The molecular basis for the autoregulation of calponin by isoform-specific C-terminal tail sequences

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Accepted 5 March 2001

Summary
The three genetic isoforms of calponin (CaP), h1, h2 and acidic, are distinguished mostly by their individual C-terminal tail sequences. Deletion of these sequences beyond the last homologous residue Cys273 increases actin filament association for all three isoforms, indicating a negative regulatory role for the unique tail regions. We have tested this hypothesis by constructing a series of deletion and substitution mutants for all three CaP isoforms. Here we demonstrate that the C-terminal sequences regulate actin association by altering the function of the second actin-binding site, ABS2, in CaP comprised of the three 29-residue calponin repeats. Removal of the inhibitory tail resulted in an increased binding and bundling activity, and caused a prominent re-localization of h2 CaP from the peripheral actin network to the central actin stress fibers in transfected A7r5 smooth muscle cells. Domain-swap experiments demonstrated that the tail sequence of h2 CaP can downregulate cytoskeletal association efficiently in all three CaP isoforms, whereas the tail of the smooth-muscle-specific h1 CaP variant had little effect. Site-directed mutagenesis further revealed that the negatively charged residues within the tail region are essential for this regulatory function. Finally we demonstrate that the tail sequences regulate the second actin-binding site (ABS2) and not the strong actin-binding ABS1 region in CaP.

Key words: Calponin, Repeats, Regulation, Localization, Actin binding

Introduction
The calponin (CaP) family of actin-binding proteins currently comprises three genetic variants: basic h1 (Takahashi et al., 1988), neutral h2 (Strasser et al., 1993) and acidic CaP (ac. CaP) (Applegate et al., 1994). The tissue-specific h1 variant is involved in the regulation of the contraction/relaxation cycle in smooth muscle, probably by blocking the weak S1 binding site for myosin on actin (EL-Mezgueldi and Marston, 1996), and plays a key role in stabilizing the structural integrity of blood vessels (Taniguchi et al., 2001). The biological function of h2 CaP is less clear but based on localization studies this CaP variant has been implicated in the organization of the actin cytoskeleton (Fukui et al., 1997; Danninger and Gimona, 2000). Acidic CaP is thought to control neurite outgrowth and branching (Ferhat et al., 2001), and neuronal regeneration (Ferhat et al., 1996). Although CaPs display a high degree of sequence similarity within the N-terminal two thirds of the molecule, they differ significantly in their C-terminal regions (reviewed by Gimona and Small, 1996).

CaP interacts in vitro with a large variety of cytoskeletal components, including myosin (Szymanski and Tao, 1993; Szymanski and Tao, 1997), cadesmon (Vancompernolle et al., 1990; Graceffa et al., 1996), desmin (Mabuchi et al., 1997), and tubulin (Fujii et al., 1997), and also with phospholipids (Bogatcheva and Gusev, 1995; Fujii et al., 1995), extracellular regulated Ser/Thr kinases (ERK) involved in MAP kinase signaling pathways (Menice et al., 1997; Leinweber et al., 1999a) and protein kinase C (Leinweber et al., 2000). One common biological property of all three CaP isoforms is binding to actin filaments; however, the subtle differences in biological functions, which can be predicted from the tissue-specific expression patterns of the individual isoforms, have not been determined.

The interaction of CaP with actin is mediated by two independent actin-binding sites: a strong (‘high affinity’) binding site (ABS1) encompassing residues 142-163 and a second, ‘low affinity’ binding site (ABS2) consisting of three copies of the 29 residue CLIK (calponin-like repeat) motif and extending to residue 266 in h1 CaP (Mino et al., 1998; Gimona and Mital, 1998). Sequences beyond Cys273 are different in, and unique for, all three mammalian CaP variants and for the Xenopus XCaP H3 molecule. In h2 and ac. CaP these C-terminal tail sequences contain a number of acidic residues that account for the observed differences in the total isoelectric points of the different CaP isoforms. The role of the N-terminal type 3 calponin homology (CH) domain in CaP is not well understood (Gimona et al., 2002) and a number of different functions have been assigned to this region including the binding to phospholipids and ERK1. In general, however, the CH domain is dispensable for actin binding in vitro and in vivo and probably plays a role in targeting CaP to the cell cortex or in recruiting additional CaP-binding partners.

