Localization and Topography of Antigenic Domains within the Heavy Chain of Smooth Muscle Myosin

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ABSTRACT We have produced and characterized monoclonal antibodies that label antigenic determinants distributed among three distinct, nonoverlapping peptide domains of the 200-kD heavy chain of avian smooth muscle myosin. Mice were immunized with a partially phosphorylated chymotryptic digest of adult turkey gizzard myosin. Hybridoma antibody specificities were determined by solid-phase indirect radioimmunoassay and immunoreplica techniques. Electron microscopy of rotary-shadowed samples was used to directly visualize the topography of individual [antibody-antigen] complexes.

Antibody TGM-1 bound to a 50-kD peptide of subfragment-1 (S-1) previously found to be associated with actin binding and was localized by immunoelectron microscopy to the distal aspect of the myosin head. However, there was no antibody-dependent inhibition of the actin-activated heavy meromyosin ATPase, nor was antibody TGM-1 binding to actin-S-1 complexes inhibited. Antibody TGM-2 detected an epitope of the subfragment-2 (S-2) domain of heavy meromyosin but not the S-2 domain of intact myosin or rod, consistent with recognition of a site exposed by chymotryptic cleavage of the S-2-light meromyosin junction. Localization of TGM-2 to the carboxyl-terminus of S-2 was substantiated by immunoelectron microscopy. Antibody TGM-3 recognized an epitope found in the light meromyosin portion of myosin. All three antibodies were specific for avian smooth muscle myosin. Of particular interest is that antibody TGM-1, unlike TGM-3, bound poorly to homogenates of 19-d embryonic smooth muscles. This indicates the expression of different myosin heavy chain epitopes during smooth muscle development.

The structural organization of myosin molecules is conserved throughout evolution and among myosins of disparate tissue origin. A pair of heavy chains comprises both carboxy-terminal α-helical domains that form a coiled-coil “tail” and amino-terminal domains that form two globular “heads.” Each head is associated with a regulatory and an essential light chain. Interaction of myosin heads with filamentous actin, activation by actin of the Mg2+-ATPase of the myosin molecule, and functional coupling between ATP hydrolysis and force generation are essential and ubiquitous processes for contraction and other motile events in muscle and nonmuscle cells (1). In vertebrate striated muscles, myosin polymorphism is apparent not only for light chains characteristic of specific muscle classes, fiber types, and developmental stages (2-4), but also for myosin heavy chains of skeletal (5-9) and cardiac (10-12) muscle myosin. In contrast, the structural and functional organization and development of myosin in vertebrate smooth muscle have received relatively little study. We have produced a series of monoclonal antibodies (mAb) directed against antigenic determinants (epitopes) of smooth muscle myosin, as probes for such investigations.

We report here results obtained with hybridoma antibodies that recognize three distinct nonoverlapping domains of the heavy chain of avian smooth muscle myosin: heavy meromyosin (HMM) subfragment-1 (S-1), subfragment-2 (S-2),...
and light meromyosin (LMM). We have studied functional aspects of smooth muscle myosin by assaying for antibody-dependent effects on the actin-activated HMM MgADP-ATPase and for actin-dependent effects on mAb binding. We have investigated the structure of the smooth muscle myosin heavy chain, not only with its purified peptide domains, immunoblot techniques, and electron microscopy of rotary-shadowed [antibody-antigen] complexes, but also in conjunction with radiophosphorylated myosin light chains (13) as a unique structural probe.

MATERIALS AND METHODS

Materials: Nitrocellulose filtration plates (pore size 0.45 μm, type STHA) were purchased from Millipore Corp. (Bedford, MA), and nitrocellulose membranes (pore size 0.20 μm, type BAKS) from Schleicher and Schuell, Inc. (Keene, NH). 125I-labeled Fab′2 fragment of affinity-purified sheep IgG directed against mouse immunoglobulins [125I-F(ab′)2, 8.5 x 10^6 Ci/mmol] was purchased from New England Nuclear (Boston, MA). Reagents for sodium dodecyl sulfate (NaDodSO4)-polyacrylamide gel electrophoresis (SDS PAGE) were 6 M urea, 0.5% NaDodSO4, 30% glycerol (Richmond, CA). Trypsin (3X crystallized, type C11), and papain (2X crystallized, type PAP) were obtained from Worthington Biochemical Corp. (Freehold, NJ). Initial stocks of P3-X63.Ag8 murine myeloma cells were obtained from Dr. Marshall Nirenberg (National Institutes of Health).

