Myosin II self-assembles to form thick filaments that are attributed to its long coiled-coil tail domain. The present study has determined a region critical for filament formation of vertebrate smooth muscle and nonmuscle myosin II. A monoclonal antibody recognizing the 28 residues from the C-terminal end of the coiled-coil domain of smooth muscle myosin II completely inhibited filament formation, whereas other antibodies recognizing other parts of the coiled-coil did not. To determine the importance of this region in the filament assembly in vivo, green fluorescent protein (GFP)-tagged smooth muscle myosin was expressed in COS-7 cells, and the filamentous localization of the GFP signal was monitored by fluorescence microscopy. Wild type GFP-tagged smooth muscle myosin colocalized with F-actin during interphase and was also recruited into the contractile ring during cytokinesis. Myosin with the nonhelical tail piece deleted showed similar behavior, whereas deletion of the 28 residues at the C-terminal end of the coiled-coil domain abolished this localization. Deletion of the corresponding region of GFP-tagged nonmuscle myosin IIA also abolished this localization. We conclude that the C-terminal end of the coiled-coil domain, but not the nonhelical tail piece, of myosin II is critical for myosin filament formation both in vitro and in vivo.

Myosin is a molecular motor that interacts with actin filaments and converts chemical energy of ATP to mechanical work. The molecular structure of conventional myosin is characterized by its globular head domain and the filament forming α-helical coiled-coil tail domain. Although the former characteristic is shared by a number of unconventional myosins, only conventional myosin, classified as the second class of myosin in conventional myosin, deletion of the N-terminal residues of LMM did not affect the solubility at low ionic strength, but deletion of 28 residues from the C terminus caused a decrease in solubility (16). Hodge et al. (17) reported that, by expressing the rod portion of nonmuscle myosin II by Escherichia coli expression system, the 35-residue C-terminal nonhelical region influences filament formation. Recently it was reported that the deletion of 29 residues near the C terminus of skeletal myosin heavy chain disrupts the filament formation (18). On the other hand, for amoeba myosin, the C-terminal 100 residues are critical for assembly of bipolar myosin minifilaments (15). In the case of Dictyostelium myosin, an expressed peptide having the C-terminal 600 residues showed the formation of paracrystals with a 14.3-nm repeat, a characteristic of thick filaments (19). Subsequently it was shown that the deletion of the C-terminal 297 amino acid residues does not affect the myosin assembly properties, whereas the deletion of an additional 35 residues significantly reduces the assembly of myosin (20). These results suggest that, whereas the critical region for myosin assembly for vertebrate skeletal myosin is near the C terminus, for Dictyostelium myosin it is away from C terminus. The difference may be related to the difference in tail lengths between the myosins. The location of the critical region for myosin assembly may be related to the regulation mechanism for myosin assembly, because the assembly of Dictyostelium myosin is regulated by heavy chain phosphorylation at sites located away from the C terminus (20). The assembly properties of vertebrate smooth muscle myosin are unique in several respects. In contrast to striated muscle myosin and amoeba myosin, smooth muscle myosin forms “side polar” filaments rather than “bipolar” filaments (21–23). Smooth muscle myosin and vertebrate nonmuscle myosin filaments are also unstable in the presence of ATP, and phosphorylation of the regulatory light chain is required to form stable filaments in vitro (24, 25). These characteristics of smooth muscle myosin assembly could be at least partly due to the difference in the structure of the region critical for filament formation. By proteolytic digestion of gizzard LMM, it was shown that the C-terminal 13-kDa domain specifies the insolvability of gizzard LMM (13); however, the region critical for thick filament formation of smooth muscle myosin both in vitro and in vivo has not yet been determined.

