(+)-Insert smooth muscle myosin heavy chain (SM-B) isoform expression in human tissues

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Léguillette, Renaud, Fulvio R. Gil, Nedjma Zitouni, Stéphane Lajoie-Kadoch, Apolinary Sobieszek, and Anne-Marie Lauzon. (+)-Insert smooth muscle myosin heavy chain (SM-B) isoform expression in human tissues. Am J Physiol Cell Physiol 289: C1277–C1285, 2005. First published July 6, 2005; doi:10.1152/ajpcell.00244.2004.—Two smooth muscle myosin heavy chain isoforms differ in their amino terminus by the presence [(+)-insert] or absence [(-)-insert] of a seven-amino-acid insert. Animal studies show that the (+)-insert isoform is predominantly expressed in rapidly contracting phasic muscle and the (−)-insert isoform is mostly found in slowly contracting tonic muscle. The expression of the (+)-insert isoform has never been demonstrated in human smooth muscle. We hypothesized that the (+)-insert isoform is present in humans and that its expression is commensurate with the organ’s functional requirements. We report, for the first time, the sequence of the human (+)-insert isoform and quantification of its expression by real-time PCR and Western blot analysis in a panel of human organs. The (+)-insert isoform mRNA and protein expression levels are significantly greater in small intestine compared with all organs studied except for trachea and are significantly greater in trachea compared with uterus and aorta. To assess the functional significance of this differential myosin isoform expression between organs, we measured the rate of actin filament movement (vmax) when propelled by myosin purified from rat organs, because the rat and human inserts are identical and their remaining sequences show 93% identity. Vmax exhibits a rank correlation from the most tonic to the most phasic organ. The selective expression of the (+)-insert isoform observed among human organs suggests that it is an important determinant of tissue shortening velocity. A differential expression of the (+)-insert isoform could also account for altered contractile properties observed in human pathology.

SMOOTH MUSCLE IS FOUND in all hollow organs of the mammalian organism, and its function ranges from tone maintenance to content propulsion. Many studies point to the smooth muscle myosin heavy chain (SMMHC) as an important element contributing to these diverse contractile properties (see Ref. 16 for review). SMMHC is made up of a globular head, containing an ATPase site and an actin binding domain, and an α-helical tail to which regulatory and essential light chains are bound. SMMHC isoforms are generated by alternative splicing from a single gene (1, 9, 33, 46). Four SMMHC isoforms have been described in various animal species. The first two isoforms identified differ in the carboxy terminus by distinct sequences of 43 (SM1) or 9 (SM2) amino acids (2, 33). The next two isoforms differ in the amino terminus by the presence [(+)-insert] or absence [(-)-insert] of a seven-amino-acid insert in a surface loop above the ATPase site (18, 46). The (+)- and (−)-insert isoforms are also commonly referred to as SM-B and SM-A, respectively. All combinations of these isoforms are possible, i.e., (+)-insert SM1, (+)-insert SM2, (−)-insert SM1, and (−)-insert SM2. No difference in molecular mechanics has been observed between SM1 and SM2, but, as shown with myosin constructs, the sole presence of the amino-terminal insert doubles the actin-activated ATPase activity and the rate of actin filament movement (vmax) in the in vitro motility assay (17, 22, 36). Because of these properties, there is considerable interest in the possibility that the selective expression of the (+)- or (−)-insert isoform could contribute to the wide range of smooth muscle contractile properties.

The expression of the (+)- and (−)-insert SMMHC isoforms is tissue specific (1, 6, 8, 18, 46). Rapidly contracting phasic muscle is predominantly composed of the (+)-insert isoform, whereas slowly contracting, tone-maintaining tonic muscle is mostly composed of the (−)-insert isoform (13, 46). This pattern of expression suggests that the (+)- and (−)-insert isoforms contribute to the different mechanical properties of phasic and tonic muscles. Furthermore, animal model studies have reported alterations in myosin isoform expression during development, pregnancy, and pathologies (1, 7, 12, 27, 30, 37, 39, 45, 47). Significantly, positive correlations between mechanical performance and the presence of the (+)-insert isoform are seen in a hypertrophied rat urinary bladder model (39), in a hypertrophied guinea pig small intestine model (27), and in a rat model of airway hyperresponsiveness (21). A (+)-insert SMMHC knockout mouse (3) confirmed the critical role of the insert in smooth muscle contractility. Notably, we showed (41) alterations in the time course of bronchoconstriction in these knockout mice. Although the presence of the fast (+)-insert isoform and its role in animal models of disease have received enormous attention, its expression in human smooth muscle has never been demonstrated.

