Assembly of Smooth Muscle Myosin by the 38k Protein, a Homologue of a Subunit of Pre-mRNA Splicing Factor-2

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Abstract. Smooth muscle myosin in the dephosphorylated state does not form filaments in vitro. However, thick filaments, which are composed of myosin and myosin-binding protein(s), persist in smooth muscle cells, even if myosin is subjected to the phosphorylation–dephosphorylation cycle. The characterization of telokin as a myosin-assembling protein successfully explained the discrepancy. However, smooth muscle cells that are devoid of telokin have been observed. We expected to find another ubiquitous protein with a similar role, and attempted to purify it from chicken gizzard. The 38k protein bound to both phosphorylated and dephosphorylated myosin to a similar extent. The effect of the myosin-binding activity was to assemble dephosphorylated myosin into filaments, although it had no effect on the phosphorylated myosin. The 38k protein bound to myosin with both COOH-terminal 20 and NH₂-terminal 28 residues of the 38k protein being essential for myosin binding. The amino acid sequence of the 38k protein was not homologous to telokin, but to human p32, which was originally found in nuclei as a subunit of pre-mRNA splicing factor-2. Western blotting showed that the protein was expressed in various smooth muscles. Immunofluorescence microscopy with cultured smooth muscle cells revealed colocalization of the 38k protein with myosin and with other cytoskeletal elements. The absence of nuclear immunostaining was discussed in relation to smooth muscle differentiation.

Key words: myosin assembly • myosin binding • myosin filament • human p32 • smooth muscle

Introduction

The thick filaments observed in various muscle cells are composed of myosin filaments that are associated with myosin-binding proteins. Assembly into filaments is an inherent property of myosin (Huxley, 1963). Myosin-binding proteins, such as myosin-binding protein-C (C-protein), myosin-binding protein-H (H-protein), M-protein, titin, and myomesin are thought to stabilize the filaments (for review, see Epstein and Fischman, 1991; Furst and Gautel, 1995).

In the case of skeletal muscle myosin, assembly is observed regardless of the phosphorylation state of myosin. However, smooth muscle myosin does not form filaments unless its regulatory light chain (RLC) is phosphorylated (for review, see Stull et al., 1991; Trybus, 1991; Gallagher et al., 1997). Smooth muscle myosin molecules, if not phosphorylated, are in a folded structure called the 10S form, and hardly assemble filaments. When the myosin is phosphorylated, its shape changes to the extended 6S form and filaments are assembled (Onishi and Wakabayashi, 1982; Trybus et al., 1982; Craig et al., 1983). Thus, the filaments formed from the phosphorylated myosin become unstable upon dephosphorylation of the RLC and, as a result, easily fall apart.

These in vitro properties of smooth muscle myosin filaments are of particular interest when we observe the persistent nature of thick filaments in smooth muscle cells. Their myosin is repeatedly subject to cycles of phosphorylation and dephosphorylation in response to contraction, followed by relaxation (for review, see Stull et al., 1991; Somlyo and Somlyo, 1994). However, there is no sign of assembly or disassembly of thick filaments, indicating that they are stable (Somlyo et al., 1981; Cooke et al., 1989; Xu et al., 1996). By raising an mAb to the 10S form, Horowitz et al. (1994) examined the intracellular form of myosin. The concentration of 10S myosin was very low, re-
gardless of contraction and relaxation states, most of the myosin being in the 6S form. Therefore, myosin filaments are expected to be stabilized as thick filaments by myosin-binding proteins in the cell. The first examination of this suggestion was by Shirinsky et al. (1993), who observed that telokin binds to dephosphorylated smooth muscle myosin to stimulate myosin assembly. However, telokin is not present in all smooth muscle cells (Gallagher and Herzig, 1991), indicating the presence of another myosin-binding protein(s) with a similar role in the cell.

We were interested in this induction and adopted a strategy to purify myosin-binding proteins from skeletal muscles (Starr and Offer, 1971). We chose a crude preparation of smooth muscle myosin as a starting material and purified a protein of 38 kD that bound to myosin, causing filament assembly.