Three dimensional image reconstructions of CaP in complex with F-actin have revealed the position of the N-terminal CH domain in a position similar to that occupied by other CH proteins such as α-actinin or fimbrin (Hodgkinson et al., 1997; Tang et al., 2001; Hanein et al., 1998). However, the remaining mass of the CaP molecule, still comprising two thirds of the molecule, remained undetected and this region has been suggested to be unstructured or disordered. Similar conclusions
were drawn from structural predictions of the CaP C-terminus, which appears to be mostly random coil. Thus, the precise location of CaP, and particularly its C-terminal region, along the filament remain to be elucidated. In our earlier studies we have shown that deletion of the C-terminal tail sequences strongly enhances cytoskeletal association, especially for the h2 variant of CaP (Danninger and Gimona, 2000). The amino acid sequence of h2 CaP differs from that of h1 or ac. CaP in two critical positions (K151 and K156) within the region of the ABS1. A K154E mutation introduced into chicken αCaP (equivalent to the K156 position in the mouse h1 CaP sequence) has been shown to significantly reduce the actin-binding capacity of recombinant chicken gizzard CaP in vitro. Together, these data point towards a non-functional ABS1 in h2 CaP and thus actin binding of h2 CaP can be achieved only via a functional ABS2. From this we concluded that the tail regions regulate the ABS2 of CaP. This hypothesis was underscored by findings of Bartegi and colleagues who demonstrated a global conformational change in the C-terminal domain of CaP upon actin binding (Bartegi et al., 1999). Their data already emphasized the potential participation of the C-terminal region (including the tandem CLIK repeats) not only in binding to actin directly, but also in the regulation of the interaction of CaP with actin filaments and possibly other, as yet unknown components of the cytoskeleton.

We have now tested our hypothesis that the tail sequences regulate the actin-binding site(s) in CaP by deletion and site-directed mutagenesis studies. We demonstrate that the tails regulate the ABS2 region in all three genetic CaP isoforms and that this regulatory function requires the negatively charged residues present in the tail sequences. These results have significant implications for the understanding of CaP function and regulation in vivo and provide important information for the interpretation of structural data.

Materials and Methods

Construction of plasmids

Standard protocols were used for all cDNA manipulations (Sambrook et al., 1989). PCR reactions for each clone were performed using Taq DNA polymerase. For construction of the h1ABS1-knockout and h2 ch-knockout mutants the following specific forward (FWD) or reverse (REV) primers were used: h1ABS1-knockout: H1 FWD: 5’-AGA GCT CGA GTC ATG TCT TCT GGC TCA AAG TTC CGC TCT

ABS1 REV: 5’-AAC GCT CGA GTC ATG TCT TCT GGC TCA AAG TTC CTT GCA GCC AGA GAC GTT.

h2.ch-knockout: H2charge FWD: 5’-CCC AAG TAC TGC CCA CAG GGA TCT GCA GCT AAG GGC GCT CCT GGG GGT AAC G; H2charge REV: 5’-GAG AGA ATT CTC AGT AAG AGG CTT GCT GGG AGG CTA GGT ACT GTG GGG CTT GCC C. Tail switch mutants were constructed by multiple digestion and ligation steps using the full length CaP cDNAs as templates. All constructs were sequenced using a LI-Cor model 4000 automated sequencer (MWG Biotech, Germany).

Cell culture, transfection and immunofluorescence microscopy

A7r5 rat smooth muscle cells were grown in low glucose (1000 mg/l) DMEM without phenol red supplemented with 10% FBS (PAA, Austria), penicillin/streptomycin (Gibco, Austria) at 37°C and 5% CO2. For transient expression, cells were grown in 60 mm plastic culture dishes and transfected using Superfect (Qiagen, Hilden) at 70% confluence, essentially as described elsewhere (Kranowitter et al., 2001). Expression and stability of the constructs was assessed by Western blotting using a monoclonal antibody against GFP (Clontech, Germany). Cells were replated onto 15 mm coverslips 16 hours post-transfection and prepared for immunofluorescence microscopy after an additional 48 hours on glass coverslips. Cells were washed three times in PBS (138 mM NaCl, 26 mM KCl, 84 mM Na2HPO4, 14 mM KH2PO4, pH 7.4), extracted in 3.7% paraformaldehyde (PFA)(0.3% Triton X-100 in PBS for 5 minutes and fixed in 3.7% PFA (Merck, Germany) in PBS for 30 minutes. Alexa 568 phalloidin was from Molecular Probes (Leiden, NL). Fluorescent images were recorded on a Zeiss AxioScope equipped with an AxioCam driven by the manufacturer’s software package (all Zeiss, Vienna) using a 63x oil immersion lens.

