Role of the COOH-terminal Nonhelical Tailpiece in the Assembly of a Vertebrate Nonmuscle Myosin Rod

Tony P. Hodge, Robert Cross, and John Kendrick-Jones
MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, United Kingdom

Abstract. A short nonhelical sequence at the COOH-terminus of vertebrate nonmuscle myosin has been shown to enhance myosin filament assembly. We have analyzed the role of this sequence in chicken intestinal epithelial brush border myosin, using protein engineering/site-directed mutagenesis. Clones encoding the rod region of this myosin were isolated and sequenced. They were truncated at various restriction sites and expressed in Escherichia coli, yielding a series of mutant myosin rods with or without the COOH-terminal tail-piece and with serial deletions from their NH2-termini. Deletion of the 35 residue COOH-terminal nonhelical tailpiece was sufficient to increase the critical concentration for myosin rod assembly by 50-fold (at 150 mM NaCl, pH 7.5), whereas NH2-terminal deletions had only minor effects. The only exception was the longest NH2-terminal deletion, which reduced the rod to 119 amino acids and rendered it assembly incompetent. The COOH-terminal tailpiece could be reduced by 15 amino acids and it still efficiently promoted assembly. We also found that the tailpiece promoted assembly of both filaments and segments; assemblies which have different molecular overlaps. Rod fragments carrying the COOH-terminal tailpiece did not promote the assembly of COOH-terminally deleted material when the two were mixed together. The tailpiece sequence thus has profound effects on assembly, yet it is apparently unstructured and can be bisected without affecting its function. Taken together these observations suggest that the nonhelical tailpiece may act sterically to block an otherwise dominant but unproductive molecular interaction in the self assembly process and does not, as has been previously thought, bind to a specific target site(s) on a neighboring molecule.
mon 28-amino acid repeating unit containing four heptad repeats, characteristic of coiled-coil molecules, with hydrophobic residues at the first and fourth positions within the heptad (Cohen and Parry, 1990). The most striking difference between these myosins is at their carboxyl termini where the smooth muscle and nonmuscle myosin rods contain a 35–43 residue nonhelical COOH-terminal tailpiece which is absent in sarcomeric myosin rods (Strehler et al., 1986; Molina et al., 1987; Babij and Periasamy, 1989; Nagai et al., 1988; chicken brush border MHC (this study); (7) human nonmuscle MHC (Seaz et al., 1990); and (8) Drosophila nonmuscle MHC (Ketchum et al., 1990).

Also shown are two of the expressed fragments; 407Δ, nonmuscle rod fragment (407 amino acids) with deleted COOH terminus (last 35 amino acids deleted); and 407T, the same rod fragment with the last 15 amino acids deleted. The sequences are grouped together according to tissue origin and aligned according to Dibb et al. (1989). Residues where an apparent consensus exists in smooth and/or nonmuscle myosins are in bold type and those residues reported to be phosphorylated are indicated (Kelley et al., 1991).

The Journal of Cell Biology, Volume 118, 1992 1086

Materials and Methods

Materials

Analytical grade reagents were used throughout and were obtained from...
BDH Chemicals Ltd. (Poole, UK) and Bethesda Research Laboratories (Gaithersburg, MD). Radioisotopes were obtained from Amersham International (Amersham, UK). Papain, chymotrypsin, and hen egg white lysozyme were from Sigma Chemical Company (Poole, UK). All other enzymes were from New England Biolabs (Beverly, MA) or from Boehringer-Mannheim GmbH (Mannheim, Germany) and were used according to the manufacturer’s standard assay conditions. Oligonucleotides were synthesized on an Applied Biosystems 380B automated DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) by Jan Fogg and Terry Smith (MRC Laboratory of Molecular Biology, Cambridge, MA). The brush border Agtl cDNA library was a generous gift from Dr. Paul Matsudaira (Whitehead Institute, Massachusetts Institute of Technology, Cambridge, MA). Chicken gizzards and intestines were obtained from G. W. Padley Ltd., (Bury St. Edmunds, UK).

**Biochemical Methods**

Chicken gizzard and brush border myosins and their light meromyosin (LMM) and rod subfragments (for control experiments) were prepared as previously described (Kendrick-Jones et al., 1971; 1985; Citi and Kendrick-Jones, 1986). The protein concentrations of the myosin, native LMM, and rod fragments were estimated spectrophotometrically using the following absorption coefficients (A_{280} nm in 1-cm cells) for myosin 0.54, rod 0.30, and LMM 0.30 mg ml⁻¹. The concentrations of the expressed fragments were determined by the Pierce BCA protein assay (Pierce Chemical Co., Rockford, IL).

