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Phosphorylation of caldesmon during smooth muscle contraction and cell migration or proliferation

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Summary

The actin-binding protein caldesmon (CaD) exists both in smooth muscle (the heavy isoform, h-CaD) and non-muscle cells (the light isoform, l-CaD). In smooth muscles h-CaD binds to myosin and actin simultaneously and modulates the actomyosin interaction. In non-muscle cells l-CaD binds to actin and stabilizes the actin stress fibers; it may also mediate the interaction between actin and non-muscle myosins. Both h- and l-CaD are phosphorylated *in vivo* upon stimulation. The major phosphorylation sites of h-CaD when activated by phorbol ester are the Erk-specific sites, modification of which is attenuated by the MEK inhibitor PD98059. The same sites in l-CaD are also phosphorylated when cells are stimulated to migrate, whereas in dividing cells l-CaD is phosphorylated more extensively, presumably by cdc2 kinase. Both Erk and cdc2 are members of the MAPK family. Thus it appears that CaD is a downstream effector of the Ras signaling pathways. Significantly, the phosphorylatable serine residues shared by both CaD isoforms are in the C-terminal region that also contains the actin-binding sites. Biochemical and structural studies indicated that phosphorylation of CaD at the Erk sites is accompanied by a conformational change that partially dissociates CaD from actin. Such a structural change in h-CaD exposes the myosin-binding sites on the actin surface and allows actomyosin interactions in smooth muscles. In the case of non-muscle cells, the change in l-CaD weakens the stability of the actin filament and facilitates its disassembly. Indeed, the level of l-CaD modification correlates very well in a reciprocal manner with the level of actin stress fibers. Since both cell migration and cell division require dynamic remodeling of actin cytoskeleton that leads to cell shape changes, phosphorylation of CaD may therefore serve as a plausible means to regulate these processes. Thus CaD not only links the smooth muscle contractility and non-muscle motility, but also provides a common mechanism for the regulation of cell migration and cell proliferation.

Abbreviations: BPM – benzophenone maleimide; CaD – caldesmon; CaM – calmodulin; EM – electron microscopy; Erk – extracellular signal-regulated kinase; FRET – fluorescence resonance energy transfer; GFP – green fluorescent protein; h-CaD – smooth muscle caldesmon; IAEDANS – 5-(iodoacetamidoethyl) aminonaphthalene-1-sulfonic acid; l-CaD – non-muscle caldesmon; MAPK – mitogen activated protein kinase; MLCK – myosin light chain kinase; MS – mass spectrometry; Pak – p21-activated protein kinase; PMA – phorbol 12-myristate 13-acetate; Raf – rat aorta fibroblast cells; Tm – tropomyosin

Remodeling of actin cytoskeleton plays a central role in a variety of cellular processes that involve shape change and movement. Malfunction of these processes could lead to pathological consequences, but how actin-mediated motility is regulated is only beginning to be understood. With recent

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advances in the field, particularly in the elucidation of signaling pathways, one likely mechanism emerges in which the regulation involves key actin-associated proteins via targeted phosphorylation. The identity of these actin-binding proteins that may serve as substrates of specific kinases upon stimulation, however, remains largely unknown. One unique candidate is caldesmon (CaD), which exists in nearly all vertebrate cell types except striated muscle cells, and binds both actin and myosin. In the smooth muscle system, CaD stabilizes the filamentous organization and inhibits actomyosin interactions [1]. In non-muscle cells CaD is involved in cell migration [2]; it also inhibits Arp2/3 assembly [3, 4] and interferes with cell cycle progression [5–7]. Binding of CaD to actin is modulated by Ca^{2+}/calmodulin (CaM) as well as phosphorylation through kinases such as MAPK, Pak and $p34^{cdc2}$ (Figure 1). The potential function of CaD raises the issue of actin filament-based, or more precisely, thin filament-based regulation of motility in non-muscle cells. Regulation involving thin filaments is well established in the striated muscle system where the troponin/tropomyosin (Tm) complex is responsible to turn on and off muscle contraction. The regulatory mechanism is more complicated in smooth muscles where myosin phosphorylation is the major on/off switch, while thin filaments are also under delicate modulation. It may very well be true that similar events take place in non-muscle cells.

The term ''thin filament regulation'' in non-muscle cells was first used by Bretscher [8], who suggested that CaD and Tm form a *contractile unit* that regulates actomyosin interactions in non-muscle cells. In that article a number of penetrat-

