Smooth muscle caldesmon binds actin and inhibits actomyosin ATPase activity. Phosphorylation of caldesmon by extracellular signal-regulated kinase (ERK) reverses this inhibitory effect and weakens actin binding. To better understand this function, we have examined the phosphorylation-dependent contact sites of caldesmon on actin by low dose electron microscopy and three-dimensional reconstruction of actin filaments decorated with a C-terminal fragment, hH32K, of human caldesmon containing the principal actin-binding domains. Helical reconstruction of negatively stained filaments demonstrated that hH32K is located on the inner portion of actin subdomain 1, traversing its upper surface toward the C-terminal segment of actin, and forms a bridge to the neighboring actin monomer of the adjacent long pitch helical strand by connecting to its subdomain 3. Such lateral binding was supported by cross-linking experiments using a mutant isoform, which was capable of cross-linking actin subunits. Upon ERK phosphorylation, however, the mutant no longer cross-linked actin to polymers. Three-dimensional reconstruction of ERK-phosphorylated hH32K indeed indicated loss of the interstrand connectivity. These results, together with fluorescence quenching data, are consistent with a phosphorylation-dependent conformational change that moves the C-terminal end segment of caldesmon near the phosphorylation site but not the upstream region around Cys595, away from F-actin, thus neutralizing its inhibitory effect on actomyosin interactions. The binding pattern of hH32K suggests a mechanism by which unphosphorylated, but not ERK-phosphorylated, caldesmon could stabilize actin filaments and resist F-actin severing or depolymerization in both smooth muscle and nonmuscle cells.

Caldesmon (CaD) is an actin-binding protein found in both nonmuscle and smooth muscle cells. In nonmuscle cells it influences contractility by interfering with focal adhesion and stress fiber assembly (1, 2). In smooth muscle, CaD is found on thin filaments within the contractile domain (3) where it suppresses basal muscle tone by inhibiting active cross-bridge cycling (4), providing fine tuning of the contractility under diverse physiological conditions. The mechanism by which CaD impinges on smooth muscle contractility and whether CaD function is subject to regulation in vivo, however, remain contentious issues (5–9).

Much of the structural information regarding CaD has been garnered from the study of the smooth muscle isoform, h-CaD, which was originally identified as a calmodulin (CaM)-binding protein that also binds filamentous actin (F-actin) (10). In native smooth muscle thin filaments, h-CaD binds lengthwise along the actin filaments with a periodicity of 38 nm (11), although its length (75 nm) (12) is sufficient to span two actin heptads. This is most likely due to staggered binding of h-CaD to the two actin strands (13). Biochemical studies of purified h-CaD demonstrate that it has three functionally distinct domains: an N-terminal domain that harbors the major myosin-binding sites (14–17), a rigid α-helical middle domain that is absent in the nonmuscle isoform, I-CaD (18–20), and a C-terminal domain that houses binding sites for actin (21–24), tropomyosin (Tm) (25, 26), and CaM (27, 28). It is the C-terminal actin-binding domain that blocks the weak binding of myosin and inhibits actomyosin ATPase activity in vitro (22–24, 29), as well as force development in Triton-skinned smooth muscle fibers when added exogenously (29).

Regulation of CaD function has been studied extensively in vitro. In the presence of Ca2+, CaM reverses the binding of CaD to actin (10) and therefore the inhibitory effect of CaD on the actomyosin interaction (30, 31). The affinity between CaD and CaM, however, is only moderate (10^6 M^-1) (32). Although it has been shown that sufficiently high local intracellular concentrations of CaM do exist in both smooth muscle (33) and nonmuscle cells (2) to allow CaD to interact with CaM in vivo, whether such an interaction plays a physiological role still remains a point of controversy. Alternatively, CaD can be phosphorylated by a number of kinases, such as protein kinase C (34, 35), CaM-dependent kinase II (36, 37), casein kinase II (38), cAMP-dependent kinase (39), P34cdc2 (40–42), and mito-

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gen-activated protein kinase (MAPK or ERK) (43). Phosphorylation, at sites primarily in the C-terminal domain of CaD, mitigates its ability to inhibit actin-Tm-activated myosin ATPase activity (44, 45), thus providing another mechanism to regulate the function of CaD.