Electrophoresis and western blotting

Analytical SDS gel electrophoresis on 8-22% gradient polyacrylamide mini-slab gels and western blotting onto

![Fig. 1](image_url) (A) The delineated functional and structural domains in calponins. Primary domains are the CH domain, the strong actin-binding site (ABS1), the adjacent triple CLIK repeats harboring the ABS2, and the C-terminal tail. (B) Amino acid exchanges in h2 CaP within the ABS1 region at positions 151 and 156. Note the characteristic h2-type exchanges in the ABS1 region of XCaPH3 from Xenopus laevis. (C) CaP mutants used in this study. All constructs carry the GFP fusion at their respective N-terminal ends. Color coding refers to h1 CaP (pink), h2 CaP (orange), and acidic CaP (brown). The non-functional ABS1 in h2 CaP is shown in gray. Mutant domains are shown as hatched blue boxes.
nitrocellulose (Amersham, Austria) was performed as described elsewhere (Gimona et al., 1990). Transferred proteins were visualized using horseradish-peroxidase-coupled secondary antibodies and the ECL chemiluminescence detection system (Amersham, Austria).

Cell extraction and fractionation
Transiently transfected A7r5 cells grown in 60 mm petri dishes were washed twice with ice-cold PBS and extracted with 200 μl extraction buffer (10 mM Hepes pH 7.5, 1.5 mM MgCl2, 1 mM DTE, 0.1 mM PMSF, 0.67 μg/ml pepstatin, 1.67 μg/ml leupeptin, 0.5% Triton X-100, containing either 10, 120 or 300 mM KCl) for 30 minutes. Cells were scraped off the dish with a cell scraper and the extract was centrifuged at 100,000 g for 30 minutes at 4°C in a Sorvall RC M150 GX centrifuge (Inula, Austria). The supernatant was centrifuged again at 100,000 g for 20 minutes. To the supernatant of this latter centrifugation one-quarter volume of 5· SDS sample buffer was added. Pellets were resuspended in an equal amount of SDS sample buffer. The distribution of GFP CaP proteins in the pellet and supernatant was estimated by densitometric scanning of western blots using a monoclonal antibody against GFP and Adobe PhotoShop 5.02 and HEROLAB Easy WinTM software (Herolab, Wiesloch, Germany).

Results
Domains of calponin
The domain organization of the three genetic isoforms of CaP is highly similar (Fig. 1A). All molecules contain an N-terminal calponin homology (CH) domain, followed by a short linker sequence connecting the high affinity binding site (ABS1), spanning residues 142-163. The three adjacent CLIK repeats constitute the second, autonomous ABS2. Beyond Cys273 the sequences display essentially no similarity, and have different lengths and a varying number of negatively charged residues. The region homologous to the ABS1 in h1 and ac. CaP features two non-conservative amino acid exchanges at positions 151 (R151N) and 156 (K156T) in mouse h2 CaP, thereby essentially neutralizing the positive charges in these regions (Fig. 1B).

Expression and subcellular localization of h1 and h2 calponin mutants
To study the influence of the C-terminal sequences on actin binding of the three CaP isoforms, we constructed a series of EGFP-tagged mutants (Fig. 1C). We have shown previously that EGFP can serve as a suitable tag for both biochemical and fluorescence studies (Danninger and Gimona, 2000; Kranewitter et al., 2001). A7r5 cells were transfected with the indicated constructs and cells were either plated on coverslips for observation by immunofluorescence microscopy, or extracted under different ionic conditions. The cytoskeletal and soluble fractions were separated by centrifugation for western blot analysis. As demonstrated before, both h1 CaP and the h1Δt mutant localize to the central stress fibers in A7r5 cells. No GFP signal is detected at the ends of stress fibers or at the cell periphery (Fig. 2). While there is no obvious difference in stress fiber association between the h1 and h1Δt mutant, the extraction profile indicates that the mutant lacking the C-terminal tail sequences is more strongly retained in the insoluble cytoskeleton fraction (Fig. 2; Fig. 3A), pointing towards an increased actin association.

