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Smooth muscle myosin filament assembly under control of a kinase-related protein (KRP) and caldesmon

DMITRY S. KUDRYASHOV[1,†], ALEXANDER V. VOROTNIKOV[1], TATYANA V. DUDNAKOVA[1], OLGA V. STEPANOVA[1], THOMAS J. LUKAS[3], JAMES R. SELLERS[2], D. MARTIN WATTERSON[3] and VLADIMIR P. SHIRINSKY[1,*]

[1]*Laboratory of Cell Motility, Cardiology Research Center, 3rd Cherepkovskaya street 15A, Moscow 121552, Russia;* [2]*Laboratory of Molecular Cardiology, NHLBI NIH, Bethesda, MD 20892;* [3]*Drug Discovery Program and Department of Molecular Pharmacology and Biological Chemistry, Northwestern University, Chicago, IL 60611, USA*

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Abstract

Kinase-related protein (KRP) and caldesmon are abundant myosin-binding proteins of smooth muscle. KRP induces the assembly of unphosphorylated smooth muscle myosin filaments in the presence of ATP by promoting the unfolded state of myosin. Based upon electron microscopy data, it was suggested that caldesmon also possessed a KRP-like activity (Katayama *et al*., 1995, *J Biol Chem* **270:** 3919–3925). However, the nature of its activity remains obscure since caldesmon does not affect the equilibrium between the folded and unfolded state of myosin. Therefore, to gain some insight into this problem we compared the effects of KRP and caldesmon, separately, and together on myosin filaments using turbidity measurements, protein sedimentation and electron microscopy. Turbidity assays demonstrated that KRP reduced myosin filament aggregation, while caldesmon had no effect. Additionally, neither caldesmon nor its N-terminal myosin binding domain (N152) induced myosin polymerization at subthreshold Mg^{2+} concentrations in the presence of ATP, whereas the filament promoting action of KRP was enhanced by Mg^{2+}. Moreover, the amino-terminal myosin binding fragment of caldesmon, like the whole protein, antagonizes Mg^{2+}-induced myosin filament formation. In electron microscopy experiments, caldesmon shortened myosin filaments in the presence of Mg^{2+} and KRP, but N152 failed to change their appearance from control. Therefore, the primary distinction between caldesmon and KRP appears to be that caldesmon interacts with myosin to limit filament extension, while KRP induces filament propagation into defined polymers. Transfection of tagged-KRP into fibroblasts and overlay of fibroblast cytoskeletons with Cy3KRP demonstrated that KRP colocalizes with myosin structures *in vivo*. We propose a new model that through their independent binding to myosin and differential effects on myosin dynamics, caldesmon and KRP can, in concert, control the length and polymerization state of myosin filaments.

Introduction

It is generally accepted that smooth muscle contraction occurs as a result of the sliding of myosin and actin filaments along each other as it was originally suggested for the cross-striated muscles (Huxley and Hanson, 1954). The primary protein constituent of smooth muscle thick filaments is smooth muscle myosin, a type II myosin that is composed of two heavy chains and two pairs of the light chains (for a review see Sellers and Goodson, 1995). Ultrastructurally, the myosin II molecule consists of the two globular heads attached to a long coiled-coil tail by the neck. Myosin heads comprise motor (ATPase) domains while the tail is involved in filament assembly.

Purified smooth muscle myosin assumes folded or 10S conformation in the presence of physiological concentrations of ATP and does not support spontaneous filament formation (Suzuki *et al*., 1978; Onishi and Wakabayashi, 1982; Trybus *et al*., 1982; Craig *et al*., 1983; Olney *et al*., 1996). The folded conformation of myosin is disfavored when the 20 kDa regulatory light chain (RCL) of myosin is phosphorylated by a Ca^{2+}-calmodulin-activated enzyme myosin light chain kinase (MLCK) (Onishi and Wakabayashi, 1982; Craig *et al*., 1983; Olney *et al*., 1996). Consequently, phosphorylated myosin adopts an extended or 6S conformation and spontaneously polymerizes into filaments. In smooth muscle tissue, myosin filaments persist regardless of the level of RLC phosphorylation (Somlyo *et al*., 1981; Gillis *et al*., 1988; Horowitz *et al*., 1994; Xu *et al*., 1997).

† Current address: Department of Chemistry and Biochemistry, ULCA, Los Angeles, CA 90095, USA

* To whom correspondence should be addressed: Tel.: +7-095-414-724616713; Fax: +7-095-414-6719/6712; E-mail: shirinsky@cardio.ru

However, substoichiometric phosphorylation is not sufficient to maintain filaments because recent studies suggest that smooth muscle myosin filaments are labile to mechanical perturbation and may be partially dissociated if not protected by phosphorylation (Qi *et al*., 2002). Altogether, these findings suggest that myosin filaments in smooth muscle are not intrinsically stable and require external stabilizing mechanisms including but not limited to RLC phosphorylation.

One such mechanism could rely upon the presence of an accessory protein known as kinase-related protein (KRP or telokin) that is expressed in smooth muscle from the MLCK genetic locus (Ito *et al*., 1989; Shirinsky *et al*., 1993). KRP is a 17 kDa non-kinase protein identical to the C-terminal domain of MLCK (Collinge *et al*., 1992; Birukov *et al*., 1998; Watterson *et al*., 1999). KRP binds at the neck region of myosin and shifts the equilibrium between 10S and 6S conformers toward unfolded state (Masato *et al*., 1997). Thus, KRP action on myosin polymerization resembles that of light chain phosphorylation by MLCK. However, KRP does not activate myosin ATPase and does not support force generation.

Another possible thick filament stabilizer is the smooth muscle protein p38 that has been suggested to stabilize unphosphorylated myosin filaments through the binding to the light meromyosin portion of myosin molecule (Okagaki *et al*., 2000). KRP and p38 do not compete for myosin and, therefore, may cooperate in providing myosin filament stability. The presence of p38 might account for the preservation of the thick filament structure in permeabilized smooth muscle fibers that have lost endogenous KRP during extraction (Walker *et al*., 2001).

A report by Katayama *et al*. (1995) suggested that caldesmon was a myosin filament stabilizing protein exhibiting activity similar to KRP. Caldesmon is smooth muscle protein capable of interacting with both myosin and actin thin filaments (for a review see Marston and Redwood, 1991; Sobue and Sellers, 1991). Caldesmon binds to the neck region of myosin within its S2 subfragment (Hemric and Chalovich, 1988, 1990; Ikebe and Reardon, 1988) a region proximal to the KRP binding site (Shirinsky *et al*., 1993; Masato *et al*., 1997; Silver *et al*., 1997). This suggests a possible competition between two myosin-binding proteins. However, the mechanism by which caldesmon induces myosin filament assembly is unclear since caldesmon was shown not to alter the equilibrium between folded and unfolded myosin monomers (Katayama *et al*., 1995).

In the present work we address the modes of KRP action on myosin filaments and its functional relationship with caldesmon. Sedimentation assays provided a simple distinction between caldesmon and KRP activities. KRP supports filament extension or propagation while caldesmon appears to function as a point for nucleation. Thus, the joint action of KRP and caldesmon could account for the assembly of uniform myosin filaments *in vivo*. Transfection and cytoskeleton overlay experiments provided evidence that myosin is an *in vivo* target for KRP. Therefore, we propose a new model for the co-involvement of KRP and caldesmon in the assembly and maintenance of the thick filaments in smooth muscle and discuss its applicability to various contractile systems.

