Actomyosin cross-linking by caldesmon in non-muscle cells

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Abstract The role of myosin-binding in cytoskeletal arrangement of non-muscle low molecular weight caldesmon (*l*-caldesmon) was studied. The N-terminal myosin-binding domain of caldesmon (N152) colocalized with myosin in transiently transfected chicken fibroblasts. When added exogenously to the Triton-insoluble cytoskeleton, N152 enhanced *l*-caldesmon displacement by exogenous C-terminal actin-binding fragment (H1). Thus, a significant fraction of *l*-caldesmon cross-links actin and myosin. In contrast, in epithelioid HeLa cells most of *l*-caldesmon was only actin-bound as H1 alone was enough for its displacement. Phosphorylation by mitogen-activated protein kinase reduced the capability of H1 to displace endogenous *l*-caldesmon, suggesting it may represent a regulatory mechanism for actin–caldesmon interaction in vivo. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Caldesmon; Cytoskeleton; Mitogen-activated protein kinase; Phosphorylation

1. Introduction

Interaction between actin and myosin underlies a wide variety of intracellular motile events including contractility, substrate attachment, shape changes, intracellular granule movement and division. Both structural and functional means exist to control the accessibility of actin by myosin motor domain in smooth and non-muscle cells. The spatial requirement for actomyosin assembly is often achieved through rearrangement of actin cytoskeleton guided by small GTP-binding proteins of the Rho family [1], while functional regulation is mediated by a specific Ca^{2+}/calmodulin-dependent phosphorylation of myosin regulatory light chains [2] and actin filament-associated protein caldesmon [3].

Caldesmon is a multifunctional actin-binding protein that inhibits actomyosin interaction and ATPase activity by preventing the strong binding of myosin heads to actin in a Ca^{2+}/calmodulin- and tropomyosin-dependent manner (reviewed in [3]). The inhibitory activity of caldesmon and the high affinity binding sites for calmodulin, actin and tropomyosin reside in the C-terminal part of the molecule, whereas the N-terminal region contains tropomyosin-binding and the major myosin-binding site of the protein [4–6]. Two isoforms of caldesmon identified in smooth muscle and non-muscle cells differ in an extended central spacer domain which links structurally and functionally conserved termini, implying that both high (*h*-caldesmon) and low molecular weight (*l*-caldesmon) isoforms have matching functions in vivo [7]. While the functional activity of caldesmon has been well documented in both ATPase assays [8,9], in vitro motility studies [4,10,11] and skinned or intact muscle fiber models [12–14], less evidence is available for the involvement of *l*-caldesmon in structural aspects of cytoskeleton dynamics and architecture.

In non-muscle cells *l*-caldesmon was localized to actin stress fibers [15–17], membrane ruffles [16] and lamellipodia extensions [17]. It was implicated in glucocorticoid-induced actin reorganization [18], association with signaling receptor complexes [19,20], vesicular transport [21], and mitosis-specific regulation of actin dynamics [22]. Transfection of cultured non-muscle cells with the full-length caldesmon cDNA resulted in either altered cell morphology and increased cell spreading [23], or decreased contractility, inhibition of stress fiber formation and focal adhesions [17]. Overexpression of the fully functional actin-binding C-terminal fragment of caldesmon also promoted cell spreading, but has been reported to inhibit attachment and to stabilize actin bundles and tropomyosin with no effect on actin dynamics and intracellular granule movement [24,25]. Hence, it is likely that the N-terminal myosin-binding activity of the full-length caldesmon adds significantly to the structural function of caldesmon.

The structural aspect of caldesmon function is thought to involve 'tethering' actin to myosin filaments. The cross-linking activity of caldesmon has been described in vitro [5,26] and in in vitro motility studies [4,11]. In permeabilized smooth muscle cells and platelets the N-terminal caldesmon fragments or peptides had dissociating effects on actomyosin and affected contraction [27,28], suggestive of a role for N-terminal myosin-binding of caldesmon in 'tethering' actomyosin in living cells. Furthermore, caldesmon is thought to be regulated by phosphorylation in smooth muscle ([29] for a review) and non-muscle cells [22]. The major caldesmon kinase in vivo has been identified as $p42/44^{erk1,2}$ mitogen-activated protein kinase (MAP-kinase) which phosphorylates sites in the C-terminus of caldesmon [30]. While the effect of MAP-kinase on caldesmon inhibitory activity is still questionable, evidence is growing that phosphorylation alters caldesmon interaction with actin

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Abbreviations: MAP-kinase, mitogen-activated protein kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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in vitro [31–33]. In this report we further address the two questions as to whether caldesmon cross-links actomyosin and if phosphorylation regulates this activity in native cytoskeleton of non-muscle cells.