Received for publication, March 5, 2001, and in revised form, May 31, 2001
Published, JBC Papers in Press, June 6, 2001, DOI 10.1074/jbc.M101969200

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*This work was supported by National Institutes of Health Grants AR41853, HL56218, and MG55834 (to M. I.), HL62468 (to R. C.), and AR41637 (to K. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: LMM, light meromyosin; GFP, green fluorescent protein; NHT, nonhelical tail of myosin; PBS, phosphate-buffered saline.
In the present study, we produced a monoclonal antibody that disassembles smooth muscle myosin filaments. The binding site of this antibody is determined to be within the C-terminal 28 residues of the coiled-coil structure of myosin rod. The GFP-tagged truncated myosin mutant lacking these 28 residues in cells failed to show evidence of filamentous structure. The results indicated that the C-terminal 28 residues of the predicted coiled-coil domain of myosin are critical for the thick filament formation of smooth muscle myosin. The corresponding region of human nonmuscle myosin II was also shown to be critical for filament formation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and modifying enzymes were purchased from New England Biolabs (Beverly, MA). The pT7-7 *E. coli* expression vector containing the T7 promoter (26) was provided by Dr. S. Tabor (Harvard Medical School). Smooth muscle myosin was prepared from frozen turkey gizzards as described (27). Actin was prepared from rabbit skeletal muscle acetone powder according to Spudich and Watt (28). Collagenase (purified from *Clostridium histolyticum*) and elastase were purchased from Worthington Biochemical Corp. (Lake-wood, NJ). Protease was purchased from Sigma. The mammalian expression vector pEGFP-C1 was purchased from CLONTECH Laboratories (Palo Alto, CA).

**Expression of the Truncated Smooth Muscle Myosin and Myosin Rod**—The mutant cDNAs were made by the site-directed mutagenesis strategy as described previously (29, 30) using the smooth muscle heavy chain cDNA in pT7-7 vector as a template. A *Nde*I site was created at codon 1396 of the heavy chain cDNA to remove a cDNA fragment encoding the amino acids 1–1395. To delete the C-terminal amino acid residues of the smooth muscle myosin rod, a stop codon was created at codons 1849, 1910, and 1937, respectively, for /H90041, /H90042, and /H9004NHL. After confirming the mutation by direct sequencing analysis, the truncated myosin rod expression vectors were transferred to BL21(DE3). The expression of the truncated rod was monitored by Western blot using various monoclonal antibodies recognizing the myosin rod. The entire coding region of chicken gizzard myosin heavy chain cDNA (smooth muscle myosin isoform 1) was created and subcloned into pFastbacHTb baculovirus transfer vector at NcoI/SalI sites in the polylinker region. A NcoI site was created at codon 859 of the heavy chain cDNA, and the vector was digested to remove a cDNA fragment encoding amino acids 1–858 and then self ligated. This construct was used to produce myosin rod. A stop codon was created at codon 1910 of the myosin heavy chain cDNA to produce the construct expressing the 28-amino acid residue deletion mutant of myosin or myosin rod. The plasmids were transformed into DH10BAC *E. coli*. Recombinant bacmids (recombinant virus DNA) were isolated and used to transfect Sf9 insect cells. The proteins were expressed by infection of fresh Sf9 cells with viral stock for 72 h. Smooth muscle myosin or myosin rod was purified using Ni2+-nitrilotriacetic acid-agarose (Qiagen).

**Expression of the GFP-tagged Smooth Muscle and Nonmuscle Myosin and Their Mutants**—A smooth muscle myosin heavy chain cDNA from chicken gizzard containing entire coding region (smooth muscle myosin isoform 1) was subcloned into pEG-FPC1 expression vector containing GFP sequence. A unique *Xho*I site was created at codon 859 of the heavy chain cDNA, and the vector was digested to remove a cDNA fragment encoding amino acids 1–858 and then self ligated. This construct was used to produce myosin rod. A stop codon was created at codon 1910 of the myosin heavy chain cDNA to produce the construct expressing the 28-amino acid residue deletion mutant of myosin or myosin rod. The plasmids were transformed into DH10BAC *E. coli*. Recombinant bacmids (recombinant virus DNA) were isolated and used to transfect Sf9 insect cells. The proteins were expressed by infection of fresh Sf9 cells with viral stock for 72 h. Smooth muscle myosin or myosin rod was purified using Ni2+-nitrilotriacetic acid-agarose (Qiagen).

**Fig. 1.** Electron micrographs of rotary shadowed smooth muscle myosin-antibody complexes, showing the site of binding. Bar, 0.3 μm.