Experimental procedures

All reagents were of analytical grade. A $MgCl_2$ standard solution was from Sigma Chemical Co. (St. Louis, MO). The quality of ATP was checked by TLC and aliquoted stock solutions were stored at −20°C.

Proteins

All proteins were prepared from frozen chicken gizzards. KRP was isolated as outlined by Ito *et al*. (1989). Caldesmon was prepared as described by Bretscher (1984) and smooth muscle myosin was prepared according to Sellers *et al*. (1981). The myosin-binding fragment of caldesmon corresponding to amino acid residues 1–152 (N152) was bacterially expressed and purified as previously described (Vorotnikov *et al*., 1997). Protein concentrations were measured spectrophotometrically using the following extinction coefficients ($E_{280\ nm}^{1mg/ml}$ and molecular masses for quantitation: 0.78 for KRP, 17 kDa (Shirinsky *et al*., 1993); 0.3 for caldesmon, 89 kDa; 0.56 for myosin, 500 kDa. The concentration of N152 (17 kDa) was measured by the method of Lowry *et al*. (1951) using chicken gizzard caldesmon as a standard. Cy3KRP was prepared using FluoroLink Cy3 monofunctional dye (Amersham Life Science) according to manufacturer's instructions. One mole Cy3 per mol KRP was incorporated.

Antibodies

Monoclonal antibodies to FLAG sequence (M2) and FITC-labeled secondary antibodies were from Sigma Chemical Co. (St. Louis, MO). Texas Red-labeled secondary antibodies were obtained from Molecular Probes Inc. (Eugene, OR). Anti-myosin antibodies were described previously (Goncharova *et al*., 2001).

Turbidity

Measurements were performed in a 1 ml quartz cuvette with 1 cm optical path at 20°C. A 4 μM myosin filament suspension in 10 mM MOPS (pH 7.0), 50 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT was titrated with a concentrated solution of KRP in the same buffer. The absorbance was recorded by a UV/VIS spectrophotometer (Yanaco UO-2000, Japan) at 400 nm. Values were corrected for sample dilution that did not

exceed 8% of the starting volume at the end of experiment.

Sedimentation assay

The assays were conducted at 20 or 4°C as previously described (Shirinsky *et al*., 1993) in a buffer containing 10 mM MOPS (pH 7.0), 50 or 100 mM NaCl, 0.1 mM EGTA, 1 mM DTT with the addition of 1–9 mM $MgCl_2$ and 0–0.5 mM ATP where specified. Sedimentation experiments were analyzed by SDS–PAGE in 9–12% gels using the system of Laemmli (1970). Gels were stained with Coomassie R-250, dried, digitized on an HP ScanJet IIcx scanner and the intensity of protein bands quantified using NIH Image 1.59 software.

Electron microscopy

Myosin preparations were diluted with the same buffer used in a corresponding experiment at 4°C to give the final myosin concentration 50 μg/ml. Diluted samples were immediately applied on the Formvar-carbon coated grids made hydrophilic by UV-irradiation and stained with 1% uranyl acetate. Electron microscopy was performed using a JEM 100CX electron microscope operating at 100 kV. Magnification was calibrated using the catalase crystal repeat. The length of filaments is expressed as average mean ± SD, n = number of filaments counted.

Molecular biology

KRP sequence was amplified by PCR using chicken MLCK cDNA clone (GeneBank Accession Number X52876) as a template and the following primers:

5′-primer: 5′-CCCAAGCTTGCCATGGCAATGATTTCTGGTATGA-3′; 3′-primer: 5′CCGAATTCTCATTCTTCCTCCTCCTCTTCA-3′; containing Hind III and EcoR I restriction sites, correspondingly (underlined). PCR product was subcloned in pFLAG CMV2 vector (Kodak Scientific Imaging Systems, New Haven, CT) at Hind III and EcoR I sites to provide for the expression of KRP with the FLAG-tag at N-terminus.

Cell culture and manipulation

Chicken embryo fibroblasts were cultured in DMEM supplemented with 10% FBS (HyClone, Europe Ltd) at 37°C and 5% CO_2, 95% air. For transient transfection with KRP-FLAG cells were grown to 70% confluency and plasmid was introduced using a Maxifectin-21 kit (Pepline, Moscow, Russia) according to the manufacturer's instructions. After 8–16 hours cells were fixed with 3.7% formaldehyde, permeabilized with 1% Triton X-100 and processed for immunofluorescence as described (Birukov *et al*., 1991).

For the cytoskeleton overlay experiments fibroblasts grown on coverslips were washed with ice-cold phosphate-buffered saline (PBS) and extracted on ice for 15 min with the buffer containing 10 mM Tris–HCl, pH 7.0, 60 mM KCl, 250 mM sucrose and 0.05% Triton X-100. Following extraction coverslips were washed three times with PBS and incubated with 0.5 μM Cy3KRP solution in PBS containing 1% BSA and 20 mM 2-mercaptoethanol for 30 min in the dark at room temperature. Coverslips were briefly rinsed in PBS and fixed for 2–3 min in 3.7% formaldehyde/PBS. After these procedures cells were stained with myosin antibodies and FITC-conjugated secondary antibodies. Cells were examined using Zeiss Photomicroscope III and photographed on Kodak Tri X-pro 400 film.

Results

Unphosphorylated smooth muscle myosin used in this study formed a turbid suspension upon dialysis from a 0.5 M NaCl buffer into a 50 mM NaCl buffer. All myosin was sedimentable under these conditions at $90000 \times g$. Addition of ATP readily clarified the myosin suspension so that no myosin sedimented. Thus, our myosin preparation demonstrated typical polymerization–depolymerization behavior characteristic of smooth muscle myosin *in vitro*.

Effects of KRP and caldesmon on myosin polymerization and aggregation

Addition of KRP to the myosin suspension (in the absence of ATP) quickly reduced its turbidity in a concentration-dependent manner (Figure 1). This effect saturated at about a 2–3-fold molar excess of KRP over myosin. When analyzed by electron microscopy, the myosin–KRP mixtures contained very fine meshes of filaments and individual filaments of an average size 0.3–0.4 μm (Figure 1, inset). The thick bundles of filaments abundant in the initial myosin suspension were only occasionally seen after the addition of KRP. Thus, KRP reduced the level of myosin aggregates, but did not cause filament depolymerization because all myosin remained sedimentable at $90,000 \times g$ (data not shown). In contrast, caldesmon over a comparable concentration range had no effect on turbidity of the myosin suspension.