5-20% acrylamide gradient SDS-PAGE gels were run as described by Matsudaira and Burgess (1978) using the Pharmacia low molecular weight marker kit (14,000-94,000) for calibration (Pharmacia Chemicals, Piscataway, NJ). Glycerol-PAGE gels were used to analyze the purified rod fragments under nondissociating conditions. Gels (7.5-14% acrylamide 40% glycerol) were polymerized and electrophoresed in the following buffer: 0.122 M glycine, 0.02 M Tris, pH 8.6, and 40 mM sodium pyrophosphate, final pH 8.9. For sample loading we included 10% 2-mercaptoethanol and 40% glycerol. Gels were run at 5 W constant power for 3 h. The proteins were visualized by staining with PAGE blue 83 (BDH Chemicals Ltd.). Western blots were carried out by the method of Burnette (1981) using brush border myosin mAbs BM1, BM3, and BM4 (Citi and Kendrick-Jones, 1987b) and were visualized using the Vector Laboratories ABC kit (Vector Laboratories Inc., Burlingame, CA).

**DNA Manipulations**

Unless otherwise stated, all DNA manipulations were performed as described in Sambrook et al. (1989). Bacteria harboring recombinant plasmids were grown in 2 × YT medium containing 50-100 μg ml⁻¹ ampicillin. The chicken epithelial brush border Agtl cDNA library was screened by the procedure described previously (Huyah et al., 1985) using brush border myosin mAbs BM1 and BM4 (Citi and Kendrick-Jones, 1987b), a biotinylated secondary antibody and an avidin-biotinylated HRP complex (Vector ABC kit, Vector Laboratories Inc.).

Initially 10 cDNA clones were identified after screening ∼6 × 10⁵ isolates while 119 have intact COOH termini; 407Δ has no tailpiece and 407T has a truncated tailpiece. The construct 407Δ is an example of a set of COOH-terminal deletions made on all the intact fragments. A restriction map of the sites used for cloning the expression constructs is also shown beneath that of L786, the EcoRI insert of an isolated Agtl cDNA clone. Clones 785, 586, and 407 are Dral-EcoRI deletions of Agtl isoform 786 and clone 407Δ is a Dral-EcoRI deletion of 407 and 407T was produced by PCR amplification of a novel 3' sequence (see Materials and Methods). A, Avall; D, Dral; E, EcoRI; H, HindIII; P, PstI; and asterisk, translation stop codon.

A battery of protease inhibitors was present in all these steps (Citi and Kendrick-Jones, 1986). After lysis of the bacterial cells and centrifugation to remove cellular debris, the proteins were precipitated from the supernatant by the addition of 5 vols of ethanol and stirred at 4°C for 30 min. A heavy, sticky precipitate formed which was resuspended in 10 ml (per liter of culture) of 25 mM Tris, pH 7.5, 1 mM MgCl₂, 1 mM DTT and then dialyzed against 2 × 2.5 liters of the same. Solid urea was added to 7 M to dissolve the precipitate which was fractionated on a DEAE-cellulose DE52 column (Whatman, Maidstone, UK) with a 0-500 mM NaCl gradient in 6 M urea, 25 mM Tris pH 7.5, 1 mM MgCl₂, and 1 mM DTT. Fractions containing the required polypeptides were identified by SDS-PAGE and checked by Western blotting. The fractions were pooled and dialyzed into 0.6 M NaCl, 25 mM sodium phosphate, pH 7.0, 10 mM Tris, pH 7.5, 0.5 mM sodium azide, and 2 mM DTT (myosin storage/dilution buffer) and stored at concentrations between 0.5 and 2 mg ml⁻¹. In later preparations all the expressed myosin fragments, with the exception of 119, were further purified by dialyzing into low salt buffer (50 mM NaCl, 25 mM sodium phosphate, pH 6.5, 5 mM MgCl₂ and 0.5 mM DTT) and collecting the precipitate by centrifugation at 30,000 g for 30 min before resuspending in storage/dilution buffer at 4-5 mg ml⁻¹.
Analysis of the Expressed Proteins

