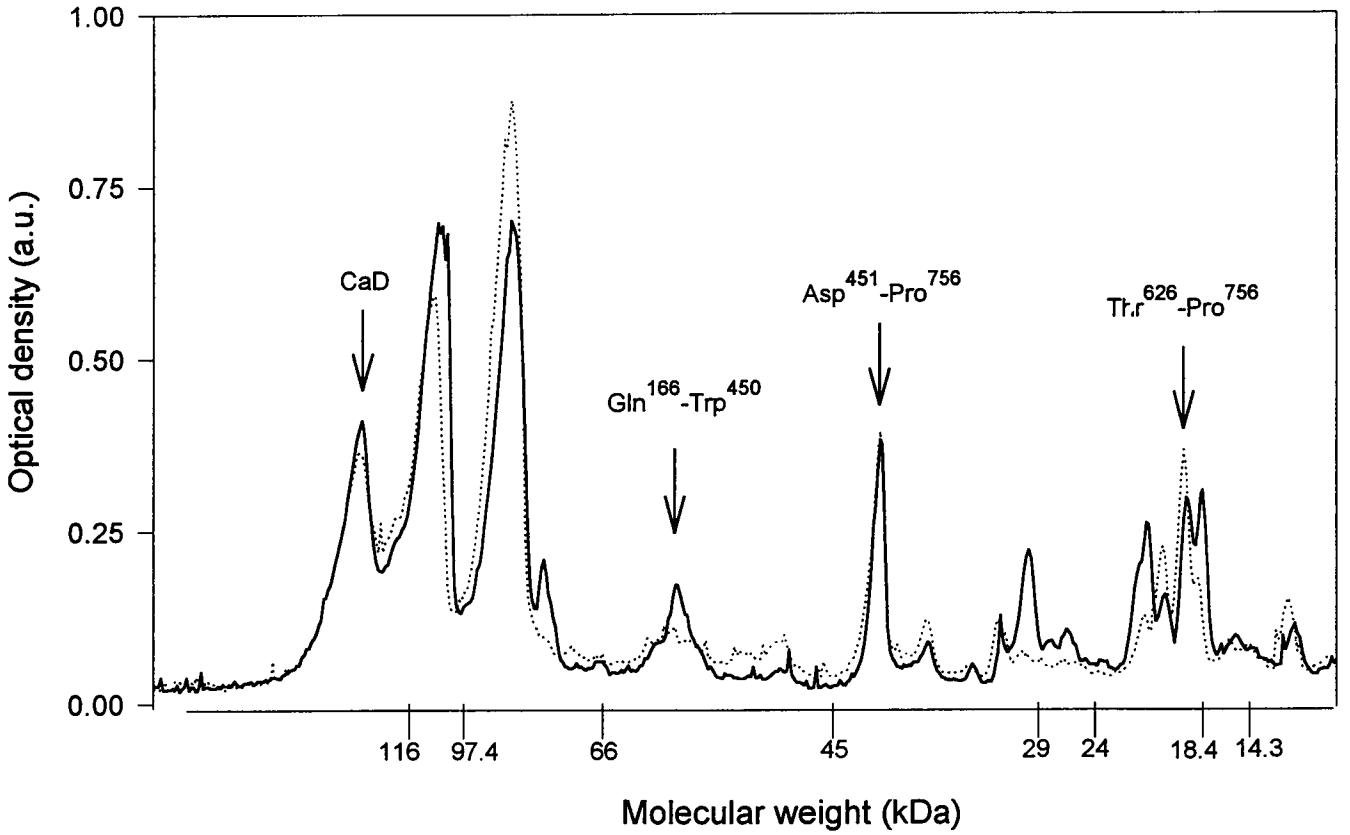


FIGURE 3 The effect of PS liposomes on the digestion pattern of chicken gizzard CaD by chymotrypsin. Proteolysis of CaD (0.2 mg) was performed at 30°C in a buffer containing 40 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 1 mM NaN_3, and 1 mM 2-mercaptoethanol in the absence (·····) and presence (——) of PS liposomes (the PS:CaD weight ratio was 1:2; the chymotrypsin:CaD weight ratio was 1:500). Samples withdrawn after 8 min of proteolysis were subjected to SDS-PAGE analysis. Gels stained with Coomassie Brilliant Blue were quantified by desitometry.