Next, we investigated the effects of KRP and caldesmon on myosin in the presence of ATP when myosin is depolymerized. As shown in Figure 2A, KRP induces myosin sedimentation in a concentration-dependent manner. About 50% of myosin is found in the pellet at a KRP:myosin molar ratio of 3:1. Caldesmon does not induce myosin sedimentation when added at similar concentrations (Figure 2B). Titration of an equimolar mixture of myosin–caldesmon with KRP demonstrated bi-phasic behavior (Figure 2C). From a 0.5- to 2-fold molar excess KRP over myosin, caldesmon partially inhibits myosin sedimentation whereas upon further

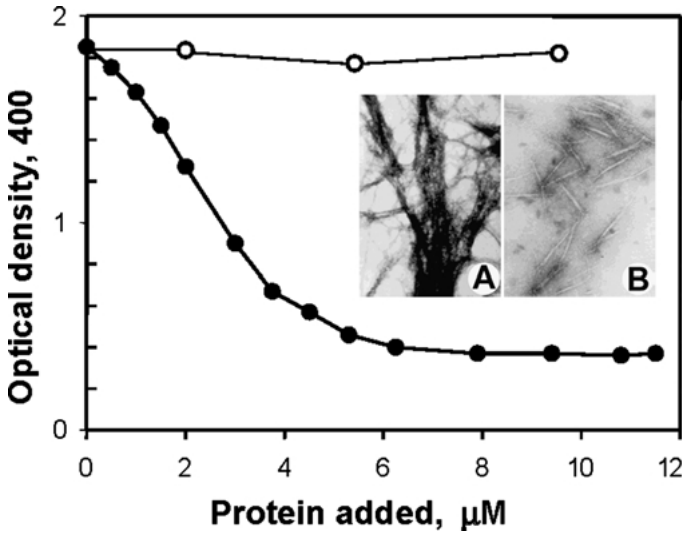

Fig. 1. Effect of KRP on turbidity of myosin suspension. Four micromolar myosin suspension in 10 mM MOPS, pH 7.0, 50 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT was titrated with concentrated KRP (●) or caldesmon (○) solutions and absorbance was recorded at 400 nm. To standardize for myosin settling, measurements were taken 30 s after sample mixing. Inset: electron microscopy of the myosin suspension before (A) and after (B) the addition of 12 μM KRP. A representative curve of three independent experiments is shown.

addition of KRP (3-fold molar excess), most of the myosin distributes in the pellet fraction. Thus, KRP promotes myosin polymerization by itself and its effect is potentiated in the presence of caldesmon.

Unphosphorylated myosin in the presence of ATP can also be polymerized by magnesium ions (Suzuki *et al.*, 1981; Ikebe and Hartshorne, 1984). Increasing the Mg^{2+} concentration progressively overcomes the depolymerizing action of ATP. Typically, in our experiments, a sharp transition from soluble to sedimentable myosin occurred at 7–8 mM $MgCl_2$ (Figure 3A). Next we investigated the effect of added Mg^{2+} on the action of KRP and caldesmon towards myosin. Figure 3A illustrates that Mg^{2+} enhances the myosin polymerizing action of KRP whereas caldesmon partially inhibits Mg^{2+}-induced myosin polymerization. An expressed 17 kDa N-terminal myosin-binding domain of caldesmon (N152) was also inhibitory towards myosin polymerization induced by Mg^{2+} (Figure 3B).

Thus, in two types of myosin polymerization experiments, we found that caldesmon and KRP operate differently. Unlike KRP, caldesmon exhibited no myosin sedimenting activity in the presence of ATP. On the other hand, caldesmon changes the threshold concentration of Mg^{2+} or KRP required for filament formation in the presence of ATP, and appears to be present in sedimentable filaments mediated by these two agents.

Electron microscopy of myosin in the presence of KRP and caldesmon

Negative contrast microscopy was used to visualize myosin polymers formed in the presence of myosin binding proteins at 4 or 9 mM Mg^{2+} (Figure 4). In accord with the sedimentation data (Figure 3) myosin remained monomeric at 4 mM Mg^{2+} and no filaments were found in myosin–caldesmon/N152 mixtures (Figure 4E, G). On the other hand, in myosin–KRP mixtures, short homogeneous filaments were observed (0.44 ± 0.08 μm; $n = 50$) (Figure 4C).

At 9 mM Mg^{2+}, myosin alone consisted of a few highly contrasted aggregates of densely packed multiple filaments interacting alongside and exhibiting a 14 nm period (Figure 4B). The size of aggregates varied from 1.5 to >10 μm and individual filaments were rarely seen in these preparations. In the presence of KRP, long filaments (1.64 ± 0.37 μm; $n = 30$) among some entangled species were present (Figure 4D), while myosin–caldesmon samples consisted predominantly of short (0.54 ± 0.08 μm; $n = 50$) homogeneous filaments dispersed among a few dense aggregates (Figure 4F) similar to myosin alone. Unlike CaD–myosin, no individual filaments were found in myosin–N152 caldesmon fragment mixtures under these conditions (Figure 4H).

When myosin polymerization was induced by the simultaneous addition of 7 mM Mg^{2+} and KRP, the presence of caldesmon shortened filaments from 1.12 ± 0.44 μm ($n = 50$) to 0.46 ± 0.08 μm ($n = 50$) while N152 left them apparently unchanged (Figure 5). Therefore, the filament shortening properties of caldesmon are independent of the presence of KRP under these conditions, and require additional amino acid sequences of caldesmon that are outside of the myosin binding domain represented by N152.

Intracellular localization of KRP

In order to reveal the interaction of KRP with filamentous myosin in the cellular context we transiently transfected chicken embryo fibroblasts with the plasmid

