similar membrane-binding properties to the native protein. This single-Trp mutant not only offers a simpler system for fluorescence studies but also can be used to help understand the spectroscopic behavior of the native protein itself. Other studies are already underway to further modify the amino acid sequence of the membrane-binding domain to correlate changes in the amino acid sequence with changes in the secondary and tertiary structure of this domain as well as with catalytic activity. Preliminary data obtained with this mutant indicate that its ability to participate in desaturation of long-chain fatty acids is the same as that of rabbit b_5 (unpublished experiments of Ladokhina-Tretyachenko and Holloway).

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Mapping the Binding Domain of a Myosin II Binding Protein†

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ABSTRACT: The way in which actin and myosin II become localized to the contractile ring of dividing cells resulting in cleavage furrow formation and cytokinesis is unknown. While much is known about actin binding proteins and actin localization, little is known about myosin localization. A 53-kDa (53K) polypeptide present in the sea urchin egg binds to myosin II in a nucleotide-dependent manner and mediates its solubility in vitro [Yabkowitz, R., & Burgess, D. R. (1987) *J. Cell Biol. 105*, 927–936]. The binding site of 53K on the myosin molecule was examined in an effort to understand the mechanism of 53K-induced myosin solubility and its potential function in myosin regulation. Blot overlay and chemical cross-linking techniques utilizing myosin proteolytic fragments indicate that 53K binds to fragments proximal to the head–rod junction of myosin. Fragments distal to the head–rod junction do not bind 53K. In addition, the binding of 53K to myosin largely inhibits protease digestion that produces the head and rod fragments. The binding of 53K to the head–rod domain of myosin may be critical in regulation of myosin conformation, localization, assembly, and ATPase activity.

The actin-based cytoskeleton of the sea urchin egg cortex plays a vital role in cytokinesis. After fertilization, changes in the cytoskeleton are induced which mediate contractile ring formation and subsequent contraction. Actin and myosin II, the chemomechanical transducer, become concentrated to the contractile ring which is responsible for cleavage furrow action (Cao et al., 1990; Fujiwara et al., 1976; Schroeder, 1987; Schroeder et al., 1988; Mittal et al., 1987; Nunnally et al., 1980; Aubin et al., 1979). Myosin II, which forms conventional bipolar filaments, has been shown to be critical for contraction of the contractile ring (Mabuchi et al., 1977). Whether myosin exists as bipolar filaments, such as in muscle, and undergoes assembly–disassembly in nonmuscle cells is not known although myosin filaments appear to form in the furrow cortex in *Dictyostelium* during division and can be induced

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to disassemble by exposure to cAMP (Yumura et al., 1985). These studies suggest that the regulation of myosin assembly is very important in the formation and mechanics of actively contracting cleavage furrows.

Regulation of smooth and nonmuscle myosin II assembly and solubility is dependent on the regulation of myosin conformation since assembly requires interaction between the extended tails of neighboring molecules to form bipolar filaments (Korn et al., 1988). The head–rod junctional region of myosin heavy chain plays a critical role in regulation by providing the binding site for the light chains (Flicker et al., 1983) and a possible site of interaction with more distal portions of the heavy-chain tail, resulting in a kinked-tail 10S conformation (Craig et al., 1983). Myosin's conformation and its enzymatic activity are also mediated by myosin light-chain phosphorylation in vitro and in vivo (Harrington et al., 1984; Ikebe et al., 1984; Kiehart et al., 1984; Kuznicki, 1986). In addition, light-chain phosphorylation increases the sedimentation coefficient and the sensitivity to proteolysis of heavy meromyosin (Kumon et al., 1984; Onishi et al., 1983). Light-chain phosphorylation also provides a regulatory mechanism for actin-activated Mg^{2+}-ATPase activity found associated with intact myosin and HMM. These data suggest the head–rod junction plays a key role in the coupling of light-chain phosphorylation to the regulation of Mg^{2+}-ATPase activity and myosin conformation.