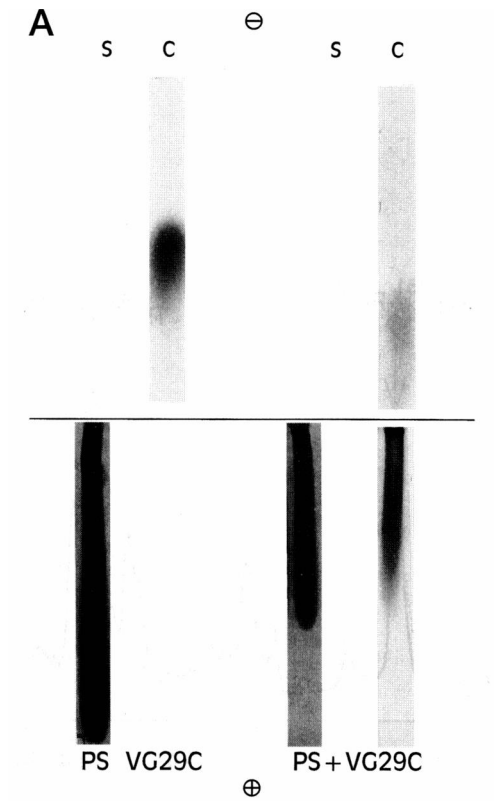
actin- and CaM-binding sites were slightly altered, and an additional peptide with an apparent molecular mass of ~30 kDa appeared. This 30-kDa fragment, the identity of which was not determined, is most likely not derived from the C-terminal region of CaD, because it did not bind to actin or CaM (data not shown).

Thus the differences in the digestion patterns clearly indicated that CaD undergoes conformational changes upon interacting with PS; such changes occurred mainly in the C-terminal region, resulting in an altered susceptibility to the enzyme. Other parts of the molecule, however, were also apparently affected, either because of additional PS-binding sites, or owing to long-ranged structural effects induced by PS binding to the C-terminal region.

Two-directional nondenaturing gel electrophoresis

To determine the PS-binding site of CaD more precisely, we have tested three synthetic peptides of CaD, GS17C (from Gly^{651} to Ser^{667}), VG29C (from Val^{685} to Gly^{713}), and MG56C (from Met^{658} to Gly^{713}), using agarose-PAGE under nondenaturing conditions, to see whether they interact with the PS liposomes. Direct association between PS and the synthetic peptides was detected by double staining with Sudan Black (for phospholipids) and Coomassie Blue (for peptides), but quantification was hampered by diffusion of the bands of the complexes. However, because under the experimental conditions all three peptides by themselves migrated toward the cathode, owing to their net positive charges, in the direction opposite that of the negatively charged PS liposomes, it was possible to separate and quantify the unbound peptide, and thereby to determine the amount of peptide interacting with PS. Representative agarose gels showing interaction of PS with VG29C are depicted in Fig. 4 *A*. Similar results were also obtained for the interaction between PS and GS17C or MG56C. Notably, the longer fragment, EK167, which contains the sequence upstream of the other synthetic peptides, did not show significant interaction with the phospholipid.

These results showed that two distinct peptide stretches in the C-terminal region of CaD, corresponding to the CaM-binding sites A and B (Marston et al., 1994; Mezgueldi et al., 1994), respectively, were able to interact with the PS liposomes. Although the electrophoretic method is usually considered qualitative in nature, laser-scanning densitometry of peptide spots in the absence and presence of increasing amounts of PS liposomes allowed us to make semiquan-



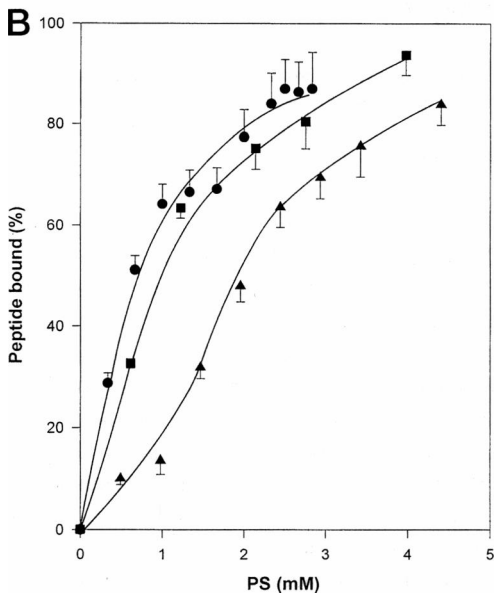


FIGURE 4 Interaction of PS liposomes with CaD peptides studied by two-directional nondenaturing agarose PAGE. (*A*) Representative gels of PS and VG29C by themselves (*left two lanes*) and the PS liposome-VG29C complex (*right two lanes*) at a molar ratio of PS:peptide = 14:1. The gel pieces are arranged in such a way that the cathode is at the top and the anode is at the bottom; the middle line denotes the origins. S and C indicate Sudan Black B and Coomassie Brilliant Blue staining, respectively. (*B*) Binding of 1 mg/ml of GS17C (▲), VG29C (■), or MG56C (●) peptide with PS liposomes, determined by desitometry (see text). In each case the amount of PS-bound peptide (in %) was calculated from the difference between the Coomassie Brilliant Blue staining of the total peptide (i.e., in the absence of PS) and that of the unbound peptide. Note that the curve for GS17C is artificially shifted to the right, because the peptide has the smallest molecular weight and, therefore, the highest molar concentration.

titative comparisons of the PS liposome-binding capacity among the three peptides (Fig. 4 *B*). The data indicated that all three peptides exhibited comparable affinities for PS, although MG56C appeared to bind PS most strongly.