In order to assess the arrangement of *l*-caldesmon in cytoskeleton of permeabilized cells we used the recombinant C-terminal (H1) and N-terminal (N152) caldesmon fragments to antagonize interaction of endogenous caldesmon with actin and myosin, respectively. First, we show that N152 colocalizes with myosin filaments in transiently transfected chicken fibroblasts thus demonstrating functionality of the isolated N-terminal myosin-binding site in vivo. Second, we find that in these cells a significant fraction of *l*-caldesmon simultaneously binds and cross-links actin and myosin as it could only be displaced by both H1 and N152, while the rest of *l*-caldesmon appears to be only microfilament-associated. In contrast, in HeLa cells most of *l*-caldesmon is bound to the actin cytoskeleton and could be displaced by H1 alone. Third, we demonstrate that phosphorylation of H1 by MAP-kinase decreased its displacing ability thus implicating the MAP-kinase in regulation of cytoskeletal dynamics and architecture via the effect on actin-binding and cross-linking properties of caldesmon.

2. Materials and methods

2.1. Expression and transfection of caldesmon constructs

The cDNA encoding the N-terminal 152 residues of chicken gizzard caldesmon (N152 fragment) was subcloned from the pMWT172-N152 plasmid [5] into pcDNA3 vector (Invitrogen) using the *Bam*HI and *Eco*RI restriction sites. Then it was subcloned into pFLAG CMV2 vector (Kodak) using the *Hin*dIII and *Eco*RI restriction sites to encode N152 with the N-terminal FLAG sequence followed by an artificial Leu-Gly-Thr-Gln-Leu-Gly-Ser sequence as a result of subcloning. Chicken fibroblasts at 50–70% confluence were transfected using the Maxifectin-21 kit (Pepline, Moscow, Russia) according to the manufacturer's protocol and after 12 h of incubation were processed for immunofluorescence. N152 and the C-terminal caldesmon fragment H1 were expressed in BL21(DE3)pLys *Escherichia coli* cells transformed with pMWT172-N152 and pMWT172-H1 plasmids as earlier [5,32]. H1 phosphorylated by recombinant GST-$p44^{erk1}$ MAP-kinase was obtained as described in [32] with modifications [33].

2.2. Cell isolation, culturing and immunofluorescence

Chicken fibroblasts (obtained by passaging the primary chicken embryo gizzard cells) and HeLa cells (ATCC# CCL-2) were cultured in DMEM (Sigma) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from HyClone). For immunofluorescence microscopy, fibroblasts were fixed in 3.7% formaldehyde and permeabilized with 1% Triton X-100. FLAG-tagged N152 was visualized with mouse monoclonal antibody to FLAG (Kodak). An anti-chicken gizzard myosin polyclonal antibody was raised in rabbits and subsequently affinity-purified. This antibody specifically cross-reacted with non-muscle (Fig. 1B) and smooth muscle myosin. FITC-labeled goat anti-mouse (Molecular Probes) and Texas red-labeled anti-rabbit secondary (Sigma) antibodies were used. Cells were examined using Zeiss Photomicroscope III and photographed on Kodak Tri X-pan 400 film.