We have identified a protein in sea urchin eggs that has a subunit molecular weight of 53000, termed 53K, which binds to myosin in a nucleotide-dependent manner and affects its solubility at low ionic strength (Yabkowitz et al., 1987). The binding site of 53K on the myosin molecule was examined in an effort to understand the mechanism of 53K-mediated myosin solubility. Egg myosin can be digested with papain to yield discrete fragments representing the head or rod regions of the molecule. Data from blot overlay techniques, chemical cross-linking studies, and proteolytic protection experiments utilizing these head–rod fragments suggest that 53K binds near to or at the head–rod junction on the myosin molecule. Therefore, the mechanism by which 53K exerts an effect on myosin solubility may depend, in part, on its binding site on the myosin molecule near this important site for myosin regulation.

Materials and Methods

Protein Purification and 53K–Myosin Binding Conditions. Myosin and 53K were purified from soluble extracts of unfertilized sea urchin *Lytechinus variegatus* or *Strongylocentrotus purpuratus* eggs as described previously (Yabkowitz & Burgess, 1987). The concentration of 53K was determined by combining a protein assay with scanning densitometry of a Coomassie Blue stained microgel lane of the 53K fraction (Yabkowitz & Burgess, 1987). The binding of 53K to myosin was accomplished using previously described conditions (Yabkowitz & Burgess, 1987) requiring ATP or ITP as nucleotide triphosphates.

Proteolytic Digestion. Egg myosin (1 mg/mL) was digested with papain (0.2% w/w) or chymotrypsin (0.1% w/w) at 25 °C in 0.1 M KCl, 10 mM imidazole, 5 mM Mg^{2+}, 0.5 mM EGTA, and 0.5 mM DTT, pH 7.0, according to methods developed for brain myosin (Mittal et al., 1987). Reactions were stopped by addition of 4× SDS–PAGE sample buffer or iodoacetamide (papain) or phenylmethanesulfonyl fluoride (chymotrypsin) to a final concentration of 1 mM. Insoluble and soluble myosin fragments were separated as described (Barylko et al., 1986). The digestions in these cases were stopped by addition of iodoacetamide for papain or soybean trypsin inhibitor for chymotrypsin. The soluble fraction was concentrated in a Centricon 30 (Amicon), and the insoluble fraction was resolubilized in 0.5 M KCl, 10 mM imidazole, and 1 mM EDTA, pH 7.2.

Blot Binding. SDS–PAGE on 12% microslab gels and electrophoretic transfer to nitrocellulose were performed as described (Yabkowitz & Burgess, 1987). Blot overlays of myosin fragments were carried out essentially as described with slight modification (Glenney et al., 1980). The blot was washed in buffer A (7.5 mM KCl, 10 mM PIPES, 0.1 mM EGTA, and 0.1 mM ATP, pH 7.0) and blocked in buffer A plus 30 mg/mL BSA and 0.1% Tween-20. The blot was incubated with 53K (25 μg/mL) in buffer A plus 0.5 mg/mL BSA for 2 h at room temperature. After unbound 53K was washed off, the nitrocellulose sheet was first incubated with anti-53K antibody previously characterized (1:250 dilution) (Yabkowitz & Burgess, 1987) and then with ^{125}I-labeled goat anti-rabbit antibody (5×10^4 cpm/mL).

Proteolytic Protection by 53K. Myosin and 53K, at a weight ratio of approximately 8:1, were allowed to associated under conditions where egg myosin is soluble by incubation at 4 °C in 0.25 M KCl, 25 mM NaP_2O_7, 25 mM NaF, 10 mM PIPES, 5 mM imidazole, 7.5 mM $MgCl_2$, 1 mM EGTA, and 2–10 mM ATP for 1.5 h. Control preincubations were performed using myosin alone under the same buffer conditions. After incubation of myosin and 53K, proteolytic digestion was performed by addition of papain or chymotrypsin (at 0.2% w/w or 0.1% w/w, respectively) and incubation at 25 °C for up to 60 min (final 0.166 M KCl). At specific time intervals, aliquots were boiled in SDS sample buffer and run on 12% SDS–PAGE.