**Fig. 2.** Localization of the binding sites of anti-smooth muscle myosin antibodies on the smooth muscle myosin molecule. Location of each antibody binding site was estimated from the electron micrographs. The distance from the tip of the tail of myosin was measured with over 50 images for each antibody, and the mean distance was calculated. The predicted flexible regions of myosin rod are indicated with red circles.
Myosin Filament Formation

The effect of antibody on filament formation was studied by mixing 0.5 mg/ml gizzard myosin with 0.3 mg/ml of each antibody in 0.5M KCl, 50 mM Tris, pH 7.0, followed by dialysis against 0.15 M KCl, 50 mM Tris, pH 7.5, at 4°C for 15 min. The mixture was then centrifuged (15,000 × g) for 15 min to precipitates the myosin filaments. The pellets and the supernatants were subjected to SDS-polyacrylamide gel electrophoresis followed by densitometry to determine the amount of precipitated myosin filaments. The recombination of the C-cap myosin rod and the C-terminal truncated rod expressed by baculovirus expression system (0.1 mg/ml) was incubated in the buffer containing 0.12 M KCl, 2 mM MgCl₂, 1 mM dithiothreitol, and 30 mM Tris-HCl, pH 7.5, at 25°C for 15 min. The samples were subjected to sedimentation assay as described above.

RESULTS

Binding Site of Monoclonal Antibodies on Smooth Muscle Myosin Rod—We produced 20 monoclonal antibodies against turkey gizzard smooth muscle myosin. These were subjected to Western blot analysis with myosin fragments digested by papain. Papain digestion of myosin produces 68-, 26-, and 130-kDa fragments. The first two peptides are derived from S1, and the last peptide is derived from the myosin rod. Among the antibodies tested, mm5, mm8, mm16, mm18, and mm19 recognized the 130-kDa fragment (not shown). To further determine the binding site, each antibody was mixed with intact gizzard myosin, and the antibody-myosin complex was examined by electron microscopy using the technique of rotary shadowing. All the antibodies tested bound to the rod portion of myosin molecule (Fig. 1). mm5 and mm8 bound at the middle of myosin tail, whereas mm18, mm16, and mm19 bound toward the C-terminal end. The position of each antibody binding site from the tip of the tail was estimated from the electron micrographs (Fig. 2). Interestingly, the antibody binding sites were nearly identical to the flexible regions of the rod that are predicted according to the amino acid sequence and electron microscopic observation (13, 35–40), suggesting that the flexible regions have high antigenicity (Fig. 2). It should be noted that most of the antibody binding sites have two antibodies bound, with one sticking out on each side.

Effect of Anti-myosin Heavy Chain Rod Antibodies on Smooth Muscle Myosin Filament Formation—It is plausible that the binding of antibodies interferes with the intermolecular interactions of myosin heavy chains, thus inhibiting filament assembly, if the antibody binding site overlaps with re-aggregation of myosin.

FIG. 3. Effect of anti-smooth muscle myosin antibodies on the aggregation of myosin. The experiments were done as described under “Experimental Procedures.” The amount of precipitated myosin (%) was obtained by dividing the amount with the total myosin. An excessive amount of antibodies was used to avoid cross-linking of myosin molecules by antibodies.

The fragment was inserted in frame in the multicloning site of pEGFP-C1 vector. Then a EcoRI/XhoI 4.0-kilobase fragment encoding the C-terminal domain of myosin heavy chain was inserted into the pEGFP-C1 vector containing the 5′ myosin heavy chain cDNA fragment at the EcoRI/XhoI sites. The sequence orientation was confirmed with restriction mapping and direct sequencing analysis, and the produced plasmid was used as a GFp-smooth muscle myosin chimera expression vector. To create the truncation myosin mutant expression vectors, a stop codon was created at the codons 1849, 1910, and 1937, termed Δ1, Δ2, and ΔNHT, respectively.