2.3. Caldesmon displacement analysis

Cells were grown in six-well plates until 80–90% confluence and washed twice with ice-cold phosphate-buffered saline. Cells were permeabilized for 15 min on ice with 10 mM Tris–HCl, pH 7.0, 60 mM KCl, 125 mM sucrose, 0.05% Triton X-100, 0.1 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 mM phenylmethylsulfonylfluoride, and washed twice with the binding buffer containing 10 mM Tris–HCl, pH 7.0, 30 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol and 0.05 mM phenylmethylsulfonylfluoride to obtain the Triton-insoluble cytoskeletons. 1 ml of the binding buffer containing appropriate concentrations of caldesmon fragments was then added to each well. After incubation on ice for 15 min the cytoskeletons were washed twice with the binding buffer and extracted with 2× concentrated loading buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were resolved by SDS–PAGE in duplicates and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were treated with 0.25% glutaraldehyde for 30 min and probed with rabbit anti-chicken gizzard caldesmon primary antibody [32] to detect *l*-caldesmon and H1 or with the fraction of this antibody purified on immobilized N152 to detect the N-terminal caldesmon fragment. Secondary peroxidase-conjugated anti-rabbit antibody (Pierce) was used and the membranes were developed with 3,3′-diaminobenzidine. HP-IIcx scanner and NIH Image software were used to quantify immunoblots.

3. Results

3.1. The N-terminal domain of caldesmon colocalizes with myosin in transfected cells

To investigate whether the N-terminal myosin-binding activity of caldesmon is functional in living cells, chicken fibroblasts were transfected with pFLAG CMV2 expression vector containing inserted cDNA encoding the N152 caldesmon fragment. Fig. 1 shows that N152 colocalized with the myosin-containing filamentous structures as revealed by double immunofluorescence using anti-FLAG M2 antibody and anti-myosin antibody that specifically recognizes non-muscle myosin on Western blot (Fig. 1C). In addition, the nuclear staining of N152-FLAG was also observed in the transfected cells, consistent with the earlier reported ability of low molecular weight proteins to penetrate into this cellular compartment [34]. Transfection of fibroblasts with the control pFLAG-BAP plasmid (Kodak) resulted in homogeneous cytoplasmic distribution of BAP (data not shown) indicating specific intracellular localization of N152. This and our previous data [5] indicate that the N-terminal domain of caldesmon contains a myosin-binding sequence, which is fully functional in the cellular context and thus may mediate the caldesmon–myosin interaction.

3.2. Caldesmon cross-links actin and myosin in chicken fibroblasts, but not in HeLa cells

Chicken gizzard fibroblasts and epithelioid HeLa cells were used to assess distribution of endogenous *l*-caldesmon within the naturally arranged cytoskeleton. The cultured cells were permeabilized with Triton X-100 to preserve cytoskeleton and the displacement of endogenous *l*-caldesmon from the Triton-insoluble cytoskeletal fraction by added bacterially expressed caldesmon fragments was investigated. As shown in Figs. 2 and 3, addition of increasing amounts of N152 alone caused no displacement of *l*-caldesmon indicating that *l*-caldesmon did not only bind to myosin. No displacement was also observed when as much as 50 μM N152 alone was applied (data not shown). Incubation of fibroblast cytoskeletons with increasing concentrations of the C-terminal actin-binding fragment H1 caused as little as 20% of endogenous *l*-caldesmon displacement suggesting it represents only the actin-bound fraction (Fig. 2). However, titration with H1 in the presence of N152 caused up to 60% dissociation of *l*-caldesmon (Fig. 2). This suggests that at least 40% of the endogenous caldesmon is simultaneously bound to actin and myosin filaments and cross-links them in fibroblast cytoskeleton.

In contrast, H1 alone was sufficient to dissociate most of the endogenous *l*-caldesmon from Triton-insoluble cytoskeleton of HeLa cells (Fig. 3). Moreover, concomitant addition of

