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Direct interaction between caldesmon and cortactin

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Abstract

Actin polymerization and depolymerization plays a central role in controlling a wide spectrum of cellular processes. There are many actin-binding proteins in eukaryotic cells. Their roles in the remodeling of the actin architecture and whether they work cooperatively await further study. Caldesmon (CaD) is an actin-binding protein present in nearly all mammalian cells. Cortactin is another actin-binding protein found mainly in the cell cortex. There have been no reports suggesting that CaD and cortactin interact with each other or work as partners. Here, we present evidence that CaD binds cortactin directly by overlay, pull-down assays, ELISA, and by column chromatography. The interaction involves the N-terminal region of cortactin and the C-terminal region of CaD, and appears to be enhanced by divalent metal ions. Cortactin competes with both full-length CaD and its C-terminal fragment for actin binding. Binding of cortactin partially alleviates the inhibitory effect of CaD on the actomyosin ATPase activity. Not only can binding be demonstrated in vitro, the two proteins also co-localize in activated cells at the cortex. Whether such interactions bear any functional significance awaits further investigation.
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Actin polymerization and depolymerization plays a central role in controlling a wide spectrum of cellular phenomena and intracellular processes. This is especially true in cells like smooth muscle cells (SMCs)[1], which undergo cell shape changes during contraction and relaxation. The ability to recover cell shape after either transient or sustained deformation, depends on the dynamic and constant rearrangement of actin cytoskeleton [1,19]. Cytoskeletal disassembly is known to contribute to the hyporesponsiveness of SMC and mesangial cells to vasopressors both in vitro and in vivo [29,3]. It was also found that under pathological conditions that mimic cytochalasin D treatment, mesangial cells respond with an up-regulation of a group of actin cytoskeleton regulatory proteins in an attempt to restore the cytoskeletal integrity [2].

The canonical mechanism of actin regulation involves cofilin (an actin disassembly factor), profilin (a G-actin sequester), Arp2/3 (a branching factor), formin (a barbed end capping protein that promotes actin assembly), and capping proteins (that control the disassembly at the pointed end). However, there are many more other actin-binding proteins in the eukaryotic cells. These include gelsolin (a severing protein) and tropomyosin (a filament stabilizer). Individual actin filaments are further organized into higher order structures by α-actinin (a bundling protein), filamin, and spectrin (crosslinking proteins), as well as myosins (motors). The actin filaments are anchored to the cell membrane to provide a framework that allows force-bearing; this is achieved by a group of membrane-associated proteins, such as dystrophin, utrophin, talin, vinculin, paxillin and integrin. While the players are identified, the interactions between them are largely unknown. Like non-muscle cells, SMCs also contain a large number of actin-binding proteins. The roles of these cytoskeleton proteins in the remodeling of the actin architecture of SMC and whether they

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[1] *Abbreviations used:* CaD, caldesmon; SMC, smooth muscle cells; RAF, rat aorta fibroblast.

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work in a cooperative fashion are intriguing questions that await further study.

Caldesmon (CaD) is an actin-binding protein present in both smooth muscle and non-muscle cells. In addition to actin, it also binds myosin and calmodulin, making it one of a few unique actin-binding proteins. Binding of actin is weakened by phosphorylation and by calmodulin in the presence of Ca^{2+} (for recent reviews, see [11,22]). These biochemical properties equipped CaD to be an ideal candidate for a regulatory element of the actin cytoskeleton. Recent studies have shown that CaD stabilizes actin stress fibers and may control the cell shape change during proliferation and migration [13].

Cortactin is also an actin-binding protein, mainly found in the cell cortex [26]. The molecular structure suggests that cortactin functions to promote actin assembly, especially at the branching site, because it also binds and activates Arp2/3 [5]. CaD, on the other hand, inhibits Arp2/3-mediated actin nucleation [28]. There have been no reports that CaD and cortactin would interact with each other, despite both bind actin and are involved with Arp2/3. Structurally CaD and cortactin share some common features too: both are elongated molecules with a central region containing repeating sequences. Here we present data to show that CaD is capable of interacting with cortactin directly. Not only binding can be demonstrated in the in vitro experiments, the two proteins are also co-localized at cell cortex. Whether such interactions bear any functional significance remains to be further investigated. It may prove to be another example that cytoskeletal proteins work together to constitute a more versatile regulatory system that allows the cell to weather a wide range of conditions and environmental impacts.

