

Tissue-specific and developmentally regulated alternative splicing of a visceral isoform of smooth muscle myosin heavy chain

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ABSTRACT

Previous work demonstrated that the rabbit smooth muscle myosin heavy chain gene showed sequence divergence at the 25kDa/50kDa junction of the S1 subfragment when compared to chicken gizzard and chicken epithelial nonmuscle myosin. RNase protection analysis with a probe spanning this region detected two partially protected fragments which were not present in RNA from vascular tissue and only found in RNA from visceral tissue. The polymerase chain reaction was used to amplify a 162bp product from primers spanning the putative region of divergence and DNA sequence analysis revealed a seven amino acid insertion not previously detected in other characterised cDNA clones. RNase protection analysis using the PCR product as probe showed that the inserted sequence was expressed exclusively in RNA from visceral tissue. Similar RNA analysis showed that the visceral isoform was not expressed in 20 day fetal rabbit smooth muscle tissues. These results indicated that the new visceral isoform was expressed in a tissue-specific and developmentally regulated manner. Genomic DNA sequencing and mapping of the exon-intron boundaries showed that the visceral isoform was the product of cassette-type alternative splicing. The inclusion of a visceral-specific sequence near the Mg-ATPase domain and at the 25kDa/50kDa junction suggests that the visceral isoform may be important for myosin function in smooth muscle cells.

INTRODUCTION

Previous analysis of protein extracts from smooth muscle tissues (1) and cultured vascular smooth muscle cells (2,3) revealed the presence of two major protein bands designated SM1 (204kDa) and SM2 (200kDa) myosin heavy chain (MHC) based on electrophoretic mobility in SDS-PAGE gels. It was subsequently demonstrated that the SM1 and SM2 isoforms of rabbit (4,5) and rat smooth muscle MHC were the products of alternative splicing of a single gene (6). The SM2 isoform differed from SM1 by an inserted sequence of nine amino acids and an in-frame stop codon at the carboxyl terminus which resulted in a shorter myosin tail. The expression of SM1 and SM2 isoforms in smooth muscle cells was verified using anti-peptide specific antibodies to the unique amino acid sequences predicted from the nucleotide

sequence of the corresponding cDNA clones (5,7). Further work showed that expression of SM1 and SM2 was tissue-specific (5,6), developmentally regulated (8) and altered by culture conditions (7).

Characterisation of the full-length rabbit uterus cDNA encoding smooth muscle MHC showed that the primary structure was more similar to the nonmuscle MHC-A and MHC-B genes than MHCs from striated muscle (9). In the S1 globular head region, rabbit smooth muscle MHC showed approximately 90% identity to the chicken gizzard (10) and chicken epithelial non-muscle MHC-A (11) genes. However, at the junction of the 25kDa/50kDa proteolytic fragments located downstream of the putative Mg-ATPase domain, the amino acid comparison revealed a completely divergent region of 13 amino acids after which the high degree of homology was restored (9). These observations raised the possibility that additional isoforms of smooth muscle MHC may be expressed in smooth muscle cells. The fact that the region of divergence was close to the functionally important ATP binding site suggested that any isoforms in this region may be important in defining the contractile properties of smooth muscle cells.

In order to obtain indirect evidence for the existence of additional isoforms of smooth muscle MHC, we constructed a probe from the area of the S1 head spanning the region of sequence divergence (9). RNase protection analysis of RNA from adult rabbit smooth muscle tissues showed that in addition to the expected fully protected fragment, two partially protected fragments were also observed but only in RNA from visceral tissues. These results strongly supported the idea that the smooth muscle MHC molecule showed heterogeneity in the S1 globular head. In the present report we show directly by RNA-PCR and genomic DNA sequencing that the rabbit smooth muscle MHC gene encodes a visceral isoform which is produced by cassette type alternative splicing. The visceral isoform encodes seven unique amino acids at the boundary of the 25kDa/50kDa junction. Expression of the visceral isoform is tissue-specific and developmentally regulated.