Sedimentation Analysis. Samples of the expressed myosin rod proteins (100 μl, 0.5 mg ml⁻¹) were dialyzed in small dialysis bags (visking dialysis tubing, size 8/32) against a range of salt concentrations (50-400 mM NaCl) in 25 mM sodium phosphate, pH 7.5, 0.5 mM DTT, and 2 mM MgCl₂, (sedimentation buffer) for 5 h with gentle agitation. For critical concentration determinations a range of protein concentrations between 0.1 and 3.0 mg ml⁻¹ were dialyzed against 150 mM NaCl in 'sedimention buffer.' Dialyzed samples were weighed to determine final volume then centrifuged at 100,000 g for 20 min in a Beckman airfuge (Beckman Instruments Inc., Fullerton, CA). Previous sedimentation velocity measurements on thymus myosin filaments (Kendrick-Jones et al., 1987) indicated that any oligomers with sedimentation coefficients larger than 155 would be sedimented under these conditions (see also Pollard, 1982). The top 40% of each supernatant was taken for analysis by SDS-PAGE. The remainder of the supernatant was removed from the tube and the pellet resuspended in 100 μl of myosin storage/dilution buffer overnight at room temperature. Samples of the pellets were taken for SDS-PAGE. The solubility of the myosin rod fragments (expressed as percent fragment in the supernatant) were determined by measuring the relative amounts of each fragment in the pellet and supernatant fractions by densitometry of the stained PAGE gel bands using either a Canon electrophoresis densitometer (Canon Instruments, Cambridge, UK) or with a Molecular Dynamics Computing Densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA).

Electron Microscopy

Samples for EM were prepared by a number of procedures: (a) Native and expressed myosin rod proteins (0.5 mg ml⁻¹) in 600 mM NaCl, 40 mM imidazole, pH 7.3, 25 mM MgCl₂ were rapidly diluted with 3 vol of water (final concentration 150 mM NaCl, 1 mM MgCl₂, 10 mM Imidazole, pH 7.3) and 10-μl aliquots taken for negative staining. Similar aggregates were formed if the proteins were dialyzed for 90 min against the 150 mM NaCl solution. (b) 10-μl aliquots of the samples from the sedimentation assays which had been dialyzed against 50 mM, 100 mM, and 150 mM NaCl in 2 mM MgCl₂, 25 mM phosphate buffer, pH 7.5, 0.5 mM DTT were taken for negative staining. (c) For paracrystal formation, 10-μl aliquots of native LMM, rod, and expressed fragments 786, 586, and 586AC (<0.5 mg ml⁻¹) were initially dialyzed against 50 mM Tris-HCl, pH 8.25, containing 50 mM potassium thiocyanate (KCNS) for native LMM, 100 mM KCNS for native rod, 65 mM KCNS for expressed fragments 786 and 586, and 60-80 mM KCNS for expressed 586AC. The solutions were centrifuged in an airfuge for 15 min at 30 psi (100,000 g) and then redialyzed against the appropriate Tris-HCl buffer/KCNS solution containing in addition 50 or 100 mM CaCl₂ (or other divalent cations) to form the paracrystals. For negative staining 10-μl samples were applied onto carbon coated, 400 mesh grids and left for 15 s. The grids were washed with six drops of the appropriate salt/buffer solution and stained with six drops of 1.5% uranyl acetate and dried. For shadowing, the samples were mixed with an equal volume of glycerol, sprayed onto freshly cleaned mica, and shadowed with platinum and carbon (Citi and Kendrick-Jones, 1987b). The specimens were examined in a Philips electron microscope (model EM400; Philips Electronic Instruments Co., Mahwah, NJ) operated at 80 kV and representative views photographed.

Results

Shotgun sequencing of the longest of our cDNA clones (X786) yielded data which agrees almost completely with the sequence already published by Shohet et al. (1989), we have read as: CGA GCC AAC GCG TCC CGC AGG (RANASRRR). Some of our clones have a longer 3' untranslated region to those described by Shohet et al. (1989) and terminate in a short poly-A tail preceded by a putative poly-A addition signal (data not shown). This would suggest a 3' untranslated region of some 1.4 kb.

Expression and Characterization of the Expressed Proteins

The CDNA expression clone 786 codes for a protein of 786 amino acids with a predicted molecular weight of 91,000 daltons, which is in good agreement with its size estimated from SDS-PAGE (Table I). Aligning the sequence with that of chicken smooth muscle MHC (Yanagisawa et al., 1987) indicates that it codes for more than two thirds of the rod region (from residue 1180 to the COOH-terminus).