Actin Binding Assays. A sample of the soluble myosin fragments after protease treatment was incubated for 30 min with F-actin (0.3 mg/mL) in 50 mM NaCl, 25 mM imidazole, 5 mM $MgCl_2$ 0.2 mM EGTA, and 0.5 mM DTT, pH 7.0, prior to centrifugation at 100000*g* for 20 min in a Beckman airfuge. Both the supernatant and pellet were processed for SDS–PAGE on 12% microslab gels.

Actin-Activated Mg^{2+}-ATPase Assays. A sample of myosin rod and head fragments was used in ATPase assays according to Agre et al. (1983). The reactions were carried out in 50 mM KCl, 10 mM Tris (pH 8.0), 5 mM Mg^{2+}, and 0.1 mM EGTA at 25 °C.

Cross-Linking Experiments. The head fragments (1 mg/mL) or rod fragments (0.5 mg/mL) of digested myosin were preincubated with 53K (0.4 or 0.2 mg/mL, respectively) for 1 h on ice in 50 mM NaCl, 25 mM imidazole, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.5 mM EDTA, and 0.1 mM ATP, pH 7.0. Cross-linking was initiated by addition of DSS (in DMSO) to 0.2 mM and incubated in a 25 °C water bath. Reactions were stopped after 30 min by the addition of 4× SDS–PAGE sample buffer.

Negative-Stain EM. Aliquots of the insoluble or soluble fragments of egg myosin digested with papain or chymotrypsin for 30 min were stained with 1% uranyl acetate on Formvar and carbon-coated grids and examined in a JEOL 100CXII microscope at 80 kV.

Results

Proteolytic Cleavage of Myosin into Discrete Fragments. Papain digestion of egg myosin in low-salt buffer yielded discrete fragments of 165, 130, 107, 80, 62, and 24 kDa (Figure 1). Papain-generated fragments of myosin were further characterized to identify which myosin domains they represented. Dialysis of digestion mixtures against low salt followed by centrifugation enabled separation of soluble (head)

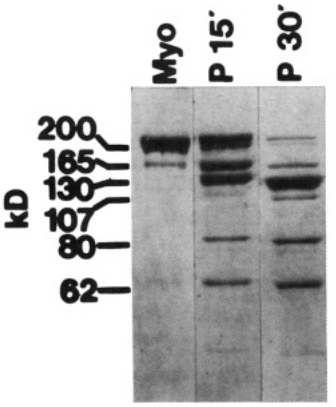

FIGURE 1: Papain cleavage of myosin results in specific fragments representing different regions of the molecule. Myosin heavy chain is a 200-kDa peptide (Myo) that when treated with papain for 15 min (P 15′) is cleaved into 165-, 130-, 107-, 80-, and 62-kDa fragments. Digestion for longer periods (P 30′) results in the further digestion of the 165-kDa peptide to form more 130- and 107-kDa fragments.

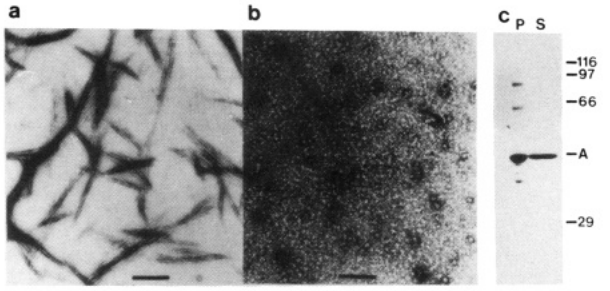

FIGURE 2: Characterization of insoluble and soluble fragments of papain-digested myosin. Negative-stain EM of the insoluble fragments (a) and soluble fragments (b) of egg myosin digested with papain for 30 min. (a) Bar = 0.4 μm; (b) bar = 0.2 μm. (c) Coomassie Blue stained SDS–PAGE of myosin soluble fragments incubated with F-actin in the absence of ATP. Material pelleted (lane P) or remaining in the supernatant (lane S) after centrifugation.

