Two Smooth Muscle Myosin Heavy Chains Differ in Their Light Meromyosin Fragment†

Thomas J. Eddinger* and Richard A. Murphy
Department of Physiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908
Received October 14, 1987; Revised Manuscript Received January 21, 1988

ABSTRACT: Smooth muscle myosin heavy chains [SM1, approximately 205 kilodaltons (kDa), and SM2, approximately 200 kDa] were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels. Peptide maps of the two heavy chains showed unique patterns. Limited proteolytic cleavage of purified swine stomach myosin was performed by using a variety of proteases to produce the major myosin fragments which were resolved on SDS gels. A single band was obtained for heavy meromyosin in the soluble fraction following chymotrypsin digestion. However, a variable number of bands were observed for light meromyosin fragments in the insoluble fraction after chymotrypsin digestion. Peptide mapping indicated that the two bands observed after short digestion times with chymotrypsin had relative mobility and solubility properties consistent with approximately 100- and 95-kDa light meromyosin (LMM) fragments. These results indicate that the region of difference between SM1 and SM2 lies in the LMM fragment.

While myosin isozymes occur in both skeletal and cardiac muscles (Hoh, 1975; Hoh & McGrath, 1978; Hoh & Yeoh, 1979), their existence in smooth muscle remains controversial. Burridge and Bray (1975) observed two myosin heavy chains (MHC's) in chicken gizzard. More recently, several groups (Beckers-Bleukx & Marechal, 1985; Lema et al., 1986; Marechal & Beckers-Bleukx, 1986; Morano et al., 1986; Rovner et al., 1986; Schildmeyer & Seidel, 1986) have reported two or more heavy chains from a variety of tissues. However, the identity, number, and ratios of the putative heavy chains are controversial. There are a number of possible explanations for the appearance of multiple bands on polyacrylamide gels. Myosin is a very large molecule, and the heavy chains are difficult to resolve on denaturing gels. Myosin aggregates at low ionic strength and is difficult to run on nondenaturing gels. Furthermore, the two phosphorylated forms of myosin migrate differently from each other and dephosphorylated myosin on nondenaturing gels (Persechini et al., 1986). Filamin, which is a prevelant smooth muscle protein, is extracted with and tends to comigrate with myosin on nondenaturing gels. In addition, partial proteolysis is a potential artifactual source of protein varients.

The purpose of this study was to confirm that there are two smooth muscle MHC's in smooth muscle and identify how they differ. By using both nondenaturing and denaturing gels, monoclonal antibodies, limited proteolytic digestion, and peptide mapping, we confirm that two different specific MHC's are present in smooth muscle and show that the region(s) giving rise to the molecular weight difference occur(s) in the light meromyosin (LMM) fragment of the molecule.

Materials and Methods

Tissue and Myosin Preparation. All tissue samples were extensively cleaned of surrounding adipose and connective tissue before homogenization. Blood vessels were stripped of their adventitia before being processed for electrophoresis. Smooth muscle myosin was purified from swine stomach according to the methods of Krisanda and Murphy (1980) with modifications (Rovner et al., 1986). The purified myosin was either immediately mixed in sample buffer for gel electrophoresis or mixed with an equal volume of cold glycerol and kept at −22 °C for up to 2 months.

Nondenaturing Gel Electrophoresis. The methods for tissue preparation and gel electrophoresis were modified from those of Hoh and co-workers (Hoh et al., 1976, 1978). Smooth muscle myosin was extracted from tissue by homogenization in 80 mM $Na_4P_2O_7$, 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 5 mM dithiothreitol (DTT) (pH 8.8). This homogenate was centrifuged (7000*g* for 15 min), and the supernatant was mixed with an equal volume of cold glycerol. The extract was kept at −22 °C until loaded on a gel. Some experiments employed the method of Persechini et al. (1986) for tissue extraction, with similar results. The gel electrophoresis buffer contained 20 mM $Na_4P_2O_7$ (pH 8.8), 2 mM cysteine, and 10% glycerol. The temperature was 1 ± 1 °C throughout the run. Slab gels (1.5 mm thick, 4.5% or 4.0% acrylamide) with 20 mM $Na_4P_2O_7$ (pH 8.8) and 10% glycerol were run on a Pharmacia Model GE 2/4 apparatus with buffer recirculation between upper and low chambers. Gels were prefocused for 75 min prior to loading samples and then run for 28–42 h at constant voltage (120–150 V). Gels were stained in 0.1% Coomassie brilliant blue R250 (CBB) and 25% trichloroacetic acid (TCA) and destained in 25% isopropyl alcohol and 10% acetic acid.

Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis. Two sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) systems were used in these studies. All low-acrylamide (3–4%) denaturing gels used for separation and study of the MHC's used the method of Laemmli (1970) with modifications as described by Rovner et al. (1986). No backing sheets were used to support these gels. Higher percentage acrylamide (8–12%)–SDS denaturing gels used the method of Giulian et al. (1983). Gels were either stained with CBB (Rovner et al., 1986) or stained with silver (Giulian et al., 1983) depending on loading conditions. Molecular weight standards were obtained from Sigma Chemical Co. and included proteins with approximate molecular weights of 205K, 116K, 97.4K, 66K, 45K, and 29K.

Limited Proteolysis and Peptide Mapping. Production of the various major fragments of myosin by enzymatic digestion

† This research was supported by NIH Grant 5-PO1-HL-19242 and by a postdoctoral fellowship to T.J.E. from the American Heart Association, Virginia Affiliate.
* Address correspondence to this author at the Department of Physiology, Box 449, University of Virginia School of Medicine, Charlottesville, VA 22908.

0006-2960/88/0427-3807$01.50/0

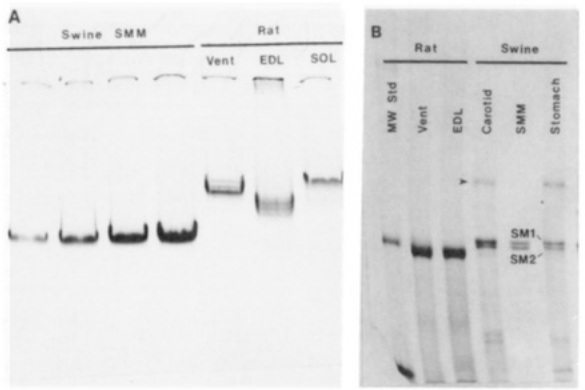

FIGURE 1: Evidence for two forms of smooth muscle MHC. (A) 4.5% nondenaturing gel: lanes 1–4, purified swine stomach smooth muscle myosin at increasing loads; lane 5, rat ventricle; lane 6, rat extensor digitorum longus (EDL); lane 7, rat soleus (SOL). Cardiac and fast-twitch myosins clearly show three isoenzymes. (B) 3–4% SDS gel: lane 1, molecular weight standard (skeletal myosin); lane 2, rat ventricle; lane 3, rat EDL; lane 4, swine carotid tissue homogenate; lane 5, purified swine stoomach smooth muscle myosin; lane 6, swine stomach tissue homogenate. The arrowhead points to filamin which is abundant in smooth muscle tissues but is not present in the purified myosin preparation.

followed the methods of Sellers and Harvey (1984) for α-chymotrypsin and trypsin and Greene et al. (1983) for papain. Soluble and insoluble fractions were prepared for gel electrophoresis and run on 10% or 12% SDS gels to separate the fragments. Protease concentrations of 5–150 μg were used for times varying from 3 to 30 min to follow fragment production with increasing digestion. Peptide mapping was done according to the methods of Cleveland et al. (1977). SM1 and SM2 were routinely run on a 3–4% SDS denaturing gel for separation and isolation, while major proteolytic fragments were separated on 10% or 12% SDS gels. Gels for peptide mapping were either 10% or 12% SDS slab gels, 1.0 or 1.5 mm thick with a 6 cm, 3.5% acrylamide stacking gel.