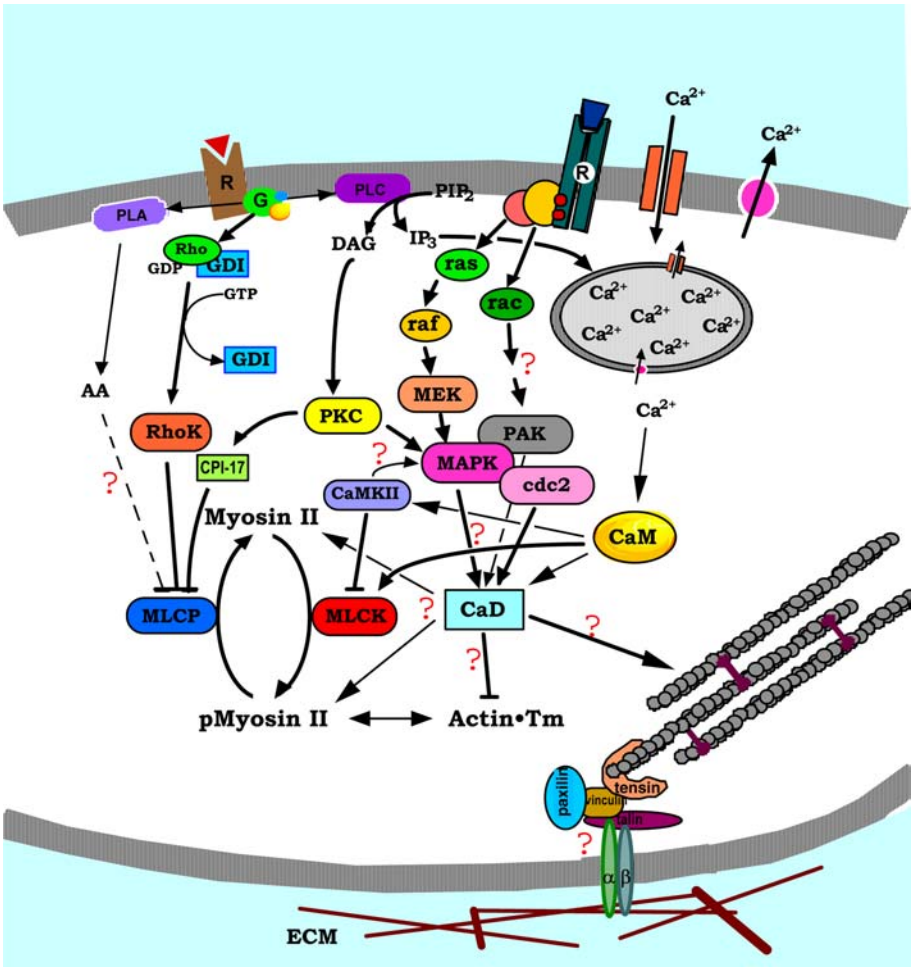

Figure 1. Regulation of actin cytoskeleton in non-muscle cells.

ing questions were raised, such as the low stoichiometry of CaD to actin, and partial dissociation for release of inhibition. Although some of these issues have indeed been clarified for smooth muscle CaD, those concerning non-muscle CaD still remain unresolved after almost two decades. Significantly, the concept of thin filament regulation in non-muscle cells is being supported by quite a few recent reports [7, 9–12]. Thus it seems timely to revisit this problem now. Furthermore, in line with Bretscher's pioneering view, another hypothesis may be considered is that the non-muscle motile system is equipped with an actin-based regulatory machinery, of which CaD is a key substrate of important kinases. By studying the role of CaD in non-muscle cells, we gain insights into the function and regulation of actin cytoskeleton during cellular processes that require cell shape changes, such as adhesion, migration and cell proliferation, and better understand the mechanism of wound healing as well as metastasis of cancer cells.

CaD is present in two isoforms (for reviews, see [1, 13, 14]: h-CaD (the smooth muscle or the *h*igh molecular weight form) and l-CaD (the non-muscle or the *l*ow molecular weight form). In mammals both forms are derived from a single gene by alternative splicing [15]. It has been well-documented that h-CaD is localized in the contractile domain of smooth muscle cells, where it is associated with a subset of actin filaments that are in the neighborhood of myosin filaments [16]. Such a specific distribution confers the postulated function for h-CaD, namely, tethering the actin and myosin filaments and modulating the interaction between them. In non-muscle cells, besides in the stress fibers, where l-CaD together with Tm and α-actinin stabilizes actin filaments [17–19], l-CaD is also present in the leading edges of the cell [20], where actin filaments undergo active assembly and disassembly. This implies the involvement of l-CaD in cell adhesion to the substratum and cell locomotion.

Most of the biochemical studies to date were done on h-CaD, which was originally isolated from chicken gizzard as a CaM- and actin-binding protein [21]. Binding of h-CaD to actin *in vitro* inhibits the actomyosin ATPase activity [22, 23]. The inhibitory effect is reversed by CaM [24, 25] in the presence of Ca^{2+}. The sites for actin and CaM binding, and inhibition are all in the C-terminal region of CaD [26–31], whereas the N-terminal region mainly interacts with myosin [32–34]. Figure 2 summarizes the domain structures of mammalian h- and l-CaD. Since both isoforms are derived from the same gene, the sequences of the

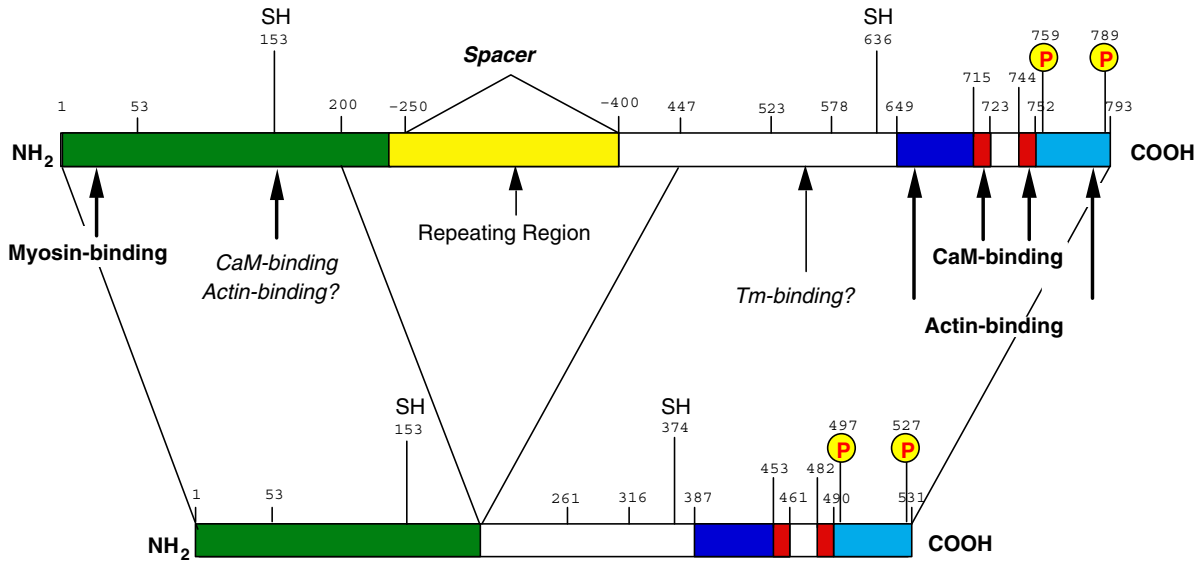

Figure 2. Domain structure of mammalian smooth muscle (top) and non-muscle (bottom) caldesmon. Note that all functional domains are shared, except that the central "spacer" is missing in the non-muscle isoform. Also shown are the two phosphorylation sites common for Erk and cdc2 kinase.