from insoluble (rod) fragments (Figure 2). SDS–PAGE analysis of the myosin head and rod fragments, characterized as above, obtained after papain digestion revealed that the rod fragments were composed of three polypeptides with apparent molecular weights of 165 000, 130 000, and 107 000 (Figure 3c). The 165-kDa fragment was highly sensitive to papain and present transiently as it rapidly broke down into the other fragments. The head fragments had apparent molecular weights of 80 000, 62 000, 24 000, and 19 000 (Figure 3c). The latter band probably represented the 19-kDa regulatory myosin light chain and was relatively resistant to papain digestion. The 62- and 24-kDa bands appeared to be the result of cleavage at a single site in the 80-kDa polypeptide fragment since there was a concomitant increase in the amount of the 62- and 24-kDa bands and a decrease in the amount of 80-kDa band upon extended papain digestion (data not shown).

When examined by negative-stain EM, the insoluble fractions (Figure 2a) were composed exclusively of assembled myosin rods. No myosin heads were observed. The soluble fraction, in contrast, contained a dispersed array of globular molecules but did not possess rods (Figure 2b). The soluble fragments decorated F-actin in negatively stained preparations with short, polarized arrowheads as expected for subfragment 1 (data not shown) and bound to and cosedimented with F-actin in the absence of ATP (Figure 2c). The actin-activated Mg^{2+}-ATPase activity of the soluble and insoluble papain-digested myosin fragments was also determined (Table I). The Mg^{2+}-ATPase activity was associated with the soluble (head) fraction, and the values of the soluble fragments were

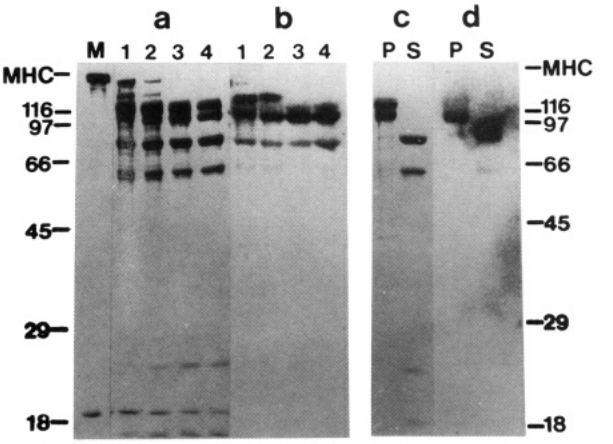

FIGURE 3: Binding of 53K to myosin fragments generated by papain digestion. (a) Coomassie Blue stained SDS–PAGE of egg myosin digested with papain for 5, 10, 20, and 30 min (lanes 1–4); M, egg myosin standard. (b) Autoradiograph of gel shown in (a) after blot overlay with 53K. (c) Coomassie Blue stained SDS–PAGE of insoluble (lane P) and soluble (lane S) myosin fragments generated by papain digestion for 30 min. (d) Autoradiograph of gel shown in (c) after blot overlay with 53K. MHC, myosin heavy chain (200 kDa). Molecular weight standards ($\times 10^{-3}$) as indicated.

Table I[a]

myosin fraction	actin	ATPase activity: papain	ATPase activity: chymotrypsin
insoluble (rod fragments)	−	0.2	1.5
insoluble (rod fragments)	+	0.2	5.7
soluble (head fragments)	−	9.1	8.2
soluble (head fragments)	+	12.6	44.7

[a] The myosin fragments were added to the reaction mixture to a final concentration of 0.1 mg/mL. The actin was added to 0.6 mg/mL. The reactions were carried out in 50 mM KCl, 10 mM Tris, 5 mM Mg^{2+}, and 0.1 mM EGTA, pH 8.0 at 25 °C. The ATPase activity is expressed as nanomoles per milligram per minute.

similar to those exhibited by myosin S1 derived from papain digestion of smooth muscle myosin (Seidel, 1978). Actin only stimulated the ATPase activity associated with the head fragments. On the basis of these data, the soluble fragments derived from limited papain digestion of egg myosin comprise the head, or S1 portion, of myosin while the insoluble fragments represent the rod domain, or tail, of myosin.