EXPERIMENTAL PROCEDURES

RNA extraction and analysis

Total RNA was isolated from adult and 20 day fetal rabbit (New Zealand White) tissues by the guanidinium thiocyanate procedure (12). The tissues studied included aorta, vena cava, uterus, small

intestine, large intestine, stomach, bladder, brain, heart and lungs. RNA samples were analysed by the RNase protection method described previously (6). The first labelled probe was a 528bp PstI-PvuII restriction fragment derived from cDNA clone PBRUC1 encoding rabbit smooth muscle MHC (9). This probe spanned the 25kDa/50kDa junction of the smooth muscle MHC molecule in the region suspected of showing sequence divergence. The PstI-PvuII fragment was subcloned into pTZ18 (Pharmacia) and linearised with HindIII. The 162bp PCR product (see below) was subcloned into the SmaI site of pBluescript SK (Stratagene) and the plasmid linearised with EcoRI.

RNA – PCR

Total RNA from adult rabbit stomach was used for RNA – PCR. Twenty micrograms of total RNA was used for first strand cDNA synthesis in a mixture containing 1×Taq polymerase buffer (Promega), 1mM dNTPs, 40 Units RNasin (Promega), 100 pmoles downstream primer and 200 Units Superscript (Gibco-BRL). The downstream primer (19mer) derived from cDNA clone PBRUC1 had the sequence 5' CAAGACGGTCAAGAA-TGAC 3' (Oswel DNA Service). The RNA was reverse transcribed at 37°C for 60 minutes and an aliquot taken for amplification. The cDNA sample was amplified in a mixture containing 1×PCR buffer (Promega), 100 pmoles downstream primer, 100 pmoles upstream primer and 0.5 Units Taq polymerase (Promega). The upstream primer (19mer) also derived from PBRUC1 had the sequence 5'GTCATCCAGTA-CCTGGCCG 3'. The product was amplified using 25 cycles of denaturation (30 sec, 95°C), annealing (60 sec, 52°C) and extension (60 sec, 72°C). In addition to the expected amplification product of 140bp equivalent to PBRUC1 (9), a second product of approximately 160bp was also observed following acrylamide gel analysis of the PCR mixture. The DNA was reamplified by a further 25 cycles and the product checked by Southern blotting. The 160bp DNA fragment was then electroeluted from acrylamide, purified by phenol extraction and the ends filled in using Klenow fragment (Gibco-BRL). An aliquot of the blunt ended DNA was then subcloned into the SmaI site of pBluescript SK.

DNA sequencing

Plasmids containing the 160bp amplified DNA insert were sequenced in both directions using Sequenase 2.0 (United States Biochemicals). Southern blotting using the PCR product as probe was used to locate the corresponding region of the gene for genomic DNA sequencing. Mapping of the exon-intron boundaries was verified by a) sequencing from the ends of genomic DNA subclones derived from the original genomic clone λ1-3 (9), b) 5' end-labelling the PCR primers with T4 polynucleotide kinase (Gibco-BRL) in order to sequence in opposite orientations across the exon-intron boundaries and c) using consensus sequences for the donor (GTA...) and acceptor (...CAG) splice sites of introns (13).

cDNA library screening

A rabbit uterus cDNA library (9) constructed in λZAP (Stratagene) was screened with a 265bp EcoRI-PstI restriction fragment from the 5' end of cDNA clone PBRUC1 (9). This 265bp sequence is common to both SM1 and SM2 type MHC isoforms. Plasmid DNA sequencing from the 5' end of cDNA clones longer than 6.0kb was used to identify clones which may contain putative inserted sequences at the 25kDa/50kDa junction.