Electroblotting. Proteins in SDS or nondenaturing gels were blotted to nitrocellulose paper (Towbin et al., 1979) in a Hoefer TE-42 apparatus. The transfer buffer was 192 mM glycine and 25 mM tris(hydroxymethyl)aminomethane (Tris) base (pH 8.3). CBB staining of the gels after blotting confirmed transfer of the protein out of the gel. Probing of blotted proteins by monoclonal antibodies allowed verification and identification of specific proteins and/or fragments. Peroxidase-conjugated antibodies were detected with 4-chloronaphthol (Hawkes et al., 1982).

Monoclonal Antibodies. Monoclonal antibodies to purified swine smooth muscle myosin were raised in BALB/C mice (Chapman et al., 1984). Purified swine stomach myosin (800 μg) dissolved in 0.5% SDS and mixed 50:50 with Freund's adjuvant was injected both intraperitoneally and subcutaneously for both the initial and booster injections. Four weeks after the booster injection, 100 μg of the antigen [in 0.6 M NaCl and 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)] was injected into the spleen and another 100 μg intraperitoneally. Four days later, the animal was sacrificed, and the spleen cells were recovered. Thirty-five clones were obtained that were specific for smooth muscle myosin. One of these (MY-8H2-D4) was used extensively throughout this study. This clone is swine smooth muscle MHC specific.

RESULTS

A single band which corresponded to myosin was observed in nondenaturing gels containing purified swine stomach smooth muscle myosin (SMM) (Figure 1A). When SMM, stomach homogenate, and carotid homogenates were loaded

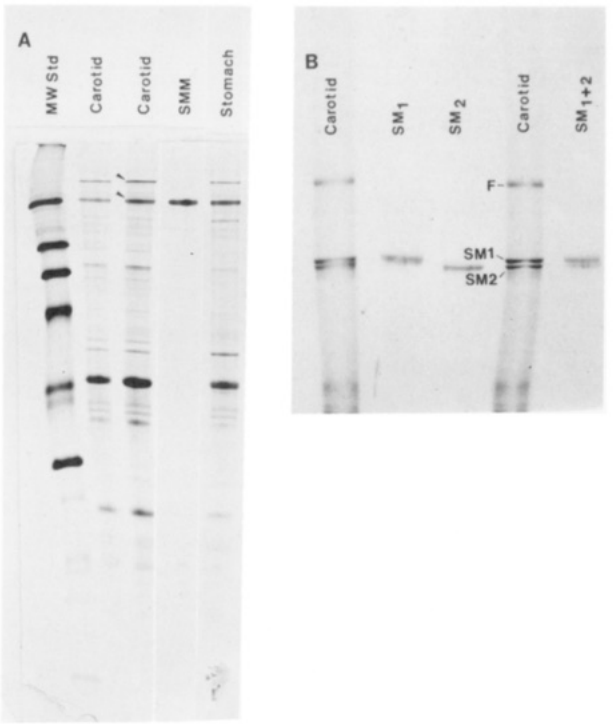

FIGURE 2: (A) Ten percent SDS gel: lane 1, molecular weight standards (205K, 116K, 97.5K, 66K, 45K, and 29K); lanes 2 and 3, swine carotid tissue homogenate (two loadings); lane 4, purified swine stomach smooth muscle myosin. (B) 3–4% SDS gel. SM1 and SM2 were cut out of a 3–4% SDS gel and rerun on the illustrated 3–4% SDS gel. SM1 and SM2 were added back singly or together. Swine carotid tissue homogenate was also run as a control to compare mobilities. Lanes 1 and 4, swine carotid tissue homogenate; lane 2, SM1 slice; lane 3, SM2 slice; lane 5, SM1 and SM2 slices together.

on 3–4% SDS denaturing gels, they showed two bands with relative mobilities suggesting that they were both MHC (Figure 1B). In order to determine if the appearance of these two bands [referred to as SM1 and SM2, SM2 having the greater mobility (Rovner et al., 1986)] was due to an artifactual band splitting in this denaturing system, we ran these samples on an SDS–PAGE system with differing acrylamide and cross-linking concentrations (Giulian et al., 1983). At 10% acrylamide where the entire protein composition of the tissue can be observed, a doublet was still apparent (Figure 2A). The individual SM1 and SM2 bands were also excised from a 3–4% SDS gel and loaded onto a second 3–4% SDS gel to test for artifactual band splitting. The results (Figure 2B) show that the individual bands run on a second gel migrated as single distinct bands with no change in molecular weight. SM1 (Figure 2B) maintained its lower mobility in relation to SM2 when they were run separately or together. All of these bands showed similar mobilities to control smooth muscle tissue homogenates run on the same gel.