Chymotrypsin digestion of myosin resulted in peptides of approximately 165, 69, and occasionally, depending on the extent of proteolysis, 52 kDa (Figure 5). The 165-kDa fragment was determined to be derived from the rod portion of myosin because of its association with the insoluble fraction under conditions for myosin assembly (Figure 5), and this fraction had much less actin-activated Mg^{2+}-ATPase activity (Table I). The 69- and 52-kDa fragments are associated with the soluble head fraction in such experiments (Figure 5) which contain the majority of the actin-activated Mg^{2+}-ATPase activity (Table I). These fractions when observed by negative-stain EM displayed a similar appearance as papain-derived fractions (data not shown).

Binding of 53K to Specific Myosin Fragments Blotted onto Nitrocellulose. Blot binding of 53K to papain-digested myosin fragments indicates that 53K binds to a combination of head and rod regions (Figure 3). The 53-kDa protein bound predominantly to the 107-kDa rod and 80-kDa head myosin fragments with apparently weaker binding to the 130- and 165-kDa rod fragments (Figure 3b). Stronger binding of 53K to the 165-kDa fragment was observed on myosin digested for 5 min but decreased concomitantly with the loss of the 165-kDa fragments by further digestion. Blot overlay performed

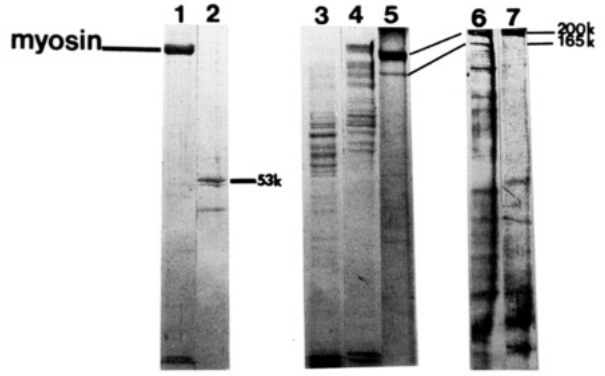

FIGURE 4: Analysis of the 53K binding site by inhibition of specific proteolysis. Myosin (lane 1) when bound to 53K (lane 2) has altered proteolytic characteristics. Papain digestion of myosin (lane 3) is greatly affected by the presence of 53K (lanes 4 and 5). ATP concentration has some effect on the degree of protection (2 mM ATP, lane 4; 10 mM ATP, lane 5). A similar protection was seen with chymotrypsin digestion (no added 53K lane 6; 53K added, lane 7).

on separated myosin head and rod fragments similarly demonstrated that 53K primarily bound the 80-kDa head fragment and the 107-kDa fragment in the rod fraction (Figure 3d).

Inhibition of Proteolytic Cleavage by 53K. The binding site of 53K on myosin was further characterized by studying the effect of 53K on the papain or chymotrypsin digestion of myosin. Myosin was mixed with 53K in solution under conditions where 53K binds to myosin before proteolytic digestion.

Generally, the presence of 53K greatly inhibited the initial cleavage of myosin by papain (Figure 4). Moreover, even the limited amount of the 165-kDa fragment produced by papain in the presence of 53K was protected from further digestion by 53K in that the amount of 130-kDa fragment resulting was also greatly diminished (Figure 4, lane 5). Protection from proteolysis was also observed using lower ATP concentrations (2 mM) but not to the same degree (Figure 4, lane 4). At higher ATP concentrations, the digestion of myosin by papain in the absence of 53K was much more extensive, resulting in the complete degradation of the 130-kDa fragments (Figure 4, lane 3).

Chymotrypsin digestion of myosin results in head and rod fragments also. The binding of 53K to myosin inhibits the chymotrypsin cleavage of myosin into these head and rod domains in a similar way as for papain (Figure 4, lanes 6 and 7). Namely, in the presence of 53K, chymotrypsin's ability to generate the 165-kDa rod fragment (Figure 5, lane 3) and the resulting head fragments (Figure 5, lane 4) is greatly reduced.