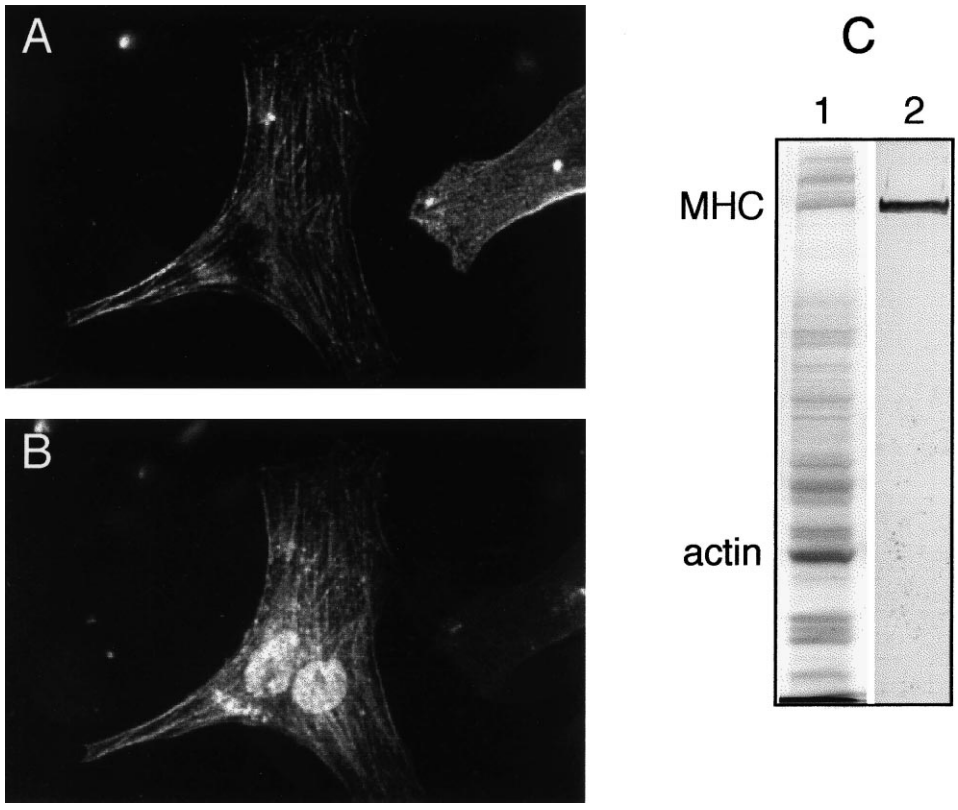

Fig. 1. Colocalization of the N-terminal caldesmon fragment with myosin in chicken fibroblasts. The cells were transiently transfected with pFLAG CMV2 vector bearing the insert encoding the N-terminal myosin-binding domain of caldesmon N152 and immunostained either against myosin (A) or FLAG tag (B). C shows that the anti-myosin antibody recognizes only myosin heavy chains (MHC) on the Western blot (lane 2) of total fibroblast protein (lane 1).

N152 produced little further effect on *l*-caldesmon displacement despite very high sequence homology between the N-terminal domains of chicken and mammalian caldesmons. Thus, most of caldesmon appears to be only actin-bound in epithelioid HeLa cells.

3.3. Phosphorylation by MAP-kinase inhibits actin–caldesmon interaction

Phosphorylation by MAP-kinase has been reported to affect the actin-binding affinity of caldesmon in vitro [31–33]. Here we further studied whether this effect is conserved in naturally arranged cytoskeleton. For this purpose, the C-terminal caldesmon fragment H1 was phosphorylated in vitro by active recombinant $p44^{erk1}$ MAP-kinase. Two serines corresponding to residues 759 and 789 of the human *h*-caldesmon sequence were found to be fully phosphorylated (Levine, B.A., Marston, S.B. and Vorotnikov, A.V., unpublished observation). The phosphorylated H1 was found much less effective in displacement of endogenous *l*-caldesmon from HeLa cytoskeleton than its unphosphorylated counterpart (Fig. 3). This illustrates that phosphorylation inhibits interaction of H1 with actin cytoskeleton per se and implies that MAP-kinase regulates actin–caldesmon interaction in cells.

4. Discussion

The mechanism by which caldesmon cross-links actin to myosin has been addressed in multiple studies in vitro and finally has been shown to involve simultaneous binding of its C-terminal part to actin and interaction of the N-terminus with myosin subfragment 2 [5]. However, the arrangement of caldesmon inside living cells has been a matter of long debate because it is impossible to visualize it within the dense actomyosin domain. Indirect evidence indicates that an impairment of caldesmon interaction with cytoskeletal proteins per se, such as microinjection of antibody interfering with tropomyosin binding [35], endogenous caldesmon phosphorylation that inhibited its actin-binding [22], treatment of permeabilized cells with the N-terminal myosin-binding fragment [27] or synthetic peptides [28], causes partial displacement of endogenous caldesmon and results in changes of cell morphology, disruption of cytoskeleton, or modulation of contractility. For most of these effects the dissociation of caldesmon-mediated cross-link and loss of 'tethering' actin to myosin filaments have been implied, albeit structural evidence has still been missing.