The two MHC's (SM1 and SM2 on SDS gels) were recognized by our antibody. No other bands on the nondenaturing gels or high-percent acrylamide gels showing the entire protein composition of the tissue were recognized (Figure 3A,B). The antibody is swine, smooth muscle, myosin heavy-chain specific.

Peptide mapping was done on each of the two MHC bands resolved on 3–4% SDS gels from both purified swine stomach myosin and stomach tissue homogenates. These bands were cut out of the gel and processed according to Cleveland's procedure (Cleveland et al., 1977). Figure 4A shows the purified SMM, SM1 and SM2, run on a 10% gel with chymotrypsin added to their lanes. SM1 and SM2 have different

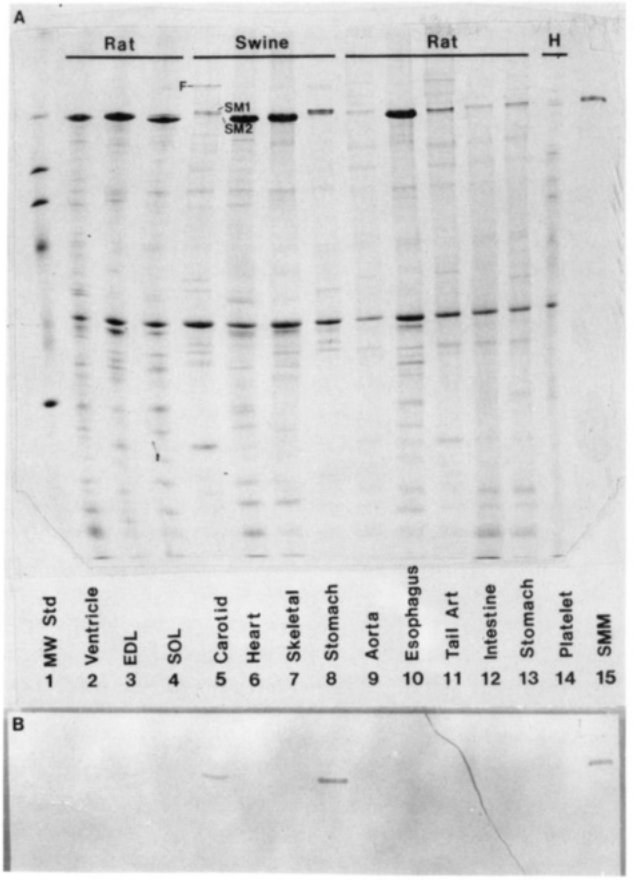

FIGURE 3: Ten percent SDS gel (A) and blot of identical gel (B) (only high molecular weight region shown) after probing with MY-8H2-D4 antibody. Gels A and B: lane 1, molecular weight standards (205K, 116K, 97.5K, 66K, 45K, and 29K); other lanes as labeled; lane 14, human platelets (H); lane 15, purified swine stomach myosin (SMM). The antibody is swine smooth muscle MHC specific.