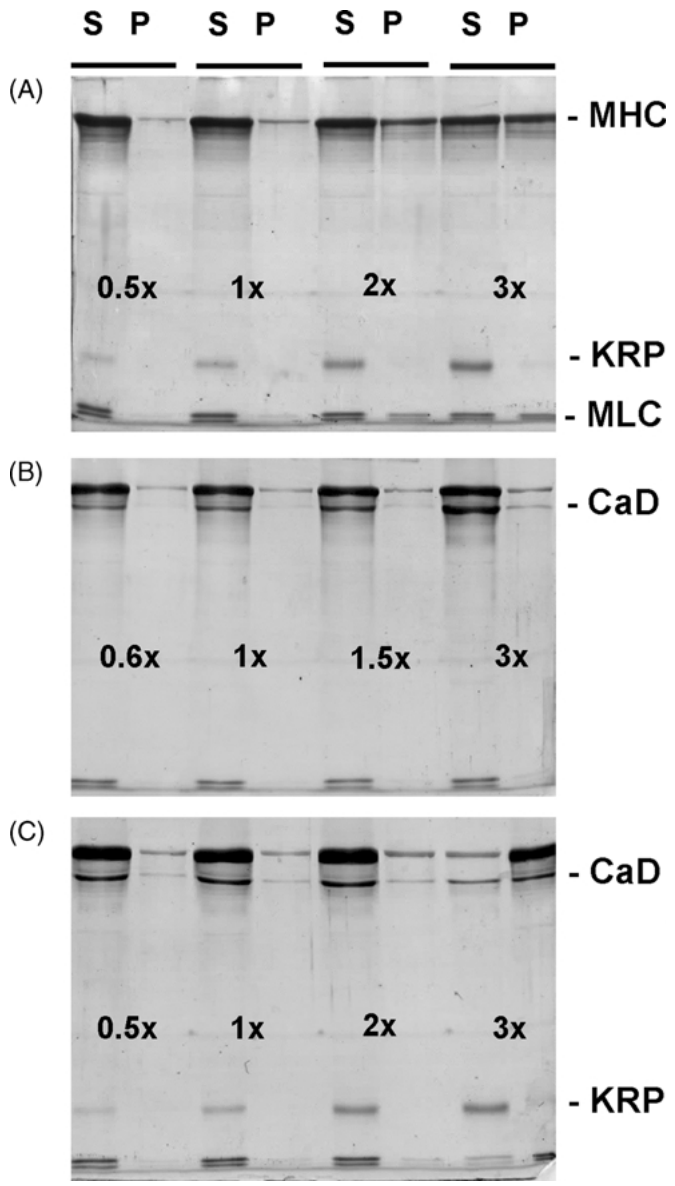

Fig. 2. Myosin polymerization in the presence of KRP and caldesmon. Four micromolar myosin in 10 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM ATP, 1 mM $MgCI_2$ was mixed with increasing concentrations of KRP (A) and caldesmon (B). In panel (C) caldesmon was kept equimolar to myosin while KRP concentration was increased. Figures in the middle of the gels denote the molar ratio of the variable myosin binding protein to myosin. Following 10 min incubation at 20°C the amount of proteins in supernatant (s) and pellet (p) was determined by a sedimentation assay and SDS–PAGE. MHC – myosin heavy chain; CaD – caldesmon; MLC – myosin light chains.

encoding wild-type KRP fused to N-terminal FLAG epitope. The rationale for this approach is that non-muscle myosin II expressed in fibroblasts is functionally similar to the smooth muscle myosin and, in particular, is capable of interacting with KRP *in vitro* (Silver *et al.*, 1997). As shown in Figure 6A and B, anti-FLAG antibodies localize KRP-FLAG with the cellular filamentous structures that are counterstained with myosin II antibodies. KRP-FLAG was also observed in the nuclei of transfected cells consistent with the ability of the low molecular weight proteins to penetrate in this cellular compartment. In a confirmatory experiment, fibroblast cytoskeletons were overlayed with Cy3-labeled KRP and the labeled protein localized to myosin positive filaments (Figure 6 C–F).

Discussion

KRP acts like a myosin filament buffer disfavoring both monomers and aggregates

The novel activities of KRP include the ability to inhibit myosin filament aggregation induced by dialysis of myosin into 50 mM NaCl containing buffer in the absence of ATP or by the addition of 7–9 mM Mg^{2+} to monomeric myosin in the presence of ATP. These properties are consistent with the interaction of KRP with the neck region of myosin molecule that favors extended myosin conformation and filament assembly (Shirinsky *et al.*, 1993; Silver *et al.*, 1997) described earlier. The results presented here suggest that KRP also

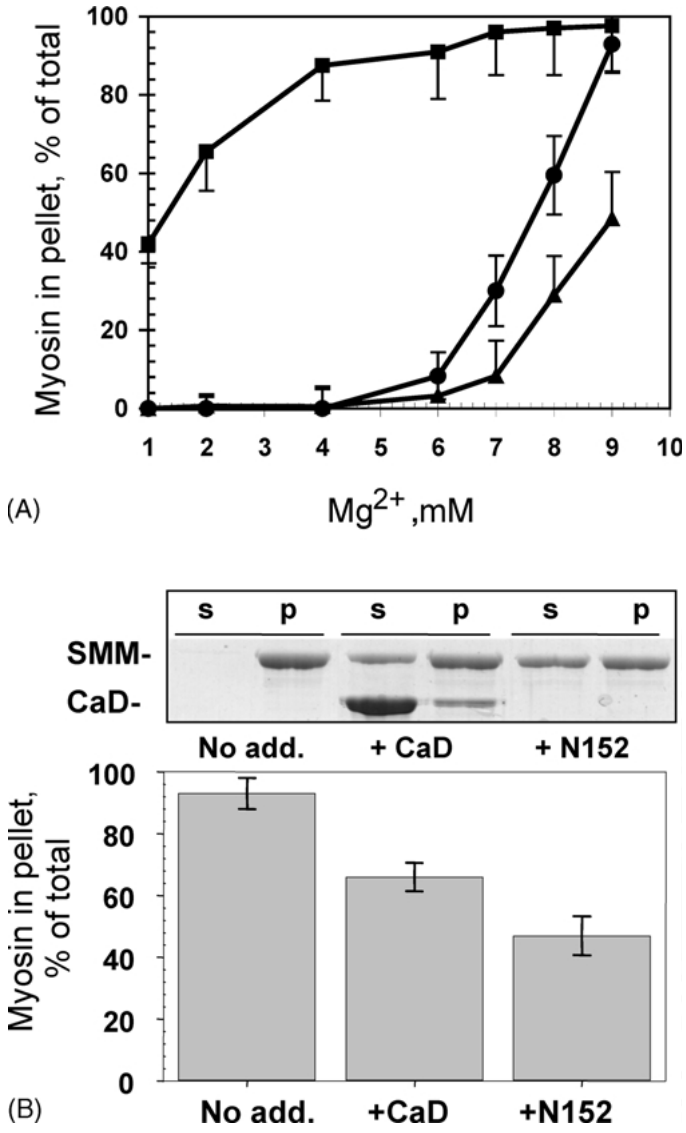

Fig. 3. Effects of KRP and caldesmon on Mg^{2+}-induced myosin polymerization. (A) Variable $MgCl_2$ concentrations were established in a solution of 1 μM myosin in 10 mM MOPS, pH 7.0, 50 mM NaCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM ATP in the presence of 14 μM KRP (squares), 14 μM caldesmon (triangles) or in the absence of KRP and caldesmon (circles). (B) Representative 10% polyacrylamide gel and the bar graph showing the effect of 10 μM caldesmon and 10 μM N152 (not included in this part of the gel) on polymerization of 1.5 μM myosin induced by 9 mM $MgCl_2$. Buffer conditions as above. Samples were processed as described in the legend to Figure 2. The mean values ± S.E.M ($n = 3$) are shown.

inhibits lateral aggregation between filaments. However, the mechanism of this process awaits further investigation. Thus, the filament organizing action of KRP may play a particularly important role in smooth muscle or other tissues where thick filament aggregation may be promoted by high myosin concentration (Ruegg, 1986). From the standpoint of muscle mechanics the clustering and masking of myosin heads achieved in aggregated filaments would be unfavorable since it would reduce the maximum force output.

Increasing Mg^{2+} concentration in myosin–KRP mixtures results in a 3- to 4-fold elongation of the assembled filaments. Perhaps, the mechanism of such elongation involves the abovementioned ability of KRP to inhibit lateral filament interactions and thus promote endwise growth. Mg^{2+} potentiates the filament stabilizing action of KRP that allows the formation, *in vitro*, of very long myosin filaments equal in length to the native thick filaments of smooth muscle (Xu *et al.*, 1996). Although it is unlikely that free Mg^{2+} concentration escalates above the typical 1–2 mM in a physiological milieu, the observed potentiating effect of Mg^{2+} on KRP-induced myosin filament assembly suggests that this activity may be relevant *in vivo*.