Using our sequence data we designed a number of expression constructs using plNII expression vectors (Nakamura and Inouye, 1982). Two types of constructs were made: those with deletions at the 5' end of the sequence to generate deletion mutants 786, 586, 407, etc. and those with deletions at the extreme 3' end of the coding sequence, which remove or truncate the nonhelical COOH-terminal tailpiece, hence clone 407 becomes 407A or 407T (Fig. 2). The constructs were expressed and after initial screening by SDS-PAGE, the required expressed proteins were purified using procedures based on the known stability properties of native myosin rod and LMM. Proteins made in this way were stable to ethanol and heat, selectively precipitated at low pH and pl and showed mobilities on PAGE gels close to those predicted (Fig. 3, Table I). The appearance of multiple bands in the expressed protein samples was noted from the outset of the purification procedure despite the presence of 10 mM EDTA and a wide spectrum of protease inhibitors (Citi and Kendrick-Jones, 1986). They were not removed by chromatography or by repeated low salt precipitation cycles. Preliminary data indicates that nicking of the coiled coil occurs at the amino terminus and the extra bands have no effect on the sedimentation assays or on the formation of paracrystals. Under non-dissociating conditions on glycerol-PAGE (40% glycerol/7.5% acrylamide) in the presence of 40 mM sodium phosphate the samples run as single bands (Fig. 3B). Thus the multiple bands are not apparent until the proteins are boiled in SDS loading buffer for the SDS-PAGE analysis.
The proteins were further tested with the brush border myosin rod mAbs BM1, BM3, and BM4 (Citi and Kendrick-Jones, 1987b), to check their identity and their approximate lengths (Table I). Earlier work (Citi and Kendrick-Jones, 1987b) had suggested that BM4 bound to the extreme COOH-terminal end of the rod but our results show it still binds to rod molecules where the last 35 residues have been deleted. Rotary shadowing the expressed proteins revealed rod-like structures of the correct average size in the electron microscope. Furthermore low angle x-ray scattering analysis (carried out by Dr. W. Faruqi, LMB, Cambridge, UK) on these proteins yielded data consistent with α-helical coiled-coil rods (data not shown).

Assembly Properties of the Nonmuscle Myosin Fragments

Sedimentation Assay. All the expressed fragments with the exception of fragment 119, showed assembly characteristics which were similar, i.e., they assembled into polymers at low salt and as the salt concentration was increased they disassembled and became soluble monomers (>300 mM) (Fig. 4 a). The effect of lowering the pH from 7.5 to 6.5 on the assembly of all the fragments was minimal. Fragment 119 however was soluble under all conditions tested, i.e., at all salt concentrations and at pH 6.5 and 7.5. It even remained completely soluble when mixed with equal concentrations of fragments 586 or 407 in high salt and dialyzed to low salt at pH 6.5, conditions under which fragments 586 and 407 readily assembled (data not shown). O'Halloran et al., 1990 have previously shown that a minimum length of rod is required for Dictyostelium myosin filament assembly. The results with 119 suggest a minimum rod length is also required for assembly of vertebrate nonmuscle myosins. Rod paracrystals assembled from COOH-terminally intact material have a 14.3-nm repeat which indicates that their constituent molecules are staggered by this amount. If this is indeed the case, then it is not surprising that rod fragment 119 did not assemble, since after taking into account the 35-residue tailpiece it is <14.3 nm long (14.3 nm of α-helix corresponds to ~98 residues, i.e., 0.1485-nm rise per amino acid residue [McLachlan and Karn, 1982]) Low angle x-ray scattering analysis on 119 confirms that it is the correct size and shape to be a short piece of coiled coil (A. R. Faruqi, unpublished data). Hence its inability to assemble is unlikely to be due to incorrect formation of the α-helical coiled-coil structure.

Deletions at the COOH-terminus lead to a dramatic alteration in the assembly properties of the rod fragments (Fig. 4 b). Removal of the entire 35-residue COOH-terminal tailpiece results in a clear shift in the solubility curves, so that at 150 mM NaCl >60% of the protein remained soluble. This change in solubility is because of a dramatic increase in the critical rod fragment concentration (Cc) required for assembly (Fig. 4 c). At 150 mM NaCl, pH 7.5, deletion of the COOH-terminal tailpiece leads to a 20–50-fold increase in critical concentration (Cc), from Cc ~20–50 µg ml⁻¹ for the rod with an intact or truncated COOH terminus to ~1 mg/ml⁻¹ for the deleted COOH-terminal (CA) fragments. Unlike fragment 119 which is assembly incompetent, the CA fragments could be forced to assemble at higher protein or at lower salt concentrations. The data indicate that the action of the tailpiece is to cause self-assembly to occur at protein concentrations considerably below those at which it would otherwise occur. This observation is consistent with the idea that the nonhelical tailpiece in some way promotes assembly, rather than being absolutely required for assembly.

Coassembly experiments. The finding that the intact and deleted COOH-terminal fragments have very different critical concentrations for assembly suggests either that the tailpiece accelerates a step or steps in the assembly pathway, or else it induces assembly to occur via a different pathway. As one way to distinguish between these possibilities, the following coassembly experiments were carried out (Fig. 5). When the intact COOH-terminal fragments 586 and 407 were mixed together at a 1:1 ratio, assembly of both of them was increased, i.e., less of each of them was present in the supernatants after dialysis and centrifugation. In contrast, when fragment 586 was mixed with the COOH-terminal-deleted fragment 407Δ in a similar ratio, then assembly of 586 and 407Δ was not significantly altered, i.e., fragment