Human myosin II A heavy chain cDNA fragments were kindly supplied by Dr. R. S. Adelestein (National Institutes of Health). These clones contain nucleotides −19 to 1200, 295 to 4155, and 4155 to 7236 of the human myosin II A heavy chain cDNA, respectively. A HindIII site was created at the side of the initiation codon, and the 0.35-kilobase fragment including the initiation codon was excised by HindIII/BsaBI digestion and subcloned into the pBluescript vector containing the myosin II A cDNA (295–4155) digested with HindIII/BsaBI. The cDNA (1–4155) was excised with HindIII/EcoRI and subcloned into pEGFP-C1 vector at HindIII/EcoRI sites in the polylinker region. The myosin II A cDNA fragment containing the nucleotides 4155–7236 was excised with EcoRI and ligated in frame with pEGFP-C1 vector at HindIII/EcoRI sites in the polylinker region. The myosin II A cDNA fragment containing the nucleotides 4155–7236 was excised with EcoRI and ligated in frame with pEGFP-C1 vector containing myosin II A cDNA (1–4155). The sequence orientation of this GFp-nonmuscle myosin II A expression vector was confirmed by restriction enzyme mapping, and the sequence was confirmed by direct sequencing analysis. To create the truncated GFp-myosin II A, a stop codon was created at codon 1899.

Cell Culture—COS-7 cells (American Type Culture Collection) were cultured with Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin (Life Technologies, Inc.).

Transfection—COS-7 cells (about 10⁶ cells/ml) on a coverslip were transfected with SuperFect™ transfection reagent (Qiagen) according to the protocol provided by the manufacturer.

Immunofluorescence—The cells were fixed with 4% formaldehyde for 10 min, followed by permeabilization with 0.1% Triton X-100 in PBS. After blocking with 3% bovine serum albumin with PBS at room temperature for 1 h, they were incubated with Texas Red-labeled phalloidin (Molecular Probes) at room temperature for 1 h. After washing with PBS three times, the samples were mounted in 3% 1,4-diazabicyclo[2.2.2]octane (Sigma), 90% glycerol in PBS.

Three-dimensional Digital Imaging Microscopy and Image Restoration—Images of labeled cells were acquired with a Nikon Diaphot 200 microscope equipped with a 100W Hg arc lamp for epifluorescence microscopy. The cells were viewed with 60× (NA 1.4) or 40× Nikon (NA 1.3) Planapoch objectives with a 2.5× or 5× camera eyepiece, and images were projected onto the face of a Photometrics thermoelectrically cooled CCD camera.

Production of Anti-smooth Muscle Myosin Monoclonal Antibodies—Monoclonal antibodies were produced against chicken gizzard myosin as described previously (31). The clones were expanded and finally grown in ascitic fluid in pristane primed BALB/c mice. IgG class anti-bodies were purified by protein A-Sepharose affinity chromatography according to the manufacturer's instructions.

Gel Electrophoresis and ATPase Assay—SDS-polyacrylamide gel electrophoresis was carried out on a 7.5–20% polyacrylamide gradient slab gel using the discontinuous buffer system of Laemmli (32). Molecular mass standards used were myosin heavy chain (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), calbindin (27 kDa), myosin regulatory light chain (20 kDa), and α-lactalbumin (14.2 kDa). The steady-state heavy meromyosin ATPase activity was measured at 25 °C as described previously (27).

Electron Microscopy—Gizzard myosin in solution containing 0.3 M KCl, 20 mM Tris-HCl, pH 7.5, 30% glycerol, was mixed with each monoclonal antibody and then absorbed onto a freshly cleaved mica surface for 30 s. Unbound proteins were rinsed away, and then the specimen was stabilized by brief exposure to uranyl acetate (33). The specimen was visualized by the rotary shadowing technique according to Mabuchi (34) with an electron microscope (model EM300; Phillips Electronic Instruments, Mahwah, NJ) at 60 kV. The C-terminal truncated smooth muscle myosin and myosin rod were also visualized by the rotary shadowing technique.

The effect of antibody on filament formation was studied by mixing 0.5 mg/ml gizzard myosin with 0.3 mg/ml of each antibody in 0.5 M KCl, 50 mM Tris, pH 7.0, followed by dialysis against 0.15 M KCl, 50 mM Tris, pH 7.5, at 4°C for 15 min. Each antibody was also added to filaments antibody formed in the absence of antibody. The samples were applied to thin carbon supported by copper mesh grids and negatively stained with 2% uranyl acetate before visualization in the electron microscope operating at 80 kV.