Cross-Linking of 53K to Myosin Fragments. Additional evidence consistent with the head–rod junction as the site where 53K binds to myosin was provided by a series of cross-linking experiments using a cross-linking agent. Cross-linking of 53K to the head fragments of papain-digested egg myosin with DSS resulted in the appearance of a 130-kDa polypeptide band on SDS-PAGE not present in the starting material (Figure 6A). This band most likely represented a cross-linked complex containing 53K and the 80-kDa polypeptide myosin fragment. There was concomitant loss of the 80-kDa polypeptide band compared to the relatively constant level of the 62-kDa band in the cross-linked gel lane (compare Figure 6A, lanes 1 and 2). Other polypeptide bands detectable on SDS–PAGE of the cross-linking experiments likely represent cross-linked oligomers composed of the 80-kDa fragment with two intact myosin light chains, a band of approximately 120 kDa seen with DSS, two self-associated 53K molecules at 105 kDa, and two self-associated 80-kDa fragments at 160 kDa. The binding of 53K to the 107-kDa rod fragment was evident by the production of a 160-kDa fragment by chemical cross-linking (Figure 6B).

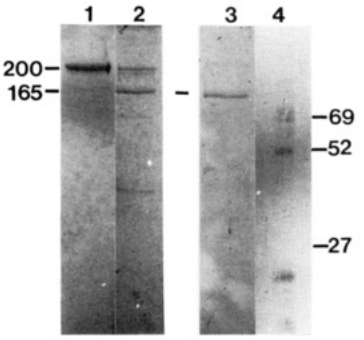

FIGURE 5: Chymotrypsin digestion of myosin (lane 1) yields high molecular weight and low molecular weight fragments (lane 2). Using methods described above to separated insoluble rod fragments from soluble head fragments, it was determined that the 165-kDa high molecular weight peptide is from the rod portion of myosin (lane 3) and that 69-kDa and 52-kDa low molecular weight peptides are derived from the head region.

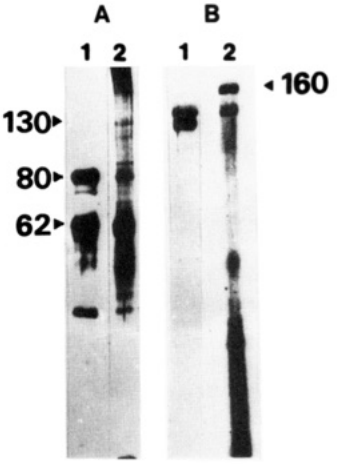

FIGURE 6: Analysis of the 53K binding site on myosin by cross-linking studies. (A) Silver-stained SDS–PAGE gels of the head fragments of papain-digested myosin (lane 1) cross-linked to 53K with DSS (lane 2). The polypeptide band in lane 1 at approximately M_r 45 000 is likely a minor cleavage product of one of eight higher molecular weight soluble fragments which is visualized only upon silver staining. (B) SDS–PAGE of the rod fragments of papain-digested myosin (lane 1) cross-linked to 53K with DSS (lane 2) (Coomassie stained, lane 1; silver stained, lane 2). Molecular weight ($\times 10^{-3}$) markers are as indicated.

Discussion

The present report provides new data consistent with the hypothesis that 53K, a mediator of myosin solubility, binds directly to myosin at a site that includes the heavy-chain head–rod junction. The results of three independent lines of experimentation (blot binding, proteolytic protection, and chemical cross-linking) are consistent with 53K binding to this important region of myosin. These data suggest that the effect of 53K on myosin's solubility may be due to direct binding of 53K to this domain on the myosin heavy chain. The head–rod junction is an integral domain for myosin conformation and, consequently, function because it possesses the light-chain binding sites, is flexible, and may mediate the interaction between the head of myosin containing ATPase activity and the tail of myosin regulating myosin assembly state. Therefore, proteins binding at the head–rod junction are likely to have an influence on myosin function.