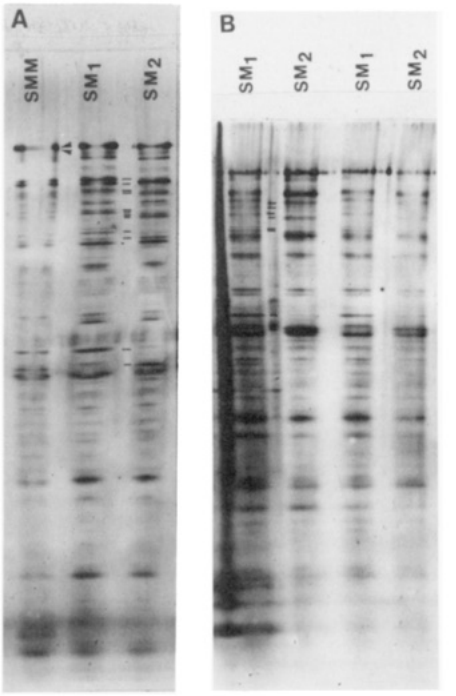

FIGURE 4: Silver-stained peptide maps of smooth muscle myosin (A) 10% SDS gel showing purified SMM, SM1, and SM2 with 8 ng of chymotrypsin (lanes 1–3). Lines point to major difference peptides. Arrowheads point to the SM1–SM2 doublet. (B) 12% SDS gel. Purified swine stomach myosin and myosin from swine stomach homogenates (not purified and therefore a control for possible proteolytic cleavage during purification) were run on 3–4% gradient SDS gels to separate SM1 and SM2. These were cut out and loaded on a 12% gel (shown) with 5 ng of chymotrypsin in each line. Lanes 1 and 2, purified SM1 and SM2; lanes 3 and 4, swine stomach homogenate SM1 and SM2. Peptide map patterns show that SM1 and SM2 are reproducibly different regardless of prior handling. Lines point to major difference peptides.

peptide maps. Figure 4B shows the SM1 and SM2 bands from both purified myosin and tissue homogenates with chymotrypsin added to their lanes. These peptide patterns show several differences between SM1 and SM2. The fact that the purified stomach SMM and that from tissue homogenates give identical results confirms that the MHC's were not altered by the purification procedure.

On the basis of the evidence that SM1 and SM2 are different polypeptides, we performed limited proteolytic cleavages on the intact purified myosin to locate the difference region. The major protein fragments were generated by using either chymotrypsin, trypsin, or papain. By alteration of the protease concentration and the digestion time, the progression of myosin breakdown with the generation of new fragments was followed. The time course studies and published data describing the primary fragments obtained with each of these proteases (Greene et al., 1983; Sellers & Harvey, 1984), their fractionation based on solubility at low ionic strength, and their molecular weight values allowed identification of the major fragments [heavy meromyosin (HMM), LMM, rod, and S1] as well as many smaller breakdown fragments (Figure 5, only chymotrypsin digestion shown). Figure 5C is a diagrammatic sketch of the MHC showing the fragments we have identified and their apparent molecular weights from SDS gels.

The initial proteolytic fragments consisted of a uniform population (as judged by a single band within a given molecular weight range) of HMM fragments in the soluble fraction. However, in the insoluble fraction, the LMM fragments appeared as two or more bands with varied digestion times (Figure 5A, chymotryptic digestion shown). These bands [LMM–SM1 and LMM–SM2 (Figure 5A)] were separated on 10% gels, and excised slices were used for peptide mapping. The peptide maps of these two bands differ (Figure 5B). SDS gels of these various proteolytic fractions were blotted to nitrocellulose paper and probed with the monoclonal antibody (Figure 6). The antibody recognized purified swine stomach myosin and all the insoluble fragments having M_r >95K (fragments of M_r <15K ran off the gel). The antibody did not recognize any of the fragments from the soluble fraction (i.e., HMM or its breakdown peptides, not shown) or any smaller proteolytic fragments of LMM–SM1 or –SM2.

Discussion

Our data confirm the results of Rovner et al. (1986) that there are two MHC's in smooth muscle tissues differing in their apparent molecular weight. The results also show that the LMM fragment of the smooth muscle myosin heavy chain contains the sequence(s) responsible for this difference in molecular weight. Numerous controls were done to assess various artifacts which could account for our results. The possibility of artifactual band splitting on the SDS gels was ruled out by cutting out each of the MHC bands and rerunning them on another SDS gel (Figure 2B). SM1 and SM2 retained their molecular weight values on the second SDS gel and remained as distinct bands, aligning with SM1 and SM2 bands from tissue homogenates run as a control. Loading both SM1 and SM2 bands into one lane produced two distinct bands.