Materials and Methods

Preparation of Proteins

Fresh chicken gizzards were minced within 2 h of killing, and kept frozen at ~80°C until use. Proteins other than myosin were purified from the frozen mince, unless otherwise specified. Dephosphorylated myosin was purified from chicken gizzard according to E bashi (1976) with modifications (Ikebe and Hartshorne, 1985a) and used as unphosphorylated myosin. We checked that myosin preparations did not contain the 38k myosin-assembling protein by SDS-PAGE or Western blotting. When required, myosin was phosphorylated with myosin light chain kinase (MLCK) in the presence of Ca²⁺ and calmodulin (Okagaki et al., 1991). Rod, light meromyosin (LMM), and heavy meromyosin (HMM) of gizzard myosin were prepared proteolytically as described (Ikebe and Hartshorne, 1985b; King et al., 1995). Myosin was also purified from chicken breast muscle and bovine heart and used as skeletal and cardiac myosin, respectively (Okagaki et al., 1993). In this paper, myosin denotes smooth muscle myosin unless otherwise mentioned. M LCK, telokin, and tropomyosin were purified from chicken gizzard according to Hayakawa et al. (1994), Ito et al. (1989), and Ishikawa et al. (1989), respectively. Monomeric actin was purified from chicken breast muscle and used as actin filaments after polymerization (Okagaki et al., 1991). The concentration of actin filaments was expressed as that of monomeric actin.

Preparation of the 38k Protein

Following our experience with C-protein, a myosin-binding protein of skeletal muscle (Okagaki et al., 1993), we used a crude myosin preparation (see below) as the starting material. From this, we purified a protein of 38 kD in SDS-PAGE using myosin-assembling activity under dark-field microscopy (see below) as a probe. Aliquots of each step of the fractionation were desalted with Biogel P-6 (BioRad Laboratories), equilibrated with buffer A (1 mM ATP, 2 mM MgCl₂, 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM DTT, and 0.5 mM PMSF) and mixed with unphosphorylated myosin at a final concentration of 0.2 μM in buffer A. The mixtures were subjected to the dark-field microscope to examine myosin-assembling activity (see Centrifugation Activity Assay). The fractions precipitated at between 55 and 80% saturation (Fig. 1, lane 4) contained the myosin-assembling activity. The fractions were dissolved in buffer B (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.5 mM PMSF, and 0.5 μg/ml leupeptin), the volume of which was selected to adjust its conductivity to be as low as that of 0.2 M NaCl. Then the solution was clarified by centrifugation at 100,000 g for 2 h. The supernatant was applied to DEAE Toyopearl 650M (Tosoh), equilibrated with buffer B supplemented with 0.2 M NaCl, followed by elution with a linear gradient of NaCl from 0.2–0.5 M. A aliquots of each eluate were desalted and tested for myosin-assembling activity as described. The activity was detected in the fractions eluted with 0.3–0.4 M NaCl. The fractions containing activity were composed of polypeptides of 38 kD, 17 kD, and minor contaminants (Fig. 1, lane 5). They were pooled, concentrated with Centricon-30 (Millipore), and applied to a Superdex H R 75 column (A merham-Pharmacia) equilibrated with buffer B supplemented with 0.3 M NaCl. Fractions corresponded to 15–30 kM, as estimated by a molecular weight marker for gel filtration (A merham-Pharmacia), showed the myosin-assembling activity. The major protein band of these fractions was 38 kD on SDS-PAGE (Fig. 1, lane 6). We used this fraction for the most of biochemical experiments. During column chromatography, the 38k protein often degraded to polypeptides of 20 and 17 kD if leupeptin was absent. We routinely obtained 0.8–1.0 mg of the 38k protein from 200 g of gizzard.

Observation of Myosin Filament Assembly by the 38k Protein

Myosin at 0.2 μM was mixed with the 38k protein or the 38k protein-containing fractions in buffer A and incubated at room temperature for 30 min. A drop of the mixture was mounted on a glass slide, sealed with a coverslip, and observed with a dark-field microscope (Okagaki et al., 1991). Myosin filaments were detected as small spots of uniform size. The fine structure of myosin filaments was confirmed by EM. A drop of the above mixture was placed on a grid coated with colloidion and carbon, stained with 1% uranyl acetate, and observed with a TEM 100C electron microscope operated at 80 kV.