Evidence for regulation of CaD by phosphorylation in vivo has come from work on nonmuscle cells and differentiated smooth muscle. Matsumura and colleagues (40) showed that phosphorylation by cdc2 kinase during mitosis caused 1-CaD to dissociate from microfilaments in proliferating fibroblasts. Working with differentiated smooth muscle, Adam et al. (46) demonstrated that 32P-labeled h-CaD, purified from phorbol 12,13-dibutyrate-stimulated canine aortic smooth muscle, was phosphorylated at sites VTS*PTKV and S*PAPK within its C terminus (Ser759 and Ser789 by the mammalian numbering scheme). Subsequent work has shown that Ser780 is the putative site of h-CaD phosphorylation in porcine carotid artery strips (47). These sequences conform to the consensus motif S(T/P)P that constitutes the preferred target site for the family of “proline-directed” kinases, of which cdc2 kinase and ERK are prototypes. ERK has been purified from smooth muscle (48), and its activation in smooth muscle has been studied (49, 50). Furthermore, it has been shown that Ca2+-free stimulation of ferret aortic smooth muscle cells, with phenylephrine, resulted in the recruitment of ERK to the plasma membrane, phosphorylation of tyrosine (thereby activating the kinase), and redistribution to CaD-decorated thin filaments (51). Taken together, these studies implicate ERK as an endogenous CaD kinase.

Further understanding of the mechanism of action of CaD has been afforded by electron microscopy and three-dimensional helical image reconstruction. Addition of a 150-residue C-terminal CaD fragment, 606C, to reconstituted actin-Tm filaments caused Tm to move from its position on the inner aspect of the outer domain of actin, toward the inner domain of actin (52). This indicates that CaD affects the conformation of actin-Tm differently than the striated muscle regulatory protein, troponin. Subsequent studies of optimally negatively stained native chicken gizzard thin filaments revealed density on the outer domain of actin on subdomains 1 and 2 that was attributed to CaD (53). However, image density was weak, likely because of incomplete saturation of actin filaments, which may have resulted from partial dissociation of CaD during the purification process. Thus although the density map was generated between CaD-bound and CaD-free filaments (after incubation with Ca2+/CaM), ambiguity remains with regard to the assignment of the binding position of CaD.

To determine the conformation of the C-terminal domain of CaD on purified actin, such that contact regions on F-actin could be assigned unambiguously, and to test whether phosphorylation of this region by ERK, a physiologically relevant event, alters its conformation on actin, we have undertaken the present study. Our data reveal actin-CaD contacts that have not been detected previously and demonstrate that phosphorylation affects the conformation of actin-bound CaD. These reconstructed images, corroborated by results from fluorescence quenching and cross-linking experiments, support a model where the C-terminal region of CaD interacts with actin via two clusters of contact points, one of which dissociates from actin upon phosphorylation, resulting in the loss of inhibition on actomyosin interaction (44). Because this C-terminal domain is shared by both CaD isoforms, the observed conformational change may serve as a common mechanism for regulating the function of CaD in smooth muscle and nonmuscle cells.

Cloning and Expression of C-Terminal Fragments of CaD—The His-tagged C-terminal region of chicken gizzard CaD (H32K, residues Met563–Pro771, using the corrected numbering system; see Ref. 54) and its variant, H32Kqc (with Gln692 mutated to Cys) were expressed as described previously (44). Gln692 is found in the unique part of H32Kqc that is in the region of, yet not too close to, the ERK phosphorylation site (Ser771). Another mutant, H32Kqca, in which Cys692 and Glu764 are simultaneously mutated to Ala and Cys, respectively, was prepared by the same procedure. Thus the wild-type H32K and the double mutant H32Kqca each contain a single cysteine, whereas H32Kqc has two cysteine residues. The mammalian homolog (hH32K) corresponding to Val579 of H32K and Val794 of H32Kqc was expressed in High-Five cells and purified on a Ni2+ column followed by a CaM affinity column (44). As in the previous work, mutagenesis in hH32K was not attempted, because Glu764 does not exist in the mammalian sequence, and there is no suitable mutation site near the phosphorylation sites.

ERK Phosphorylation of C-Terminal Fragments of CaD—Phosphorylation of both chicken and human CaD fragments was carried out using purified proteins and recombinant ERK2 (New England BioLabs, Inc.) in the manufacturer-supplied 1× MAPK buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM EDTA, 2 mM DTT, and 0.01% Brij58), and assayed by mass spectrometric analysis as described previously (44). Although ERK phosphorylates hH32K at both Ser759 and Ser779, H32Kc is only phosphorylated at Ser771 (which corresponds to Ser780 in the mammalian sequence), because the other site is absent in the chicken sequence.

