Identification of Two Types of Smooth Muscle Myosin Heavy Chain Isoforms by cDNA Cloning and Immunoblot Analysis*

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We previously reported the characterization of a rabbit uterus cDNA clone (SMHC29) which encoded part of the light meromyosin of smooth muscle myosin heavy chain (Nagai, R., Larson, D. M., and Periasamy, M. (1986) Proc. Natl. Acad. Sci. U. S. A. 85, 1047-1051). We have now characterized a second cDNA clone (SMHC40) which also encodes part of the light meromyosin but differs from SMHC29 in the following respects. Nucleotide sequence analysis demonstrates that the two myosin heavy chain mRNAs are identical over 1424 nucleotides but differ in part of the 3'-carboxyl coding region and a portion of the 3'-nontranslated sequence. Specifically, SMHC40 cDNA encodes a unique stretch of 43 amino acids at the carboxyl terminus, whereas SMHC29 cDNA contains a shorter carboxyl terminus of 9 unique amino acids which is the result of a 39-nucleotide insertion. Recent peptide mapping of smooth muscle myosin heavy chain identified two isotypes with differences in the light meromyosin fragment that were designated as SM1 (204 kDa) and SM2 (200 kDa) type myosin (Eddinger, T. J., and Murphy, R. A. (1988) Biochemistry 27, 3807-3811). In this study we present direct evidence that SMHC40 and SMHC29 mRNA encode the two smooth muscle myosin heavy chain isoforms, SM1 and SM2, respectively, by immunoblot analysis using antibodies against specific carboxyl terminus sequences deduced from SMHC40 and SMHC29 cDNA clones.

Myosin heavy chain isoforms of striated muscle are encoded by a highly conserved multigene family, the expression of which is regulated developmentally, hormonally, and in a tissue-specific manner (1-6). The complexity of myosin heavy chains and their phenotypic expression in smooth muscle tissues, however, are less well understood. Several investigations have reported that at least two myosin heavy chain isoforms are present, based on polyacrylamide gel analysis of MHC from a variety of smooth muscle tissues (7-11). The two proteins SM1 (approximately 204 kDa) and SM2 (approximately 200 kDa) migrate very closely on SDS-polyacrylamide (5-4%) gels (10, 11). Recently Eddinger and Murphy (11) used limited proteolysis and peptide mapping of purified swine stomach myosin to show that the region of difference between SM1 and SM2 lies in the light meromyosin portion of the myosin molecule.

The characterization of cDNA clones encoding a chicken gizzard (12) and a rabbit smooth muscle myosin heavy chain (13) have recently been reported. In our report (13) based on S1 mapping analysis results using SMHC29 cDNA, we indicated that there might be a second MHC mRNA transcript present in smooth muscle tissues. Following this observation, we performed an extensive rescreening of a rabbit uterus cDNA library and succeeded in isolating and characterizing a second type of smooth muscle MHC cDNA clone. The two types of smooth muscle MHC cDNA clones encode part of the light meromyosin of the myosin molecule and have identical nucleotide and amino acid sequences except at the very carboxyl terminus where SMHC29 has an inserted sequence of 39 nucleotides which is not found in SMHC40-type myosin. This inserted sequence which encodes 9 unique amino acids in SMHC29 confers on the cDNAs two different types of carboxyl termini. Based on the deduced amino acid sequences of carboxyl termini of SMHC29- and SMHC40-type MHC, we synthesized two short peptides and raised specific antibodies against those MHCs. The immunoblot analysis using these two antibodies clearly demonstrated that SMHC40- and SMHC29-type MHC correspond to SM1 and SM2, respectively.

MATERIALS AND METHODS

Construction and Screening of λgt11 cDNA Library—Construction of a rabbit uterus cDNA library into λgt11 expression vector and isolation of a cDNA clone SMHC29 were described previously (13). The library was rescreened using SMHC29 as a probe. Forty-four clones were positively identified and 11 subsequently chosen for restriction endonuclease mapping and nucleic acid sequencing.

Nucleic Acid Sequence Analysis—λgt11 cDNA inserts were subcloned into the EcoRI site of puC18 and M13 mp18 cloning vectors and sequenced by the chemical cleavage method of Maxam and Gilbert (14) and the dyeoxy method of Sanger et al. (15).

S1 Nuclease Mapping—The procedures of S1 nuclease mapping have been described previously (15, 16). A 407-bp PstI restriction fragment from the 3' end of SMHC40 was 3' end-labeled with [γ-32P]ATP (Amersham Corp.), strand-separated, and the strand complementary to the mRNA was used for S1 analysis. This probe contains 209 nucleotides of coding region, 158 nucleotides of the 3'-untranslated region, and 40 nucleotides of the polylinker of the puC18 cloning vector.