Centrifugation Assay for Myosin-assembling, Myosin-binding, and Actin-binding Activities

Myosin (1.1 μM), either in phosphorylated or unphosphorylated form, was mixed with various concentrations of the 38k protein in buffer A, and the mixture was incubated at room temperature for 30 min, followed by centrifugation at 100,000 g for 40 min. The supernatant and precipitate were separately subjected to SDS-PAGE. The SDS-PAGE gels were
stained with Coomassie brilliant blue and the protein bands were densitometrically scanned. The density of the protein band was calculated by NIH image (version 3.0). The density of myosin heavy chain (MHC), the 38k protein, and telokin were considered, respectively, as assembled myosin, the 38k protein bound to myosin, and telokin bound to myosin.

The relationship of the amount of the 38k protein bound to unphosphorylated myosin is shown (see Fig. 3). Unphosphorylated myosin remains in the supernatant if insufficient 38k protein is added. Therefore, it is possible that part of the 38k protein remains in the supernatant, despite its binding to myosin. However, we believe that this is unlikely and that the 38k protein bound to unphosphorylated myosin is mostly recovered in the precipitate (compare open and closed circles in Fig. 3). The binding of 5.4 μM of 38k protein to 10 μM actin filaments in the presence and absence of tropomyosin was examined by a similar centrifugation assay (Ishikawa et al., 1989).

The centrifugation assay of the 38k protein bound to insoluble myosin fragments was also performed in a similar way. Various concentrations of the 38k protein were mixed with 1.1 μM rod or LMM in buffer A, centrifuged, and analyzed as above. The interaction of the 38k protein to soluble myosin-fraction of HMM was examined by the gel filtration (see Fig. 5). Inset), i.e., 8.5 μM HMM and 40 μM 38k protein in 300 μl buffer A was applied to the column Superose 6 (A merck-sharpePharma- cia).

The molecular weights used for calculation based upon their primary structure are 440,000 for MHC, 24,000 for the 38k protein (or human p32, its human isoform, see below), 18,000 for telokin, 42,000 for actin, and 35,000 for tropomyosin. The molecular weights of myosin fragments used for calculation are: 250,000, 150,000, and 260,000 for rod, LMM, and HMM, respectively.

Transformation of Partial Amino Acid Sequence

The partial amino acid sequence of the 38k protein was determined after cleavage with Staphylococcus V8 protease (Piecer Chemical Co.). The weight ratio of the 38k protein to V8 protease was 100:1 and the digestion was for 30 min at room temperature at the boundary between the stacking and running gels of SDS-PAGE. The 38k protein was degraded to only a few fragments of 95 kDa. The cleaved protein was subjected to running gels of SDS-PAGE. The 38k protein was degraded to only a few fragments of 95 kDa.

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Expression of the Human p32 and the 38k Protein

A cell line was identified, the 38k protein had a high sequence homology to human p32. pT7 plasmid containing cDNA encoding human p32, a gift from D. A. Kainer (Cold Spring Harbor Laboratory, N.Y.), was transformed into Escherichia coli strain BL21(DE3) (Kainer et al., 1991). Cells expressing human p32 after induction with 1 mI of isopropylthio-β-D-galactoside (IPTG) were collected by centrifugation at 10,000 × g for 10 min and resuspended in 0.3 M NaCl, 20 μM Tris, pH 7.5, 0.5 M KCl, 0.1 M MgCl₂, and 0.1 mg/ml leupeptin. The cells were frozen, thawed, sonicated (SONI-FIER 200, Branson) and centrifuged at 10,000 × g for 30 min. Human p32 was purified from the supernatant by the ammonium sulfate fractionation, followed by DEAE chromatography as described for the purification of the 38k protein from chicken gizzard. The recombinant protein was used in the present experiments as human p32.