Fig. 2. Subcellular localization of h1 CaP constructs in A7r5 cells. Both h1 CaP and h1 CaPΔt localize preferentially to the central portion of the stress fibers. Note the lack of overlap for EGFP (green) and phalloidin (red) signals at the ends of stress fibers, corresponding to sites of focal adhesions (arrows). Disruption of the ABS1 by site-directed mutagenesis (h1ABS1-k.o.) causes a significant change in the localization pattern. This effect is reversed by the additional deletion of the tail (h1Δt ABS1-k.o.). Top row, actin visualized by Alexa 568 phalloidin; middle row, GFP fluorescence; bottom row, merged images.
By contrast, removal of the tail sequence in h2 CaP causes a significant shift in the localization pattern of the protein from the cell periphery to the central actin stress fibers (Fig. 4), resembling the h1 and h1Δt variants. At the same time, extractability of the h2Δt CaP construct is decreased significantly (Fig. 3B), again arguing for an increase in cytoskeletal association due to the removal of an autoinhibitory sequence.

Since deletion of the tail apparently increased the association of CaP with the actin cytoskeleton, we asked whether the inhibitory action depends merely on the presence of a C-terminal extension, or whether the individual tail sequences have isoform-specific function(s). To address this question, we exchanged the tails of h1 and h2 CaP with that of one of the three CaP variants and assayed for localization and cytoskeleton association as above. In the case of h1 CaP, fusion to either h2 CaP or ac. CaP tails reduces the amount of h1 CaP retained in the cytoskeletal fraction, with the more negatively charged h2 CaP sequence having a more pronounced effect (Fig. 3A). The localization pattern of the GFP constructs in transfected A7r5 cells is also altered: the previously observed, strictly central stress fiber localization is lost and a fraction of the h1 CaP mutant protein is localized in the more peripheral regions of the cells (Fig. 5).

Again, the opposite effect is seen with the tail-switched constructs of h2 CaP. Neither the h1 CaP nor the ac. CaP tail can cause a decrease in the actin association in h2 CaP
equivalent to the native h2 CaP sequence (Fig. 3B), and the cellular localization is likewise altered in comparison to full length h2 CaP (Fig. 6). Notably, the ac. CaP tail, containing a significantly greater number of negatively charged residues and exhibiting an isoelectric point closer to that of h2 CaP (Table 1), is more efficient in ‘downregulating’ CaP than the neutral h1 CaP tail at high ionic strength (300 mM KCl) extraction conditions (Fig. 3B), but not at the lower ionic conditions of 10 and 120 mM KCl, respectively. Despite a longer tail sequence, containing a larger number of negatively charged amino acids, the net isoelectric point of the ac. CaP tail is slightly more basic than that of h2 CaP tail. Thus, the regulatory potential of the tails followed the calculated net charge of the tail sequences, the most acidic h2 CaP tail (pI 3.09) being more efficient than the less negatively charged ac. CaP tail (pI 3.53), whereas the h1 CaP tail (pI 5.88) had little effect in downregulating actin association.

Since the three CaP variants differ essentially only in their sequences C-terminal of Cys273, the results of the ‘tail-switch’ experiments suggest that there may be specific information within the individual CaP tails, sufficient for differential regulation or isoform-specific function(s). We tested this hypothesis by introducing six point mutations in the h2 CaP tail, changing the aspartate and glutamate residues to asparagine and glutamine, respectively (mutant h2 ch-k.o.*). The isoelectric point of this mutant sequence is basic at 7.99 (Table 1). This ‘neutralized’ tail now causes h2 CaP to associate preferentially with the central actin stress fibers in transfected A7r5 cells (Fig. 4) and significantly increases the actin association in the extraction assay (Fig. 3B). Notably, the effect is almost identical to that observed with the h2-h1t mutant, supporting our initial theory that the negatively charged residues indeed play a role in the autoinhibition of the actin-binding site(s).