respective functional domains are identical, and hence, the same biochemical properties should apply to both. For instance, a peptide segment (residues 1–53) has been identified in h-CaD that binds smooth muscle myosin [34]. Synthetic peptides (IK29C and MY27C) derived from this myosin-binding sequence were able to induce force in permeabilized smooth muscle cells [35], presumably by displacing the endogenous h-CaD from the myosin filaments. In addition, a C-terminal peptide (GS17C, which binds both actin and CaM; [36]) was also shown to induce force in vascular smooth muscle cells [37]. In this case it was thought that the added peptide competes off the endogenous h-CaD from actin filaments, and alleviates its inhibitory effect. As the sequences for actin and myosin (in the S-2 region where CaD binds) are largely conserved, one expects that similar interactions exist between l-CaD and its non-muscle partners.

In intact smooth muscle the phosphorylation level of h-CaD increases upon stimulation [38]. The kinase responsible for the *in vivo* phosphorylation has been identified to be mitogen activated protein kinase (MAPK; see [39, 40]). In cultured smooth muscle cells where dedifferentiation renders expression switchover of many contractile proteins to the non-muscle isoforms, phosphorylation of l-CaD was also detected upon serum stimulation [41]. Such an increase in phosphorylation was markedly reduced by the MEK inhibitor PD98059, but not by the p38 MAPK inhibitor SB203580 [41]. These results strongly point to extracellular signal-regulated kinase (Erk)-induced phosphorylation of l-CaD during cell proliferation. It has been demonstrated that the sites of phosphorylation are at Ser759 and Ser789 in the mammalian h-CaD sequence [42], which correspond to Ser497 and Ser527 in the l-CaD sequence [41, 43].

Both binding of Ca^{2+}/CaM and Erk phosphorylation result in the reversal of CaD's inhibitory action; it is of interest to see whether or not the molecular mechanisms of such reversal are the same. To investigate this problem we have expressed His_6-tagged fragments containing the sequence of the C-terminal region of human (M563-V793) or chicken (M563-P771) CaD, and a variant of the chicken isoform with a Q766C point mutation. It was shown that within the C-terminal region of CaD there are multiple actin contact points that can be grouped into two clusters. Intramolecular fluorescence resonance energy transfer (FRET) between probes attached to cysteine residues engineered in these two clusters revealed that this C-terminal fragment is elongated in shape, but becomes more compact when bound to actin [44]. Binding of CaM restores the elongated conformation and facilitates dissociation of the C-terminal CaD fragment from F-actin. When the CaD fragment is phosphorylated with Erk (at a single position S717, which corresponds to S759 in the mammalian sequence), the fragment assumes an intermediate length and binding analysis shows that only one of the two actin-binding clusters dissociates from F-actin, whereas the other remains bound (Figure 3). Taken together, these results demonstrate that Ca^{2+}/CaM and Erk phosphorylation govern CaD's function via a similar, but not exactly the same conformational change [44].

To further test this model, photo-crosslinking experiments were carried out using a C-terminal fragment of chicken CaD (H32K) and two mutants, H32Kqc (with Q766 replaced by Cys, thus containing 2 Cys) and H32Kqc/ca (a double mutant with Cys595 also replaced by Ala; containing only one Cys at position 766). When both Cys residues are labeled with a photo-crosslinker, benzophenone maleimide (BPM), H32Kqc is capable of crosslinking actin subunits to form high molecular weight adducts, indicating that it spans at least 2 actin subunits when bound to F-actin. Erk-phosphorylated H32Kqc, however, no longer crosslinks actin to polymers. Similar results were obtained by disulfide crosslinking between H32Kqc and actin (Figure 4). The single-Cys mutant H32Kqc/ca only cross-links to one actin monomer; such crosslinking is also diminished after phosphorylation by Erk. Acrylamide quenching experiments further showed that the solvent accessibility of probes attached to one site (C766), but not the other (C595), is affected by Erk treatment (Figure 5). These results are consistent with a phosphorylation-dependent conformational change that moves the C-terminal segment of CaD, but not the region more upstream, away from F-actin, conferring the observed removal of its inhibitory effect.

The contact sites of CaD on actin was also examined by low-dose electron microscopy and 3D reconstruction of actin filaments decorated with

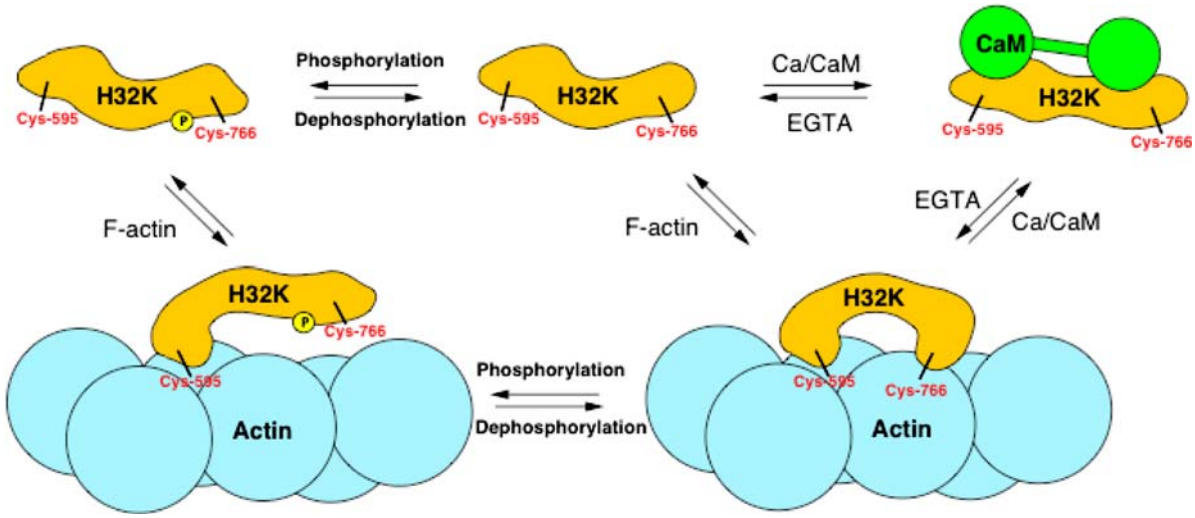

Figure 3. A model depicting the two-pronged binding of the C-terminal region of chicken CaD and the conformational changes induced by CaM and Erk-phosphorylation. Note the actin subunits are drawn only for illustrative purpose; it was shown later that the two clusters do not land on the same actin subunit.