Intrinsic tryptophan fluorescence study

It has been shown that binding of the PS liposomes affects the tryptophan fluorescence of full-length CaD (Czurylo et al., 1993). In an attempt to characterize the interaction between PS and the C-terminal segments of CaD, we have measured the quantum yields of the tryptophan emission for GS17C, VG29C, and MG56C (containing Trp^{659}, Trp^{692}, and both residues, respectively) as well as that of EK167 (containing Trp^{450}) in the absence and presence of the PS liposomes. The addition of PS resulted in an increase in the quantum yield of tryptophan fluorescence in the case of GS17C, MG56C, and EK167, but a slight decrease in the case of VG29C (Fig. 5).

The apparent binding parameters of PS for GS17C, MG56C, and EK167 peptides were calculated based on the best fitted curves ($\Delta Q/Q_o$ versus [PS]) (Table 1). GS17C exhibited about an eightfold lower value of the dissociation constant ($K_d = 1.4 \times 10^{-4}$ M) when compared with the longer peptide (MG56C, $K_d = 1.8 \times 10^{-5}$ M; assuming two

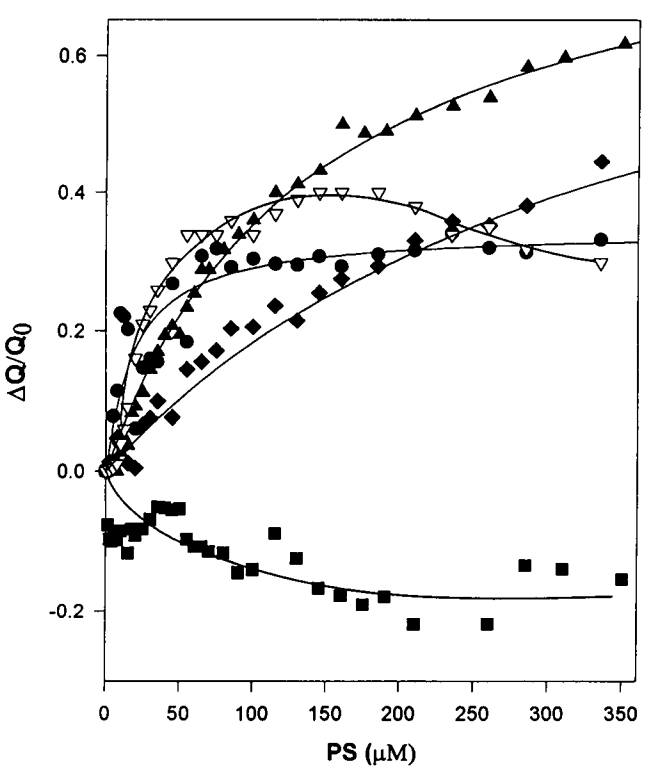


FIGURE 5 The effect of liposomes on tryptophan fluorescence of CaD peptides. The excitation wavelength was set at 295 nm. The fluorescence quantum yield was measured in a buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 1 mM NaN_3, and 1 mM 2-mercaptoethanol at 2 μM concentration of each peptide/fragment. ▲, GS17C; ■, VG29C; ●, MG56C; ♦, EK167; ▽, CaD.

TABLE 1 Binding parameters of caldesmon peptides to phosphatidylserine liposomes

Peptide	$10^4 \times K_d$ (M)	Number of sites*
GS17C	1.43 ± 0.12	1
VG29C	Not fitted	Not fitted
MG56C	0.18 ± 0.02	2
EK167	3.85 ± 0.59	1
CaD#	0.069 ± 0.006	1
	2.17 ± 0.19	1

*The data were fitted with a fixed number of sites.
#Data taken from Czurylo et al. (1993).

PS-binding sites), which also contains a second CaM-binding site. The change of quantum yield obtained for VG29C was too small to allow accurate estimation of binding parameters. Nevertheless, data presented here agreed well with those obtained from the native agarose-PAGE experiments, and showed similar affinities of both CaM-binding sites for PS. EK167 had a lower affinity for PS binding ($K_d = 3.85 \times 10^{-4}$ M) and probably corresponded to a second class of PS-binding sites observed earlier by Czurylo et al. (1993). It should be noted that all peptides studied exhibited affinities lower than that of native CaD (6.90×10^{-6} M and 2.17×10^{-4} M; Czurylo et al., 1993), again suggesting that binding of phospholipid to CaD cannot be fully accounted for by its interaction with the C-terminal region alone, and other parts of the molecule are likely to be involved as well.