In this study, we identified a human (+)-insert SMMHC and sequenced both the (+)- and (−)-insert isoforms. We showed that the human (+)-insert isoform is expressed at both the mRNA and protein levels, in proportion to the organ’s functional requirements. This was independently confirmed in our in vitro motility assay of myosin purified from multiple organs. Thus our study is not only the first report of the presence of the

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(+)-insert SMMHC isoform in humans but also provides essential information for our understanding of human smooth muscle function in normal and pathological conditions.

METHODS

Sequencing of Human SMMHC Insert cDNA

To determine the sequence of the human SMMHC insert, the region hypothesically coding for the insert was amplified by conventional PCR and the products were sequenced. Briefly, 1 µg of human bladder total RNA (BD Clontech), in a total reaction volume of 20 µl, was reverse transcribed with oligo(dT)12–18 primer, Superscript II, and RNAguard ribonuclease inhibitor (Amersham Pharmacia). Primers flanking the hypothetical region containing the code for the insert were designed from human (−)-insert SMMHC (GenBank accession no. NM-002474): sense primer 5′-CCGAAAACCAAAGAAGGTC-3′ (nucleotides 642–661), antisense primer 5′-GTTGGCTC-CCACGATGTAC-3′ (nucleotides 849–868). These primers amplified both myosin isoforms [(+) and (−)-insert]. It should be noted that we designed these primers to bind to two different exons, separated by two large introns (4 and 3.5 kb). This strategy rendered amplification of genomic DNA unlikely while making it easily noticeable by the large difference in size of amplicons. The PCR mixture consisted of 1.5 mM MgCl2, 10× PCR buffer (Invitrogen), dNTPs each at 0.2 mM, 2 units of Platinum Taq DNA polymerase (Invitrogen), sense and antisense primers each at 0.4 µM, and 1 µl of cDNA. The samples were amplified in a Programmable Thermal Controller (PTC-100; MJ Research, Watertown, MA) for 40 cycles (45 s of denaturation at 94°C, 45 s of annealing at 58°C, 45 s of extension at 68°C) and 8-min final extension at 68°C. The PCR amplification products were resolved on 0.8% TAE-agarose gel electrophoresis and visualized by ethidium bromide. The two amplicons obtained were purified (Qiagen QIAquick kit) and ligated into pGEM-T Easy (Promega) according to the manufacturer’s instructions. Ligation products were transformed into DH5α-competent cells. Plasmids were extracted with a miniprep kit (Qiagen). Plasmids were sequenced commercially to verify their identity and integrity.

Sequencing of Complete SMMHC cDNA

To determine the sequence of the complete SMMHC isoform, the cDNA generated as above was amplified in four segments (≈1.8 kb each) by conventional PCR, and the products were sequenced. The entire (+)-insert SMMHC transcript was amplified, and the product was then used in nested and heminested PCR to yield four overlapping segments. The primers were designed from human (−)-insert SMMHC (GenBank accession no. NM-002474): sense primer 5′-CCGAAAACCAAAGAAGGTC-3′ (nucleotides 642–661), antisense primer 5′-GTTGGCTC-CCACGATGTAC-3′ (nucleotides 849–868). These primers amplified both myosin isoforms [(+) and (−)-insert]. It should be noted that we designed these primers to bind to two different exons, separated by two large introns (4 and 3.5 kb). This strategy rendered amplification of genomic DNA unlikely while making it easily noticeable by the large difference in size of amplicons. The PCR mixture consisted of 1.5 mM MgCl2, 10× PCR buffer (Invitrogen), dNTPs each at 0.2 mM, 2 units of Platinum Taq DNA polymerase (Invitrogen), sense and antisense primers each at 0.4 µM, and 1 µl of cDNA. The samples were amplified in a Programmable Thermal Controller (PTC-100; MJ Research, Watertown, MA) for 40 cycles (45 s of denaturation at 94°C, 45 s of annealing at 58°C, 45 s of extension at 68°C) and 8-min final extension at 68°C. The PCR amplification products were resolved on 0.8% TAE-agarose gel electrophoresis and visualized by ethidium bromide. The two amplicons obtained were purified (Qiagen QIAquick kit) and ligated into pGEM-T Easy (Promega) according to the manufacturer’s instructions. Ligation products were transformed into DH5α-competent cells. Plasmids were extracted with a miniprep kit (Qiagen). Plasmids were sequenced commercially to verify their identity and integrity.