Sample Preparation for Electron Microscopy—Filamentous rabbit skeletal actin (5 μl of 1 μM; prepared as described in Ref. 55) in 5 mM PIPES, pH 7.5, 50 mM KCl, 3.5 mM MgCl₂, 0.1 mM EDTA, 0.02% NaN₃, and 0.5 mM DTT was applied to carbon-coated microscope grids. The actin solution was wicked down to a volume of ~0.5 μl, allowing F-actin to adsorb weakly to the grid surface before a solution of H32K (5 μl of ~25 μM in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride, and 5 μl leupeptin) was added to the grid. The grids were then allowed to stand for 5–15 min at room temperature (22 °C) in a chamber, maintained at a relative humidity of 70–80% to minimize sample evaporation prior to staining. The samples were stained with 1% uranyl acetate. The resulting grids were then examined at 80 kV in a Philips CM120 electron microscope at 60,000× magnification under low dose conditions (12 e⁻/Å²). The micrographs were digitized using a SCAI scanner at a pixel size corresponding to 0.7 nm in the filaments (56). In the current study, filaments were chosen for analysis if the stain surrounding them was well spread and even and if the filaments lacked distortions, discontinuities, or overlying contaminants. Areas displaying astigmatism or specimen drift were not processed, and curved filaments were straightened by applying spline-fitting algorithms (57). Helical reconstruction was carried out using standard methods (58–60) as described previously (61, 62). Layer line data extended to a resolution of ~25–30 Å, and no data were collected beyond 23 Å. The maps of actin-hH32K and actin-phospho-hH32K filaments were each generated by calculating the average amplitudes and phases along layer lines of Fourier transforms determined for 19 filaments from two hH32K and two phospho-hH32K preparations. Maps of individual filaments were averaged after aligning them to each other by iterative rotation and translation in reciprocal space to attain a common phase origin (63).
reaction mixture was dialyzed against 20 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl.
BPM-labeled H32K fragments and actin were mixed typically in a 1:5 ratio in F-buffer (50 mM NaCl, 0.2 mM CaCl₂, 0.4 mM ATP, 2 mM MgCl₂, 2 mM DTT, 2 mM HEPEs, pH 7.5). Ultraviolet irradiation was carried out in a Rayonet RPR-100 photochemical reactor equipped with sixteen 3500 lamps (Southern New England Ultraviolet, Hamden, CT) at 4 °C for 15 min, and the thin filaments were centrifuged at 85,000 rpm for 30 min at 4 °C. The cross-linked products in both pellet and supernatant fractions were analyzed with 10% or 4–20% gradient SDS-polyacrylamide gels (Bio-Rad). The apparent molecular mass of the gel bands was calculated using the mobility of the molecular mass markers (Bio-Rad) on the same gel as standards.

Disulfide Cross-linking Experiments—To disulfide cross-link H32K mutants to actin, we have made use of the ability to cross-link Cysθ to Cad Cysθ with the reagent NbS₂ (64). NbS₂ can catalyze disulfide bond formation between two nearby thiol groups by means of disulfide exchange. G-actin Cysθ was first activated by reacting with NbS₂, as described previously (64, 65), except that G-actin monomer was used in place of filamentous F-actin. The resulting NbS₂-G-actin was then polymerized to F-actin by adding NaCl to 40 mM and MgCl₂ to 2 mM (F-buffer). Unphosphorylated or ERK2-phosphorylated Cad fragments (H32K, H32Kq, and H32Kq/ca) were reduced with 10 mM DTT, then extensively dialyzed against a buffer containing 40 mM NaCl, 5 mM Mops, pH 7.5, 0.2 mM EDTA, and 0.01% NaN₃. The disulfide reaction between NbS₂-F-actin (~14 μm) and Cad fragments, together with gizzard smooth muscle Tm, was carried out at room temperature in F-buffer with a molar ratio of 1:2:14 Cad fragment/Tm:actin. The reaction was quenched at specific times with 2 mM N-ethylmaleimide to block all available cysteine residues. The reaction products were separated on SDS-PAGE with the running gel containing 2 mM CaCl₂, which results in the resolution of the αTm band from actin (66, 67). The bands of the cross-linked products were excised from the gel, incubated with 100 mM DTT, and reapplied to SDS-PAGE. A reaction mixture without Cad was used as a control.