Caldesmon is not a KRP-like myosin filament stabilizer

The effects of caldesmon on myosin filament assembly were different from those observed for KRP. No filaments were formed in the presence of caldesmon at the subthreshold Mg^{2+}, e.g. under the conditions when KRP induced a significant myosin polymerization (Figures 2, 3).

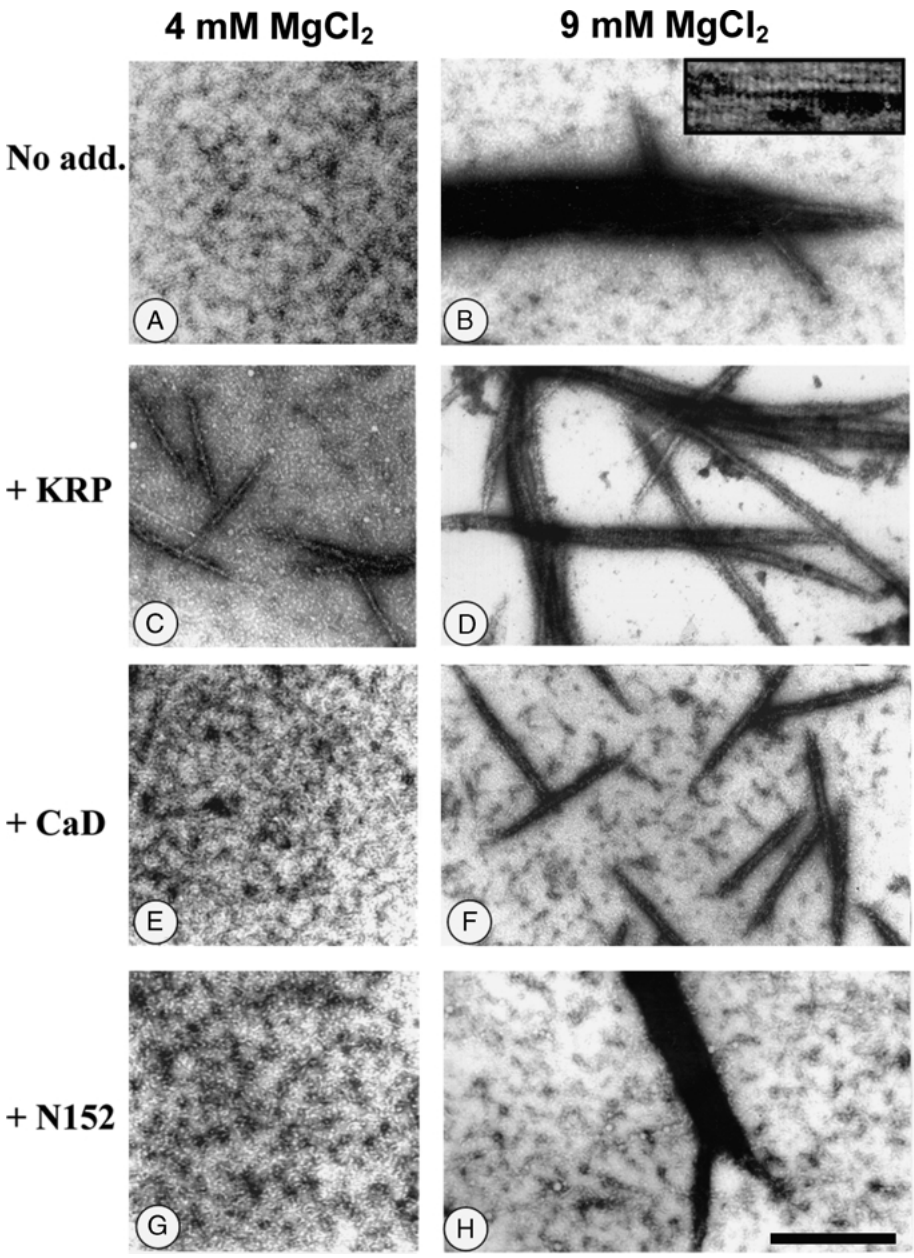

Fig. 4. Electron microscopy analysis of myosin polymerization in the presence of KRP, caldesmon and N152. 1.5 μM unphosphorylated smooth muscle myosin was incubated in the absence (A, B) or in the presence of 15 μM of KRP (C, D), 15 μM of caldesmon (E, F) or 15 μM of N152 (G, H) in a buffer containing 10 mM MOPS, pH 7.0, 50 mM NaCl, 0.1 mM EGTA, 1 mM DTT, 0.5 mM ATP and either 4 mM $MgCl_2$ (A, C, E, G) or 9 mM $MgCl_2$ (B, D, F, H). Inset in (B): the area of filament aggregate demonstrating 14 nm repeat. Scale bar: 0.5 μm (A–H), 0.25 μm (inset in (B)).

Furthermore, at Mg^{2+} concentrations sufficient to polymerize myosin caldesmon partially inhibited this process and N-terminal myosin binding domain of caldesmon, N152, demonstrated similar behavior.

Electron microscopy, however, revealed differences in the action of caldesmon and N152. At high Mg^{2+} caldesmon favored the formation of short filaments that were distinct from Mg^{2+}-induced aggregates or long filaments induced by Mg^{2+} and KRP whereas N152 failed to alter the appearance of myosin from control.

Comparison of caldesmon and N152 activities suggests a possible molecular mechanism of caldesmon action on myosin polymerization. It has been shown that the N-terminal domain of caldesmon interacts with the S2 subfragment of myosin (Hemric and Chalovich, 1988, 1990; Ikebe and Reardon, 1988). Therefore, to a limited (by concentration) extent, it may trap myosin monomers and reduce the fraction of myosin molecules incorporated in filaments by shifting the equilibrium of this process. In addition, caldesmon possesses a second C-terminal myosin-binding site (Yamakita *et al.*, 1992; Huber *et al.*, 1993) that may also act as a trap. As we found that the N152 fragment of caldesmon did not have filament shortening properties it is possible that using a second myosin binding site, caldesmon brings together myosin monomers and acts as a nucleating

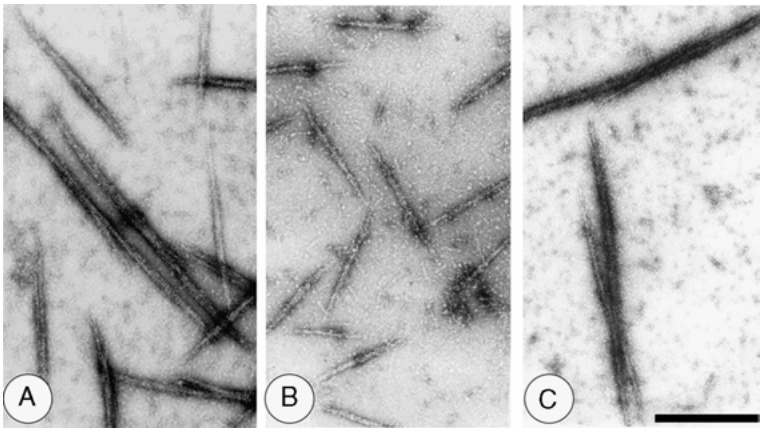

Fig. 5. Combined action of KRP and caldesmon or N152 on myosin filament assembly. Polymerization of 1.5 μM myosin was induced by the addition of 15 μM KRP alone (A) and in the presence of 15 μM caldesmon (B) or 15 μM N152 (C). Buffer conditions as in Figure 4 except that 7 mM $MgCl_2$ was used. Scale bar: 0.5 μm.