In this report we used a transfection technique and directly demonstrated that the N-terminal domain of caldesmon interacts with myosin in intact cytoskeleton. These results agree with the recent observation that myosin-binding peptides of the N-terminus of caldesmon label actomyosin-like structures within the contractile domain of permeabilized ferret smooth muscle cells [28] and thus justify the potential use of N152 to dissociate endogenous caldesmon from myosin filaments in permeabilized cells.

Direct evidence is now provided that a significant fraction of *l*-caldesmon is involved in cross-linking of actomyosin in fibroblast cytoskeleton. This correlates with the high degree of

actin and myosin colocalization in these cells as revealed by immunofluorescent microscopy and their prominent motile activity. On the other hand, our results on *l*-caldesmon displacement from HeLa cytoskeletons do not reveal myosin-bound caldesmon. Perhaps, the fraction of juxtapositioned actin and myosin filaments in these cells is smaller than in fibroblasts due to the peculiarities of epithelioid cell cytoskeleton organization and the relatively low level of actomyosin found in many transformed cells.

The finding that caldesmon significantly contributes to 'tethering' actin to myosin in permeabilized cells suggests that it may be directly involved in modulation of cytoskeletal

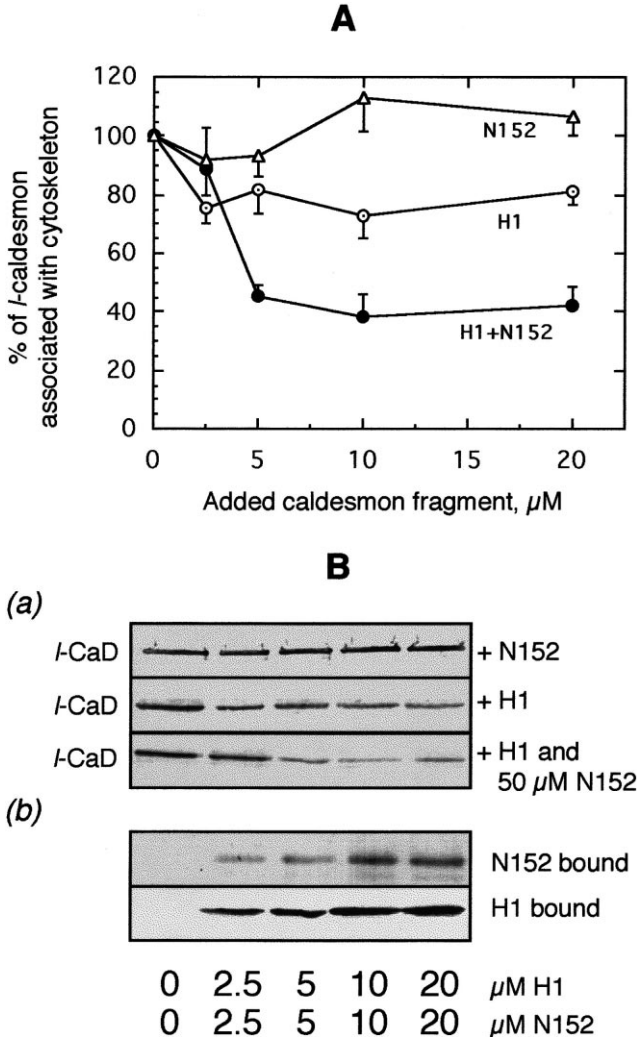

Fig. 2. Dissociation of caldesmon from Triton-insoluble cytoskeletons of chicken fibroblasts. A: The displacement of *l*-caldesmon isoform by increasing amounts of exogenously added actin-binding (H1, open circles), myosin-binding (N152, triangles) recombinant fragments of caldesmon, or by increasing H1 in the constant presence of 50 μM N152 (H1+N152, closed circles) is shown. Each point is the mean ± S.E.M. from two determinations for 2.5 μM N152 alone and four independent experiments otherwise. B: Representative immunoblots showing *l*-caldesmon (*l*-CaD) content left in Triton-insoluble cytoskeletons of fibroblasts incubated with exogenous caldesmon fragments depicted on the right (a) and relative binding of these fragments to cytoskeletons (b). The applied concentrations of individual caldesmon fragments are indicated below, H1+50 μM N152 denotes indicated concentrations of H1 in the constant presence of 50 μM N152. The actin band staining and quantification (not shown) confirmed equal loading onto the gels.