Quenching Experiments—Unphosphorylated and ERK-phosphorylated H32K fragments were first treated with 10 mM DTT for 1 h at room temperature and extensively dialyzed to remove DTT against 20 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl, 1 mM EDTA. A 5-fold molar excess of 1,5-IAEDANS was added from a 20 mM stock solution in dimethylformamide, and the samples were rotated for 4 h at room temperature. The reaction was quenched with 5 mM DTT, and the samples were dialyzed against 20 mM Tris-HCl buffer, pH 7.5, 50 mM NaCl. After labeling, the H32K fragments were mixed with F-actin in F-buffer. Aliquots of acrylamide solution were then added to the mixture, and the fluorescence intensity was measured in a 1-cm-path length cuvette (λmax = 335 nm; λem = 494 nm). Analysis was done with KaleidaGraph software.

RESULTS

Electron Microscopy of F-actin-hH32K Complexes—F-actin was complexed with a polypeptide containing the C-terminal 189 residues of human b-CaD (hH32K), under conditions to maximize saturation of F-actin filaments with the protein. Electron micrographs of negatively stained filaments showed that hH32K caused F-actin to form tight bundles. Bundling was minimized, but not eliminated, by applying F-actin to the sample grids prior to incubation with hH32K or phospho-hH32K (see “Materials and Methods”). Only unbundled filaments were analyzed. Actin substructure, although evident, was frequently obscured by the binding of the hH32K on the surface of filaments (Fig. 1, a and c), which also caused them to appear wider than pure F-actin. Globular structures were occasionally seen projecting from filaments, but no details on the shape, orientation, and periodicity of the hH32K were not discernable. To detect the hH32K binding and determine its position on F-actin, image processing and three-dimensional reconstruction were therefore necessary.

Three-dimensional Reconstructions of Reconstituted Thin Filaments—Filaments bearing hH32K, from two preparations, were negatively stained as described under “Materials and Methods.” The data arising from different hH32K preparations were pooled because they were highly similar. Density maps of reconstituted filaments were calculated from the averages of the Fourier transform layer line data (not shown). All of the maps obtained showed typical two-domain actin monomers that could be further divided into identifiable subdomains 1, 2, 3, and 4 (see labeling in Fig. 2a). When compared with the maps generated from pure F-actin, each separately calculated reconstruction of F-actin-hH32K showed obvious extra density lying on subdomain 1, reaching around the back of the subdomain and ultimately spanning to the inner domain of the neighboring monomer (n – 1) down in the adjacent long pitch helical strand of F-actin. Inspection of the surface views averaged from 19 hH32K-bearing actin filaments (Fig. 2b) showed that hH32K makes broad contact with subdomain 1, and to a less degree with subdomain 2, with a protuberance of density on the top edge of subdomain 1. The hH32K density also extends from the backside of subdomain 1 and spans the “interstrand” gap (Fig. 2a, b, and c, green arrows) to make contact with subdomain 3 of the previous actin monomer on the other long pitch helix (Fig. 2b, red ellipse). hH32K therefore appears to bridge the two strands of the right-handed long pitch helices of F-actin, acting as a “molecular staple.”

Effect of ERK Phosphorylation on Reconstructed Images—In the reconstruction of phospho-hH32K-decorated F-actin (Fig. 2c) averaged over 19 filaments, one sees a number of differences when compared with that of the unphosphorylated sample (Fig. 2b). The mass density over subdomain 1 and subdomain 3’ (of the n – 1 actin monomer) shifts more toward subdomain 3 in such a manner that the “molecular bridge” between adjacent long pitch F-actin strands (Fig. 2b) is no longer visible (Fig. 2c, compare c with f). Subdomain 1 still retains some density that is not observed on F-actin alone (Fig. 2, compare d with f), although it is more diffuse than that observed for F-actin-hH32K (Fig. 2b), and the protrusion at the top edge of subdomain 1 disappears. The weaker or more diffuse density observed in reconstructions of F-actin-phospho-hH32K filaments may reflect both lower saturation of the fil-
aments because of weakened binding of phospho-hH32K to F-actin and/or greater flexibility of F-actin-bound phospho-
hH32K. Greater flexibility would suggest that part of the phos-
phorylated CaD fragment is no longer strongly bound to actin 
filaments. Concomitantly, we have observed that phospho-
hH32K caused less actin bundling (not shown), a phenomenon 
consistent with weakened binding.