Production of Monoclonal Antibodies: To prepare smooth muscle myosin as immunogen and as antigen for initial screening assays, myosin was purified from adult turkey gizzards (14). Gizzard myosin was phosphorylated by myosin light chain kinase (14) to –0.5 mol PO4/mol 20-kD myosin light chain (LC20) and subsequently subjected to limited proteolysis with chymotrypsin (1 mg/100 mg myosin) at 25°C in 500 mM NaCl, 10 mM MgCl2, 50 mM Tris-HCl, pH 7.4. The reaction was stopped after 10 min with the addition of phenylmethylsulfonyl fluoride to 0.5 mM. Female 6- to 8-week-old BALB/c mice were inoculated intraperitoneally at 0 and 8 d with 75 μg of myosin digest in complete Freunds' adjuvant, and at 15 d with 75 μg of myosin digest intravenously without adjuvant. Cell fusion at 18 d produced detectable antibody directed against a chymotrypsin slice of adult turkey gizzard myosin. We report results obtained with three hybridoma cell lines, each recloned three times at limiting dilution to ensure homogeneity and adapted to growth in roller bottles yielding ~150 mg/iter of monoclonal immunoglobulin. The binding specificity of each mAb was first determined in the presence of 10^-4 ~10^-5 g/well of myosin purified from adult turkey gizzard, chicken pectoralis, or calf aorta (Fig. 1). Antibodies TGM-1 and TGM-3, under the conditions shown, recognized intact turkey gizzard myosin but neither avian skeletal muscle myosin nor mammalian smooth muscle myosin. Antibody was comparably bound to myosin purified from gizzards of adult turkey versus adult chicken (data not shown).

RESULTS

Specificity for Avian Smooth Muscle Myosin: Purified Myosin

63 of 173 hybridoma colonies assayed 14–24 d after cell fusion produced detectable antibody directed against a chymotryptic digest of partially phosphorylated adult turkey gizzard myosin. We report results obtained with three hybridoma cell lines, each recloned three times at limiting dilution to ensure homogeneity and adapted to growth in roller bottles yielding ~150 mg/iter of monoclonal immunoglobulin. The binding specificity of each mAb was first determined in the presence of 10^-4 ~10^-5 g/well of myosin purified from adult turkey gizzard, chicken pectoralis, or calf aorta (Fig. 1). Antibodies TGM-1 and TGM-3, under the conditions shown, recognized intact turkey gizzard myosin but neither avian skeletal muscle myosin nor mammalian smooth muscle myosin. Antibody was comparably bound to myosin purified from gizzards of adult turkey versus adult chicken (data not shown).

A. TGM-1 O

FIGURE 1 Specificity of mAbs for avian smooth muscle myosin.

Myosin purified from adult turkey gizzard (a), chick pectoralis (c), or calf aorta (a) was immobilized by evaporation onto 96-well nitrocellulose plates (10^-4 ~10^-3 g/well) and reacted with hybridoma antibodies by indirect radioimmunoassay. Results [mol 125I-F(ab′)2 specifically bound per well], corrected as discussed in Materials and Methods, are shown for three monoclonal antibodies that bind the chymotryptic gizzard myosin digest used as immunogen: (A) TGM-1, (B) TGM-2, and (C) TGM-3.

B. TGM-3 O

C. TGM-2 O

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bind to intact myosin. None of the antibodies tested bound to rabbit skeletal muscle HMM or to nonmuscle myosin purified from chicken intestinal brush border.

**Tissue Homogenates**

To determine the tissue specificity of epitopes recognized by antibodies TGM-1 and TGM-3, SDS-denatured homogenates of 19-d chicken embryo tissues were analyzed by indirect radioimmunoassay (Fig. 2). Antibody TGM-3 bound to homogenates of 19-d embryonic chicken gizzard, aorta, and intestine, but not to cardiac ventricle, pectoralis, or cerebellum. The binding of mAb TGM-3 was specific for the 200-kD smooth muscle myosin heavy chain as demonstrated in the accompanying Western blot (Fig. 3). In contrast, using antibody TMG-1, little binding to myosin in homogenates of 19-d embryonic chicken smooth muscle was detected by indirect radioimmunoassay (Fig. 2). Results demonstrating binding of each antibody in the presence of purified adult turkey gizzard myosin are included for comparison. Thus, antibody TGM-1 may identify a region of smooth muscle myosin that undergoes developmental regulation and is preferentially expressed in the adult. None of the antibodies bound SDS-denatured homogenates of uterus, stomach, cardiac ventricle, pectoralis, cerebellum, or spleen of adult rat (data not shown), indicating specificity for avian smooth muscle myosin.