RESULTS

Evidence for sequence heterogeneity at the smooth muscle MHC 25kDa/50kDa junction

RNase protection analysis using a probe spanning the 25kDa/50kDa junction was used to search for smooth muscle myosin heterogeneity in the S1 subfragment. As expected, the probe detected a fully protected fragment of approximately 530bp, equivalent to the isoform encoded by cDNA clone PBRUC1 (Fig. 1). This isoform (corresponding to SM1 MHC) was expressed in all adult smooth muscle tissues studied and was highly tissue-specific. The same probe, however, detected the presence of two partially protected fragments of approximately 400bp and 130bp which were only present in visceral type smooth muscle tissues (Fig. 1). Longer film exposure failed to reveal the presence of these fragments in RNA from aorta and vena cava samples. The sizes of the partially protected fragments appeared to match with the length of the probe used and the region of divergence at the 25kDa/50kDa junction.

A unique isoform of smooth muscle MHC is expressed in visceral smooth muscle cells

PCR was used to amplify DNA encoding the putative visceral isoform from adult rabbit stomach RNA. Southern blotting was used to identify positive clones which were then sequenced to provide direct evidence for the existence of the new isoform. The 162bp DNA sequence in Figure 2(a) designated PBVISPCR2 was that derived from cloning the amplified DNA. The bracket shows the location of the seven amino acid insertion which is unique to the visceral isoform. The position of the isoform sequence was then mapped to its location in the previously reported genomic clone λ1-3 (9) by Southern blotting. Genomic DNA sequencing confirmed that the sequence was genuine (Fig. 2b). The DNA sequence in Fig. 2b shows the position of

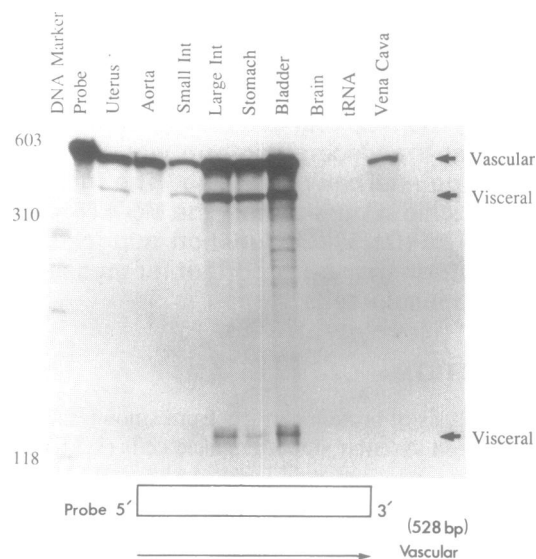


Figure 1. RNase protection analysis of adult rabbit total RNA (15µg) using a ³²P uniformly labeled RNA probe (528bp) spanning the 25kDa/50kDa junction of the smooth muscle MHC molecule. A fully protected fragment is found in all types of smooth muscle tissues and two partially protected fragments are present only in visceral tissues. A signal at 132bp can be detected in uterus and small intestine samples following prolonged film exposure.

the exon encoding the visceral isoform with respect to the immediate upstream and downstream introns containing the consensus sequences for acceptor (cag) and donor (gta) splice sites. A consensus branch point sequence (YNYURAY) for lariat formation is located 25bp upstream of the 3' splice site.

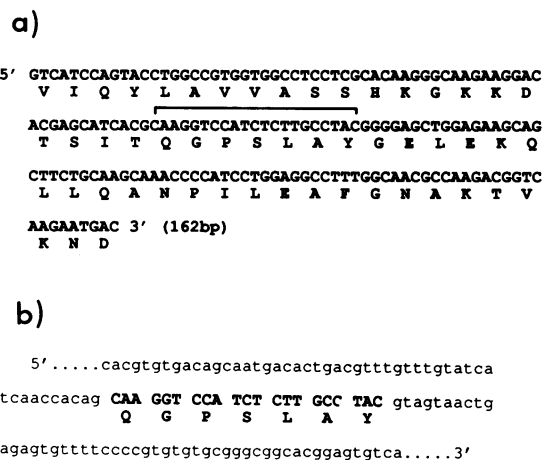


Figure 2. DNA sequence analysis of the visceral isoform of smooth muscle MHC. a) DNA sequence of the mRNA-derived PCR product PBVISPCR2 showing the location (in brackets) of the visceral-specific 21bp insertion encoding seven amino acids. b) DNA sequence of the corresponding region in the rabbit genomic clone λ 1-3, showing 50bp of intervening sequence (lower case letters) immediately upstream and downstream of the visceral-specific exon.