Antibodies and Immunoblotting—Two short peptides were synthesized based on the deduced amino acid sequences (Fig. 1) at the carboxyl terminus of SMHC29 (Gly-Pro-Pro-Pro-Glu-Thr-Ser-Gln) and SMHC40-type MHC (Asp-Ala-Asp-Phe-Asn-Gly-Thr-Lys-Ser-Glu). These amino acid sequences are isoform-specific to SMHC29 or SMHC40 and do not show any homology to any sequences in the common coding region. The peptides were conjugated with bovine serum albumin, and 200 μg of conjugates were injected intradermally into Wistar rats at biweekly intervals. Myosin from

*The abbreviations used are: MHC, myosin heavy chain; bp, base pair(s); SDS, sodium dodecyl sulfate.
by 3.5% SDS-polyacrylamide gel electrophoresis as described by Towbin et al. (10). The gel was transblotted onto a nitrocellulose membrane and immunologically stained by a standard protocol of Towbin et al. (18) using antisera against SMHC40 peptide (C1) and SMHC29 peptide (C2).

**RESULTS**

Isolation of the Two Types of Smooth Muscle MHC cDNA Clones—Forty-four positive clones were isolated by rescreening a rabbit uterine cDNA library (4 x 10^6/11 cDNA clones) with 32P-labeled SMHC29 peptide (12). Out of these, 11 clones which had inserts of >0.5 kilobase were chosen for further characterization. These cDNA clones showed identical restriction maps when digested with PstI, PvuII, SacI, and BglII. All 11 clones were subjected to sequencing analyses, and attention was focused at the 3' end of the coding region where we predicted a heterogeneity of MHC mRNA on the basis of sequence analysis. The new set of clones designated as SMHC40-type MHC extended the same distance the carboxyl terminus of the SMHC29-type myosin (Fig. 2). When compared to chicken gizzard smooth muscle MHC protein (12) SMHC40-type MHC extended the same distance (unlike SMHC29) and exhibited 87% amino acid sequence homology indicating an evolutionary conservation (Fig. 2).

When we analyzed the nucleotide sequences of SMHC40 and SMHC29 cDNAs, we found that the first part of the untranslated sequence (except for the first nine nucleotides) of cDNA SMHC29 was translated in frame into the 43 amino acids present at the carboxyl terminus of the SMHC40-type MHC (Fig. 1). The remainder of the 3'-untranslated region in cDNA SMHC29 was also present in cDNA SMHC40 (Fig. 1). The cDNA SMHC40 therefore contains essentially the same sequence as SMHC29 cDNA except that SMHC29 cDNA contains an extra 39 nucleotides (nucleotides 1425-1464 in SMHC29) which encodes a specific carboxyl terminus of 9 amino acids and the first eight nucleotides of the 3'-untranslated region. This inserted sequence is not present in the carboxyl terminus of the SMHC29-type myosin (Fig. 2).

**Expression of SMHC40 and SMHC29 mRNAs in Smooth Muscle Tissues**—The tissue-specific expression of SMHC29 mRNA was previously studied in smooth muscle types by S1 nuclease mapping analysis (13). We found that this MHC mRNA was expressed in all smooth muscle tissues tested. In addition, the detection of a partially protected fragment in this analysis indicated that there might be a second MHC isoform coexpressed in these smooth muscle tissues. To further test the possibility that SMHC40 corresponds to the second MHC isoform, we investigated the expression of SMHC40 mRNA in various smooth muscle tissues by S1 nuclease mapping analysis. For this purpose a 407-nucleotide single-stranded probe was derived from the 3' end of cDNA SMHC40. This probe contained both coding (70 amino acids) and 3'-untranslated sequences (Figs. 1 and 3) but was different from SMHC29 only by not containing the unique 39-nucleotide insertion. The expected full protection of SMHC40 probe (367 nucleotides) was observed in all smooth muscle tissues (Fig. 3). Weak hybridization was also evident with a partial protected fragment which should correspond to SMHC29 as a probe, we were not able to detect any partially protected fragment which should correspond to SMHC29.
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**FIG. 2.** Comparison of deduced amino acid sequences at the carboxyl terminus between chicken gizzard and rabbit uterus smooth muscle myosin heavy chains. Chicken gizzard myosin amino acid sequence was taken from Yanagisawa et al. (12). Amino acid differences are indicated by asterisks.

**FIG. 3.** Expression of SMHC40 cDNA in smooth muscle tissues by S1 nuclease mapping. The schematic diagram at the bottom of the figure indicates the origin and length of probe used. Full protection results in a fragment of 367 nucleotides (nt).

mRNA. This is because the entire nucleotide sequence in SMHC40 probe is contained in SMHC29 mRNA and therefore instead of a partial protection of SMHC29 mRNA, we observe an apparent full protection. On the basis of S1 nuclease mapping therefore using specific probes from SMHC29 (13) and the recent isolation of the SMHC cDNA clone, it seemed evident that two types of MHC mRNA transcripts were expressed in adult rabbit smooth muscle tissues, possibly encoding two different MHC proteins. In order to demonstrate unequivocally that this was the case, we performed immunoblot analysis with specific antibodies to the two different carboxyl termini of MHC29 and SMHC40 cDNA clones.