Constructions of truncated forms of the 38k protein were made by PCR reactions using primers containing a 5′ NcoI restriction site including ATG and a 3′ EcoRI site. The primers used were: M1, 5′-GATCATGGAC- TGGAAAGCTTATTACGGG-3′; M2, 5′-GATCATGGAC-TGATCCCTCCTGGG-3′; M3, 5′-GCTGAGATTCTTCTTGCGTTCGTAAAGC-3′; M4, 5′-GCTGAGATCTCTAGCTAGTTGGAC-3′; M5, 5′-GCTGAGATCTCTAGCTAGTTGGAC-3′; M6, 5′-GCTGAGATCTCTAGCTAGTTGGAC-3′; M7, 5′-GCTGAGATCTCTAGCTAGTTGGAC-3′; and M8, 5′-GCTGAGATCTCTAGCTAGTTGGAC-3′. The CDNA fragment of S3 (see above) was used as a template for the PCR reactions. The primers were used in the following combinations: full-length protein (1–207 residues), M1 and M4; ΔN28 (29–207 residues), M7 and M4; ΔN63 (64–207 residues), M2 and M4; ΔN88 (89–207 residues), M6 and M4; ΔNc (64–141 residues), M2 and M3; ΔCC20 (1–141 residues), M1 and M8. PCR was as described, PCR products were cut with NcoI and EcoRI, and subcloned to the NcoI/EcoRI site of pET21d (Novagen). These plasmids were transformed into BL21(DE3) and the truncated and full-length forms of the 38k protein were expressed as described. The expressed proteins were purified as described above and used as the recombinant 38k protein and the truncated 38k protein. The 38k protein contains native protein purified from smooth muscle.

Immunoochemical Experiments

Rabbits and guinea pigs were injected with human p32. Their sera were used as anti-38k protein antibodies after affinity purification through NHS-sepharose (a merck-sharpePharma- cia) conjugated with human p32. For Western blotting, samples after SDS-PAGE were electrotransferred onto PVDF membrane to react with the anti-38k protein antibody. Immunoreaction was detected using goat anti-rabbit IgG conjugated with HRP (55689; Cappel) as secondary antibody, coupled with the ECL system (a merck-sharpePharma- cia).

To estimate the content of the 38k protein in various smooth muscle tissues, they were excised from a freshly killed adult rat, frozen in liquid nitrogen, broken into small chips with a frozen air hammer, and then mixed with an SDS-containing buffer. The cells were subjected to Western blotting, together with specified amounts of human p32 as a standard. Intensities of the immunoreacted bands were quantitated by densitometry as described above.

For indirect immunofluorescence microscopy, we used the smooth muscle cell line, ACO1, recently established from mouse aorta (Ohmi et al., 1997). This cell line shows the immunopositive staining for smooth muscle actin.
Results

Myosin Filaments Formed by the 38k Protein

Purified myosin hardly assembled to form filaments when unphosphorylated (Fig. 2 a). However, when the 38k protein was present, unphosphorylated myosin could form filaments (Fig. 2 c). The preparation of the 38k protein did not show kinase activity for myosin when incubated with myosin in the presence of ATP for 1 h (data not shown). Thus, the myosin assembling activity could not be due to an elevation of phosphorylation level of myosin, but to an inherent property of the 38k protein.

As shown in the histograms (Fig. 2, e and f), the length of assembled filament was from 0.4–1.2 μm and the diameter was ~20 nm. The length and diameter of these filaments were almost identical to those of filaments formed by phosphorylated myosin without the 38k protein (Fig. 2 b). The average length of the filaments formed with the 38k protein was 0.65 ± 0.15 μm, SEM (n = 150; Fig. 2 e, arrow), whereas that of phosphorylated myosin was 0.65 ± 0.16 μm, SEM (n = 150; Fig. 2 f, arrow).

Thus, the 38k protein keeps myosin in filaments, even though myosin is unphosphorylated. As will be discussed later, we expect that the 38k protein could prevent disassembly of thick filaments when myosin is dephosphorylated by phosphatase in vivo.

Quantification of Myosin-binding and Myosin-assembling Activities of the 38k Protein

To quantify the myosin filament assembling activity of the 38k protein, unphosphorylated myosin was incubated in the presence of various concentrations of the 38k protein and the assembled unphosphorylated myosin was precipitated by centrifugation. In the absence of the 38k protein, >90% of unphosphorylated myosin remained in the supernatant after centrifugation (Fig. 3 a, lanes 1 and 2). The amount of unphosphorylated myosin recovered in the precipitate was increased by increasing concentration of the 38k protein, as shown by the open circles in Fig. 3 d. Concentration to give half-maximal assembly was 0.8 ± 0.2 μM, SEM (n = 3), with the maximal effect being observed in the presence of ~3 μM of the 38k protein. The amount of the 38k protein precipitated together with unphosphorylated myosin also increased, as is shown by open circles in Fig. 3 e. The maximum amount of the 38k protein bound to 1.1 μM myosin was 1.2 ± 0.1 μM, SEM (n = 3) and the concentration giving half-maximal binding was 1.7 ± 0.2 μM, SEM (n = 3).