Interaction of PS liposome-bound CaD with actin

Because CaD exhibits both actin-binding and PS-binding abilities, we have tested whether a ternary complex of PS with these two proteins can be formed, i.e., whether CaD is capable of anchoring actin filaments to the membranes. This was done by cosedimentation with actin, coupled with densitometric analysis of proteins in the supernatants and pellets as well as the unpelleted liposomes. We found that actin sedimented with liposomes even in the absence of CaD, the molar ratio of PS to actin in the pellet being ~9:1 (Table 2).

TABLE 2 Actin cosedimentation of PS liposomes, CaD, and PS-bound CaD*

Additions	Content in pellet (nmol)		
	Actin	CaD	PS
Actin	1.51 ± 0.03	—	—
Actin + PS#	1.50 ± 0.04	—	13 ± 3
Actin + CaD#	1.50 ± 0.02	0.22 ± 0.02	—
Actin + (PS · CaD)	1.52 ± 0.04	0.09 ± 0.01	26 ± 5

*Samples containing 4 μM actin, 150 μM PS, and 0.8 μM CaD were prepared and centrifuged as described in Materials and Methods. The amounts of proteins and PS in the pellets were determined by SDS-PAGE and laser scanning desitometry, and by phosphate assay, respectively. Differences between the average values of PS and CaD content in the pellet of samples containing PS-bound CaD were significant when compared with those of samples containing PS or CaD alone ($p < 0.01$, Student's t-test for paired comparisons).
#PS and CaD were all centrifuged in the same way before use.

The amount of PS pelleted with actin was doubled when PS liposomes were incubated with CaD before ultracentrifugation. These observations indicated that membrane-bound CaD indeed promotes anchoring of additional actin filaments, most likely by tethering the liposomes to F-actin. There was, however, a decrease in the amount of CaD bound to actin caused by PS. The molar ratio was ~1:17 in the ternary complex, whereas it was 1:7 in the binary complex of CaD with actin. This may be explained by steric hindrance due to the large liposome particles that block the binding of consecutive CaD molecules along the actin filament. Alternatively, some of the liposome-bound CaD may have remained in the supernatant and therefore may have been left unaccounted for.

Finally, negatively stained electron microscopic images of F-actin in the presence of liposomes alone and liposomes with bound CaD confirmed the complex formation in both cases (Fig. 6). The general appearance of such complexes was quite similar: a network of actin filaments decorated by liposome "knots." In the case of the binary complexes of PS liposomes and actin (Fig. 6 *b*), a larger fraction of actin filaments were not decorated by the liposomes. Because in the presence of CaD nearly all actin filaments were covered with liposomes (Fig. 6 *a*), it may be concluded that binding of PS liposomes to actin is enhanced by CaD, and that in the ternary complex PS liposomes are most likely bound to actin via CaD.

DISCUSSION

Polymerization of actin and formation of a meshwork of microfilaments, which accompany receptor-mediated responses in many cells, are regulated by a large number of actin-associated proteins (Cooper, 1991; Janmey, 1994). One of these proteins may be CaD (the low-molecular-weight isoform), because it is present in the subplasmalemmal region of many nonmuscle cells (Bretscher and Lynch, 1985; Burgoyne et al., 1986; Walker et al., 1989). CaD is able to induce actin polymerization (Galazkiewicz et al., 1989; Galazkiewicz et al., 1985), and it is able to assemble actin filaments into meshwork or bundles (Bretscher, 1984; Dabrowska et al., 1985). Like several other actin-binding proteins (for a review, see Isenberg, 1991), CaD interacts with anionic plasma membrane phospholipids (Czurylo et al., 1993; Vorotnikov et al., 1992; Vorotnikov and Gusev, 1990). This interaction may be important for the promotion of cellular activities, such as secretion, endocytosis, filopodia formation, and receptor capping.

The results presented in this work indicate that the major PS-binding region of CaD overlaps the CaM-binding sites. This part of the molecule also contains several serine residues (Ser^{657}, Ser^{686}, and Ser^{717}) recognized by PKC (Vorotnikov et al., 1994) (Fig. 1). It is therefore possible that the interaction between PS and CaD may be reversed by CaM binding and/or PKC phosphorylation. Indeed, it has been