**SMHC40 and SMHC29 cDNAs Encode SM1 and SM2 Myosin Heavy Chains, Respectively, at the Protein Level—**To show that SMHC40 and SMHC29 mRNA encoded, respectively, SM1 and SM2 myosin polypeptides, we performed immunoblot analysis using purified myosin from adult rabbit aorta and uterus tissues. Specific antisera were raised against two synthetic peptides corresponding to the unique carboxyl termini of SMHC40- and SMHC29-type MHC as described under “Materials and Methods.” Rat antisera raised against the SMHC40-type carboxyl terminus reacted specifically with the SM1 myosin band (Fig. 4). On the other hand, SM2 myosin only reacted with SMHC29 antisera. Similarly SM1 and SM2 myosin bands from other smooth muscle tissues such as stomach, intestines, or urinary bladder were also specifically recognized by anti-SMHC40 and anti-SMHC29 (data not shown).

**DISCUSSION**

In this communication, we have presented evidence for the presence of two types of smooth muscle MHC isoforms by cDNA cloning and immunoblot analysis using isotype-specific antibodies. The SMHC29 MHC mRNA previously characterized (13) has an extra sequence of 39 nucleotides with a stop codon in its reading frame when compared to SMHC40 cDNA. Thus at the 3’ end of the coding region, the SMHC29 mRNA encodes 9 amino acids following the common coding sequence, whereas SMHC40 mRNA reported in this study encodes 43 amino acids after the point of divergence from SMHC29 mRNA. The fact that all of the 1425 5’-nucleotides and deduced amino acid sequences are identical in both SMHC29 and SMHC40 mRNAs raises the possibility that the two types of smooth muscle MHC mRNA are products of alternative RNA processing of a single gene. Genomic Southern analysis performed with specific probes from the region of divergence of the two cDNA clones (Fig. 1) resulted in identical patterns of hybridization (data not shown).

In our previous report (13), we presented multiple bands after genomic Southern blotting using SMHC29 cDNA as a probe. Although in this report we present a possible alternative splicing mechanism to generate SM1 and SM2 from a single gene, this does not necessarily contradict our previous genomic Southern blotting because MHC molecules in non-
muscle tissues have been found to be highly homologous with smooth muscle MHC (19). The evidence for smooth muscle MHC isoform diversity comparable to that characterized for cardiac and skeletal muscle tissue has been equivocal. Based on SDS-nondenaturing pyrophosphate gel electrophoresis or denaturing polyacrylamide gels, several investigators have reported two or more smooth muscle myosin bands which were present in varying amounts (7-10). Rovner et al. (10) found two myosin bands, SM1 and SM2, on 3.5% polyacrylamide gels, both of which cross-reacted with polyclonal smooth muscle myosin antibody. More recently, Eddinger and Murphy (11) have used limited proteolytic peptide mapping to show that the two myosins have a molecular mass difference of approximately 30 residues or about 3–5 kDa and that the structural difference lies in the light meromyosin fragments. Our analysis shows that the difference of 34 amino acid residues between SMHC40 and SMHC29 MHC isotypes at the carboxyl terminus amounts to a difference in molecular mass equivalent to about 2.6 kDa. In the present study, we predicted that this is indeed the case since antibodies specific for antibodies against SMHC29 and SMHC40 have demonstrated that this is the case since antibodies specific for SMHC40- and SMHC29-type MHCs selectively recognized SM1 and SM2 as examined by immunoblotting. It is presently unknown whether these two types of MHCs are coexpressed in the same smooth muscle cell as hetero- or homodimers or are expressed exclusively in individual cells.

Drosophila melanogaster is the only other species so far to demonstrate alternative splicing of an MHC gene (20–23). In the case of the Drosophila muscle MHC gene, at least three sets of alternatively spliced exons are present in the NH2 terminus generating multiple functional domains of the MHC head (22). In addition, diversity at the carboxyl terminus is produced by alternative splicing of the Drosophila MHC gene (23). It is interesting to note that only Drosophila muscle and vertebrate smooth muscle MHC genes so far demonstrate an alternative splicing mechanism to generate isoform diversity. In smooth muscle MHC, however, the presence of additional splice sites at the 5' end yet remains to be demonstrated. These observations may provide important insights into understanding the evolution and divergence of MHC genes as well as smooth muscle cell contractility.

The functional significance of the two types of smooth muscle MHC carboxyl terminus is presently unknown. Heterogeneity in length and sequence at the carboxyl terminus has been observed for other myosin heavy chain proteins (23–25). Several lines of evidence indicate that differences in the carboxyl terminus may affect assembly of myosin filaments. For example, mutations in the carboxyl terminus of Caenorhabditis elegans unc-54 MHC protein disrupt thick filament assembly (26). In addition phosphorylation of the carboxyl terminus of Acanthamoeba and Dictystelium nonmuscle MHC appear to inhibit thick filament assembly (27, 28). Recently it was shown that the 204-kDa species of smooth muscle MHC protein is phosphorylated, but the site of phosphorylation could not be identified (29). Another interesting example of MHC tail function is seen in molluscan smooth muscles (30) where a "catch" state (prolonged tension development with minimal ATP consumption) is associated with MHC rod phosphorylation by an endogenous kinase. Although these examples of MHC tail functions are in lower animals, our new findings on the presence of two types of smooth muscle MHC isoforms in vertebrates may help to elucidate the regulatory mechanisms of smooth muscle contractility.

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