Hodge et al. Role of Myosin COOH-terminal Tailpiece
Figure 5. Coassembly of myosin rod fragments. Mixtures of equal concentrations of 586 alone or with either 407 or 407Δ, or 407Δ alone or with 586 (total protein concentration 0.5 mg/ml) were dialyzed against sedimentation buffer at 150 mM NaCl, centrifuged and the supernatant and pellet fractions analyzed by SDS-PAGE. The bar graphs are grouped according to the fragment whose solubility was measured and any other fragment present in the dialysis bag is shown after the bar. The lower solubility of 586 in the presence of 407 suggests some cooperative effect on filament formation. The minimal effect of 586 on the solubility of 407Δ shows that intact molecules are unable to ‘rescue’ COOH-terminal deleted molecules. Identical results were obtained at 200 mM NaCl and when other mixtures of intact and COOH-terminal deleted rod fragments were used.

407Δ still remained mainly soluble. Thus, fragment 586 with an intact COOH-terminal tailpiece is not able to ‘rescue’ the COOH-terminal–deleted fragment 407Δ by interacting with it to assemble into copolymers. There is apparently a requirement for symmetry in the self-assembly process, suggesting that the growing polymer senses in some way whether incoming molecules have the COOH-terminal tailpiece or not. This is consistent with earlier work with proteolytic rod fragments (Cross and Vanderkerckhove, 1986), which indicated that a mixture of chicken gizzard LMM fragments with intact and deleted COOH-termini segregated to the pellet.

Figure 4. The assembly properties of the expressed myosin rod fragments. (a) The assembly behavior of expressed fragments 586 (– - -), 407 (– o o), and 119 (– - -) as a function of the salt concentration. At these protein concentrations (0.5 mg ml⁻¹) all the fragments including 786 (not shown) show similar assembly profiles with the exception of fragment 119 which is completely soluble. (b) Comparison of the assembly behavior of expressed fragments with the COOH-terminal tailpiece (407 [– o o] and 586 [– - -]) and without the COOH-terminal tailpiece (407Δ [– o o] and 586Δ [– - -]) as a function of the salt concentration. Deletion of the COOH-terminal tailpiece increases the solubility of fragments 407 and 586. 407T (X) (last 15 amino acids deleted). (c) The critical monomer concentration for assembly of fragment 407 (●), 407Δ (○) and 407T (X) (last 15 amino acids deleted) at 150 mM NaCl, 2 mM MgCl₂, pH 7.5. The change in solubility brought about by deletion of the tailpiece is due to a dramatic increase in the critical monomer concentration required for assembly.
and supernatant fractions, respectively, after ultracentrifugation. These results mean that provision of nuclei composed of intact, assembly competent rod molecules does not allow molecules with deleted COOH-termini to coassemble, indicating that the nonhelical COOH-terminus promotes both polymer initiation and polymer growth.

**Electron Microscopy.** The ability of the expressed fragments to assemble was compared by examining the assembled forms in the electron microscope after negative staining (Fig. 6). Under approximately physiological conditions (150 mM NaCl, 2 mM MgCl₂, pH 7.3) the expressed fragments 586 and 786 assembled to form numerous short symmetrical filaments with pointed ends (Fig. 6, a and d) which were very similar to those formed by the native rod and LMM fragments (Fig. 6, b and c). Fragment 786 showed a slightly elevated solubility in the sedimentation assay (data not shown) but formed filaments which were similar in structure although variable in size compared with those formed by the other fragments. Under the same conditions the COOH-terminal-deleted fragment 586Δ formed only a few small

*Figure 6.** Electron micrographs of the ordered aggregates formed by native and expressed myosin rod fragments at approximately physiological salt, pH, and MgCl₂ concentrations (150 mM NaCl, 1 mM MgCl₂ and pH 7.3). (a) expressed 786; (b) native myosin rod; (c) native myosin LMM; (d) expressed 586; (e) expressed 586Δ at ~0.5 mg ml⁻¹; (f) expressed 586Δ (at >1.0 mg ml⁻¹) left for ~16 h. Fragments 786 and 586 (and fragment 407 not shown) form structures very much like those formed by the native rod and LMM control fragments. Deletion of the nonhelical tailpiece disrupts formation of these needle-like structures. However, close examination of e reveals numerous small parallel aggregates in the background. By increasing the protein concentrations of these COOH-terminal deleted fragments, they will, given time, form large well ordered structures with prominent 14.3-nm periodicities as seen in f. Note the conditions used were similar to those used in the sedimentation assay (at 150 mM NaCl) shown in Fig. 4. All the 'aggregates' were formed by the rapid dilution from high salt into low salt buffer procedure. Very similar structures were observed when the rod fragments were dialyzed into the low salt buffer conditions or samples were taken directly from the sedimentation assay. The results are very reproducible with different preparations of the rod fragments. The micrographs shown are representative of all the material observed on the grids. Bar, 0.1 μm.
“carrot-shaped” arrays and a high concentration of protein was evident in the background (Fig. 6 e). When higher concentrations of 586Δ (>1 mg/ml) were used and the samples were left for ~16 h at 4°C, then the small loosely packed arrays aggregated to form large paracrystalline structures with prominent 14.3-nm periodicities (Fig. 6 f). The formation of these large aggregates which require high protein concentrations and time suggests that they assemble by a less favorable pathway with few stable nuclei and a slow filament growth rate.