The binding of 53K to both papain-derived head (80 kDa) and rod (107, 130, and 165 kDa) fragments, directly dem-

onstrated by blot binding, indicates that 53K's binding site is at or near the head–rod junction of myosin. The 53K protection of myosin from papain or chymotrypsin proteolytic digestion at rod–head junction specific sites is also consistent with 53K binding to the head–rod junction of myosin. The same effect could be achieved if 53K caused some aggregation of the myosin leading indirectly to inhibition of myosin cleavage. This is proably not the case if one considers the concentration of KCl in the digestion mixture and the nature of the effect of 53K on myosin. The concentration of KCl in the reaction is 0.166 M which is unfavorable for myosin assembly with or without 53K. In addition, the 53K–myosin complex aggregates only under conditions that myosin alone would aggregate, whereas 53K induces myosin to be soluble in low ionic strength buffers. So, if in these experiments 53K induced myosin aggregation and inhibited digestion, then the control myosin digest under the same conditions would also be aggregated and show inhibited digestion. Finally, chemical cross-linking of 53K to both head and rod fragments is used as supportive evidence to the protection and blot binding studies. This also suggests that the binding site for 53K is in the head–rod junctional region. Because of the low yield of cross-linked products and the inherent problems with interpreting chemical cross-linking experiments, this is not compelling data by itself. However, taken into consideration with the other experimental data, it adds strength to their conclusions.

While myosin assembly into structures in nonmuscle cells can be dynamic (McKenna et al., 1989; Mittal et al., 1987; Yumura & Fukui, 1985), the question is raised as to how transitions in myosin supramolecular organization occur. Smooth muscle and nonmuscle myosins are able to exist in a soluble 10S form, in which the tail is folded into approximately equal thirds (Craig et al., 1983; Trybus et al., 1982). Rotary-shadowing of 10S myosin molecules suggests that the tail region near the distal kink interacts with the myosin head or neck. It is possible that, in vivo, unassembled myosin might be stabilized into a soluble complex by 53K, similar to the 10S form seen in vitro. A complex between 53K and myosin is formed in vitro which has a sedimentation coefficient of 24 S (Yabkowitz & Burgess, 1987). The effect of 53K on myosin solubility in vitro (Yabkowitz & Burgess, 1987) could be explained by conformational constraints imposed by the binding of 53K to myosin, as suggested by the location of the 53K binding site in the head–rod junction region of the myosin molecule. Binding of 53K to myosin might physically prevent the myosin tail from unfolding and stabilize a 10S-like structure. Therefore, 53K may stabilize a myosin conformation which maintains myosin solubility under low ionic strength conditions.

Myosin-light chain phosphorylation in vertebrate nonmuscle cells promotes filament formation and increases the percentage of myosin molecules in the extended 6S form (Craig et al., 1983; Scholey et al., 1980). However, if 53K's effect was mediated by phosphorylation of myosin alone and subsequent conformational changes of myosin to the 6S extended form, then one would expect to observe an increase in the susceptibility of myosin to papain cleavage (Ikebe & Hartshorne, 1984). This is contrary to what this study shows at least with respect to proteolysis. Moreover, 53K binds to myosin and affects its solubility in the presence of ITP (Yabkowitz & Burgess, 1987), which is not a substrate for myosin light-chain kinases. However, no evidence exists at present on the phosphorylation state of myosin light chains in the proteolysis protection assays. ATP concentration also has an influence on the ability of 53K to inhibit myosin proteolysis. A possible explanation for the greater inhibition of papain cleavage at higher ATP concentrations may be a more enhanced binding of 53K to myosin at 10 mM ATP than at 2 mM. The precipitation of myosin filaments from extract in the presence of 53K at higher concentrations of ATP is consistent with a possible involvement of 53K-associated light-chain kinase. Alternatively, 53K may directly induce myosin to assume the 10S kinked conformation, keeping it soluble and resistant to proteases. Structural and enzymatic studies should resolve this issue.

Acknowledgments

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Registry No. Papain, 9001-73-4; chymotrypain, 9004-07-3.

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