H32K [45]. Helical reconstruction of negatively stained filaments demonstrates density attributable to H32K on subdomain 1 of actin, in good agreement with earlier reconstructions of full-length CaD [46] and with biochemical data. In the reconstructed image H32K is localized on the inner aspect of subdomain 1 and then traverses the upper surface of the subdomain towards actin's C-terminus, and finally forms a bridge to the neighboring actin monomer of the adjacent long-pitch helical strand, by connecting to its subdomain 3. This "staple-like" binding mode suggests a mechanism by which CaD could stabilize actin filaments and resist F-actin severing or depolymerization in both smooth muscle and non-muscle cells. In the image of Erk-phosphorylated H32K the density over subdomain 1 is much less and the inter-strand connectivity is lost. These results are

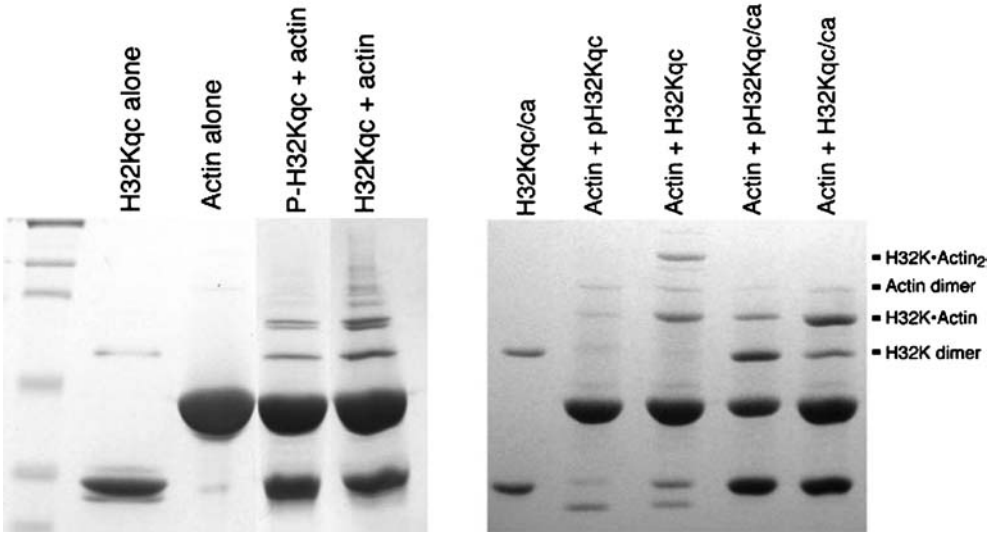

Figure 4. Photo-crosslinking (left) and disulfide crosslinking (right) between H32K and actin. In both cases Erk-induced phosphorylation resulted in less H32Kactin crosslinking and almost no H32Kactin$_2$ species.

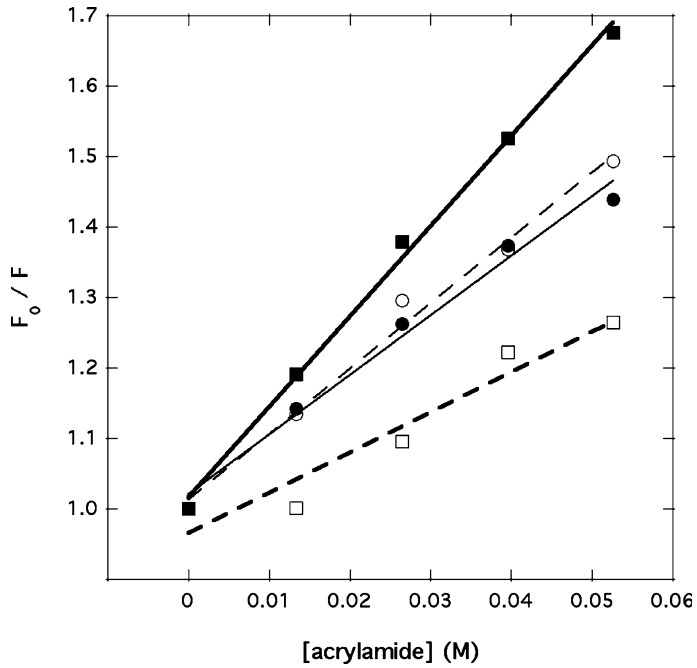

Figure 5. Stern–Volmer plot of unphosphorylated (open symbols; dash lines) and Erk-phosphorylated (closed symbols; solid lines) AEDANS-labeled H32K (circles; thin lines) and H32Kqc/ca (squares; heavy lines) quenched by acrylamide.

consistent with partial dissociation of phosphorylated CaD that deduced from the crosslinking and fluorescence quenching data (see above).