To further probe the interactions involved in assembly, attempts were made to induce the expressed fragments to form paracrystalline segments in the presence of high concentrations of divalent cations (Fig. 7). As previously demonstrated (Kendrick-Jones et al., 1971) native LMM readily forms these bipolar segments where the molecules are staggered by 43 nm as shown in Fig. 7 d. Under similar conditions, fragments 786 and 586 formed similar structures although the number observed were few and they tended to be less well ordered and rather ragged. We were unable to induce the 586Δ fragment to form such paracrystalline arrays despite testing a variety of conditions, for example, differing pHs from 7.5 to 8.6, using differing amounts of the solubilizing agents potassium thiocyanate and sodium chloride and different divalent cations such as Ca2+, Mg2+, or Cd2+ at high concentrations. These results provide further proof that the COOH-terminal tailpiece is crucial for generating bipolar assemblies with a 43-nm stagger which may reflect one of the axial staggerers between myosin rods involved in assembling thick filaments.

In both the segments and filament aggregates the electron-dense striations in the assemblies are due to stain accumulation at the ends of the molecules. Hence the COOH-terminal nonhelical domain enhances the assembly of rod fragments into two types of polymers having two different molecular overlaps. One can therefore exclude that the tailpiece recognizes and binds to a single target site on a neighboring molecule. We note further that filaments formed from rod fragments with intact COOH termini and (at higher concentrations) those with deleted COOH termini both have 14.3 nm striations, suggesting that the tailpiece does not alter the overlap between molecules in the filaments. It remains possible however that two or more recognition sites for the tailpiece exist on neighboring molecules. To test this possibility we made further truncations in the tailpiece region, and assayed the assembly of the resulting constructs (Fig. 4 b). Truncation of the brush border myosin tailpiece sequence down to 40% of its original length (407T) did not affect its function, again consistent with there being no specific sequence requirements in this region. This truncation removes the last 15 amino acids and most of the region of negative charge within the tailpiece (Fig. 1), yet it does not appear to affect the assembly of the rod fragments.

Discussion

The most significant difference between the α-helical coiled coil rod regions of vertebrate smooth muscle/nonmuscle and sarcomeric (skeletal) muscle myosins is the presence in the former of nonhelical carboxyl-terminal tailpieces, ~35–43 amino acids long (Fig. 1). Although the lengths and sequences of the COOH-terminal tailpieces vary in the different smooth muscle/nonmuscle myosins, the distribution of hydrophobic, basic, and acidic amino acids is preserved (Fig. 1). It is not clear from the sequences however whether the conformation of the tailpiece is a true random coil or whether some nonhelical secondary structure is present.

Previous experiments involving the removal of this COOH-terminal nonhelical domain from various myosins, either by proteolysis or site-directed mutagenesis (Cross and Vanderkolkhove, 1986; Sinard et al., 1990; Ikebe et al., 1991) have suggested that this domain is required for self-assembly. In addition work on chicken epithelial brush border myosin (Citl and Kendrick-Jones, 1988) showed that mAbs binding to the COOH-terminal tip of the rod blocked filament assembly while those binding to regions one third and two thirds along the rod, gave rise to assembly intermediates, suggesting that some interaction involving the
nonhelical tailpiece is a primary step in filament assembly. Antibodies to *Acanthamoeba* and *Dictyostelium* myosin IIs have similarly demonstrated the importance of the distal tail in the initial steps in the assembly of these myosins (Pagh and Gerisch, 1986; Rimm et al., 1990). Recently Ikebe et al. (1991) made the interesting observation that after assembly of vertebrate smooth muscle myosin filaments, the tailpiece can be proteolytically cleaved away, without inducing filament disassembly. The present series of experiments were designed to address the question of which steps in the assembly pathway of a vertebrate nonmuscle myosin are promoted by the presence of the tailpiece.