The binding experiment was also performed using phosphorylated myosin. Phosphorylated myosin could assemble into filaments without the 38k protein, as was seen by microscopic observation (Fig. 2 b). When quantified by the centrifugation assay (Fig. 3 b), >90% of the myosin was sedimented (Fig. 3 b, lane 2). There was no obvious effect on the assembly when the 38k protein was added (Fig. 3 d, closed circles).

The closed circles in Fig. 3 e show the binding of the 38k protein to phosphorylated myosin. Similar to unphosphorylated myosin, the half-maximal binding was observed at 1.4 ± 0.2 μM, SEM (n = 3). The binding to phosphorylated myosin is distinct from telokin, which does not show any binding to phosphorylated myosin (Shirinsky et al.,
The distinction may derive from the difference in the binding site of myosin (see Fig. 5).

The binding of the 38k protein to skeletal muscle myosin was much weaker than that of smooth muscle myosin (see Fig. 5). The same was observed with cardiac myosin (see Fig. 5). These observations indicate that its binding is specific for smooth muscle myosin and agree with the observation that it is absent from skeletal muscle cells (see Fig. 6 d).

To examine whether the protein can bind to myofibrillar components other than myosin, we studied its binding to actin filaments in the presence and absence of tropomyosin. The 38k protein bound to neither actin filaments (Fig. 3 c, lanes 3 and 4) nor actin filaments associated with tropomyosin (Fig. 3 c, lanes 5 and 6).

**Absence of Cross-Talk between the 38k Protein and Telokin**

To determine whether the 38k protein and telokin share the same binding site(s) in the myosin molecule, we allowed the 38k protein to compete with telokin for myosin binding. We initially confirmed that telokin induced assembly of unphosphorylated myosin under our experimental conditions (Figs. 2 d and 4 a). The effect was saturated when >2 μM of telokin was mixed with 1 μM of myosin. To this mixture, various amounts of the 38k protein were added. A’s shown in Fig. 4 c, even when the assembly was maximally induced by telokin, the addition of the 38k protein further stimulated assembly. The maximal binding of the 38k protein to myosin did not change whether or not telokin was present (Fig. 4 d, open circles). Accordingly, the maximal binding of telokin to myosin was not affected by the 38k protein (Fig. 4 d, closed circles).

The possibility that the 38k protein binds directly to telokin without affecting its myosin-binding activity can be rejected because telokin did not interact with the 38k protein when the interaction was examined by gel filtration (data not shown).
Binding of the 38k Protein to Myosin Fragments

To examine which portion of the myosin molecule the 38k protein binds to, we allowed it to bind to rod, LMM, or intact myosin. The fragments themselves are insoluble and easy to precipitate under the conditions for myosin binding. As shown in Fig. 5, the 38k protein could bind to LMM and rod, as well as myosin, although the maximum extent of binding to LMM was lower than those to rod and to myosin.

We then mixed the 38k protein with HMM of a soluble myosin fragment, and the mixture was subjected to the gel filtration. Both proteins were eluted in the different fractions, and their interaction was not detected (Fig. 5, inset).

Taken together, the above data indicate that the major site of the binding of the 38k protein should be in LMM. The indication is compatible with the absence of cross-talk between the 38k protein and telokin, because telokin binds to myosin at subfragment-2 (Shirinsky et al., 1993).

Both NH2-terminal and COOH-terminal Parts of the 38k Protein Are Required for Myosin Assembly

The myosin-assembling activity of the recombinant 38k protein was confirmed by observing that myosin filaments formed in its presence were the same as those formed by the native 38k protein under EM (data not shown). To test which part of the 38k protein contributes to the myosin-assembling activity, we produced several constructs of deletion mutants comparable to those of telokin (Silver et al., 1997).

As shown in Table I, when full-length, recombinant 38k protein at 4 µM was mixed with 1.1 µM myosin in either phosphorylated or unphosphorylated form, the amount of the 38k protein bound to the myosin was ~1.0 µM. However, the NH2-terminal–deleted mutants ΔN28, ΔN63, and ΔN88 did not bind to phosphorylated or unphosphorylated myosin. Similarly, the COOH-terminal–deleted mutants ΔC20 and ΔC66, could not bind to either type of myosin. Thus, deletion of the NH2-terminal 28 or COOH-terminal 20 amino acid residues of the 38k protein completely abolished the interaction with myosin. The significance of these data will be discussed below with reference to the crystal structure of human p32, which was published recently by Jiang et al. (1999).