Sedimentation—Smooth muscle myosin was dialyzed against buffer A containing 10 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, and 30 mM Tris-HCl, pH 7.5, to form filaments. 0.4 μM myosin was then mixed with 2 μl of each antibody or buffer A at room temperature for 5 min. The mixture was then centrifuged (15,000 × g) for 15 min to precipitates the myosin filaments. The pellets and the supernatants were subjected to SDS-polyacrylamide gel electrophoresis followed by densitometry to determine the amount of precipitated myosin filaments. The recombinant smooth muscle myosin rod and the C-terminal truncated rod expressed by baculovirus expression system (0.1 mg/ml) was incubated in the buffer containing 0.12 M KCl, 2 mM MgCl₂, 1 mM dithiothreitol, and 30 mM Tris-HCl, pH 7.5, at 25°C for 15 min. The samples were subjected to sedimentation assay as described above.
regions critical to the filament assembly. To examine the effect of the antibodies on myosin filament assembly, we added each monoclonal antibody to gizzard myosin at low ionic strength, where myosin molecules assemble to form filaments, and the myosin-antibody mixture was subjected to sedimentation analysis. As shown in Fig. 3, mm19 dramatically diminished the fraction of myosin sedimented by centrifugation, suggesting that mm19 inhibited myosin filament assembly. To further confirm this notion, the effect of antibodies on myosin filament formation was directly monitored by visualization of the filaments with electron microscopy (Fig. 4). Two different methods were used to evaluate the effect of the antibodies on filament formation. First, smooth muscle myosin was mixed with antibodies at high ionic strength where myosin exists as a monomer, and then the mixture was dialyzed against low ionic strength buffer to induce filament formation. Second, smooth muscle myosin was initially dialyzed against low ionic strengths buffer to form thick filaments, and then the antibodies were added. For both methods, mm19 antibody markedly inhibited smooth muscle myosin filament formation (Fig. 4B), and most of the myosin remained depolymerized in the background. On the other hand, mm18, mm8, and mm5 did not prevent myosin filament formation (Fig. 4C–E), but the resulting filaments were generally shorter than those formed in the absence of antibody (Fig. 4A). Some aggregation and cross-linking of filaments occurred in the presence of these antibodies, particularly in the case of mm5 and mm8, which also produced the shortest filaments. It appears that, with the exception of mm19, the antibodies studied had a significant effect on the process of filament formation even though they did not prevent filaments from forming. However, mm19 was the only antibody to essentially prevent filament formation from occurring and to also dissolve preformed filaments.

**Determination of mm19 Binding Site on Smooth Muscle Myosin**—To determine the mm19 binding region, various C-terminal fragments of smooth muscle myosin rod were produced. The expressed fragments were subjected to Western blot analysis using the mm19 antibody as a probe. As shown in Fig. 5, mm19 recognized both the fragment containing entire C-terminal sequence (AA1396–1979) and that which contained the entire C-terminal coiled-coil region but not the nonhelical tail piece (AA1396–1935). On the other hand, mm19 failed to recognize the fragments of AA1396–1907 and AA1396–1856. The results clearly indicate that the 28 residues at the C-terminal end of the α-helical coiled-coil domain, but not the C-terminal nonhelical tail, is the mm19 binding site and suggest that this region is critical for the filament formation of smooth muscle myosin.

**Effect of the Deletion of the C-terminal Residues on the Coiled-coil Formation and Filament Formation of Myosin Rod**—One potential explanation for the disruption of the filament formation by C-terminal deletion would be that the C-
terminal residues are critical for formation of the coiled-coil structure. To test this possibility, we produced myosin and myosin rod in which the 28 residues from the end of the coiled-coil as well as the nonhelical tail are deleted and visualized by rotary shadowing technique. As shown in Fig. 6A, the C-terminal residue deleted myosin showed the two heads connected with a long rod that is indistinguishable to native myosin. The tail length of the C-terminal 28-residue truncated myosin was nearly identical to the nontruncated myosin. On the other hand, the C-terminal residue deleted myosin rod also showed an elongated rod without the head domain. The images were practically identical to those of nontruncated rod, and there was no indication of the disruption of the coiled-coil (Fig. 6B).