**Subfragments of Smooth Muscle Myosin Heavy Chain**

Binding of each antibody was determined in the presence of purified intact adult turkey gizzard myosin or its purified subunits by indirect radioimmunoassay (Fig. 4) and by Western blot (Fig. 5). mAb TGM-1 identified intact myosin, HMM, and S-1, but not S-2, rod, or LMM. mAb TGM-3 recognized intact myosin, rod, and LMM, but not HMM, S-1, or S-2. Thus, the TGM-1 epitope was localized to S-1, and the TGM-3 epitope to LMM.

mAb TGM-2, which did not bind to intact myosin, bound to HMM and S-2 but not S-1, indicating that a free carboxy-terminus of S-2 must be present for recognition. To confirm this localization of the TGM-2 binding site, HMM was digested with papain, and myosin rod was digested with chymotrypsin (Fig. 6, A and E). The resultant peptides were analyzed by Western blot. mAb TGM-2 bound to undigested HMM, to a number of intermediate fragments, and to S-2 produced in a time-dependent manner from the carboxy-terminus of HMM (Fig. 6C). Whereas no binding to undigested rod or its intermediate fragments was detected, mAb TGM-2 did bind to S-2 cleaved from the amino-terminus of...
FIGURE 5 Specificity of mAbs for identified domains of smooth muscle myosin heavy chain by Western blot. Purified subunits of adult turkey gizzard myosin (1 µg/lane) were subjected to SDS PAGE (panels A–D, 7.5% acrylamide; E–H, 12.5% acrylamide). Lanes: (a) HMM, (b) HMM produced by chymotryptic cleavage of myosin in the presence of ATP, (c) S-1, (d) LMM, (e) S-2, and (f) mixed LC2o and LC7. Panel E shows the Coomassie Blue-stained gel; B–D and F–H are autoradiograms of nitrocellulose replicas using mAbs TGM-1 (B and F), TGM-2 (C and G), or TGM-3 (D and H). The heavy chain of both 130-kD HMM and 97-kD S-1 undergo secondary proteolysis at a site ~70 kD from the amino-terminus (see Fig. 11). This gives rise to the additional bands seen below the main heavy chain bands in the HMM and S-1 preparations. (See reference 13 for a discussion of the digestion patterns.)

FIGURE 6 mAb TGM-2 recognizes HMM, S-2 derived from either HMM or from rod, but not intact rod. (A–D) HMM was digested with papain (0.01 U/mg HMM) and (E–H) rod with chymotrypsin (1 mg/100 mg rod) at 25°C for the times shown. Peptides (1 µg/lane) were separated by SDS PAGE (12.5% acrylamide). A and E show the Coomassie Blue-stained gel; B–D and F–H are autoradiograms of nitrocellulose replicas with mAbs TGM-1 (B and F), TGM-2 (C and G), or TGM-3 (D and H).

rod (Fig. 6G). Thus, [125I-F(ab')2]TGM-2-S-2 complexes were not formed when the S-2:LMM junction was intact, and we postulate that the TGM-2 binding site is at or near the S-2:LMM junction. mAb TGM-1 (Fig. 6, B and F) and TGM-3 (Fig. 6, D and H) identified distinct families of proteolytic peptides derived only from HMM or from rod, respectively, and are shown for comparison.

The globular S-1 head produced after digestion of myosin with papain retains the actin-binding and actin-activated Mg2+-ATPase activity of the intact myosin molecule (29). Controlled proteolysis of skeletal myosin S-1 with trypsin (30–32) results in formation of an amino-terminal 25-kD adenine nucleotide-binding fragment, a 50-kD central fragment containing one of two actin cross-linking sites, and a carboxy-terminal 20-kD fragment that contains the second actin cross-linking site, the S-1:S-2 junction, and a site of probable association with myosin light chains (13, 33, 34). Smooth muscle myosin S-1 similarly digested with trypsin results in homologous fragments of apparent molecular weight 29-, 50-, and 26-kD (references 13 and 35; Fig. 7A). The Western blot employing mAb TGM-1 localizes the TGM-1 epitope (Fig. 7C) to the 50-kD tryptic fragment; the antibody bound neither to the amino-terminal 29-kD fragment, nor to the carboxy-terminal 26-kD fragment that binds radiophosphorylated 20-kD light chains of gizzard myosin (see reference 13 and Fig. 7 B). The 28-kD band detected in the immunoblot only at 7.5 and 30 min-digestion does not correspond with the 29-kD fragment, as shown in the 1.5-min lanes.