agent facilitating the initial step of myosin polymerization. Increased nucleation in the system with the constant amount of myosin supports the formation of more filaments but reduces their average length. This is exactly what we observed in our experiments (Figures 4, 5). Importantly, caldesmon does not promote the elongation step of filament formation and additional agents such as KRP or Mg^{2+} are required to support filament growth from preexisting nuclei. Our results on myosin polymerization in the presence of KRP and caldesmon allow us to propose a mechanistic model for their concerted action on myosin filaments. In this model (Figure 7), CaD participates in the early steps of myosin monomer nucleation. KRP promotes the formation of a dynamic pool of myosin filaments containing attached CaD that may coordinate interaction with actin filaments (Marston *et al*., 1992; Katayama and Ikebe, 1995). Of course, myosin phosphorylation displaces KRP (Shirinsky *et al*., 1993) providing the filaments with the mobility required for movement along the actin scaffold.

Based on the results of this study, our view of the role of caldesmon in myosin polymerization disagrees with that of Katayama *et al*. (1995). These authors proposed that caldesmon is analogous to KRP in its ability to induce myosin filament assembly. Our interpretation of these data differs in that we suggest that the filaments presented by these authors as an evidence of a KRP-like caldesmon activity (Figure 2B in Katayama *et al*., (1995)) may be the product of Mg^{2+}-induced filament assembly because the concentrations of myosin and Mg^{2+} employed are near the threshold for myosin filament formation (Figure 3). We rationalize that these disparate results may be due in part to differences in myosin isolation and purification that change the myosin polymerization properties.

Initial observations of smooth muscle myosin polymerization in the presence of caldesmon made by Ikebe and Reardon (1988) could also be explained on the basis of our proposed mechanism. These authors found that phosphorylated myosin more efficiently formed filaments in the presence of caldesmon than unphosphorylated myosin. To the extent that phosphorylated myosin mimics the structural effect of KRP binding, these data are consistent with our results. Additionally, Ikebe and Reardon (1988) demonstrated that filament formation was inhibited by Ca^{2+}-calmodulin, presumably through binding to the carboxyl-terminal domain of caldesmon. This result is also consistent with our mechanism because the attenuation of C-terminal myosin binding activity of caldesmon would be expected to decrease its nucleating capacity as we found with the N152 fragment of caldesmon that does not contain the carboxyl-terminal calmodulin binding domain.

Myosin is the relevant intracellular target for KRP

We complemented *in vitro* studies of myosin polymerization under control of KRP and caldesmon with the demonstration that KRP can interact with myosin II positive fibers in cells. This has been done by two independent approaches, transfection of KRP-FLAG into fibroblasts and fibroblast cytoskeleton overlay with the Cy3KRP. Similar studies have been done previously with the caldesmon N-terminal domain N152 (Goncharova *et al*., 2001). Although cytoskeletal bundles contain other potential binding proteins, the lack of documented interaction of KRP and N152 with proteins other than myosin suggests it is a relevant target.

Several questions arise regarding general applicability of proposed mechanisms of myosin polymerization with the involvement of accessory proteins. First, the amount of KRP is variable in smooth muscles. KRP is approximately equimolar to myosin in phasic smooth muscles but is less abundant in tonic muscles such as arteries (Krymsky *et al*., 2001). Perhaps, there is no need for constitutively high KRP expression in tonic muscle since other myosin stabilizing mechanisms may be operative in this tissue. One could expect that continuous stretching of the vessel wall due to blood pulsation would keep

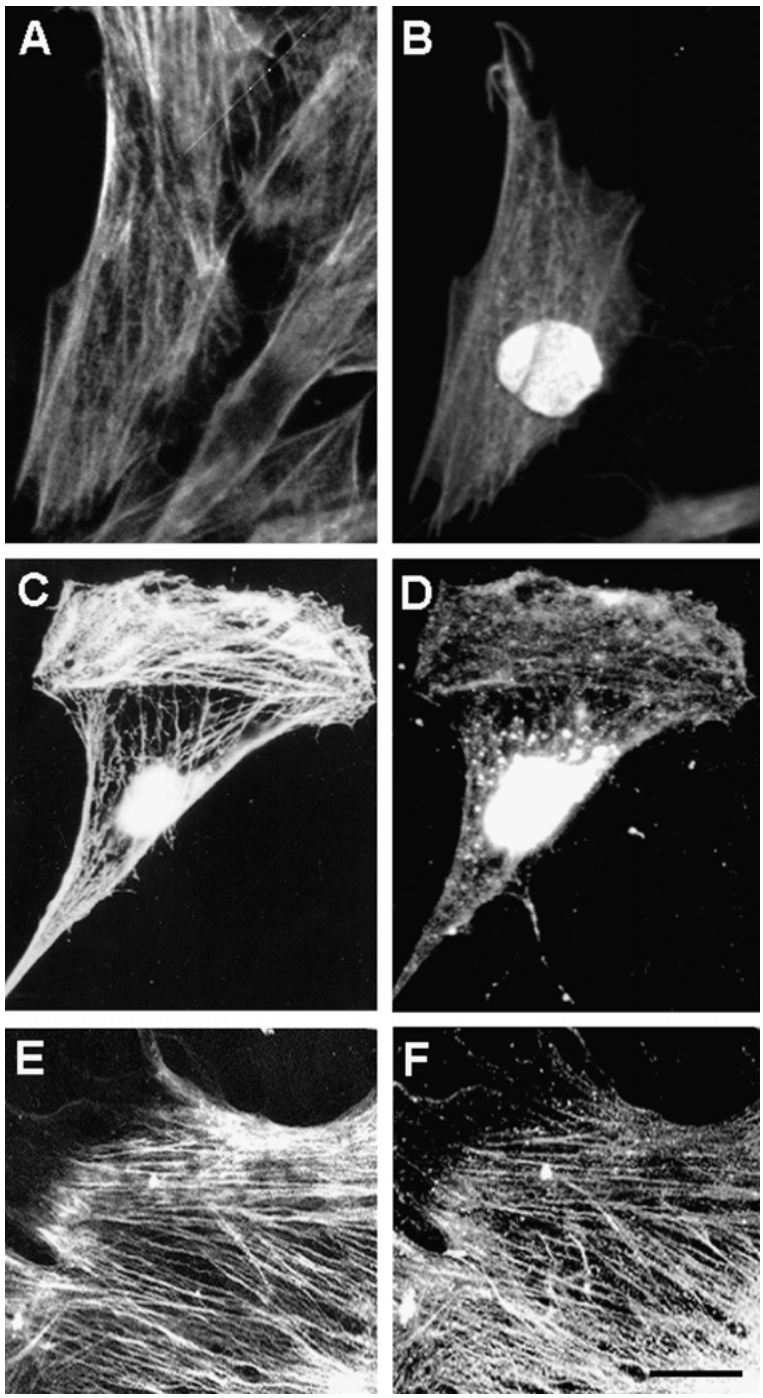

Fig. 6. Immunofluorescent localization of KRP in cultured cells. Chicken embryo fibroblasts were transiently transfected with KRP-FLAG construct and processed for immunofluorescent detection of myosin (A) and KRP-FLAG (B) using anti-FLAG M2 monoclonal antibody. (C–F) chicken embryo fibroblast cytoskeletons were overlayed with Cy3KRP (D, F) solution and stained with myosin antibodies (C, E). (E) and (F) represent enlarged area of peripheral cytoplasm. Scale bar; 50 μm (A–D), 15 μm (E, F).