While the above experiments document an autoinhibitory role for the tail in h2 CaP, the situation is less clear for h1 CaP. This is primarily due to the presence of two autonomous functional actin-binding sites, ABS1 and ABS2, in this isoform. Gong et al. have shown that a mutation in the critical lysine residue at position 154 in the chicken sequence (156 in mouse) causes a significant reduction in actin binding (Gong et al., 1993). This residue and the more N-terminal arginine 151 are both absent in the ABS1 sequences of mouse h2 CaP (Fig. 1B), suggesting that this site is non-functional in this variant. Therefore, we introduced the identical mutations into

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**Table 1. Calculated isoelectric points of CaP isoforms and individual domains**

<table>
<thead>
<tr>
<th></th>
<th>Full length</th>
<th>Tail</th>
<th>Tailless mutants</th>
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<tr>
<td>h1 CaP</td>
<td>8.99</td>
<td>5.88</td>
<td>9.35</td>
</tr>
<tr>
<td>h2 CaP</td>
<td>7.58</td>
<td>3.09</td>
<td>8.77</td>
</tr>
<tr>
<td>ac CaP</td>
<td>5.50</td>
<td>3.53</td>
<td>8.99</td>
</tr>
<tr>
<td>h2 CaP ch-k.o.*</td>
<td>8.67</td>
<td>7.99</td>
<td>n.a.</td>
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</table>

*Note the dramatic change in pI in the charge mutant of h2 CaP.*
the ABS1 sequence of h1 CaP in order to eliminate the function of this site. In accordance with the previous report (Gong et al., 1993), this mutant displays a significantly reduced cytoskeletal association in extraction experiments, and the localization on actin stress fibers is less defined in transfected A7r5 cells (Fig. 2; Fig. 3A). However, actin binding is not abolished completely, indicating that the ABS2 is intact and also functional in h1 CaP. With this latter mutation (h1 ABS1-knockout) in hand, we were then able to further test the influence of the specific tail sequence of h1 CaP on this isoform’s actin-binding and localization behavior. In agreement with the results obtained with the h2 CaP mutants, we expected that deletion of the tail would release the presumptive block on the ABS2. Indeed, additional deletion of the tail sequence from the h1 ABS1-knockout mutant completely restored both the extraction profile and subcellular localization to almost wild-type levels, despite the mutations in the ABS1.

We also attempted to verify the increased actin association of the Δt mutants by direct biochemical analysis employing actin co-sedimentation assays. For this purpose, we re-cloned the three constructs into a vector suitable for expression in bacteria. However, the tailless constructs for h2 and ac CaP were unstable in solution, thus preventing detailed analysis (not shown). However, note that h1 Δt CaP bound more strongly to purified smooth muscle CaP than its wild-type counterpart and showed increased actin-bundling activity (G.B., W.J.K. and M.G., unpublished), supporting our data obtained in the localization and extraction experiments.

**Discussion**

Calponin is involved in the regulation of actomyosin interactions in several tissues, most prominently in smooth muscle contraction/relaxation cyles and in neuronal outgrowth. However, little is known about the regulation of CaP at the molecular level. Ca2+-binding proteins such as calmodulin or S100 proteins have been demonstrated to release the inhibitory function of CaP on actin filaments sliding over myosin-coated surfaces in vitro (Kolakowski et al., 1995). Likewise, phosphorylation has been shown to potently inhibit CaP binding to actin in vitro (Winder et al., 1993; Uyama et al., 1996; Jin et al., 2000). Nevertheless, it remains to be demonstrated whether phosphorylation of CaP on Ser175 causes CaP to release the filament in vivo, and whether phosphorylation is indeed the major mechanism for regulating the actin-binding function of CaP. This point is particularly noteworthy, since the Xenopus XCaP H3 protein, which contains both h1 and h2-specific sequences, but features also a unique (acidic) tail motif, contains a proline residue at position 175 (Morgan et al., 1999). The different experimental data on the subcellular localization and in vitro functions of CaP impede on the formulation of a consistent mechanistic view on the possible in vivo scenario of regulated CaP-actin interactions.