Although demonstrated by the *in vitro* experiments, the regulatory role of h-CaD phosphorylation by MAPK in smooth muscle cells still remains controversial, primarily because inhibition of MAPK failed to produce detectable differences in contractility of smooth muscle tissues [47]. On the other hand, there is little doubt that phosphorylation of l-CaD is involved in non-muscle cell division and locomotion. Matsumura's group made a number of pioneering discoveries on the phosphorylation of l-CaD. Of particular interest are the findings that phosphorylated l-CaD is transiently dissociated from actin filaments during mitosis [48] and that $p34^{cdc2}$ is responsible for regulating this process [49]. Subsequent immunofluorescence images indicate that, in contrast to other actin-binding proteins such as myosin, α-actinin and Tm, l-CaD is *not* concentrated at the cleavage furrow until at later stages of cytokinesis [50]. Despite this, it was postulated that l-CaD inhibits activation of the contractile ring at early stages of assembly, presumably by either blocking the actomyosin interaction, or blocking severing activities of agents such as gelsolin, both being required for cytokinesis. Upon phosphorylation by cdc2 kinase, l-CaD dissociates from actin filaments, thus removing such inhibitory effects, and allowing activation of the contractile ring.

The regulatory role of l-CaD during cytokinesis is an attractive idea, especially in view of the fact that a similar role is played by h-CaD in smooth muscles. Since cdc2 kinase is also a proline-directed Ser/Thr kinase, both h-CaD and l-CaD may be regulated by similar mechanisms, i.e. phosphorylation at Ser/Thr residues in the C-terminal region, leading to a weakened affinity toward the actin filament. Furthermore, prevention of l-CaD phosphorylation at all potential cdc2 sites by mutagenesis indeed slows down, although does not stop, the progression of cell cycle [5, 51]. Nevertheless, several important gaps remain: Direct evidence is missing for CaD phosphorylation in individual cells at various cell cycle stages, since in these studies anti-CaD antibodies were used in microscopic imaging, and phosphorylation of l-CaD was inferred only by subsequent analysis of ^{32}P incorporation of cells arrested at the corresponding stages [48–50]. Likewise the relative distribution of phosphorylated l-CaD and actin

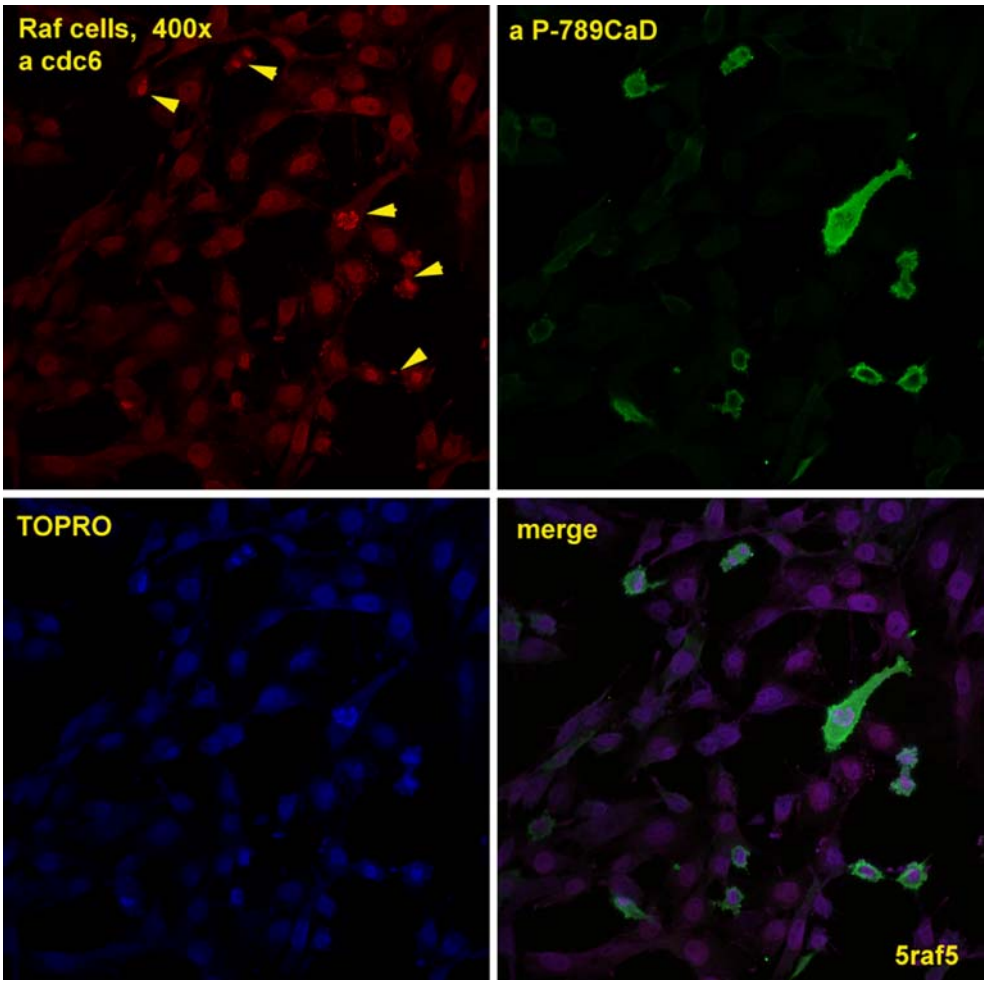

Figure 6. Only dividing cells have CaD prominently phosphorylated. Shown here Raf cells are stained with anti-cdc6, marker of proliferating cells (red), TOPRO (blue) and anti-phospho-S789/527) of CaD (green). Same results are obtained with anti-phospho-S759/497) antibodies. Clearly, not all cells are stained to the same level by the anti-phospho-CaD antibodies. The strong phospho-CaD staining is not due to the rounded-up cell shape, since actin staining with phalloidin does not appear much different among cells (not shown), but is rather closely associated with dividing cells (yellow arrowheads) and by the nuclear aggregation. Cells grown on coverslips are washed in PBS, fixed with 3.7% paraformaldehyde (PFA) and permeabilized with 0.3% Triton X-100 (in PFA and PBS). After antibody treatments the coverslips are rinsed, mounted on glass slides in Mowiol, and examined with a Bio-Rad confocal microscope.

filaments is not known, such that the relationship between l-CaD phosphorylation and actin cyto-skeleton assembly still remains as a mystery.