SM1 and SM2 isolated from purified myosin showed unique peptide maps for each MHC with chymotrypsin (Figure 4A). These patterns were observed consistently in both stomach and

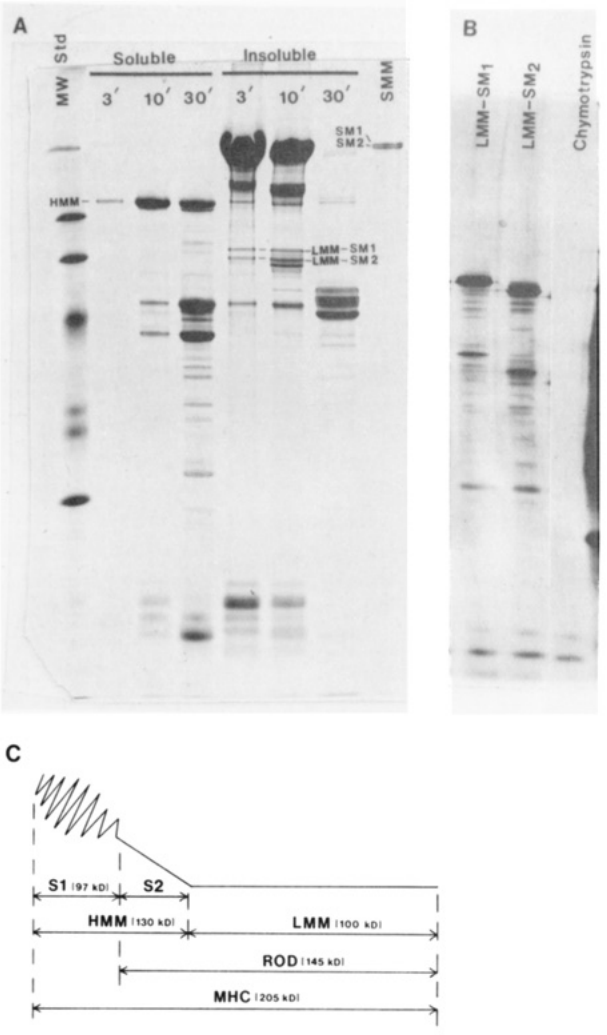

FIGURE 5: (A) Ten percent SDS gel showing major fragments following limited chymotryptic digestion of purified swine stomach myosin: lane 1, molecular weight standards (205K, 116K, 97.5K, 66K, 45K, and 29K); lanes 2–4, soluble fraction after 3-, 10-, and 30-min digestion; lanes 5–7, insoluble fraction after 3-, 10-, and 30-min digestion; lane 8, SMM. Digestion was at room temperature with 50 ng of chymotrypsin added to start the digestion, and an additional 50 ng was added to the remaining protein solution after each timed aliquot was removed. (B) Silver-stained 10% SDS gel peptide map of LMM–SM1 and LMM–SM2. The two polypeptides were separated and cut out of a 10% SDS gel as illustrated in panel A. LMM–SM1 (lane 1) and LMM–SM2 (lane 2) show different peptide maps. (C) Diagrammatic sketch of MHC showing proteolytic fragments and their apparent molecular weights on this gel system.

carotid arterial myosins. Figure 4B shows that the map patterns for SM1 and SM2 isolated directly from SDS gels of purified myosin and myosin from tissue homogenates are the same, arguing against any artifactual degradation during protein purification. By use of varied digestion times and protease concentrations, the peptides could be pieced together to determine their origin. LMM–SM1 and LMM–SM2 were identified in this fashion.