**Mutational analysis in h1 CaP**

Most of the subtle changes concerning the actin-binding/cytoskeletal association in this major CaP isoform are obscured by the function of the strong binding ABS1. Deletion of the tail sequences had little detectable effect on actin binding, owing to the high intrinsic actin affinity of the intact molecule. However, altering the critical residues K151 and K156 in the ABS1 from the h1 to the h2 type severely affected both the subcellular localization and extraction behavior for this mutant, but did not abolish actin binding. Notably, the extraction profile for the h1 ABS1-knockout mutant resembled that of the ‘charge-neutralized’ h2 ch-knockout construct. Thus, as for h2 CaP, the ABS2 appears to function as an autonomous binding site in this variant. In accordance with our original hypothesis, additional deletion of the regulatory tail largely restored binding back to levels similar to that found in the h2 ch-knockout and h2Δt mutant. Thus, these mutations allow us to separate the relative contributions of the two ABSs in h1 CaP. Kolakowski et al. proposed the existence of two independent actin-binding sites on the calponin molecule and hypothesized further that one site may be involved in the binding/bundling activity of the CaP molecule, whereas the second may be involved in regulating the actomyosin ATPase activity (Kolakowski et al., 1995). As discussed below, these functional requirements may be performed by the ABS2 and ABS1, respectively, as outlined in this study. However, the resolution of our immunofluorescence studies is not sufficient to answer these questions unequivocally and the localization experiments must thus be seen together with the data obtained from the extraction studies. Although the ABS2 can clearly function as an actin-binding site in h2 CaP, it remains to be shown whether the ABS1 forms an independent site or contributes to an overall extended actin-binding interface in the h1 CaP isoform.

**Mutational analysis in h2 CaP**

For h2 CaP the consequences of deletion of the tail were much more evident due to the nonfunctional ABS1. Removal of the C-terminal 35 amino acid residues unmasked the ABS2, significantly increasing the actin association of this mutant. In addition, neutralization of the negative charges in the tail had a similar, yet weaker effect suggesting that the negative charges in the CaP tails are essential for the regulation of the ABS2. Additional tail switch analysis further support our hypothesis that the tails regulate only the ABS2, and not the ABS1, the latter being functional only in h1 and ac CaP. Whereas both h1 CaP and ac CaP tails were capable of downregulating actin association for the h2 CaP variant, the ac CaP tail had almost no effect on the extraction of h1 CaP. Surprisingly, however, the tail sequences of h2 CaP caused a reduction in actin association of h1 CaP, best seen at the high ionic extraction conditions. These latter results are in good agreement with those obtained from the h1 CaP mutants discussed above: altering the ABS1 sequence to an h2 CaP type results in a mutant resembling h2 CaP fused to the h1 CaP tail. Indeed, the extraction profiles and subcellular localization of the h2-h1t and h1 ABS-knockout mutant are almost indistinguishable. These data clearly point towards an autoregulatory function of the isoform specific CaP tails.

Regulation of protein function by structurally flexible tail regions is a common theme in cytoskeletal proteins. The focal adhesion protein vinculin exposes a cryptic actin-binding site in its C-terminus upon binding to actin and phosphatidylinositol (4,5)-bisphosphate, and/or phosphorylation by protein kinase C (Weeks et al., 1996; Steimle et al., 1999). This F-actin-binding
site is regulated by an intramolecular interaction between the acidic, globular head region and the basic vinculin tail. ERM proteins (ezrin, radixin, moesin and merlin) undergo a similar structural transition upon phosphorylation of a threonine residue in the C-terminal tail region by Rho-kinase, which releases a block on the F-actin-binding site residing in the C-terminal part (Tsukita et al., 1997; Matsu et al., 1998; Pearson et al., 2000). It remains to be shown whether the interactions of the CaP tails also involve the globular, all helical N-terminal CH domain of the molecule. A molecular domain arrangement similar to that of CaP (an N-terminal CH domain and a negatively charged C-terminal tail domain) is present in the microtubule plus-end binding protein EB-1, suggesting that head-to-tail interactions play a role in the regulation of CH domain family proteins.