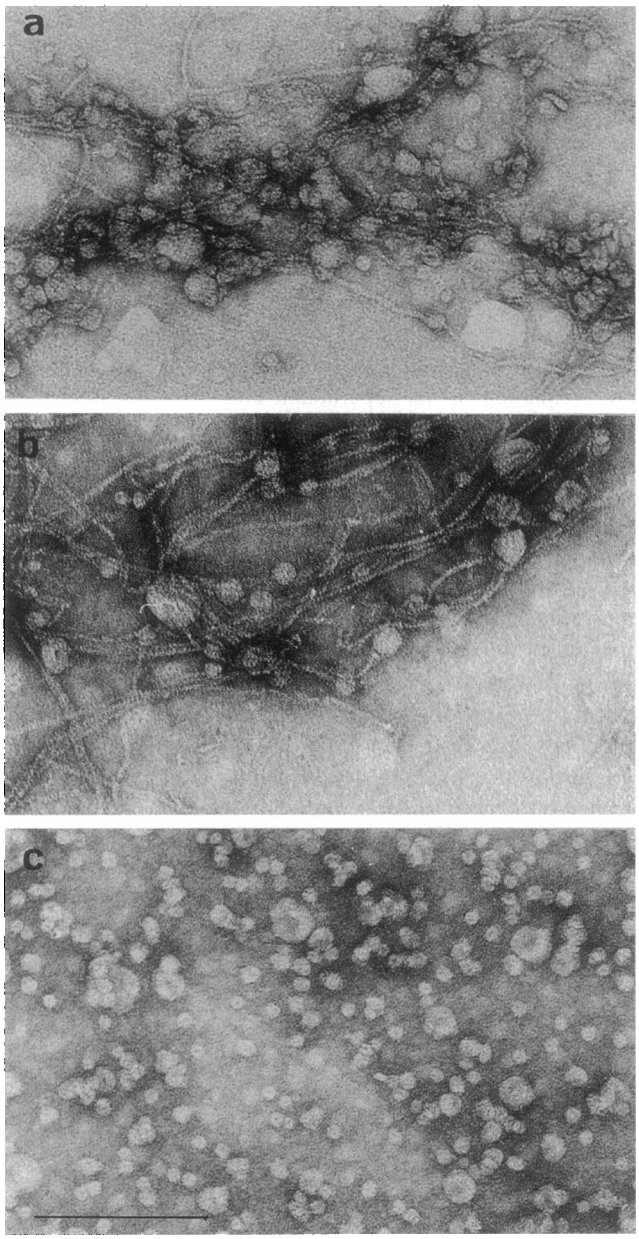


FIGURE 6 Electron microscopy of negatively stained F-actin and PS liposome-CaD complexes (*a*), actin and PS liposomes without CaD (*b*), and liposome-CaD alone (*c*). Samples were prepared as described in Materials and Methods. The scale bar represents 200 nm.

shown previously that PS and CaM compete with each other for CaD binding (Czurylo et al., 1993; Vorotnikov et al., 1992). As for the effect of phosphorylation on lipid binding, although more experimental evidence is needed, reversal of the membrane association of proteins in addition to CaD has been reported. Upon PKC phosphorylation, for example, the membrane-bound peptides of MARCKS (myristoylated, alanine-rich C kinase substrate; see Aderem, 1992) that anchor actin filaments to membranes move away from cell membranes and phospholipid vesicles (Kim et al., 1994). Phosphorylation of CaD by PKC may also reverse the electrostatic association of the cluster of basic residues in the protein with acidic PS (Vorotnikov et al., 1992).

We also identified a second, lower-affinity site that interacts with PS. The significance of this second class of lipid-binding site is unclear, but the apparent higher affinity of intact CaD for PS compared to each of the individual peptides strongly suggests the involvement of additional regions of CaD in its interaction with lipid membrane. Based on the effect of phospholipids on the proteolytic digestion pattern (Fig. 3), and the binding mode of an anti-N-terminal antibody to the CaD-liposome complexes (Fig. 2), such additional PS-binding sites may be located in the N-terminal region of CaD.

Because the major actin-binding sites of CaD are located in the C-terminal region in close proximity to the CaM/phospholipid-binding sites (Mezgueldi et al., 1994; Wang et al., 1991), it is of interest to see whether CaD bridges actin filaments and membranes as in the case of MARCKS (Hartwig et al., 1992). Actin cosedimentation experiments and electron microscopy showed that the PS liposome-CaD complex bound actin filaments, resulting in cross-linking of the filaments. Moreover, CaD enhanced interactions of actin filaments with PS liposomes (Fig. 6). It is generally believed that actin filaments interact with cell membrane, either directly by binding to the phospholipids of the membrane, or indirectly via actin-binding proteins (Gicquaud, 1993, and references therein). Direct interactions were observed in the case of positively charged liposomes, whereas negatively charged or neutral liposomes interacted with actin only in the presence of Mg^{2+} or Ca^{2+} ions (in the millimolar range) (St-Onge and Gicquaud, 1989). The PS liposome-actin complex was also formed in the absence of divalent cations, although to a lesser extent. CaD increased the amount of sedimenting PS liposomes, apparently through mediation in their interaction with actin filaments by forming ternary complexes.