**Models to Explain the Role of the Tailpiece**

We have considered two types of possible model to explain our results (Fig. 8). Both postulate that the tailpiece produces its effect on the critical monomer concentration for assembly by enhancing the ability of molecules to bind productively to one or more partners in such a way that the molecules build into a filament. In Fig. 8, Model 1, the tailpiece is postulated to recognize and bind to a target site on one or more neighboring molecules, thereby increasing the binding constant, and specifying a particular overlap between molecules. This was suggested by E. D. Korn and his col-
leagues to be the way in which the COOH terminus of Acanthamoeba myosin II promoted myosin filament assembly (Atkinson et al., 1989). Recently Kalbitzer et al. (1991) showed by nuclear magnetic resonance spectroscopy that even the COOH terminus of rabbit skeletal muscle myosin contains a short unfolded mobile region which they propose might function as a 'kink of cement' to stabilize the thick filament structure. In Fig. 8, Model 2, the nonhelical tailpiece is postulated to block an otherwise dominant, but unproductive mode of intermolecular binding (Fig. 8A). In this model the tailpiece promotes assembly by diverting molecules out of this futile cycle, and on to a productive pathway.

The lack of any obvious sequence anomaly within the rod, which would indicate a specific binding site, argues against Fig. 8, Model 1. The finding that the tailpiece enhances both filament and segment assembly, and hence enhances two different molecular overlaps, also argues against a specific single binding site in the myosin rod. Finally, we found that the tailpiece could be deleted down to half its wild type length, substantially altering its charge, without affecting function. This again renders it unlikely that specific sequence features in the tailpiece are required for function. Rather, the data indicate that the COOH-terminal tailpiece must have a minimum size, but that its length and sequence can vary substantially without affecting function (see Fig. 1).

We find this is much more consistent with a steric blocking role (model 2) than with there being one or more specific tailpiece-binding sites within the rod molecule. Further support is provided by the observations of Ikebe et al. (1991) that the tailpiece can be selectively removed from myosin filaments by proteolytic cleavage without inducing filament disassembly, indicating that it is accessible and not buried in the spaces in the filament structure.

For a steric blocking mechanism to be effective in promoting productive assembly, there must be at least one competing, unproductive pathway, which sequesters molecules lacking a tailpiece. Our experiments do not reveal details of this competing pathway, but it might for example involve rod molecules lacking a COOH-terminal nonhelical tailpiece tending to bind to one another with full overlap (see Fig. 8B). The model requires that this mode of assembly becomes unstable after the addition of a few molecules. Steric interference by the tailpieces with one another would force a stagger to occur between molecules, thereby promoting productive filament assembly. In this model, myosin rod fragments without tailpiece would interact rapidly to form small aggregates, possibly only dimers or trimers, which once formed are able only slowly to reorganize (dissociate or slip) to form protofilaments. The action of the tailpiece would be to promote this reorganization. In this connection, it is interesting to note that Egelhoff et al. (1991) have demonstrated that a COOH-terminal tailpiece promotes the disassembly of Dictyostelium myosin filaments.

In conclusion, we have shown that the COOH-terminal nonhelical tailpiece of a vertebrate nonmuscle myosin II acts to lower the critical concentration of myosin required for assembly by 20–50-fold at physiological ionic strength and have presented evidence consistent with a role for this tailpiece in the steric blocking of unproductive modes of assembly. By extending the approach described, it should now be possible to dissect in molecular detail those steps of the assembly reaction which are promoted by the presence of the tailpiece.
Kendrick-Jones, J., W. Z. Cande, P. J. Tooth, R. C. Smith, and J. M. Scholey. 1983. Studies on the effect of phosphorylation of the 20,000 Mr light chain of vertebrate smooth muscle myosin. J. Mol. Biol. 165:139–162.

Kendrick-Jones, J., R. C. Smith, R. Craig, and S. Citri. 1987. Polymerisation of vertebrate nonmuscle and smooth muscle myosins. J. Mol. Biol. 198:241–255.

Ketchum, A. S., C. T. Stewart, M. Stewart, and D. P. Kiehart. 1990. Complete sequence of the Drosophila nonmuscle myosin heavy chain transcript: conserved sequences in the myosin tail and differential splicing in the 5' untranslated sequence. Proc. Natl. Acad. Sci. USA. 87:6316–6320.

Maeda, K., A. Rösch, Y. Maeda, H. R. Kalbitzer, and A. Wittinghofer. 1991. Rabbit skeletal muscle myosin. Unfolded carboxyl-terminus and its role in molecular assembly. FEBS (Fed. Eur. Biochem. Soc.) Lett. 281:23–26.

Matsudaira, P. T., and D. R. Burgess. 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. Anal. Biochem. 87:386–396.

Mclachlan, A. D. 1984. Structural implications of the myosin amino basic sequence match crossbridge spacings in muscle. Annu. Rev. Biophys. Biophys. Chem. 13:167–189.