Comparison of the Amino Acid Sequences of the 38k Protein and Human p32

Two partial amino acid sequences (LHTEGDKAFQAFLTDSEL and REVSPQPTGE5D) were obtained by cleavage with V8 protease. The former sequence was also obtained from undigested 38k protein, suggesting that the sequence is at the NH2 terminus of the protein. The amino acid sequence deduced from the cDNA sequence of the 38k protein (see Materials and Methods) is shown in Fig. 6 a. The calculated molecular weight was 23,681. Comparison with the GenBank/EMBL/DBJ data base revealed that the sequence is highly homologous to human p32, which was originally identified as a protein copurified with human splicing factor-2 (Krainer et al., 1991; Honore et al., 1993). A ligament of the 38k protein to p32 showed that 78% of the amino acid sequence of chicken 38k protein was identical to that of human p32. Mouse Y L2 (Luo et al., 1994), a homologue of p32, was also highly homologous (74%) with the 38k protein.

Characterization of Expressed Human p32 as a Homologue of the 38k Protein

Human p32 is known to contribute to various cellular functions, such as splicing of pre-mRNA (Krainer et al., 1991; M ayeda et al., 1992; Honore et al., 1993), binding of rev protein of the HIV virus to enhance duplication of the genomic DNA of the virus (Luo et al., 1994; Tange et al., 1996) and cell surface receptors for complements (Hebrewi et al., 1994; Lim et al., 1996), and for high molecular weight kininogen (Joseph et al., 1996). However, to our

Table I. Binding of the 38k Protein and its Deletion Mutants to Myosin

| Mutants | Unphosphorylated myosin | Phosphorylated myosin |
|---------|--------------------------|------------------------|
| Full-length (1–207) | 1.00 | 1.09 |
| ΔΔN28 (20–207) | 0.02 | 0.02 |
| ΔΔN63 (64–207) | 0.01 | 0.02 |
| ΔΔN88 (89–207) | 0.01 | 0.01 |
| ΔΔC66 (1–141) | 0.00 | 0.00 |
| ΔΔC29 (1–187) | 0.02 | 0.03 |

Mutants of the 38k protein (4.0 µM) were mixed with myosin (1.1 µM) in buffer A and precipitated with myosin as described in Materials and Methods. A mounts of the 38k protein or its mutants recovered in the precipitate were quantified and expressed in micromoles (µM). Data are the average of two sets of independent experiments. The numbers in parentheses refer to the number of the amino acid residue from the NH2 terminus of the full-length 38k protein.
knowledge, it has not yet been characterized as a cytoskeletal protein.

To clarify any functional difference between the 38k protein and human p32, we examined assembly of myosin by human p32. As shown in Fig. 7a, it induced formation of filaments of unphosphorylated myosin, as well as the 38k protein did. The length distribution of the myosin filaments (Fig. 7b) was similar to that of the 38k protein (Fig. 2e), the average length of the filaments being 0.69 ± 0.16 μm, SEM (n = 150; Fig. 7b, arrow). The myosin-assembling activity was also quantified by the centrifugation assay. The concentration of human p32 required to give half-maximal assembly estimated from Fig. 7c was 0.9 ± 0.2 μM, SEM (n = 3), with the concentration giving half-maximal binding at 1.8 ± 0.2 μM, SEM (n = 3). These figures are similar to those of the 38k protein (compare Fig. 7c with Fig. 3d). Thus, the human p32 appears to possess similar activity to that of the 38k protein.

We stained the blot of SDS-PAGE of total homogenate of chicken gizzard and the purified 38k protein with anti-38k protein antibody. As shown in Fig. 6c, the sole immunoreacting band is 38 kD. Because the antibody was raised against human p32 (see Materials and Methods), we conclude that the human p32 is a homologue of the 38k protein.