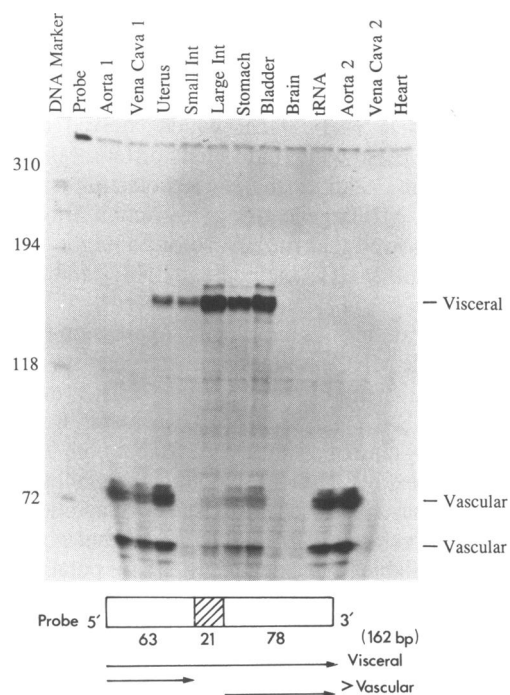


Figure 3. RNase protection analysis of adult rabbit total RNA (15 μ g) using the PCR product PBVISPCR2 as probe. Full protection is shown by the 162bp fragment present only in visceral tissues. Vascular cells do not express the visceral isoform and therefore show two partially protected fragments corresponding to the fully protected fragment shown in Fig. 1.

To check expression of the visceral isoform, the PCR product was used as a probe in RNase protection analysis of RNA from smooth muscle tissues. The results in Fig. 3 confirm that the isoform is only expressed in visceral smooth muscle tissues. The probe fully protects a fragment of 162bp which is only present in visceral smooth muscle. Expression of the isoform appears to be strongest in RNA from large intestine and bladder. No expression was detected in aorta, vena cava and other rabbit tissues. Since the probe in Fig. 3 contains the exon-specific sequence for the visceral form, it can be used to reliably determine the ratio of visceral:vascular mRNAs in smooth muscle tissues. As expected, the probe partially protects two fragments of 63bp and 78bp corresponding to the vascular isoform (PBRUC1) which, although it is detected in all smooth muscle tissues, is only weakly expressed in visceral tissues. The uterus appears to express similar amounts of both isoforms.

To determine whether the visceral-specific exon was incorporated into an MHC molecule associated with either the SM1 or SM2 type MHC isoforms, a cDNA library was screened to isolate cDNA clones encoding the visceral isoform. Thirteen cDNA clones greater than 6.0 kb in length were isolated and shown by DNA sequencing to begin further 5' upstream of the 25kDa/50kDa junction (data not shown). However, Southern blotting using an SM2 isoform-specific 5' end-labelled primer as probe (6) failed to detect the presence of the SM2 specific sequence and indicated that all the new cDNA clones were of the SM1 type. Furthermore, none of the new SM1 type cDNA clones hybridized to a 5' end-labelled primer encoding the visceral-specific isoform. These results therefore suggested that the visceral-specific exon was not associated with the SM1 type MHC. It was also apparent that both the SM2 and visceral-specific MHC isoforms were poorly represented in the rabbit uterus cDNA library, probably reflecting the fact that the ratio of SM1:SM2 in rabbit uterus is approximately 80:20.