Cross-linking between CaD and Actin—To test the “staple-
like” binding mode of CaD fragment on F-actin biochemically, 
we have performed cross-linking experiments. The photo-cross-
linking results (Fig. 3) showed that H32Kqc (a mutant of the 
chicken isoform of hH32K with Gln766 converted to Cys), which 
has two Cys residues at positions 595 and 766, cross-linked 
more than one actin subunit and formed higher order products. 
In addition to the H32Kqc dimer (%/H11011 70 kDa) and the 1:1 adduct 
(%/H11011 80 kDa) of H32Kqc and actin, there were also protein 
bands, albeit weak, on the gel that could be attributed to such 
species as H32Kqc2 %/H18528 actin (%/H11011 110 kDa), H32Kqc 
%/H18528 actin2 (%/H11011 120 kDa), etc. Notably, the 80-kDa band is a doublet. These two 
bands may correspond to the cross-linked products through the 
two Cys, or simply two different sites on actin being hit by the 
cross-linker. The cross-linking yield was only moderate, espe-
cially for the high molecular mass products. Photo-cross-link-
ers are known to form intramolecular cross-linking; they can 
also be quenched by water molecules. Such alternative reaction 
pathways may explain the observed low yield for intermolecu-
lar cross-linking. When the reaction was allowed to last for an 
hour or longer, a smear of high molecular mass species deve-
loped with a concomitant decrease in the 80-kDa species (data 
not shown), indicating that H32Kqc acts as a cross-linker to 
covalently polymerize actin subunits.

The ability of H32Kqc to cross-link two actin monomers was 
further demonstrated by disulfide cross-linking. When Cys374 
of actin was activated by NbS2, cross-linking between H32Kqc 
and actin occurred instantaneously and almost quantitatively, 
resulting in two cross-linked species (Fig. 4). When these two 
products, at 80 kDa (%/band A) and 120 kDa, (%/band B), were 
excised from the gel, reduced with DTT, and applied to SDS-
PAGE again, they were resolved into two, and only two, protein 
species, H32Kqc and actin, the molar ratios between which 
being close to 1:1 and 1:2, respectively. This clearly and un-
equivocally showed that cross-linking did occur and occurred 
only between the CaD fragment and actin, in a more complete 
thin filament with Tm also present. The stoichiometry of the 
reduced protein bands demonstrated that the 120-kDa species 
indeed contained one H32Kqc and two actin monomers. Both 
wild-type H32K and the double mutant H32Kqc/ca also formed 
disulfide-cross-linked products with actin, each giving rise to a 
single species of 80 kDa (similar to %/band A in Fig. 4; data not 
shown). Although these results are consistent with the ex-
pected binding mode, it is somewhat surprising that both 
Cys595 and Cys766 were able to form disulfide linkages with 
Cys374 of actin, despite the fact that each binds to a separate 
actin monomer. Clearly, our results indicated that the two
actin-binding clusters of H32K target individual regions on the actin surface that are both close to Cys374 (see “Discussion”).

**Effect of ERK Phosphorylation on Cross-linking between CaD and Actin**—After H32Kqc was phosphorylated by ERK2, the photo-cross-linked bands of 80 kDa and greater were diminished (Fig. 3), indicating a weakened ability of this CaD fragment to bridge two actin monomers. Thus phosphorylation induces a conformational change in H32Kqc such that one of the two cysteines moves farther away from actin, as depicted in our previously proposed model (44). To determine which cysteine is affected, we have carried out cross-linking experiments using wild-type H32K and the double mutant H32Kqc/ca.

The H32K-actin photo-cross-linking results showed that there was no obvious difference between phosphorylated and unphosphorylated H32K-BPM (Fig. 5A), indicating that phosphorylation does not significantly affect the environment near position 595, which is the only Cys residue in the wild-type H32K and is relatively far away from the phosphorylation site (Ser717) of CaD. On the other hand, H32Kqc/ca, which contains a single Cys at position 766, showed that the H32Kqc/ca-activated F-actin, whereas the cross-linking efficiency of the wild-type H32K with actin was not affected by phosphorylation (data not shown). Thus the physical separation of Cys595 and is relatively far away from the phosphorylation site (Ser717) of CaD is affected, we have carried out cross-linking experiments to test the conformational change in the C-terminal end of CaD.