In conclusion, present and earlier results from this and other laboratories (Bretscher, 1984; Czurylo et al., 1993; Dabrowska et al., 1985; Galazkiewicz et al., 1985, 1989; Vorotnikov et al., 1992; Vorotnikov and Gusev, 1990) all suggest the participation of CaD in the cascade of reactions that triggers actin-based motility, but determination of its precise role in these processes requires further work. Binding of CaD to both actin filaments (Dabrowska et al., 1985; Vorotnikov et al., 1994) and the membrane (Czurylo et al., 1993; Vorotnikov et al., 1992) is disrupted by Ca^{2+}/CaM (or other Ca^{2+}-binding proteins) or phosphorylation by PKC or other kinases (for a review, see Matsumura and Yamashiro, 1993). As a result, CaD is released from the membrane to cytosol as the Ca^{2+} concentration is increased, so that it could not be used for the nucleation of monomeric actin (recruited, for example, from profilactin; see Galazkiewicz et al., 1991) or interaction with cortical filamentous actin. Moreover, overlapping of sites for phospholipid binding and CaM binding and sites for phosphorylation was observed not only in CaD, but also in other proteins: neuromodulin, neurogranin, plasma membrane Ca^{2+}-ATPase,

and brush border myosin I (Baudier et al., 1991; Carafoli, 1994; Houbre et al., 1991; Swanljung-Collins and Collins, 1992). Notably, all of these proteins are substrates of PKC. It is thus tempting to speculate that these common features may be related to the regulation of their activities in the cell.

CaD is known to inhibit in vitro actomyosin ATPase activity, and this forms the basis for its putative modulatory effect on the interaction between myosin and actin filaments in smooth muscle cells. In nonmuscle cells the contractile apparatus is recruited and assembled upon stimulation to conduct various motile and transporting functions, many of which occur near the cell membranes. As suggested by our data, reversible binding of CaD to phospholipids as well as actin filaments, governed by Ca^{2+}/CaM or phosphorylation by PKC, is consistent with a role in such subplasmalemmal dynamics of actin filament architecture, as it has been shown that the cytoskeleton-membrane link can be disrupted by cell stimulation and increasing Ca^{2+} concentrations (Linstedt and Kelly, 1987). Because many unconventional myosins are also associated with the membranes, CaD may thus guide actin filaments to regions where interaction with myosin can be facilitated. Whether CaD in nonmuscle cells still functions as an inhibitor of the motile machinery would be an intriguing question, and more studies in this direction will undoubtedly be needed.

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REFERENCES

Aderem, A. 1992. The MARCKS brothers: a family of protein kinase C substrates. *Cell.* 71:713–716.

Baudier, J., J. C. Deloulme, A. Van Dorsselaer, D. Black, and H. W. D. Matthes. 1991. Purification and characterization of a brain-specific protein kinase C substrate, neurogranin. *J. Biol. Chem.* 266:229–237.

Bogatcheva, N. V., and N. B. Gusev. 1995. Computer-assistant prediction of phospholipid binding sites of caldesmon and calponin. *FEBS Lett.* 363:269–272.

Bogatcheva, N. V., P. A. J. Huber, I. D. C. Fraser, S. B. Marston, and N. B. Gusev. 1994. Localization of phospholipid-binding sites of caldesmon. *FEBS Lett.* 342:176–180.

Bretscher, A. 1984. Smooth muscle caldesmon. *J. Biol. Chem.* 259: 12873–12880.

Bretscher, A., and W. Lynch. 1985. Identification and localization of immunoreactive forms of caldesmon in smooth and nonmuscle cells: a comparison with the distributions of tropomyosin and alpha-actinin. *J. Cell Biol.* 100:1656–1663.

Bryan, J., M. Imai, R. Lee, P. Moore, R. G. Cook, and W.-G. Lin. 1989. Cloning and expression of a smooth muscle caldesmon. *J. Biol. Chem.* 264:13873–13879.

Bryan, J., and C.-L. A. Wang. 1993. Caldesmon. *In* Guidebook to the Cytoskeletal and Motor Proteins. Oxford University Press, Oxford. 29–31.

Burgoyne, R. D., T. R. Cheek, and K.-M. Norman. 1986. Identification of a secretory granule-binding protein as caldesmon. *Nature.* 319:68–70.

Carafoli, E. 1994. Biogenesis: plasma membrane calcium ATPase: 15 years of work on the purified enzyme. *FASEB J.* 8:993–1002.

Cohen, A. M., S.-C. Liu, L. H. Derick, and J. Palek. 1986. Ultrastructural studies of the interaction of spectrin with phosphatidylserine liposomes. *Blood.* 68:920–926.

Cooper, J. A. 1991. The role of actin polymerization in cell motility. *Annu. Rev. Physiol.* 53:585–605.

Czurylo, E. A., J. Zborowski, and R. Dabrowska. 1993. Interaction of caldesmon with phospholipids. *Biochem. J.* 291:403–408.

Dabrowska, R. 1994. Caldesmon. *In* Airways Smooth Muscle: Biochemical Control of Contraction and Relaxation. Birkhäuser Verlag, Basel, Boston, Berlin. 31–59.

Dabrowska, R., A. Goch, B. Galazkiewicz, and H. Osinska. 1985. The influence of caldesmon on ATPase activity of the skeletal muscle actomyosin and bundling of actin filaments. *Biochim. Biophys. Acta.* 842:70–75.