**Distribution of the 38k Protein in Smooth Muscle Tissues**

To examine the expression of the 38k protein in various...
smooth muscle tissues, their homogenates were subjected to Western blots. The 38k protein was detected in almost every smooth muscle tissue, including esophagus, stomach, small intestine, cecum, uterus, aorta, and airway as shown in Fig. 6 d. It was especially highly expressed in stomach and digestive tracts. Thus, the 38k protein can be thought of as a universal component of smooth muscle. It was also detected in cardiac muscle, but not clearly in skeletal muscle (Fig. 6 d, lanes 10 and 11). It could also be detected in nonmuscle tissues, such as liver, lung, spleen, and brain (data not shown), giving good conformity with the presence of human p32 in a variety of cells (Simon and Gorgatos, 1994; Deb and Datta, 1996; Dedia and Muller-Esterl, 1996; Joseph et al., 1996; Tange et al., 1996), but we cannot exclude the possibility that the reaction was due to contamination by smooth muscle cells from blood vessels.

**Colocalization of the 38k Protein with Myosin in Cultured Smooth Muscle Cells**

To determine whether the 38k protein is associated with myosin in smooth muscle cells, we examined its localization in the cultured smooth muscle cell line, AC01, by indirect immunofluorescence. Double staining (Fig. 8, a and b), followed by the image analysis (Fig. 8 c), indicates that the localization of the 38k protein coincides with myosin. Together with the same staining as shown in Fig. 8, a and b, the analysis indicates that the 38k protein is associated with myosin in the whole cells. In the case of the double staining of the 38k protein (Fig. 8 d) and mitochondria (Fig. 8 e, see below), the overlapping staining is limited to the central part of the cell (Fig. 8 f).

To examine whether the 38k protein is localized in the contractile apparatus composed of myosin and/or actin, we performed further double staining with antibodies against components of the contractile apparatus other than myosin. At least two types of actin isoforms are detected in A C01 cells by Western blotting, α- and β-actin (data not shown). Localization of the 38k protein in the cells is close to that of α-actin (Fig. 9, c and d), but does not necessarily match that of β-actin (Fig. 9, e and f). The localization of the 38k protein overlaps that of desmin, a major component of dense bodies (Fig. 9, g and h).

**Discussion**

In this report, we identified and characterized a novel myosin-binding protein of smooth muscle, a protein of 38 kD that assembles myosin into filaments. The myosin-assembling activity is similar to the activity reported for telokin (Shirinsky et al., 1993). Telokin binds at the NH2-terminal end of subfragment-2 of unphosphorylated myosin to change myosin from the 10S form to the 6S form (Masato et al., 1997; Silver et al., 1997). Thus, unphosphorylated myosin can be assembled into filaments. However, the localization of the 38k protein overlaps that of desmin, a major component of dense bodies (Fig. 9, g and h).

**Figure 8.** Localization of the 38k protein in the cultured smooth muscle cell line A C01. a–c, Staining pattern with anti-38k protein (a) and that with anti-MHC (b) antibodies. To amplify colocalized region of the 38k protein and MHC, images of a and b were multiplied in c, i.e., c = a × b. d–f, The cell was stained with MitoTracker Red CMXRos to visualize position of mitochondria. Staining pattern with anti-38k protein (d) and that with MitoTracker Red CMXRos (e). To demonstrate colocalized region of the 38k protein with mitochondria, images of d and e were multiplied in f, i.e., f = d × e. Magnification is the same in all pictures. Bar, 30 μm.
binding site for the 38k protein in the myosin molecule is different from that of telokin (Figs. 4 and 5).

It must be noted that telokin binds to only unphosphorylated myosin (Shirinsky et al., 1993). Therefore, it is expected in vitro that telokin may dissociate from myosin filaments to the cytosol when smooth muscle myosin is phosphorylated upon excitation of smooth muscle cells. However, because the 38k protein binds to both phosphorylated and unphosphorylated myosin, the 38k protein is thought to remain as a component of myosin filaments, whether or not smooth muscle cells are excited. The other distinction is that expression of telokin is limited to only a few types of smooth muscles (Gallagher and Herring, 1991; Herring and Smith, 1996), but the 38k protein is expressed in various types of smooth muscle cells, as shown in Fig. 6d.