Quantitative Real-Time PCR Analysis

To quantify human tissue total SMMHC as well as the (+) and (−)-insert SMMHC isoforms, human total RNA was obtained commercially (BD Clontech) for the following tissues: skeletal muscle, bladder, aorta, heart, trachea, lung parenchyma, stomach, small intestine, uterus, placenta, testis, kidney, spleen, thyroid, salivary glands, spinal cord, brain, bone marrow, fetal liver, and fetal brain. The company pooled the RNA from 5–59 Caucasian subjects (depending on the organ) aged from 15 to 75 yr, with the exception of fetal tissues, which were pooled from 22- to 40-wk-old fetuses. The company also assessed the RNA by electrophoresis on denaturing gel and certified its quality and quantity. One microgram of RNA from each of these tissues was reverse transcribed simultaneously, to minimize variability, in a total reaction volume of 20 µl. Total SMMHC was first quantified in absolute terms, using standard curve analysis to determine which organs contained sufficient smooth muscle to pursue a relevant investigation of the (+) and (−)-insert isoforms. Quantification of splice variants in absolute terms is, however, very difficult because of the additive errors introduced in generating two reliable standard curves for two sets of primers (42). The (+) and (−)-insert isoforms were therefore quantified in relative terms for the selected organs, using a previously validated algorithm (24–26) that expresses the content of each isoform normalized to total SMMHC, with respect to a calibrator organ. The calibrator organ was chosen to be the one that contained the greatest amount of each isoform. Primers for total SMMHC were designed by targeting a region common to all SMMHC isoforms as follows: total SMMHC sense primer 5′-GCCCTAGGCCTGAAGTCA-3′ (nucleotides 2904–2923) and antisense primer 5′-GGAGGATGAGTTCCTG-GTC-3′ (nucleotides 3036–3055). Because total SMMHC was quantified in absolute terms, we generated standard curves as follows. Total SMMHC PCR products were extracted from several amplicons and pooled. cDNA concentration was measured by spectrophotometry and was considered accurate only when the optical density was >0.1 (relative units). Ten dilutions were then assayed by real-time PCR, and the best five contiguous dilutions were chosen for the standard curve. For the (+) and (−)-insert SMMHC isoform relative quantification, primers were designed with the insert sequence determined above and the human (−)-insert SMMHC sequence (GenBank accession no. NM-002474). The 21-bp insert sequence itself served as the (+)-insert sense primer. The (−)-insert sense primer spanned the insert junction. The sequences of the primers were as follows: (+)-insert isoform: sense primer 5′-CAAGGCCCATCTTTGCTGACT-3′ (nucleotides 703–723) and antisense primer 5′-GTGGGTCTCCACGAT-GTAC-3′ (nucleotides 849–868); (−)-insert isoform: sense primer 5′-CAAGAAGACACAGTGATCCAGG-3′ (nucleotides 700–722) and antisense primer same as for (+)-insert isoform. It should be noted that, to keep efficiencies close, the primers were designed to obtain amplicons of similar length (1-bp difference) and the same antisense primer was used. PCR reactions were performed in a volume of 20 µl containing 1 µl of cDNA, 10 µl of 2× QuantiTect SYBR Green PCR (Qiagen), 7 µl of nuclelease-free H2O, and 1 µl of both the forward and reverse primers (final concentration 0.1 µmol each). The samples were amplified in a LightCycler system (Roche Diagnostics). The real-time PCR conditions consisted of a denaturation step of 15 min at 95°C, followed by an amplification of 45 cycles (denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and extension at 72°C for 20 s) and 1 melting curve cycle. PCR reactions were performed in triplicate, i.e., three repeats on the same RNA samples pooled from several subjects. Each primer set generated only one PCR product, and the identity and integrity of these products were confirmed by commercial sequencing. PCR reaction efficiencies were calculated for each reaction, following a previously described method (24, 25), and were used in the quantitative analysis that was performed with LightCycler software (version 3.5). The results are reported as means ± SE.