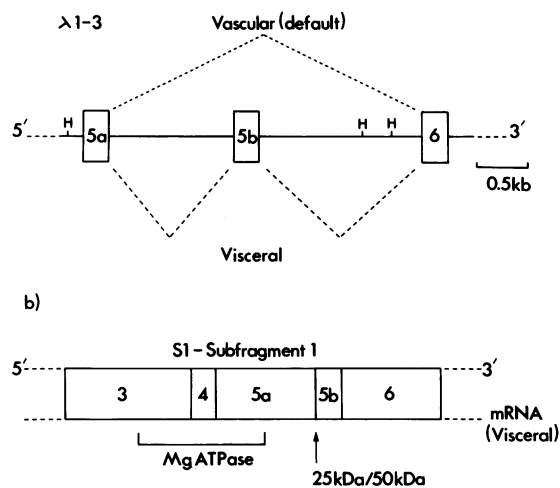


Figure 4. Scheme showing alternative splicing pathway of the visceral isoform. a) Exon-intron organisation of the visceral-specific exon represented by exon 5b in a 4.0kb region of rabbit genomic clone λ 1-3. Exons 5a and 6 are the contiguous exons previously reported for genomic clone λ 1-3 (9). Dotted lines show the likely splicing pathways producing the vascular (constitutive) and visceral-specific isoforms. H = HindIII restriction sites in this region of the genomic clone. b) Organisation of exons in the S1 subfragment of smooth muscle MHC incorporating the Mg-ATPase domain and 25kDa/50kDa junction. The presence of the visceral-specific exon 5b is shown located at the 25kDa/50kDa junction.

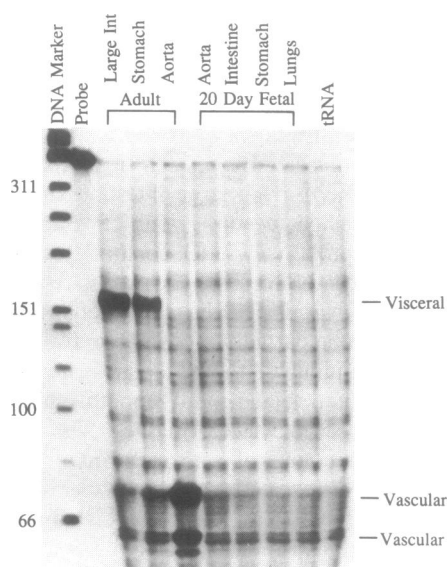


Figure 5. RNase protection analysis of total RNA (15 μ g) from adult rabbit and 20 day fetal rabbit tissues using the visceral isoform probe as shown in Fig. 3. No expression of the visceral isoform can be detected in fetal tissue of this age. (Prolonged film exposure appeared to indicate a very low level of expression in fetal tissue above the level of the tRNA background).

The visceral isoform of smooth muscle MHC is produced by alternative splicing

A combination of Southern blotting and DNA sequencing was used to locate the position of the visceral-specific exon in the corresponding genomic clone. The exon-intron organisation of the visceral isoform is shown in the HindIII partial restriction map in Fig. 4(a). Exons designated number 5a and 6 represent the contiguous exons originally mapped to a 4.0kb region of genomic clone λ 1-3 after comparison with the cDNA clone PBRUC1 (9). Exon 5b represents the visceral isoform mapped to a position between exons 5a and 6. This arrangement of exons suggests that the most likely mechanism of producing the visceral mRNA is by cassette type alternative splicing. The observation that the vascular form is expressed in all types of smooth muscle suggests that the mechanism linking exon 5a to 6 is the preferred splicing pathway.

The diagram in Fig. 4b shows the location of the visceral-specific exon with respect to its neighbouring exons which form the Mg-ATPase domain and 25kDa/50kDa junction as part of the myosin S1 subfragment. This arrangement of exons presumably arises as a result of the positive pressure to select this particular splicing pathway in visceral smooth muscle cells.

Expression of the visceral isoform is developmentally regulated

To further study the factors regulating expression of the visceral isoform, RNase protection analysis was used to measure mRNA transcript levels for the visceral isoform in fetal tissue. The results in Fig. 5 show that when the visceral isoform was used as probe, expression could not be detected in smooth muscle tissues from 20 day old fetal rabbits. No expression could also be detected in 20 day fetal bladder tissue (data not shown). Previously it was shown that SM1 and SM2 isoforms were developmentally regulated in vascular tissue, with the SM1 isoform appearing first

in 25–28 day old fetal rabbits (8). The results of these studies put together confirm that expression of both vascular and visceral isoforms of smooth muscle MHC occurs during late fetal development in the rabbit.