MLCK constantly activated and myosin RLC phosphorylated providing similar to KRP effect on myosin filament stabilization. In addition, there could be p38-related pathways of myosin stabilization (Okagaki *et al*., 2000) and mechanisms dependent on myosin RLC phosphorylation by alternative protein kinases like integrin-linked kinase (Deng *et al*., 2001) or ZIP kinase (Murata-Hori *et al*., 1999) that could substitute for the low amount of KRP in arterial muscle and its absence in non-muscle cells.

Second, caldesmon can localize with the actomyosin fibers in smooth muscle and non-muscle cells through the interaction of its C-terminal domain with actin filaments (Sobue and Sellers, 1991). The caldesmon N-terminal myosin binding domain is therefore available to link to myosin (Goncharova *et al*., 2001) and may serve as myosin monomer trap. However, it is unknown whether C-terminal myosin binding site of caldesmon cooperates with its N-terminal site in cross-linking myosin to form nuclei. Further studies are required to

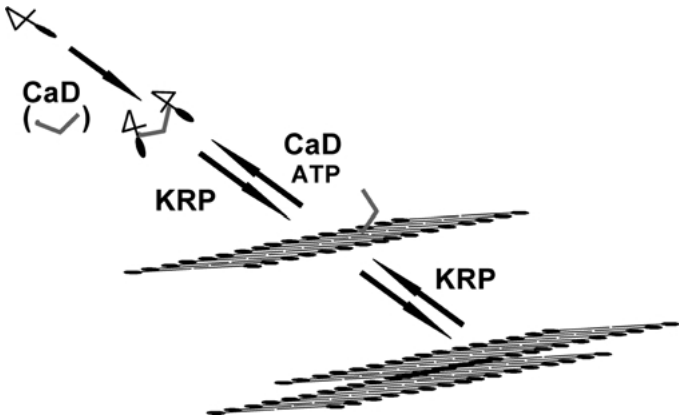

Fig. 7. Potential role of KRP and caldesmon in smooth muscle myosin polymerization. Unphosphorylated or otherwise unprotected smooth muscle myosin is monomeric in the presence of physiological ATP concentrations. In the initial step caldesmon links myosin monomers using two myosin binding sites and enhances dimer formation that may serve as nuclei for filament growth. KRP promotes elongation of the nuclei into the filaments favoring the extended conformation of myosin monomers. KRP also prevents filaments from aggregation with each other. A portion of the caldesmon may stay bound to stabilized myosin filaments and link them to actin filaments as suggested by previous studies (Marston *et al.*, 1992; Katayama and Ikebe, 1995). For simplicity one myosin head is shown instead of two.

support this idea. However, one may speculate that C-terminal site can be made available for myosin binding when caldesmon interaction with actin is disrupted by phosphorylation (Shirinsky *et al.*, 1999).

Taking into account the presence and relative abundance of caldesmon in smooth muscles and non-muscle cells it is plausible to hypothesize that this protein represents the 'nucleation agent' of the controlled thick filament assembly in these systems whereas the 'elongation and stabilization agents' could be cell specific but functionally interchangeable.

In summary, our results suggest distinct functions for the well-known myosin binding proteins, KRP and caldesmon, and provide a model for their involvement in myosin polymerization.

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References

Birukov KG, Schavocky JP, Shirinsky VP, Chibalina MV, Van Eldik LJ and Watterson DM (1998) Organization of the genetic locus for chicken myosin light chain kinase is complex: multiple proteins are encoded and exhibit differential expression and localization. *J Cell Biochem* **7:** 402–413.

Birukov KG, Stepanova OV, Nanaev AK and Shirinsky VP (1991) Expression of calponin in rabbit and human aortic smooth muscle cells. *Cell Tissue Res* **266:** 579–584.

Bretscher A (1984) Smooth muscle caldesmon. Rapid purification and F-actin cross-linking properties. *J Biol Chem* **259:** 12,873–12,880.

Collinge M, Matrisian PE, Zimmer WE, Shattuck RL, Lukas TJ, Van Eldik LJ and Watterson DM (1992) Structure and expression of a calcium-binding protein gene contained within a calmodulin-regulated protein kinase gene. *Mol CelI Biol* **12:** 2359–2371.

Craig R, Smith R and Kendrick-Jones J (1983) Light-chain phosphorylation controls the conformation of vertebrate non-muscle and smooth muscle myosin molecules. *Nature* **302:** 436–439.

Deng JT, Van Lierop JE, Sutherland C and Walsh MP (2001) Ca^{2+}-independent smooth muscle contraction. A novel function for integrin-linked kinase. *J Biol Chem* **276:** 16,365–16,373.

Gillis JM, Cao ML and Godfraind-DeBecker A (1988) Density of myosin filaments in the rat anococcygeus muscle, at rest and in contraction. II. *J Muscle Res Cell Motil* **9:** 18–28.

Goncharova EA, Shirinsky VP, Shevelev AY, Marston SB and Vorotnikov AV (2001) Actomyosin cross-linking by caldesmon in non-muscle cells. *FEBS Lett* **497:** 113–117.

Hemric ME and Chalovich JM (1988) Effect of caldesmon on the ATPase activity and the binding of smooth and skeletal myosin subfragments to actin. *J Biol Chem* **263:** 1878–1885.

Hemric ME and Chalovich JM (1990) Characterization of caldesmon binding to myosin. *J Biol Chem* **265:** 19,672–19,678.

Herring BP and Smith AF (1996) Telokin expression is mediated by a smooth muscle cell-specific promoter. *Am J Physiol* **270:** C1656–C1665.

Horowitz A, Trybus KM, Bowman DS and Fay FS (1994) Antibodies probe for folded monomeric myosin in relaxed and contracted smooth muscle. *J Cell Biol* **126:** 1195–1200.

Huber PA, Fraser ID and Marston SB (1995) Location of smooth-muscle myosin and tropomyosin binding sites in the C-terminal 288 residues of human caldesmon. *Biochem J* **312:** 617–625.

Huber PA, Redwood CS, Avent ND, Tanner MJ and Marston SB (1993) Identification of functioning regulatory sites and a new myosin binding site in the C-terminal 288 amino acids of caldesmon expressed from a human clone. *J Muscle Res Cell Motil* **14:** 385–391.

Huxley HE and Hanson J (1954) Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. *Nature* **173:** 973–976.

Ikebe M and Hartshorne DJ (1984) Conformation-dependent proteolysis of smooth-muscle myosin. *J Biol Chem* **259:** 11,639–11,642.