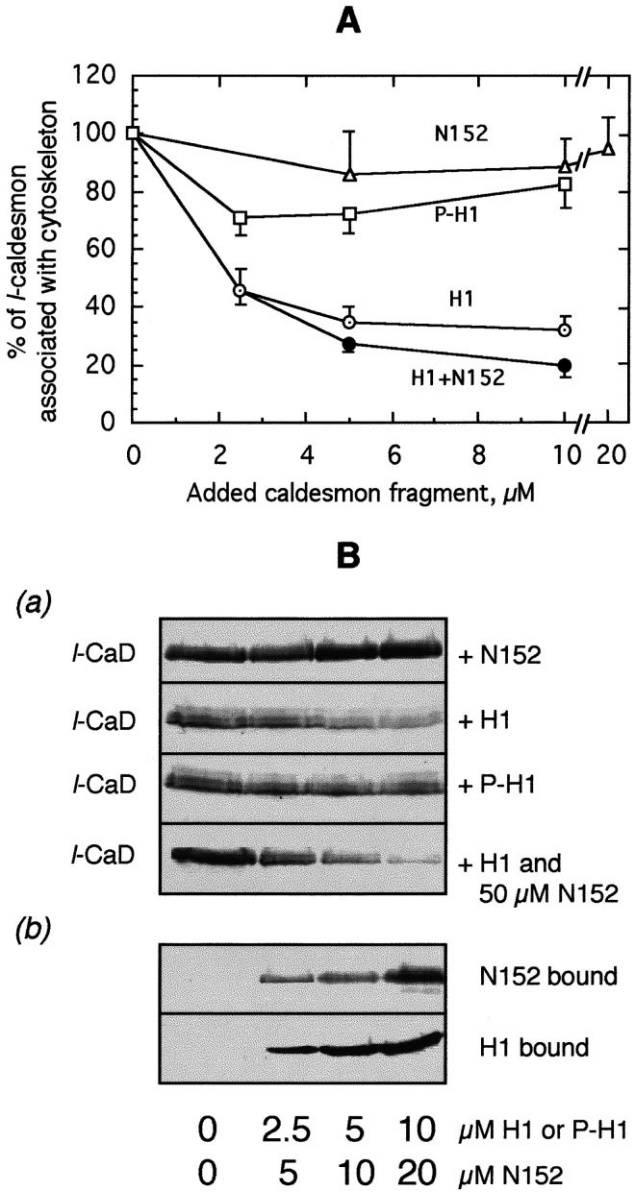

Fig. 3. Displacement of *l*-caldesmon from Triton-insoluble cytoskeletons of HeLa cells by exogenous caldesmon fragments and the effect of MAP-kinase phosphorylation. Abbreviations are as in the legend for Fig. 2, P-H1 (squares) denotes H1 phosphorylated in vitro by the recombinant $p44^{erk1}$ MAP-kinase. A: Quantification of immunoblots represented in (B), the mean values ± S.E.M. from two determinations for 5 μM N152 alone and four independent experiments otherwise are shown.

dynamics during such events as cell motility, chemotaxis and cytokinesis. Reversible phosphorylation of actin-associated proteins may constitute a mechanism for modulating their activity and lead to changes in cytoskeletal dynamics. We hypothesized that caldesmon is similarly regulated. The binding of caldesmon to myosin is affected by phosphorylation within its N-terminal domain in vitro, however, the responsible kinases are unlikely to phosphorylate caldesmon in vivo (reviewed in [29]). Only $p42/44^{erk1,2}$ MAP-kinase and protein kinase C were shown to phosphorylate sites within the C-terminal actin-binding domain of caldesmon in intact smooth muscle [30,32] and non-muscle cells [36,37], respectively. Both inhibit actin–caldesmon interaction in vitro [29]

while no significant effect of MAP-kinase on other caldesmon activities has been described so far [32,38–40]. This report confirms the in vitro results that phosphorylation by MAP-kinase inhibits caldesmon interaction with isolated actin filaments [31,33] and provides the first indication that this effect is conserved in the native Triton-insoluble cytoskeleton suggesting it can serve as a major function of caldesmon phosphorylation in vivo.

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