Chymotryptic digestion of myosin has been used by numerous laboratories as a standard procedure for generating HMM/LMM fragments. The presence of a single HMM band after chymotryptic digestion does not rule out possible heterogeneity in this region of the molecule. However, the difference in molecular weight observed between SM1 and SM2 can be fully accounted for by the molecular weight difference between LMM–SM1 and LMM–SM2. The insolubility of the LMM fragments in low salt, their equal stoichiometry, their apparent molecular weights (approxi-

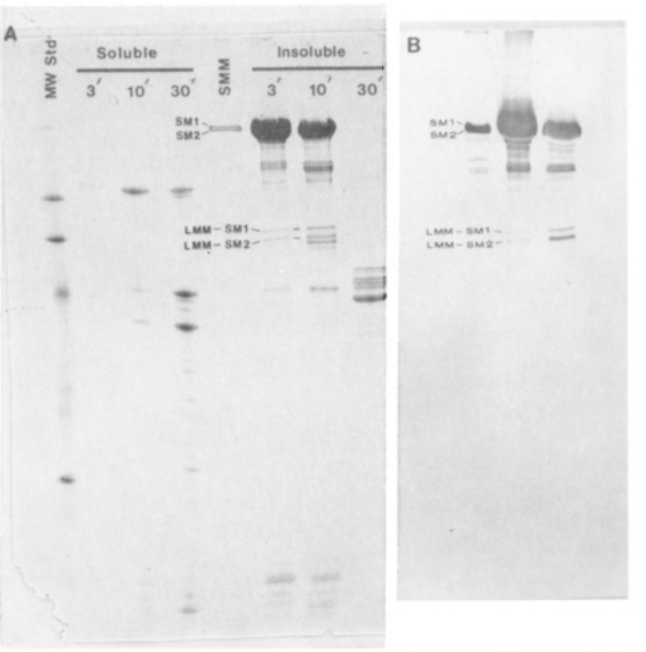

FIGURE 6: SDS (10%) gels (A): lane 1, molecular weight standards (205K, 116K, 97.4K, 66K, 45K, and 29K); lanes 2–4, soluble fraction after 3-, 10-, and 30-min chymotrypsin digestion; lanes 6–8, insoluble fraction after 3-, 10-, and 30-min chymotrypsin digestion; lane 5, purified SMM. (B) Nitrocellulose blot of gel identical with gel A (lanes 5–8 shown) after probing with myosin MY-8H2-D4 antibody. The antibody did not recognize any peptide in lanes 1–4 (not shown). See text for discussion.

mately 100K and 95K), their recognition by the monoclonal antibody, and their different peptide maps all lead to the conclusion that the major difference sequence(s) distinguishing the SM1 and SM2 heavy chains reside(s) within this fragment. The simultaneous appearance and disappearance of these bands in the insoluble fraction with increasing digestion times, coupled with the absence of detectable differences in HMM derived from SM1 or SM2 heavy chains, also supports this conclusion.

Our results suggest that LMM–SM2 is about 3–5-kDa smaller than LMM–SM1. The functional implications are currently unknown. However, an intriguing possibility is suggested by the results of Strehler et al. (1986) describing the total sequence for the rat embryonic skeletal MHC. Their results when compared with those of Karn et al. (1983) for the nematode unc-54 MHC indicate that the MHC rod sequence is highly conserved. There is not a single amino acid insertion or deletion (there are substitutions) in an alignment of the rat and nematode MHC rod sequences (Strehler et al., 1986). The rod sequence for many MHC's shows an internal repeat with a 28-residue pattern typical for an α-helical coiled-coil (Strehler et al., 1986; Dibb et al., 1985; Karn et al., 1983). This uniformity does not extend to the S2 hinge region in both rat and rabbit (Lu & Wong, 1985) and suggests that the stability of this region is lower.

The molecular weight difference between SM1 and SM2 is approximately 30 residues, or potentially an additional 28 amino acid repeat unit in LMM–SM1. Because of the strict sequence restraints required for an α-helical coiled-coil, the extra segment may have to be near either the S2 hinge region or the tail for an SM1–SM2 heterodimer to form (this would not be necessary if the heavy chains combined only as homodimers). Ueno and Harrington (1986a,b) reported that the hinge of the rod shows conformational changes (as suggested by susceptibility to enzymatic digestion) which are coupled to cross-bridge cycling. They hypothesize that these confor-

mational changes may play a major role in force generation. Thus, a difference located in this region could play a role in cross-bridge function which has unique aspects in smooth muscle (Dillon et al., 1981; Siegman et al., 1986). Alternatively, an extra segment in LMM–S1 could form an extended tail piece at the carboxyl terminal where differences in length and sequence are common (Strehler et al., 1986). In either case, sequences near the carboxyl-terminal end of the rod, at the S2 hinge region of MHC, or anywhere else in the LMM fragment may be important for the proper assembly of myosin molecules into thick filaments (Dibb et al., 1985; Peltz et al., 1985).