**Solvent Accessibility Assessed by Fluorescence Quenching**—If ERK treatment indeed causes a conformational change in H32K sufficient to differentially affect the proximity between actin and the two cysteine residues, one might expect that the environment of these two residues is also changed. To test this we have used fluorescence quenching to probe the solvent accessibility of labels attached at these two positions. The two single-Cys fragments, H32K and H32Kqc/ca, were labeled with 1,5-IAEDANS for this purpose. When the quencher, acrylamide, was added to a solution containing F-actin and labeled H32K, the AEDANS fluorescence intensity decreased because of collisional quenching. The slope of the Stern-Volmer plot (the reciprocal of fluorescence intensity plotted as a function of the quencher concentration; Fig. 6) reflects the solvent accessibility of the probe at this position (68). ERK phosphorylated H32K yielded essentially the same slope as that of the unphosphorylated fragment, indicating that the solvent accessibility of Cys595 is not affected by phosphorylation at Ser717. The experiment with H32Kqc/ca, however, showed that after ERK2 phosphorylation, the AEDANS label at Cys766 became more exposed (with a greater slope in the Stern-Volmer plot; Fig. 6), whereas the Cys766 accessibility of the unphosphorylated H32Kqc/ca is much more restricted. Thus the region harboring Cys766 is more sensitive to ERK2-mediated phosphorylation than that around Cys595. In the unphosphorylated state both Cys595 and Cys766 are situated in similar environments, but the latter dissociates from F-actin and becomes more exposed to the solvent after phosphorylation. These results are again consistent with the cross-linking results and also agree well with the phosphorylation-induced flexibility observed in three-dimensional image reconstruction.
phosphorylation at the ERK sites occurred during labor (78). It was recently reported that an increase in rat myometrial CaD induced contractility in vascular smooth muscle (77). Moreover, it resulted in a significant reduction in serotonin-in-duced contraction of canine colonic smooth muscle (75), again suggesting that CaD phosphorylation and inhibition of ERK activity by PD98059 abolished the inhibitory effect of H32K on actin.

The mechanism of reversing the putative inhibition by CaD of smooth muscle contraction is not fully understood. In vitro, the inhibition of actomyosin ATPase activity can be alleviated by CaD/CaM. Such a mechanism may also operate in vivo because the local level of intracellular free CaM can be high enough to regulate the activity of CaD (2, 33). However, some reports have shown that CaM levels may not always be sufficient to fully activate targets for which it has a higher affinity, such as myosin light chain kinase (69–71), arguing against a general model of CaD regulation by CaM. An alternative mechanism calls for phosphorylation of CaD, which might be more important in view of the fact that smooth muscles can contract at low levels of CaM.

Smooth muscle CaD is indeed phosphorylated in vivo upon stimulation; incorporation of 32P increases from 0.35–0.45 mol phosphate/mol CaD at rest to 0.52–1.45 mol/mol upon stimulation depending on the agonists used (72). Although many kinases can phosphorylate CaD in vitro, MAPK (or ERK) emerged as the most likely candidate responsible for CaD phosphorylation in intact smooth muscle in vitro (46, 73, 74). However, the role of MAPK-mediated phosphorylation of CaD remains elusive. Findings that phosphorylation at neither Ser759 nor Ser789 correlates well with the contractile state argue against such a regulatory role (47). Similar conclusions were reached in an earlier report using recombinant ERKs (75). Furthermore, blocking the ERK activity by PD98059 abolished CaD phosphorylation yet did not prevent smooth muscle contraction (50), again suggesting that CaD phosphorylation and contraction are not coupled. On the other hand, acetylcholine-induced contraction of canine colonic smooth muscle was accompanied by CaD phosphorylation (76), and inhibition of ERK pathways resulted in a significant reduction in serotonin-induced contractility in vascular smooth muscle (77). Moreover, it was recently reported that an increase in rat myometrial CaD phosphorylation at the ERK sites occurred during labor (78).

Finally, an ERK-CaD pathway is known to play a role in chemotactic migration of cultured smooth muscle cells (79). Thus the question of whether or not CaD phosphorylation by ERK/MAPK is regulatory is far from settled. In the present study we have sought to determine whether there is a structural basis for such a proposed mechanism.

We previously suggested (44) that the C-terminal domain of CaD undergoes a phosphorylation-dependent conformational change whereby one of the two actin-binding clusters (near the phosphorylation sites) becomes detached from F-actin, whereas the other cluster is largely unaffected. Although the proposed regional dissociation was supported by mass spectrometric analyses coupled with proteolytic digestion and actin cosedimentation and was also consistent with the observed reversal of the actomyosin ATPase inhibition, direct evidence for the conformational change was lacking. Furthermore, it was unclear whether the bound CaD fragment spanned one actin monomer or two. The results of this study resolve both issues.