Ikebe M and Reardon S (1988) Binding of caldesmon to smooth muscle myosin. *J Biol Chem* **263:** 3055–3058.

Ito M, Dabrowska R, Guerriero Jr, V and Hartshorne DJ (1989) Identification in turkey gizzard of an acidic protein related to the C-terminal portion of smooth muscle myosin light chain kinase. *J Biol Chem* **264:** 13,971–13,974.

Katayama E and Ikebe M (1995) Mode of caldesmon binding to smooth muscle thin filament: possible projection of the amino-

terminal of caldesmon from native thin filament. *Biophys J* **68:** 2419–2428.

Katayama E, Scott-Woo G and Ikebe M (1995) Effect of caldesmon on the assembly of smooth muscle myosin. *J Biol Chem* **270:** 3919–3925.

Krymsky MA, Kudryashov DS, Shirinsky VP, Lukas TJ, Watterson DM and Vorotnikov AV (2001) Phosphorylation of kinase-related protein (telokin) in tonic and phasic smooth muscles. *J Muscle Res Cell Motil* **22:** 425–437.

Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T-4. *Nature* **227:** 680–685.

Lehman W, Denault D and Marston S (1993) The caldesmon content of vertebrate smooth muscle. *Biochim Biophys Acta* **1203:** 53–59.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the folin pnenol reagent. *J Biol Chem* **193:** 265–275.

Marston S, Pinter K and Bennett P (1992) Caldesmon binds to smooth muscle myosin and myosin rod and crosslinks thick filaments to actin filaments. *J Muscle Res Cell Motil* **13:** 206–218.

Marston SB and Redwood CS (1991) The molecular anatomy of caldesmon. *Biochem J* **279:** 1–16.

Masato T, Numata T, Katoh T, Morita F and Yazawa M (1997) Crosslinking of telokin to chicken gizzard smooth muscle myosin. *J Biochem (Tokyo)* **121:** 225–230.

Murata-Hori M, Suizu F, Iwasaki T, Kikuchi A and Hosoya H (1999) ZIP kinase identified as a novel myosin regulatory light chain kinase in HeLa cells. *FEBS Lett* **451:** 81–84.

Okagaki T, Nakamura A, Suzuki T, Ohmi K and Kohama K (2000) Assembly of smooth muscle myosin by the 38k protein, a homologue of a subunit of pre-mRNA splicing factor-2. *J Cell Biol* **148:** 653–664.

Olney JJ, Sellers JR and Cremo CR (1996) Structure and function of the 10 S conformation of smooth muscle myosin. *J Biol Chem* **271:** 20,375–20,384.

Onishi H and Wakabayashi T (1982) Electron microscopic studies of myosin molecules from chicken gizzard muscle I: the formation of the intramolecular loop in the myosin tail. *J Biochem* (*Tokyo*) **92:** 871–879.

Qi D, Mitchell RW, Burdyga T, Ford LE, Kuo KH and Seow CY (2002) Myosin light chain phosphorylation facilitates *in vivo* myosin filament reassembly after mechanical perturbation. *Am J Physiol Cell Physiol* **282:** C1298–C1305.

Ruegg JC (1986) *Calcium in Muscle Activation*: *A Comparative Approach*. Springer-Verlag, Berlin.

Sellers JR and Goodson HV (1995) Motor Proteins 2: myosin. *Protein Profile* **2:** 1323–1423.

Sellers JR, Pato MD and Adelstein RS (1981) Reversible phosphorylation of smooth muscle myosin, heavy meromyosin, and platelet myosin. *J Biol Chem* **256:** 13,137–13,142.

Shirinsky VP, Biryukov KG, Vorotnikov AV and Gusev NB (1989) Caldesmon150, caldesmon77 and skeletal muscle troponin T share a common antigenic determinant. *FEBS Lett* **251:** 65–68.

Shirinsky VP, Vorotnikov AV, Birukov KG, Nanaev AK, Collinge M, Lukas TJ, Sellers JR and Watterson DM (1993) A kinase-related protein stabilizes unphosphorylated smooth muscle myosin minifilaments in the presence of ATP. *J Biol Chem* **268:** 16,578–16,583.

Shirinsky VP, Vorotnikov AV and Gusev NB (1999) Caldesmon phosphorylation and smooth muscle contraction. In: Kohama K and Sasaki Y (eds) Molecular Mechanisms and Their Disorder in Smooth Muscle Contraction (pp. 59–79) LANDES Bioscience, Austin, TX USA.

Silver DL, Vorotnikov AV, Watterson DM, Shirinsky VP and Sellers JR (1997) Sites of interaction between kinase-related protein and smooth muscle myosin. *J Biol Chem* **272:** 25,353–25,359.

Sobue K and Sellers JR (1991) Caldesmon, a novel regulatory protein in smooth muscle and nonmuscle actomyosin systems. *J Biol Chem* **266:** 12,115–12,118.

Somlyo AV, Butler TM, Bond M and Somlyo AP (1981) Myosin filaments have non-phosphorylated light chains in relaxed smooth muscle. *Nature* **294:** 567–569.

Suzuki H, Onishi H, Takahashi K and Watanabe S (1978) Structure and function of chicken gizzard myosin. *J Biochem* (*Tokyo*) **84:** 1529–1542.

Suzuki H, Suzuki S and Watanabe S (1981) High concentrations of magnesium for formation of thick-filaments of chicken gizzard myosin. *J Biochem* (*Tokyo*) **89:** 871–878.

Trybus KM, Huiatt TW and Lowey S (1982) A bent monomeric conformation of myosin from smooth muscle. *Proc Natl Acad Sci USA* **79:** 6151–6155.

Vorotnikov AV, Marston SB and Huber PA (1997) Location and functional characterization of myosin contact sites in smooth muscle caldesmon. *Biochem J* **328:** 211–218.

Walker LA, MacDonald JA, Liu X, Nakamoto RK, Haystead TA, Somlyo AV and Somlyo AP (2001) Site-specific phosphorylation and point mutations of telokin modulate its Ca^{2+}-desensitizing effect in smooth muscle. *J Biol Chem* **276:** 24,519–24,524.

Watterson DM, Collinge M, Lukas TJ, Van Eldik LJ, Birukov KG, Stepanova OV and Shirinsky VP (1995) Multiple gene products are produced from a novel protein kinase transcription region. *FEBS Lett* **373:** 217–220.

Watterson DM, Schavocky JR, Guo L, Weiss C, Chlenski A, Shirinsky VP, Van Eldik LJ and Haiech J (1999) Analysis of the kinase-related protein gene found at human chromosome 3q21 in a multigene cluster: organization, expression, alternative splicing, and polymorphic marker. *J Cell Biochem* **75:** 481–491.

Xu JQ, Gillis JM and Craig R (1997) Polymerization of myosin on activation of rat anococcygeus smooth muscle. *J Muscle Res Cell Motil* **18:** 381–393.

Xu JQ, Harder BA, Uman P and Craig R (1996) Myosin filament structure in vertebrate smooth muscle. *J Cell Biol* **134:** 53–66.

Yamakita Y, Yamashiro S and Matsumura F (1992) Characterization of mitotically phosphorylated caldesmon. *J Biol Chem* **267:** 12,022–12,029.