These results clearly indicate that the deletion of the C-terminal residues does not disrupt the coiled-coil formation of the myosin rod. The C-terminal residue deleted rod was examined for the filament formation by sedimentation analysis (see “Experimental Procedures”). The result demonstrated that the deletion of the C-terminal sequence significantly diminished the filament formation. Only 8% of the nontruncated myosin rod remained in the supernatant after centrifugation in physiological ionic strength, whereas 95% of the truncated rod was found in the supernatant.

**Filament Formation of Smooth Muscle Myosin Variants in Living Cells**—To examine the importance of the 28 residues from the end of the coiled-coil domain of the rod in the thick filament formation in vivo, we produced GFP-tagged smooth muscle myosin variants and expressed them in COS-7 cells. As shown in Fig. 7, the wild type smooth muscle myosin tagged with GFP at the N-terminal end showed stress fiber localization and submembrane localization in interphase cells that coincided with the actin localization. Similar filamentous localization of GFP-myosin was also observed for ΔNHT myosin in which the entire nonhelical tail sequence is deleted. In contrast, the two other constructs, in which 28 and 79 residues from the C-terminal end of the coiled-coil sequence were deleted, showed completely diffuse localization. The results clearly indicate that the C-terminal 28 residues of the coiled-coil domain are critical for the formation of functional myosin filaments in vivo.

It is known that myosin is recruited at the equator of cells at late telophase of mitosis to form a contractile ring that is important for cytokinesis. We examined whether the 28 residues defined above are critical for the incorporation of myosin into the contractile ring during cytokinesis. The wild type GFP-tagged smooth muscle myosin localized at the equator of the cells at the beginning of cytokinesis to form the contractile ring (Fig. 8A). ΔNHT myosin showed the same localization during cytokinesis (Fig. 8D). On the other hand Δ1 and Δ2 myosin constructs both showed diffuse localization throughout mitosis (Fig. 8, B and C). The results are consistent with the finding in interphase cells and indicate that myosin forms thick filaments during cytokinesis at the equator of the cells and the C-terminal end of the coiled-coil region is critical for the filament formation of myosin. The results also show that smooth muscle myosin can be recruited into the contractile ring during cytokinesis.

**Filament Formation of Nonmuscle Myosin Variants in Living Cells**—To clarify whether the finding is specific to smooth muscle myosin or is in common with nonmuscle type myosins, nonmuscle myosin IIA and its variants were tagged with GFP and expressed in COS-7 cells. The wild type GFP-tagged myosin IIA showed filamentous localization that is superimposed on actin filaments in interphase (Fig. 9). Deletion of 32 residues...
from the end of the coiled-coil sequence completely abolished the filamentous localization of myosin IIA (Fig. 9), whereas the deletion of the nonhelical tail sequence (34 residues from the C terminus) did not affect the filamentous localization (not shown).

**DISCUSSION**

The present study clearly shows that the 28 residues from the C-terminal end of the coiled-coil domain of smooth and nonmuscle myosin rod are critical for filament formation both in vitro and in vivo. The deletion of this region, but not the deletion of the C-terminal nonhelical tail piece, completely abolished the filamentous localization of myosin in both interphase and cytokinesis. The results strongly suggest that the filament formation of myosin is disrupted by the deletion of this region in vivo. It may be possible that the deletion interferes with the recruitment of myosin filaments into larger structures but not filament formation itself, but this is less likely because mm19 binding to this region, but not the binding of other antibodies to various other regions of the rod, completely abolished filament formation. It is possible that the inhibition of the filament formation by C-terminal deletion is due to the disruption of the coiled-coil formation, but this is not the case because both the C-terminal truncated myosin and rod showed an elongated rod structure that is indistinguishable to the nontruncated ones (Fig. 6).

It is known that there is a nonhelical region present at the C-terminal end of the myosin II molecule that is very diverse among different myosins. Several groups have shown that this region may modulate filament assembly in smooth, skeletal, and nonmuscle myosins (16, 17, 41). Recently, it was found that
Mts1, a 9-kDa S100 family protein, binds to the nonhelical tail of nonmuscle myosin II, thus destabilizing filaments (42). The present finding is consistent with these earlier reports, and it is plausible that the nonhelical tail influences the stability of myosin II filament formation because it resides adjacent to the region critical for filament formation.