The three-dimensional reconstruction of hH32K on actin clearly demonstrates that the unphosphorylated C-terminal fragment of CaD binds to two neighboring actin monomers and spans the gap between the strands of the long pitch helix of actin (Fig. 2). The two essential contact points appear to be on subdomain 1 and subdomain 3 of the n1th and the (n − 1)th actin subunit, respectively. Previous studies have revealed that H32K and similar recombinant constructs are elongated molecules (44, 80). Specifically, a similar fragment (2K, which is 31 amino acid residues shorter than H32K) was shown to be 105 Å long, as determined by small angle x-ray scattering (80), long enough to span two actin subunits. The distance between the two cysteine residues at positions 595 and 766 in actin-bound H32K was −45 Å (44), comparable with the separation between two azimuthally adjacent actin subunits. The fact that Cys734 of actin is situated at the junction of subdomains 1 and 3 on a flexible branch of peptide may allow it to form disulfide linkages with both Cys residues near the two actin-binding sites in H32K (Fig. 4) (64, 81). Furthermore, bridging of two adjacent actin subunits by the C-terminal CaD fragment is also consistent with the observation that H32K is able to cross-link F-actin into polymers (Fig. 3). The “stable-like” connectivity between the helical strands may help to explain the observations that CaD and its isoform confer a greater actin filament stability (82, 83). Finally, the H32K-binding regions, particularly subdomain 1, are close to the weak binding site of myosin to actin, consistent with the observation that the C-terminal fragment alone (like the full-length CaD) inhibits the actomyosin ATPase activity (84–86).

Upon ERK2 phosphorylation, the inhibitory effect of H32K on the actomyosin ATPase activity was lifted, which was accompanied by an increase in the distance between Cys595 and Cys766 and lost binding of peptides near Cys766 (44). The result of this conformational change, detachment of one contact point from F-actin, was visualized in this study. Based on the acrylamide quenching experiments, the solvent accessibilities of the AEDANS-labeled H32K and similar recombinant constructs are elongated molecules (44, 80).

**FIG. 6.** Stern-Volmer plot of AEDANS-labeled H32K and H32Kq/c/a quenched by acrylamide. The reciprocal of the relative fluorescence intensity (F/F0) of both unphosphorylated (open symbols, dashed lines) and ERK-phosphorylated (closed symbols, solid lines) AEDANS-labeled H32K (circles, light lines) or H32Kq/c/a (squares, heavy lines) were plotted as a function of acrylamide concentration. All of the data sets have the initial value of 1.0. The straight lines are the best fits obtained by assuming linearity for the changes.
segment of CaD near position 766, but not the region around Cys956, moves away from F-actin.

Interestingly, the “horn-like” protrusion near subdomain 1 seen in the reconstruction of hH32K disappeared when hH32K was phosphorylated, whereas the more diffuse extra mass around subdomain 1 remained. This can be best explained by the proposed conformational change, because the dissociated portion of the fragment is more mobile, and therefore its densities are diminished upon averaging. If this is true, then the part remaining on actin at subdomain 3 must be the N-terminal region of the CaD fragment, and the other end (near the phosphorylation sites) must be interacting with subdomain 1 of the next actin subunit. Such an assignment would also be consistent with the previous findings that it is the C-terminal extreme segment of CaD that contains the inhibitory elements (24, 84–86).

Not many actin-binding proteins assume the same binding mode as has been observed for CaD fragments. Scrin is one such rare case (87), which also “braces” the two actin strands. As pointed out previously (87), subdomains 1 and 3 of actin are structurally homologous, both containing a helix-loop-loop motif (residues 107–137 in subdomain 1 and residues 304–335 in subdomain 3); this feature may enable proteins such as scrin and CaD that contain multiple homologous but nonidentical actin-binding domains to bind across two consecutive actin subunits along the genetic filament helix. But unlike scrin, which is an actin-bundling protein, full-length CaD does not bundle actin filaments in vivo, although it does show bundling activity in vitro.