To address these issues we have made use of anti-phosphopeptide antibodies that had been developed previously in our laboratory [41, 52]. These antibodies recognize phosphorylation at S759 and S789 of h-CaD, but since the C-terminal sequences are identical for the two isoforms, they also recognize S497 and S527 of l-CaD (Figure 1). With the aid of these antibodies we were able to monitor l-CaD phosphorylation in individual cells during the process of mitosis [12]. In agreement with the earlier works by Matsumura (e.g. [48] and Li [51], we found l-CaD indeed undergoes phosphorylation in mitotic cells (Figure 6). Our results further revealed that the level of CaD phosphorylation varies in a reciprocal manner with the level of actin filaments and/or bundles in such a way that it is minimum during the interphase, and reaches maximum at meta/ana-phase (Figure 7). Both the spatial and temporal distributions of phospho-CaD strongly suggest that non-muscle CaD phosphorylation plays an

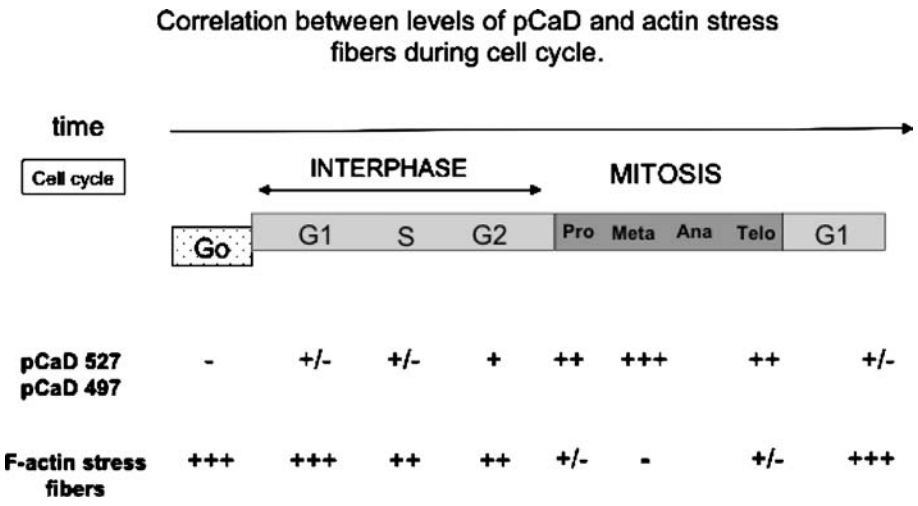

Figure 7. The level of phospho-CaD staining reciprocates that of actin filaments/bundles.

important role in facilitating the remodeling of cytoskeleton during cell division, rather than just regulating cytokinesis *per se*. This conclusion is supported by the observations that CaD phosphorylation is required during cell migration and cell adhesion [54].

Now the process can be understood as follows: Like h-CaD in smooth muscle cells, l-CaD in non-muscle cells is unphosphorylated at the resting state, where it binds to actin filaments and stabilizes the filamentous structure by strengthening the linkage between neighboring actin subunits, and by preventing binding of severing proteins. It may also tether to myosin II and stabilize actin bundles in the stress fibers. Upon stimulation, l-CaD becomes phosphorylated in the C-terminal domain, depending on the kinase, either at Ser497 and Ser527 by Erk in spreading and migrating cells [53, 54], or at additional sites by cdc2 in dividing cells [43], leading to partial or complete dissociation from actin, allowing actin filament severing and/or disassembly. Several recent reports raised the possibility that both MAPK and cdc2 kinase are simultaneously activated under a wide range of conditions [10, 11, 55]. CaD phosphorylation may thus provide a common mechanism to link cell proliferation and cell migration, both requiring cell shape change.

When Ser497 and Ser527 are replaced by alanine residues, so that phosphorylation at these positions no longer takes place, cells tend to be more spread and exhibit much more robust stress fibers (Figure 8). These mutant cells also do not respond as promptly as wild-type cells to stimulants such as phorbol ester, and are more resistant to trypsin-induced cell rounding [12]. Such phenomena are consistent with the idea that unphosphorylatable l-CaD remains bound to actin filaments even when pertinent kinases are activated, rendering the overly stabilized actin cytoskeleton more difficult to remodel itself. However, like in the case when all seven cdc2 kinase sites were mutated mitosis was only delayed, yet not stopped [5, 7], the mutation at Erk sites fails to prevent the cell from eventually changing its shape. Apparently, there are other sites in this region that can be modified by alternative signaling pathways. Notably CaD is known to be phosphorylated by the p21-activated protein kinase (Pak; [56]). The major Pak-phosphorylated sites (Ser672 and Ser702, in the corrected chicken h-CaD sequence, [57]; or Ser452 and Ser482 in the mammalian l-CaD sequence) are also in the C-terminal region, but different from the Erk sites. More recently, it was reported [58] that constitutively active Pak causes Ca^{2+}-independent contractility in smooth muscle fibers accompanied by an increase in the level of h-CaD phosphorylation at the same position as Ser672 (chicken sequence). Since Pak is a downstream effector in the Rac1/Cdc42 signaling pathways [59] under a variety of agonist stimulations, it is not at all surprising that Pak-induced phosphorylation affects migratory behaviors. It can therefore be predicted that when these residues phosphorylatable by Pak are simultaneously mutated to Ala along with the Erk sites,