Dabrowska, R., H. Hinssen, B. Galazkiewicz, and E. Nowak. 1996. Modulation of gelsolin-induced actin-filament severing by caldesmon and tropomyosin and the effect of these proteins on the actin activation of myosin Mg^{2+}-ATPase activity. *Biochem. J.* 315:753–759.

Eisenberg, D., E. Schwartz, M. Komaromy, and R. Wall. 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* 179:125–142.

Galazkiewicz, B., J. Belagyi, and R. Dabrowska. 1989. The effect of caldesmon on assembly and dynamic properties of actin. *Eur. J. Biochem.* 181:607–614.

Galazkiewicz, B., F. Buss, B. M. Jockusch, and R. Dabrowska. 1991. Caldesmon-induced polymerization of actin from profilactin. *Eur. J. Biochem.* 195:543–547.

Galazkiewicz, B., M. Mossakowska, H. Osinska, and R. Dabrowska. 1985. Polymerization of G-actin by caldesmon. *FEBS Lett.* 184:144–149.

Gicquaud, C. 1993. Actin conformation is drastically altered by direct interaction with membrane lipids: a differential scanning calorimetry study. *Biochemistry.* 32:11873–11877.

Graceffa, P., C.-L. A. Wang, and W. F. Stafford. 1988. Caldesmon: molecular weight and subunit composition by analytical ultracentrifugation. *J. Biol. Chem.* 263:14196–14202.

Hartwig, J. H., M. Thelen, A. Rosen, P. A. Janmey, A. C. Nairn, and A. Aderem. 1992. MARCKS is an actin filament crosslinking protein regulated by protein kinase C, and calcium-calmodulin. *Nature.* 356: 618–622.

Houbre, D., G. Duporthail, J. C. Deloulme, and J. Baudier. 1991. The interactions of the brain-specific calmodulin-binding protein kinase C substrate, neuromodulin (GAP 43), with membrane phospholipids. *J. Biol. Chem.* 266:7121–7131.

Houk, T., and K. Ue. 1974. The measurement of actin concentration in solution: a comparison of methods. *Anal. Biochem.* 62:66–74.

Isenberg, G. 1991. Actin binding proteins-lipid interactions. *J. Muscle Res. Cell Motil.* 12:136–144.

Ishikawa, R., S. Yamashiro, and F. Matsumura. 1989a. Annealing of gelsolin-severed actin fragments by tropomyosin in the presence of Ca^{2+}—potentiation of the annealing process by caldesmon. *J. Biol. Chem.* 264:16764–16770.

Ishikawa, R., S. Yamashiro, and F. Matsumura. 1989b. Differential modulation of actin-severing activity of gelsolin by multiple isoforms of cultured rat cell tropomyosin—potentiation of protective ability of tropomyosin by 83-kDa nonmuscle caldesmon. *J. Biol. Chem.* 264: 7490–7497.

Janmey, P. A. 1994. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annu. Rev. Physiol.* 56:169–191.

Kim, J., P. J. Blackshear, J. D. Johnson, and S. McLaughlin. 1994. Phosphorylation reverses the membrane association of peptides that correspond to the basic domains of MARCKS and neuromodulin. *Biophys. J.* 67:227–237.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Lin, J. J.-C., E. J. Davis-Nanthakumar, J.-P. Jin, D. Lourim, R. E. Novy, and J. L.-C. Lin. 1991. Epitope mapping of monoclonal antibodies against caldesmon and their effects on the binding of caldesmon to Ca^{2+}/calmodulin and to actin or actin-tropomyosin filaments. *Cell Motil. Cytoskeleton.* 20:95–108.

Linstedt, A. D., and R. B. Kelly. 1987. Overcoming barrier to exocytosis. *Trends Neurosci.* 10:446–448.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

Mabuchi, K. 1991. Heavy-meromyosin-decorated actin filaments: a simple method to preserve actin filaments for rotary shadowing. *J. Struct. Biol.* 107:22–28.

Mabuchi, K., J. J.-C. Lin, and C.-L. A. Wang. 1993. Electron microscopic images suggest both ends of caldesmon interact with actin filaments. *J. Muscle Res. Cell Motil.* 14:54–64.

Mabuchi, K., and C.-L. A. Wang. 1991. Electron microscopic studies of chicken gizzard caldesmon and its complex with calmodulin. *J. Muscle Res. Cell Motil.* 12:145–151.

Marston, S. B., I. D. C. Fraser, P. Huber, A. J., K. Pritchard, N. B. Gusev, and K. Torok. 1994. Location of two contact sites between human smooth muscle caldesmon and Ca^{2+}-calmodulin. *J. Biol. Chem.* 269: 8134–8139.

Marston, S. B., and P. A. J. Huber. 1996. Caldesmon. *In* Biochemistry of Smooth Muscle Contraction. Academic Press, San Diego, CA. 77–90.