Together with previous biochemical fluorescence data (Bartegi et al., 1999), our results allow new interpretations of some of the important and controversial findings about calponin. One possibility is that the tail sequences interact with the positively charged residues of the CLIK repeats and cause a conformational change in this region of the molecule. Consequently, the ABS2 becomes regulated and inaccessible/nonfunctional. Upon deletion of the tail in our mutant constructs (or the regulated removal of the tail by conformational alterations), a further conformational change in the CLIK repeats causes the formation of a functional second actin-binding site. These interpretations support the conjecture of Bartegi and co-workers, who suggested that the C-terminal tail region and the actin-binding site(s) in CaP communicate with each other and that deletion of the C-terminal sequences can transmit information to the actin-binding region (Bartegi et al., 1999). Our data indicate that this ‘information’ prevents autoinhibition of the ABS2 by the tail sequences. The ABS2 probably occupies a novel site along the actin filament, away from the CH domain docking site (J. L. Hodgkinson and M.G., unpublished), as the interactions of both CaP and the C. elegans protein UNC-87, which contains seven copies of the CLIK module, are not perturbed by saturating amounts of CaP (an N-terminal CH domain and a negatively charged C-terminal tail domain) is present in the microtubule plus-end binding protein EB-1, suggesting that head-to-tail interactions play a role in the regulation of CH domain family proteins.

Although several studies have provided evidence for the specific sorting of β-cytoplasmic actin and of β-actin mRNA to the cell periphery and the ruffling membrane regions of motile cells (Herman, 1993; Bassell et al., 1998), stationary cells such as REF-52 fibroblasts or A7r5 smooth muscle cells appear to incorporate both β- and γ-cytoplasmic, as well as smooth muscle α-actin into their stress fibers, without detectable regional preference. Hence, the observed specific interaction of CaP with the central stress fibers may reflect a structural difference of the actin filament itself (Egelman and Orlova, 2001) rather than actin isotype sorting. Katoh and colleagues (Katoh et al., 2001a; Katoh et al., 2001b) have argued that in human fibroblasts myosin light chain kinase (MLCK) and Rho kinase impact differentially on the structural organization of the actin cytoskeleton. Whereas MLCK may regulate peripheral stress fibers, Rho-kinase is involved in the modulation of the central stress fibers. The two types of stress fibers exhibit differential contractile properties, but potential differences in their molecular composition remain to be determined. In addition, Lehman (Lehman, 1991) demonstrated that caldesmon and calponin are present on distinct types of thin filaments in avian smooth muscle in vivo, suggesting a functional segregation of thin filament subpopulations in smooth muscle. Taken together, these results provoke the speculation that CaP may play a role as a signal transduction molecule for only a certain subset of actin filaments under the control of the Rho/Rho-kinase pathway and primarily involved in cytoskeleton stabilization.

Conclusions

While most recent studies on the molecular interactions of the subdomains in CaP have focused on the N-terminal CH domain, our efforts in this study aimed at understanding the C-terminal, actin-binding modules. Together, these studies support a view of CaP’s molecular domain organization that features an N-terminal ‘signaling domain’, represented by the CH module, and a dynamic, primarily structural, actin-binding region in the C-terminal part (Leinweber et al., 1999b). Both parts appear to function autonomously, as (1) deletion of the CH domain has essentially no influence on the actin-binding and stress fiber association (Gimona and Mital, 1998), and (2)
ERK-binding to the CH domain does not require C-terminal sequences (Leinweber et al., 1999a). While the functional implication of the CaP signaling domain remains to be determined, the actin filament-stabilizing function of the C-terminal actin-binding domains has been well documented both in vivo and in vitro. Most importantly, recent studies using h1 CaP-deficient mouse strains have revealed an important role for this molecule in the integrity of blood vessels (Taniguchi et al., 2001). The observations that the loss of the smooth-muscle-specific CaP variant resulted in increased fragility of the blood vessels accompanied by decreased cell adhesion and causing frequent leakage of the vessels significantly underscore the hypothesis that CaP’s function as a structural component of the actin cytoskeleton is of considerable biological significance for smooth muscle function and regulation.

The authors are grateful to Ulrike Tischler and Maria Schmittner for expert technical assistance. Work in the M.G. laboratory is supported in part by the Austrian Science Foundation.

References