Despite the fact that hH32K binds F-actin obliquely to the longitudinal filament axis, one would not expect that H32K would interfere with the binding of Tm, because the binding sites of the two proteins show little overlapping on the surface of actin. As shown previously, Tm binds to the actin groove formed between subdomains 1 and 3 in the absence of CaD and moves further toward the inner domains (3 and 4) when CaD is present (52). In the same study the binding position of the added C-terminal CaD fragment (606C, which encompasses residues 621–771 of the chicken sequence) could not be determined unequivocally, partly because its weak density was masked by Tm (52, 53). In the present study, Tm was not included, thus avoiding this problem. These CaD densities did not overlap with the proposed binding position of Tm, yet CaD can modulate the binding of Tm to the actin filament. The position of CaD does, however, at least partially overlap with the binding site of myosin light chain kinase (56). Although direct competition between CaD and myosin light chain kinase for actin binding has not been reported, any potential steric interference may not cause serious problems in vivo anyway, in view of the low intracellular concentration of myosin light chain kinase compared with CaD.

In summary, our data provide a structural basis for the observed biochemical properties of CaD, including its effects on the actomyosin interactions, and the stability and growth of actin filaments. The phosphorylation-dependent conformational change also explains the weakened affinity for actin and reversal of H32K-mediated ATPase inhibition by phosphorylation. Given that we have used in this study a C-terminal fragment of CaD common to both the smooth muscle and the nonmuscle isoforms, our data should be equally salient to studies of the regulation of cytoskeletal structure in nonmuscle systems.

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52. Hodgkinson, J. L., Marston, S. B., Craig, R., Vibert, P., and Lehman, W. (1997) Biophys. J. 72, 2398–2404
53. Lehman, W., Vibert, P., and Craig, R. (1997) J. Mol. Biol. 274, 310–317
54. Guo, H., Bryan, J., and Wang, C.-L. A. (1999) J. Muscle Res. Cell Motil. 20, 725–726
55. Pardue, J. D., and Spudich, J. A. (1982) Methods Cell Biol. 24, 271–289
56. Hatch, V., Zhi, G., Smith, L., Stull, J. T., Craig, R., and Lehman, W. (2001) J. Cell Biol. 154, 611–617
57. Egelman, E. H. (1986) Ultramicroscopy 19, 367–373
58. DeRosier, D. J., and Moore, P. B. (1970) J. Mol. Biol. 52, 355–369
59. Amos, L. A., and Klug, A. (1975) J. Mol. Biol. 99, 51–64
60. Owen, C. H., Morgan, D. G., and DeRosier, D. J. (1996) J. Struct. Biol. 116, 167–175
61. Vibert, P., Craig, R., and Lehman, W. (1993) J. Cell Biol. 123, 313–321
62. Amos, L. A. (1975) J. Mol. Biol. 99, 65–73
63. Graceffa, P., and Jancso, A. (1991) J. Biol. Chem. 266, 20305–20310
64. Graceffa, P. (1995) J. Biol. Chem. 270, 30187–30190
65. Nishida, W., Abe, M., Takahashi, K., and Hiwada, K. (1990) FEBS Lett. 268, 165–168
66. Graceffa, P. (1999) Biochemistry 38, 11984–11992
67. Lehrer, S. S., and Lewis, P. C. (1978) Methods Enzymol. 49, 222–236
68. Isotani, E., Zhi, G., Lau, K. S., Huang, J., Mizuno, Y., Persechini, A., Geguchadze, R., Kamm, K. E., and Stull, J. T. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6279–6284
69. Tran, Q. K., Black, D. J., and Persechini, A. (2003) J. Biol. Chem. 278, 24247–24250
70. Bartley, B., and Barany, K. (1996) in Biochemistry of Smooth Muscle Contraction (Barany, M., ed) pp. 321–339, Academic Press, San Diego, CA
71. Adam, L. P., Haeberle, J. R., and Hathaway, D. R. (1989) J. Biol. Chem. 264, 7699–7703
72. Cook, A. K., Carty, M., Singer, C. A., Yamakawa, I. A., and Gerthoffer, W. T. (1998) Am. J. Physiol. 278, G429–G437
73. Nixon, G. F., Iizuka, K., Haystead, C. M., Haystead, T. A., Somlyo, A. P., and Somlyo, A. V. (1995) J. Physiol. (Lond.) 487, 283–289
74. Krueger, J. K., Gallagher, S. C., Wang, C.-L. A., and Reisler, E. (1997) J. Biol. Chem. 272, 20001–20006
75. Bartegi, A., Fattoum, A., and Kassab, R. (1990) J. Biol. Chem. 265, 2231–2237
76. Adams, S., DasGupta, G., Chalovich, J. M., and Reisler, E. (1990) J. Biol. Chem. 265, 19672–19677
77. Schmid, M. F., Agris, J. M., Jakana, J., Matsudaira, P., and Chiu, W. (1994) J. Cell Biol. 124, 341–350