We compared the amino acid sequence of the 38k protein to other myosin-binding proteins. The similarity of the amino acid sequence of the 38k protein to telokin (Gallagher and Herring, 1991; Yoshikai and Ikebe, 1992) is unexpectedly low, <5%. The 38k protein also showed little sequence homology with myosin-binding proteins of striated muscle, such as C-protein (Furst et al., 1992; Okagaki et al., 1993; Weber et al., 1993), H-protein (Vaughan et al., 1993), M-protein (Noguchi et al., 1992), and titin (Labeit et al., 1990), or other components of smooth muscle that interact with myosin, such as the 130 kD subunit of myosin phosphatase (Shimizu et al., 1994) and MLCK (Olson et al., 1990). Thus, the primary structure of the 38k protein is unique amongst myosin-binding proteins, suggesting that its myosin-binding, and hence myosin-bundling, activities are by a novel mode.

Recent analysis of the crystal structure of human p32 showed that it consists of seven consecutive antiparallel β-sheets flanked by one NH2-terminal and two COOH-terminal α helices, and that the α helix at the NH2 termi-

Figure 9. Colocalization of the 38k protein with components of contractile apparatus in the cultured smooth muscle cell line, A C01. a–h, Double staining of A C01 cell with anti-38k protein antibody, as detected by FITC-labeled second antibody and with various antibodies as detected by rhodamine-labeled second antibody. Staining pattern with anti-38k protein antibody (a, c, e, and g) and with antibodies against M H C (b), α-actin (d), β-actin (f), or desmin (h). Magnification is the same in all pictures. Bar, 30 μm.
The 38k protein is at the contact, because deletion of either the NH$_2$-terminal or COOH-terminal residues (Table I) leads to the loss of myosin-binding activity of the 38k protein.

We quantified the intracellular concentration of the 38k protein with an antibody as described in Materials and Methods. Its concentration in gizzard was 6.4 ± 0.5 μM, SD (n = 3). Since the concentration of myosin is reported as 46 μM (Yamazaki et al., 1987), the molar ratio of myosin to the 38k protein is ~1 to 0.14. Cellular myosin in the relaxed state is mostly unphosphorylated (for review, see Somlyo and Somlyo, 1994). From the kinetic profile shown in Fig. 3 d, the assembly of 1.1 μM unphosphorylated myosin was maximal in the presence of ~3 μM of the 38k protein, indicating that 2.7-fold molar excess of the protein over myosin was required for full assembly. Therefore, the cytoplasmic concentration of the 38k protein is too low to explain myosin assembly in vivo. The Prosite database predicts that the 38k protein has residues that can be phosphorylated with protein kinase C at Thr$^{130}$ and with casein kinase-2 at Thr$^{75}$, Ser$^{185}$, and Thr$^{86}$. It is possible that the myosin-assembly activity may be enhanced upon phosphorylation of the 38k protein. A further explanation is that, like thick filaments of skeletal muscle, a few myo-
sin-binding proteins, including telokin (Shirinsky et al., 1993) and the 38k protein, are working at the same time to assemble myosin into thick filaments of smooth muscle.

As explained in relation to Fig. 9, the 38k protein has been reported to be present in a variety of cellular organelles, including mitochondria, nuclei, vesicles, and cell surfaces. The present study describes additional localization of cytoskeleton. We realized that the variety may be attributable to that of the cells/organisms used for the respective experiments and speculated that all of them may not function at once. Rather, they might change according to the cellular states, e.g., differentiation. The first step in testing our speculation, we stained smooth muscle cells with antibodies against the 38k protein, are working at the same time to assemble myosin into thick filaments of smooth muscle.

The results are compatible with our speculation, we stained smooth muscle cell lines differentiated type, as shown in Figs. 8 and 9, the staining of myosin (Fig. 8 d, the assembly of 1.1 μM unphosphorylated myosin was maximal in the presence of ~3 μM of the 38k protein, indicating that 2.7-fold molar excess of the protein over myosin was required for full assembly. Therefore, the cytoplasmic concentration of the 38k protein is too low to explain myosin assembly in vivo. The Prosite database predicts that the 38k protein has residues that can be phosphorylated with protein kinase C at Thr$^{130}$ and with casein kinase-2 at Thr$^{75}$, Ser$^{185}$, and Thr$^{86}$. It is possible that the myosin-assembly activity may be enhanced upon phosphorylation of the 38k protein. A further explanation is that, like thick filaments of skeletal muscle, a few myo-
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