It has been known that the α-helical coiled-coil structure carries a characteristic seven-residue repeat pattern with hydrophobic residues that create a major stabilization energy for the coiled-coil structure (7). The myosin rod also contains this feature, thus forming a coiled-coil, but, in addition to this heptapeptide pattern, there is a 28-residue periodicity (4.1 nm) with alternating bands of positive and negative charges (9–11). This feature, present throughout the rod, has been thought to be essential for rod aggregation. Recently, it has become evident that the C-terminal domain of the rod is important for the aggregation of myosin (18, 43). The results obtained in this study are consistent with this earlier work and further define the critical region for filament formation as a very short stretch of the coiled-coil structure at the C-terminal end.

The sequence alignment of the C-terminal end of the coiled-coil domain shows that most of the charged residues are well conserved among myosin II from nonmuscle and smooth muscle tissues (99, 44, 45). The charged residues are clustered on the outside of the coiled-coil dimer with alternate positive and negative charges. This pattern of the charged residues in the coiled-coil structure could be responsible for the filament stabilization. However, it is unclear why the C-terminal end of the predicted coiled-coil region is so critical for filament formation, because the charged residues are present throughout the rod domain of myosin molecule. Therefore, it is likely that this region adopts a specific structure. One possibility is that it is not rigidly incorporated into the coiled-coil structure but is more flexible. The charged residues in this region could interact with the counterpart of the adjacent myosin with an appropriate conformation that is not readily possible for the charged residues present in the interior of the rigid coiled-coil domain. This is consistent with the fact that all the produced monoclonal antibodies against LMM domain recognized “flexible” patches of LMM. Considering that mm19 recognizes the C-terminal end of the coiled-coil, this suggests some structural freedom of this region.

Another possibility is that this region adopts a unique structural feature that favors intermolecular interaction to form filaments. Recently Cohen and Parry (43) analyzed the structure of the C-terminal region of paramyosin and myosin. They reported that the 63-residue domain from the C-terminal end of the coiled-coil structure has a structural distinct from the rest of the coiled-coil region. This region has a unique charge profile that lacks the periodic variation in charge found in the rest of the coiled-coil region and a high proportion of large apolar residues in surface position. The importance of large hydrophobic residues for the interaction between molecules was also suggested for Caenorhabditis elegans myosin assembly (46). The results of the present study are consistent with this structural consideration for the intermolecular association of the coiled-coil.

Sohn et al. (18) reported that, in striated muscle myosin, the amino acids between 54 and 25 residues from the C-terminal end of the predicted coiled-coil are important for filament formation. They expressed various rod fragments in E. coli and examined the solubility and the formation of paracrystals in vitro. Paracrystal formation was diminished by deleting residues 1874–1902. Furthermore, deletion of this segment disrupted the needle-shaped structures that are thought to represent arrays of thick filaments (47). This region is closer to the N terminus of the coiled-coil compared with the region identified in the present study. This difference could be due to the difference in the type of myosin, because it is known that the filament structure of smooth muscle myosin is quite different from that of striated myosin (21–23). Another possibility is that they examined paracrystal formation of partial segments of the rod but not filament formation of the whole myosin molecule. Although the deletion of this segment from striated myosin diminished the needle-shaped structure in cells, it is likely that the deletion disrupts the entire downstream structure of myosin including the region homologous to the sequence identified in the present study.

In contrast to the earlier model (17), the present results clearly show that the nonhelical tailpiece of the rod does not play a significant role in filament formation. In the earlier model, the tailpiece favors the formation of staggered intermediates for filament assembly as opposed to the accumulation of unstaggered intermediates. It is plausible that the C-terminal 28 residues identified in this study may play a role as the tailpiece originally proposed by Hodg et al. (17), although the identity of such a putative tailpiece was possibly misassigned as the “nonhelical tailpiece.”

Acknowledgment—We thank Dr. R. S. Adelstein (National Institutes of Health) for providing us with the cDNA fragments encoding human nonmuscle myosin II heavy chain.

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The Tip of the Coiled-coil Rod Determines the Filament Formation of Smooth Muscle and Nonmuscle Myosin
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J. Biol. Chem. 2001, 276:30293-30300.
doi: 10.1074/jbc.M101969200 originally published online June 6, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101969200

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