Acknowledgments

We thank Dr. A. S. Rovner for protocols and advice that saved much time and effort and Dr. W. M. Sutherland for generating the monoclonal antibodies. Swine tissues were donated by Smithfield Packing Co., Smithfield, VA.

References

Beckers-Bleukx, G., & Marechal, G. (1985) *Eur. J. Biochem. 152*, 207–211.

Burridge, K., & Bray, D. (1975) *J. Mol. Biol. 99*, 1–14.

Chapman, M. D., Sutherland, W. M., & Platts-Mills, T. A. E. (1984) *J. Immunol. 133*, 2488–2495.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.

Dibb, N. J., Brown, D. M., Karn. J., Moerman, D. G., Bolten, S. L., & Waterston, R. H. (1985) *J. Mol. Biol. 183*, 543–551.

Dillon, P. F., Aksoy, M. O., Driska, S. P., & Murphy, R. A. (1981) *Science (Washington, D.C.) 211*, 495–497.

Giulian, G. G., Moss, R. L., & Greaser, M. L. (1983) *Anal. Biochem. 129*, 277–287.

Greene, L. E., Sellers, J. R., Eisenberg, E., & Adelstein, R. S. (1983) *Biochemistry 22*, 530–535.

Hawkes, R., Niday, E., & Gordon, J. (1982) *Anal. Biochem. 119*, 142–147.

Hoh, J. F. Y. (1975) *Biochemistry 14*, 742–747.

Hoh, J. F. Y., & Yeoh, G. P. S. (1979) *Nature (London) 280*, 321–323.

Hoh, J. F. Y., McGrath, P. A., & White, R. I. (1976) *Biochem. J. 157*, 87–95.

Hoh, J. F. Y., McGrath, P. A., & Hale, P. T. (1978) *J. Mol. Cell. Cardiol. 10*, 1053–1076.

Karn, J., Breener, S., & Barnett, L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4253–4257.

Krisanda, J. M., & Murphy, R. A. (1980) *J. Biol. Chem. 255*, 10771–10776.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lema, M. J., Pagani, E. D., Shemin, R., & Julian, F. J. (1986) *Circ. Res. 59*, 115–123.

Lu, R. C., & Wong, A. (1985) *J. Biol. Chem. 260*, 3456–3461.

Marechal, G., & Beckers-Bleukx, G. (1986) *J. Muscle Res. Cell Motil. 7*, 65.

Peltz, G., Spudich, J. A., & Parham, P. (1985) *J. Cell Biol. 100*, 1016–1023.

Persechini, A., Kamm, K. E., & Stull, J. T. (1986) *J. Biol. Chem. 261*, 6293–6299.

Rovner, A. S., Thompson, M. M., & Murphy, R. A. (1986) *Am. J. Physiol. 250*, C861–870.

Schildmeyer, L. A., & Seidel, C. L. (1986) *Biophys. J. 49*, 70a.

Sellers, J. R., & Harvey, E. V. (1984) *J. Biol. Chem. 259*, 14203–14207.

Siegman, M. J., Butler, T. M., Mooers, S. U., & Davies, R. E. (1976) *Science (Washington, D.C.) 191*, 383–385.

Strehler, E. E., Strehler-Page, M.-A., Perriard, J.-C., Periasamy, M., & Nadal-Ginard, B. (1986) *J. Mol. Biol. 190*, 291–317.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Ueno, H., & Harrington, W. F. (1986a) *J. Mol. Biol. 190*, 59–68.

Ueno, H., & Harrington, W. F. (1986b) *J. Mol. Biol. 190*, 69–82.