After incubation of gizzard HMM or S-1 with rabbit skeletal muscle F-actin in the absence of ATP, such that all actin-binding sites were occupied, as determined by airfuge sedimentation of [S-1:actin] complexes, little or no inhibition of mAb TGM-1 binding was detected (Fig. 8). Phosphorylated gizzard HMM was incubated with mAb TGM-1 and then assayed for actin-activated Mg2+-ATPase activity. Little or no inhibition was detected under the conditions tested. In contrast, incubation of HMM with mAb TGM-2 resulted in a slight increase in the actin-activated Mg2+-ATPase activity (see Table I).

Topography of mAb Epitopes

Fig. 9 shows rotary-shadowed specimens of HMM and HMM reacted with TGM-1 and TGM-2. HMM molecules...
Fig. 8 Binding of mAb directed against myosin S-1 and HMM in the presence and absence of actin. Turkey gizzard HMM (circles) or myosin S-1 (squares) was incubated at 25°C for 30 min in the presence (solid symbols) or absence (open symbols) of rabbit skeletal muscle F-actin, as previously described (19). The proteins were immobilized on nitrocellulose for solid-phase indirect radioimmunoassay (see Materials and Methods) at the antigen concentration indicated. Results shown are fmol [125I]-Fab'2 specifically bound per well. (A) Reaction of HMM and S-1 with TGM-1. (B) Reaction of HMM and S-1 with TGM-2.

Fig. 9 Low-angle rotary shadowing of [mAb·HMM] complexes with platinum. Gallery of individual unlabeled myosin (A) or HMM molecules (B) for reference. (C) HMM molecules treated with anti-S-1 TGM-1, labeled on the distal aspect of a single S-1 head, or labeled symmetrically between two heads. (D) HMM molecules treated with anti-S-2 TGM-2, labeled at the carboxy-terminus of S-2. ×125,000.

Fig. 10 Schematic diagram indicating the organization of smooth muscle myosin heavy chain domains. Subunits generated after limited proteolysis with papain (P), yielding S-1 and rod, or with chymotrypsin (CT), yielding HMM and LMM, are labeled above the figure. T, principal sites of tryptic cleavage. Secondary sites of cleavage within S-1 by papain (P') or chymotrypsin (CT') are also shown, as well as an amino-terminal S-1 site of ATP-dependent chymotryptic digestion (CTAP). For clarity, only one heavy chain is illustrated, and subfragment lengths are not to scale. The topographic specificity of each mAb is indicated below the figure; the 32P-LC20 binding site is labeled for comparison.

DISCUSSION

We have produced and characterized monoclonal antibodies that label epitopes distributed among three distinct, non-overlapping identified peptide domains of the 200-kD heavy chain of avian smooth muscle myosin. Though turkey gizzard myosin shares similar native structure and domain organization with myosin not only from mammalian smooth muscle but also from striated muscle, the antibodies reported here exhibit specificity for avian smooth muscle myosin alone and do not cross-react with purified myosin from calf aorta, chicken pectoralis, or with avian nonmuscle myosin(s). Localization of the smooth muscle myosin epitopes (Fig. 10)
was established by indirect radioimmunoassay employing pu-
perified myosin subunits as well as by Western blot analysis of 
these subunits or of fragments generated by further proteoly-
sis. Assignment of mAb TGM-1 to S-1 and TGM-2 to the 
carboxy-terminus of S-2 based on peptide mapping is ad-
ditionally supported by the topography of [mAb-HMM] com-
plexes visualized by electron microscopy after low-angle ro-
mary shadowing with platinum.

mAb TGM-1 recognizes a 50-kD tryptic peptide of S-1 but 
not the amino-terminal 20-kD fragment or carboxy-terminal 
26-kD LC2jo-binding fragment. However, actin fails to inhibit 
TGM-1 binding, and conversely, antibody fails to inhibit 
actin-activated Mg²⁺-ATPase of HMM. Thus, we have de-
tected no functional interaction between mAb TGM-1 and 
the actin-binding site within the 50-kD peptide.

mAb TGM-2 recognizes an epitope of S-2 that is created by 
chymotryptic cleavage of the S-2:LMM junction. Thus, 
TGM-2 recognizes HMM and S-2 but not intact myosin or 
myosin rod. Analogous results have been reported for pol-
yclonal antibodies produced in rabbits immunized with S-1 
derived from chicken pectoralis myosin that precipitate S-1 
but not intact myosin (36). We postulate, therefore, that the 
antibody-combining site is located at or near the carboxy-
terminus of S-2 and that [TGM-2-S-2] complexes are not 
formed when the S-2:LMM junction is intact. This is con-
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