Marston, S. B., and C. S. Redwood. 1991. The molecular anatomy of caldesmon. *Biochem. J.* 279:1–16.

Matsumura, F., and S. Yamashiro. 1993. Caldesmon. *Curr. Opin. Cell Biol.* 5:70–76.

Mezgueldi, M., J. Derancourt, B. Calas, R. Kassab, and A. Fattoum. 1994. Precise identification of the regulatory F-actin- and calmodulin-binding sequences in the 10-kDa carboxyl-terminal domain of caldesmon. *J. Biol. Chem.* 269:12824–12832.

Mizushima, Y., K. Kanda, T. Hamaoka, H. Fujiwara, and K. Sobue. 1987. Redistribution of caldesmon and tropomyosin associated with concanavalin A receptor capping on splenic T-lymphocytes. *Biomed. Res.* 8:73–78.

Prat, J. P., J. N. Lamy, and J. D. Weill. 1969. Colortation des lipoprotéines après electrophorèse en gel de polyacrylamide. *Bull. Soc. Chim. Biol.* 51:136739.

Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* 5:494–496.

Sobue, K., and J. R. Sellers. 1991. Caldesmon, a novel regulatory protein in smooth muscle and nonmuscle actomyosin systems. *J. Biol. Chem.* 266:12115–12118.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction: biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

St-Onge, D., and C. Gicquaud. 1989. Evidence of direct interaction between actin and membrane lipids. *Biochem. Cell Biol.* 67:297–300.

Surgucheva, I., and J. Bryan. 1995. Over-expression of smooth muscle caldesmon in mouse fibroblasts. *Cell Motil. Cytoskeleton.* 32:233–243.

Swanljung-Collins, H., and J. H. Collins. 1992. Phosphorylation of brush border myosin I by protein kinase C is regulated by Ca^{2+}-stimulated binding of myosin I to phosphatidylserine concerted with calmodulin dissociation. *J. Biol. Chem.* 267:3445–3454.

Szoka, F., and D. Papahadjoboulos. 1980. Comparative properties and methods of preparations of lipid vesicles (liposomes). *Annu. Rev. Biophys. Bioeng.* 9:467–4.

Szpacenko, A., and R. Dabrowska. 1986. Functional domain of caldesmon. *FEBS Lett.* 202:182–186.

Vorotnikov, A. V., N. V. Bogatcheva, and N. B. Gusev. 1992. Caldesmon-phospholipid interaction. *Biochem. J.* 284:911–916.

Vorotnikov, A. V., and N. B. Gusev. 1990. Interaction of smooth muscle caldesmon with phospholipids. *FEBS Lett.* 277:134–136.

Vorotnikov, A. V., N. B. Gusev, S. Hua, J. H. Collins, C. S. Redwood, and S. B. Marston. 1994. Phosphorylation of aorta caldesmon by endogenous proteolytic fragments of protein kinase C. *J. Muscle Res. Cell Motil.* 15:37–48.

Walker, G., W. G. L. Kerrick, and L. Y. W. Bourguignon. 1989. The role of caldesmon in the regulation of receptor capping in mouse T-lymphoma cell. *J. Biol. Chem.* 264:496–500.

Walsh, M. P. 1990. Calcium-dependent mechanisms of smooth muscle contraction. *Biochem. Cell Biol.* 69:771–800.

Wang, C.-L. A., L.-W. C. Wang, S. Xu, R. C. Lu, V. Saavedra-Alanis, and J. Bryan. 1991. Localization of the calmodulin- and the actin-binding sites of caldesmon. *J. Biol. Chem.* 266:9166–9172.

Warren, K. S., J. L.-J. Lin, D. D. Wamboldt, and J. J.-C. Lin. 1994. Overexpression of human fibroblast caldesmon fragment containing actin-Ca^{2+}/calmodulin-, and tropomyosin-binding domains stabilizes endogenous tropomyosin and microfilaments. *J. Cell Biol.* 125: 359–368.

Yamashiro, S., Y. Yamakita, H. Hosoya, and F. Matsumura. 1991. Phosphorylation of non-muscle caldesmon by p34cdc2 kinase during mitosis. *Nature.* 349:169–172.

Yamashiro, S., Y. Yamakita, R. Ishikawa, and F. Matsumura. 1990. Mitosis-specific phosphorylation causes 83k non-muscle caldesmon to dissociate from microfilaments. *Nature.* 344:675–678.

Zhan, Q., S. S. Wong, and C.-L. A. Wang. 1991. A calmodulin-binding peptide of caldesmon. *J. Biol. Chem.* 266:21810–21814.

Zhuang, S., E. Wang, and C.-L. A. Wang. 1995. Identification of the functionally relevant calmodulin binding site in smooth muscle caldesmon. *J. Biol. Chem.* 270:19964–19968.