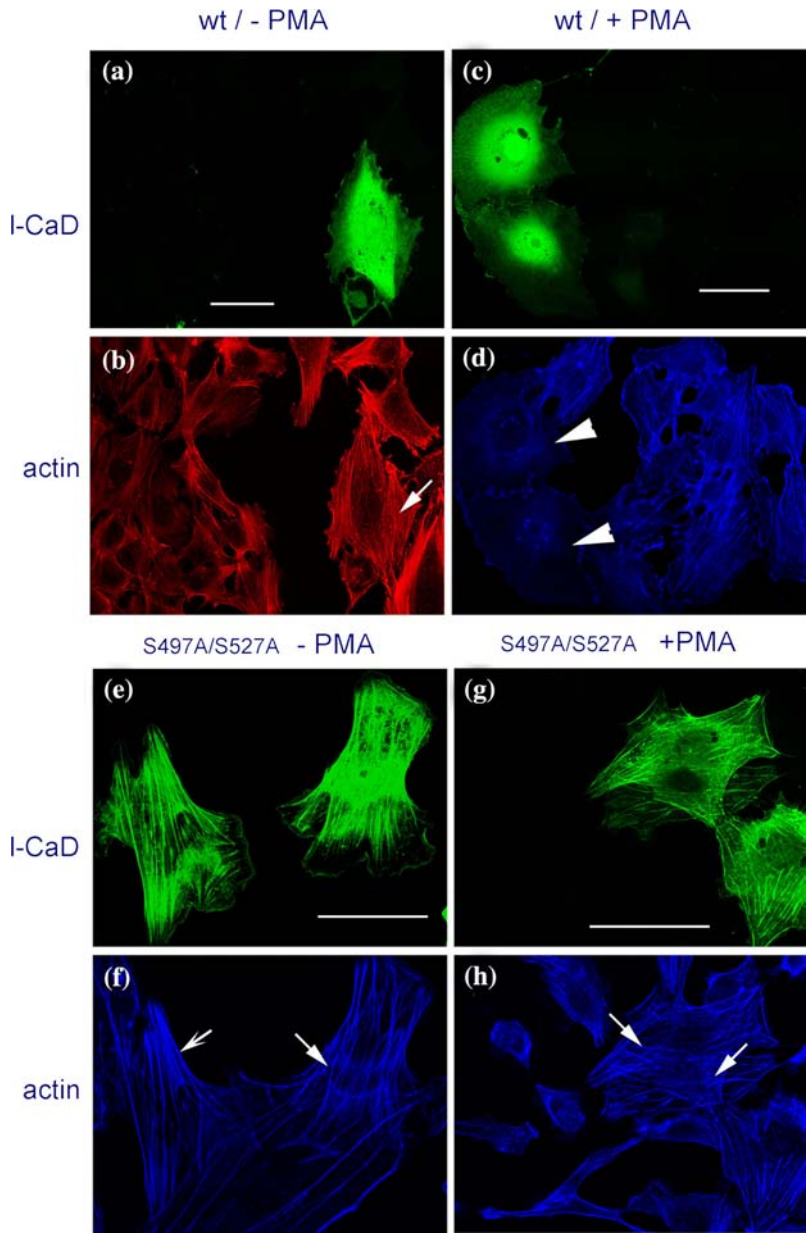

Figure 8. Overexpression of unphosphorylatable CaD slows down stress fibers disassembly in migratory cells. In resting Raf cells transfected with GFP-fused wild-type l-CaD (a, b) exogenous l-CaD localizes to actin stress fibers and cytoplasm. Upon PMA stimulation (c, d) cells become rounder and start to form lamellipodia. In unstimulated cells transfected with double mutant S497A/S527A (e, f) stress fibers appear to be more robust (f, arrows) as compared with wild-type CaD-expressing cells (b, arrow). Upon PMA stimulation, mutant cells (g, h) remain unchanged (h, arrow), while not-transfected cells round up and form lamellipodia. Scale bar: 50 μm.

cell motility would be more effectively inhibited. Whether l-CaD is indeed subject to Pak regulation and what the structural and functional consequences would be are of great interest. These issues should be addressed in parallel with the investigation of the Erk-phosphorylation. Such studies may also reveal potential cross talks between the two signaling pathways.

Another interesting finding is that when rat aorta fibroblast (Raf) cells are transfected with wild-type l-CaD, the stress fibers in these cells disassemble more extensively than in untransfected

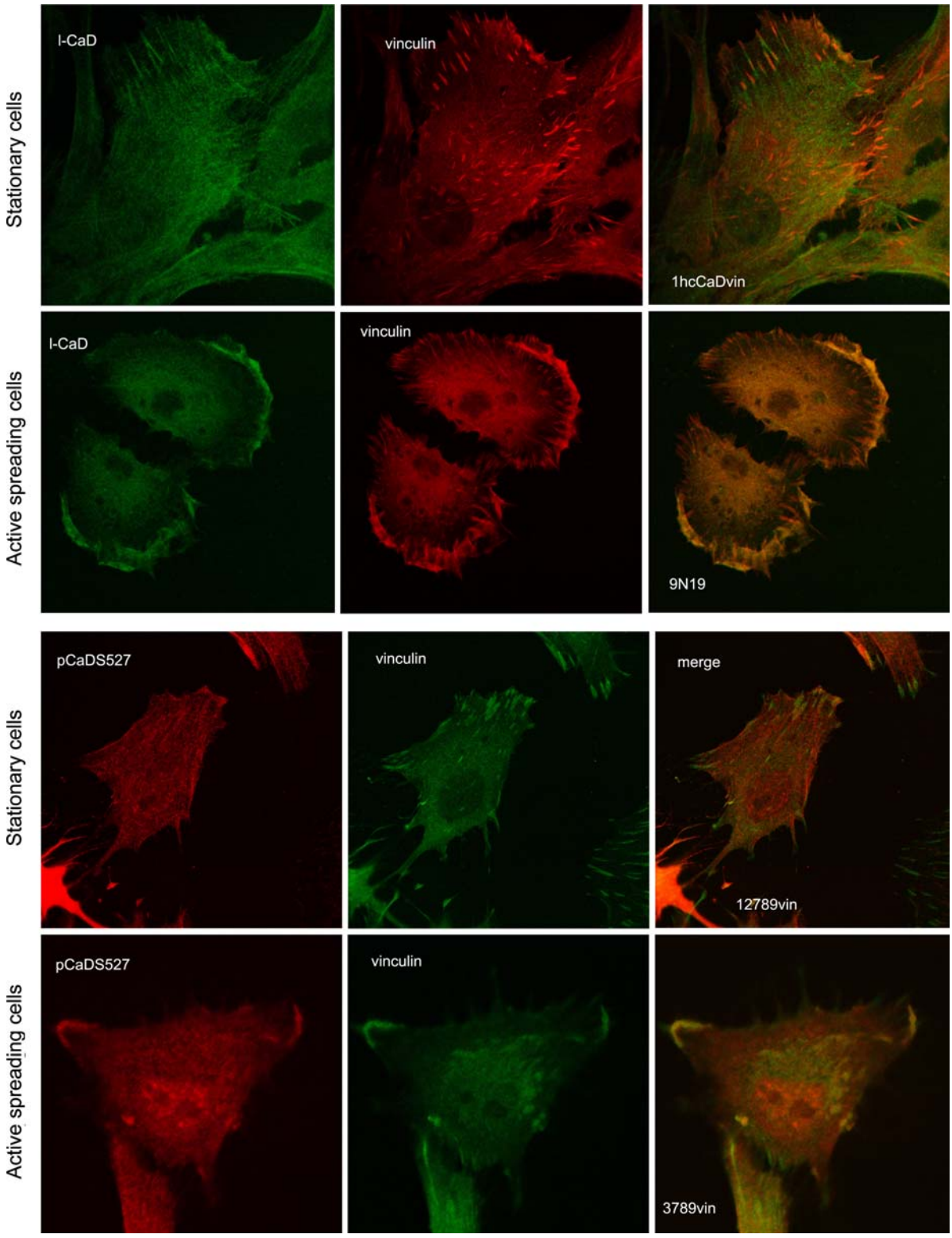
Stationary cells
l-CaD
vinculin
1hcCaDvin
Active spreading cells
l-CaD
vinculin
9N19
Stationary cells
pCaDS527
vinculin
merge
12789vin
Active spreading cells
pCaDS527
vinculin
3789vin