Mclachlan, A. D., and J. Karn. 1982. Charge distributions in the myosin rod amino basic sequence match crossbridge spacings in muscle. Nature (Lond.) 299:226–231.

Megerman, J., and S. Lowey. 1981. Polymerisation of myosin from smooth muscle of calf aorta. Biochemistry. 20:2099–2110.

Molina, M. I., K. E. Kropp, J. Oulick, and J. Robbins. 1987. The sequence of an embryonic myosin heavy chain gene and isolation of its corresponding cDNA. J. Biol. Chem. 262:6478–6488.

Nagai, R., D. M. Larson, and M. Periasamy. 1988. Characterization of a mammalian smooth muscle myosin heavy chain cDNA clone and its expression in various smooth muscle types. Proc. Natl. Acad. Sci. USA. 85:1047–1051.

Nakamura, K., and M. Inouye. 1982. Construction of versatile expression cloning vehicles using the lipoprotein gene of Escherichia coli. EMBO (Eur. Mol. Biol. Organ.) J. 1(6):771–775.

O’Halloran, T., S. Ravid, and J. A. Spudich. 1990. Expression of Dictyostelium myosin tail segments in Escherichia coli: domains required for assembly and phosphorylation. J. Cell Biol. 110:63–70.

Pagh, K., and G. Gerisch. 1986. Monoclonal antibodies binding to the tail of Dictyostelium discoideum myosin: their effects on antiparallel and parallel assembly and actin-activated ATPase activity. J. Cell Biol. 103:1527–1538.

Pollard, T. D. 1982. Structure and polymerization of Acanthamoeba myosin II filaments. J. Cell Biol. 95:816–825.

Quinlan, R. A., and M. Stewart. 1987. Crystalline tubes of myosin subfragment-2 showing the coiled-coil and molecular interaction geometry. J. Cell Biol. 105:403–415.

Reisler, E., C. Smith, and G. Seegan. 1980. Myosin minifilaments. J. Mol. Biol. 143:129–145.

Rimm, D. L., D. A. Kaiser, D. Bhanardi, P. Maupin, D. P. Kiehart, and T. D. Pollard. 1990. Identification of functional regions on the tail of Acanthamoeba myosin-II using recombinant fusion proteins. I. High resolution epitope mapping and characterisation of mononclonal binding sites. J. Cell Biol. 111:2405–2416.

Saez, C. G., J. C. Myers, T. B. Shows, and L. A. Leinwand. 1990. Human nonmuscle myosin heavy chain mRNA: generation of diversity through alternative polyadenylation. Proc. Natl. Acad. Sci. USA. 87:1164–1168.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Second edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sathyamoorthy, V., M. A. L. Atkinson, B. Bowers, and E. D. Korn. 1990. Functional consequences of the proteolytic removal of regulatory serines from the non-helical tailpiece of Acanthamoeba myosin II. Biochemistry. 29:3793–3797.

Shohet, R. V., M. A. Conti, S. Kawamoto, Y. A. Preston, D. A. Brill, and R. S. Adelstein. 1989. Cloning of the cDNA encoding the myosin heavy chain of a vertebrate cellular myosin. Proc. Natl. Acad. Sci. USA. 86:7726–7730.

Sinard, J. H., and T. D. Pollard. 1989. The effect of heavy chain phosphorylation and solution conditions on the steady state assembly of Acanthamoeba myosin II. J. Cell Biol. 107:1529–1535.

Sinard, J. H., W. F. Stafford, and T. Pollard. 1989. The mechanism of assembly of Acanthamoeba myosin II minifilaments. Minifilaments assemble by three successive dimerisation steps. J. Cell Biol. 109:1537–1547.

Squire, J. M. 1981. The Structural Basis of Muscle Contraction. Plenum Publishing Corp., New York. 276 pp.

Strehler, E. E., M.-A. Strehler-Page, J.-C. Perriard, M. Periasamy, and B. Nadal-Grinard. 1986. Complete nucleotide and encoded amino acid sequence of a mammalian myosin heavy chain gene. Evidence against Intron-dependent Evolution of the rod. J. Molec. Biol. 190:291–397.

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

Trybus, K. M., T. W. Huiatt, and S. Lowey. 1982. A bent monomeric conformation of myosin from smooth muscle. Proc. Natl. Acad. Sci. USA. 79:6151–6155.

Warwick, H. M., and J. A. Spudich. 1987. Myosin structure and function in cell motility. Annu. Rev. Cell Biol. 3:379–421.

Yanagisawa, M., Y. Hamada, Y. Katsuragawa, M. Imamura, T. Mikawa, and T. Masaki. 1987. Complete primary structure of vertebrate smooth muscle myosin heavy chain deduced from its complementary DNA sequence. J. Mol. Biol. 198:143–157.