DISCUSSION

The present results provide direct evidence for the existence of a visceral-specific isoform of MHC in rabbit smooth muscle cells. The visceral isoform contains an inserted sequence of seven amino acids located at the 25kDa/50kDa junction of the MHC molecule.

Expression of the visceral isoform is limited exclusively to visceral type smooth muscle tissue thus suggesting a link between expression of the isoform and MHC function in smooth muscle cells. Differences in the contractile properties between vascular and visceral smooth muscle cells are well known (eg 14) but little evidence is available to implicate differences in the MHC molecule as a possible mediator of some of these events. Although the significance of the inserted sequence for MHC function remains to be determined, the location of the small peptide sequence at the 25kDa/50kDa junction indicates that it may be important for folding of the MHC molecule and/or activity in the Mg-ATPase domain. The inserted sequence contains both a serine and tyrosine residue but it is unknown if the MHC molecule is phosphorylated in this region in vivo.

Although the present results suggest that the visceral-specific exon is most likely to be associated with the SM2 type MHC, the functional significance of this arrangement of exons remains to be determined. The production of a visceral-specific anti-peptide antibody should permit a further investigation of its expression in smooth muscle cells. The finding that the smooth muscle MHC gene encodes multiple isoforms suggests that the proteins originally designated as SM1 and SM2 MHC probably represent multiple forms which cannot be separated by one dimensional SDS-PAGE analysis. A similar but more complicated arrangement of alternatively spliced exons was reported for the *Drosophila* muscle MHC (15), which showed that multiple isoforms could exist with only small differences in apparent molecular weight. Additional RNase protection analysis of the smooth muscle MHC gene using restriction fragments from PBRUC1 (9) showed that further isoforms might exist in other regions of the MHC molecule (Babij and Periasamy, unpublished).

The demonstration of tissue-specific expression of an isoform of smooth muscle MHC provides a new mechanism of generating MHC isoform diversity in smooth muscle cells. Previously it was shown that the SM1 and SM2 MHC isoforms were produced by alternative splicing of a single gene (6) but both isoforms were shown to be co-expressed in all smooth muscle cell types (4,5,6). Other workers have proposed the existence of additional isoforms of MHC in smooth muscle cells (16,17,18) but no information is presently available to decide whether these putative forms are encoded by new genes or further alternative splicing mechanisms. The present results lend further support to the idea that alternative splicing is the major mechanism for generating MHC isoform diversity in smooth muscle cells. Furthermore the data suggests that an additional level of complexity characterises regulation of MHC expression in that a positive pressure is required for selection of the visceral splicing pathway (Fig. 4). The observation that the vascular isoform is expressed in all smooth muscle cells suggests that it may represent a constitutive type of splicing pathway which has similarities to the default pattern

shown by other muscle genes. The expression of the 21bp visceral-specific exon only in visceral cells suggests that tissue-specific splicing factors may regulate its non-constitutive inclusion during RNA processing. The existence of putative tissue-specific splicing factors in muscle cells has gained support particularly through work on such genes as α -tropomyosin (19) and troponin T (20) which are known to produce tissue-specific and developmentally regulated isoforms.

Recently it was shown that two inserted sequences of 21 and 10 amino acids were present near the actin binding site and Mg-ATPase domain respectively of the chicken nonmuscle MHC-B gene (21). RNA analysis revealed that both inserted sequences were expressed in a tissue-dependent manner, with expression being restricted to nonmuscle cells of the nervous system. It was suggested that the isoforms were the likely result of alternative splicing although this awaits confirmation. If this is the case, then it suggests that remarkable similarities exist between the vertebrate smooth muscle (9) and nonmuscle MHC genes (11,21) in terms of their ability to generate isoform diversity by the mechanism of alternative splicing. These results therefore support the idea that common evolutionary pathways were involved in the formation of this branch of the MHC gene family.

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