Figure 9. (a) In stationary and well-spread Raf cells (upper panels), l-CaD (anti-CaD, green) is on the actin stress fibers, and not present in the matured focal adhesions, which are stained with anti-vinculin (red). Note the separation of color in the merged image (right panels). On the other hand, in cells shortly after division, which are undergoing post-mitotic spreading (lower panels), l-CaD, which is likely to be phosphorylated, is co-localized with vinculin at the newly formed focal contacts at the leading edges. (b) The well-spread Raf cells (upper panels) and the cells undergoing post-mitotic spreading (lower panels) are stained with anti-phospho-CaD antibody (red) and anti-vinculin (green). Clearly, it is the phosphorylated CaD that is co-localized with vinculin at the leading edges of the spreading cell. Note that although CaD in the cell at the center of the upper panels has only moderate level of phosphorylation, the other cell at lower right corner (of the upper panels; white arrow) has well-established focal adhesions, but is totally absent of phospho-CaD.

cells when stimulated with PMA (Figure 8) [12]. Since the over-expressed l-CaD is apparently all phosphorylated at the Erk sites, this would indicate that phospho-CaD not just passively dissociates from the actin filament, thus allowing disassembly to occur, but is able to *actively* promote the process. The mechanism by which this postulated active role of phosphorylated l-CaD is accomplished remains unknown. One possibility is that phosphorylated CaD recruits some elements (e.g. severing proteins) to the actin filament. More research should be carried out along this line.

It has been documented that in stationary, well-spread cells l-CaD is found on actin stress fibers [17], and not at focal contacts [60]. In fact, ectopic expression of l-CaD in cultured cells was found to inhibit focal adhesion formation [60]. Since Rho induces cell adhesion, it has been suggested that focal adhesion involves actomyosin-based contractility. What has not been examined is the phosphorylation state of such CaD. Whether, for example, phospho-CaD plays a role in recruiting essential partners to the focal adhesions remains to be seen. Furthermore, it was reported that l-CaD is associated with Grb2, Shc and Sos, and in this complex l-CaD was found to be tyrosine-phosphorylated [9, 61]. The significance of this also awaits further investigation. When anti-phospho-CaD antibodies are used to probe Raf cells, l-CaD in non-dividing cells is not phosphorylated and exhibits primarily a filamentous distribution, which coincides well with actin staining, but distinctly different from vinculin staining. Interestingly, in cells shortly after division, where the level of l-CaD phosphorylation remains high, phospho-CaD is also found to co-localize with vinculin at nascent focal adhesions and the leading edges of spreading cells (Figure 9) [12]. This suggests that phosphorylated CaD is involved in the early formation of focal adhesions. It should be pointed out that small and faint adhesions near the leading edge have been reported to transmit strong propulsive tractions, whereas large, mature focal adhesions exerted weaker forces [62]. As focal adhesions mature, changes in their structure, protein composition, or phosphorylation state may convert their function from transmitting strong propulsive forces, to providing a passive anchorage for maintaining a spread morphology [62–65]. If one assumes that l-CaD exerts its function in the contractile domains of the cell, this could be the reason why l-CaD is not detected in mature "anchoring" focal adhesions. Although myosin II patches are often seen in the rear of the moving cell, punctate and transitional myosin II accumulation are also observed in the protruding edges of spreading cells [66, 67]. Myosin I, too, exhibits a punctate distribution within initial protrusions of migrating fibroblasts [68]. Interestingly it was proposed that for cells to probe the rigidity of the environment they must invoke appropriate contractile mechanism [69]: focal adhesions with myosin II could be such a sensing device. More data are required to clarify such a transient localization of phospho-CaD at nascent focal adhesion and the eventual relationship between pCaD and myosin at these loci. Phosphorylation at Ser497 and Ser527 may very well facilitate l-CaD to recruit myosin, and other cellular components to these foci during post-mitotic spreading and would suggest a new role for phosphorylated CaD in the formation of new sites of cell attachment to the substratum. Such an intriguing possibility should be further investigated.

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