Materials and methods

Protein preparations

Cortactin was cloned from mouse smooth muscle RNA (stomach) mixture by RT-PCR using primers derived from the amino acid sequence at both N- and C-terminal segments. After DNA sequencing it was found that the repeating motif was shorter (the 6th kelch repeat is missing), and therefore the isoform was identified as cortactin-B. The cDNA of the His-tagged protein was subcloned into an expression vector (pET28a) and transformed into Escherichia coli cells for expression. Recombinant cortactin-B was purified by Ni^{2+}-column, and further purified by using a CaD–Sepharose affinity column that was prepared by coupling 10 mg C-terminal CaD fragment (H32K) to 1 g CNBr-activated Sepharose 4B according to the manufacturer's instructions. Crude preparation of cortactin was loaded onto the H32K–Sepharose column in the presence of 1 mM $CaCl_2$ or Mg Cl_2. After washing with the same buffer, the column was eluted with a buffer containing 5 mM EDTA, and further eluted with a buffer containing 0.5 M NaCl or 50 mM glycine (pH 2.3) and 250 mM NaCl. All fractions were analyzed by SDS–PAGE. Recombinant full-length smooth muscle CaD was expressed in insect cells and purified without heating [30]. Recombinant fragment of the N-terminal region (N240) and the C-terminal region (H32K) of CaD were prepared according to the chicken sequence as previously described [15,10]. Purified cortactin-B was also coupled to CNBr-activated Sepharose 4B beads for pull-down experiments.

Antibodies

Anti-CaD (H-C) and anti-phospho-CaD (anti-pS789CaD) polyclonal antibodies, both being raised in this laboratory, have been described previously [17,4]. Monoclonal anti-cortactin (#05-180) was obtained from Upstate.

Western analysis

The eluted fractions collected from H32K-Sepharose affinity column were separated on SDS–PAGE and immunoblotted with monoclonal anti-cortactin followed by affinity purified anti-mouse secondary antibodies, conjugated with IRDyeTM 800. For detection we used Odyssey Infrared Imaging System by LI-COR, Biosciences (Lincoln, NE) allowing quantitative data presentation by measuring direct infrared fluorescence.

Overlay assays

Recombinant cortactin-B (20 μg) was first run on 10% SDS–PAGE, and electrophoretically transblotted to PVDF membrane. After washing with PBS buffer, the membrane was blocked with LI-COR Odyssey blocking buffer, cut into strips, and incubated separately with 0.5 μM CaD, H32K or N240 in a solution containing 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 5 μM leupeptin, 1 mM dithiothreitol (DTT), and 1 mM PMSF in the presence of 1 mM $CaCl_2$ or EDTA after being washed with the same buffer. After further washing, the membrane strips were rinsed with PBS–Tween, and allowed to react with anti-CaD polyclonal antiserum, followed by fluorescent probe labeled anti-rabbit secondary antibodies and then scanned with the LI-COR Odyssey Infrared Image system.

Pull-down assay

Interaction between proteins was assayed by incubating H32K- (or cortactin-) coupled Sepharose beads with cortactin (or full-length CaD, H32K or N240) in a buffer containing 20 mM Tris–HCl, pH 7.5, 50 mM NaCl and 1 mM $CaCl_2$ for 1 h, washing with the same buffer four times, each time the beads were spun down at 3000 rpm for 5 min, followed by addition of a buffer containing 2 mM EDTA to dissociate the complex and centrifugation again after 10 min incubation.

ELISA assay

Cortactin was coated on a 96-well microtiter plate at 4 °C overnight, followed by blocking with 10% milk. After five times of 5 min washing with PBS–Tween, different concentrations of CaD or H32K were added to the plate in the presence of 1 mM $CaCl_2$ and incubated at 37 °C for an hour. After another five times PBS–Tween washing, 1:1000 diluted anti-CaD (H-C) was added into the wells, and incubated at 37 °C for 1 h. The antibody was washed thoroughly with PBS–Tween, and the plate was incubated with anti-rabbit secondary antibody at 37 °C for 1 h. H_2SO_4 was added to stop the tetramethylbenzidine staining reaction. The plate was then placed in the Labsystems Multiskan Plus to read the OD at 490 nm.

Mass spectrometric analysis

V8 protease (1:20, w/w; from Sigma) digestion of H32K or cortactin was performed at room temperature for 10 min in a buffer containing 20 mM Tris–Cl (pH 7.5), 50 mM NaCl, 1 mM $CaCl_2$, 1 mM DTT, 5 μM leupeptin, and 1 mM PMSF and stopped with diisopropyl fluorophosphate. The digested fragments were mixed with cortactin- or H32K-sepharose resin in the same buffer at room temperature for 1 h. After several times of washing, the resin was boiled for 10 min and the supernatant was collected by centrifugation. The solution was mixed with α-cyano-4-hydroxycinnamic acid in an acetonitrile/trifluoroacetic acid mixture and spotted onto a 100-well stainless steel plate. Mass spectrometric analysis was then performed on a MALDI-TOF mass spectrometer (PerSeptive Biosystems Model Voyager-Elite with Delayed Extraction Technology) in linear mode.

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