(+)insert and Total SMMHC Semiquantitative Protein Analysis

Western blot analysis was performed to determine the (+)-insert SMMHC isoform and total SMMHC protein expression in human and rat tissues.

Human. Human small intestine, stomach, trachea, bladder, uterus, aorta, and brain (negative control) protein medley were obtained from BD Biosciences (Clontech). The company pooled the proteins from 16–49 Caucasian subjects (depending on the organ) aged from 16 to

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65 yr. Electrophoresis was done through 6% SDS polyacrylamide gel with a Laemmli buffer system. Protein concentration was determined by a standard Bradford assay. For detection of total SMMHC 60 μg of protein was loaded on the gel, whereas for the (+) insert isoform gels were loaded with either 100 or 160 μg. Proteins were then electroblotted onto nitrocellulose membrane (Bio-Rad). The membrane was probed with the polyclonal (+) insert antibody (kindly provided by Dr. A. Rovner, University of Vermont, Burlington, VT), which specifically recognizes the seven-amino acid insert GQPSFAY (47), or with the polyclonal BT-562 (Biomedical Technologies), which recognizes all SMMHC isoforms. Antibody detection was done by enhanced chemiluminescence (Amersham). Six Western blot repeats were done on the same protein samples pooled from several subjects. Because different organs contain different amounts of muscle, it is important to normalize the myosin isoform content to the total amount of myosin in a given organ. However, to overcome the inevitable differences in affinities of the antibodies for the (+) insert isoform and total myosin, we normalized the densitometric values with respect to a calibrator organ in the following way:

\[
\text{(+ insert content} = \frac{\text{(+ insert in target organ}}{\text{total myosin in target organ}} / \frac{\text{(+ insert in reference organ}}{\text{total myosin in reference organ}}
\]

The results are reported as means ± SE.

Rat. Four Lewis female rats, ~16 wk of age, were purchased from Harlan. All experimental protocols involving animals were approved by the McGill University Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care. Animals were killed with an overdose of pentobarbital sodium (100 mg/kg ip). The small intestine, stomach, trachea, bladder, uterus, and aorta were harvested, powdered in liquid nitrogen, and homogenized at 4°C in sodium pyrophosphate extraction buffer (in mM: 10 sodium pyrophosphate, 3 MgCl₂, and 2 DTT, with 0.3 M KCl; pH 6.8). Electrophoresis was performed as above except that 50 μg of total protein was loaded onto the gel. Organs from the four rats were pooled, and measurements were repeated three times. The quantification was performed as described above for the human Western blots.

A) (+) and (−) SMMHC in Vitro Rate of Movement

To quantify at the molecular level the differences in mechanics between tonic and phasic smooth muscle, we measured with the in vitro motility assay the rate of actin filament movement (v\text{max}) when propelled by smooth muscle myosin purified from rat organs. Sixteen Lewis rats were killed as described above, and their small intestine, stomach, trachea, bladder, uterus, and aorta were rapidly harvested and frozen in liquid nitrogen. (Note that the motility of stomach myosin was not assessed because of the difficulty in purifying functional myosin from that organ.) Myosin extraction was carried out in folding conditions (90 mM KCl, 2 mM Na₂ATP), followed by filamentation at 50 mM MgCl₂. Separation of myosin from actin was performed by ammonium sulfate fractionation. Myosin fractions were then dissolved and dialyzed against low-salt buffer (40 mM KCl, 25 KCl, 25 imidazole, 1 EGTA, 4 MgCl₂, and 25 DTT; pH 7.4). The myosin in solution was flushed through a chamber (44) and allowed to attach randomly onto the nitrocellulose-coated glass. Inactive myosin heads were further inhibited by adding monomeric unlabeled actin in actin buffer. Labeled actin filaments in actin buffer were then added to the chamber, followed by motility buffer (in mM: 2 MgATP, 25 KCl, 25 imidazole, 1 EGTA, 4 MgCl₂, and 25 DTT, with 0.5% methycellulose and 0.3 M KCl; pH 6.8). Measurements were made at 30°C. Actin movement was recorded by a SIT camera, digitized, and analyzed (Scion Image software). The v\text{max} measurements were repeated five times with myosin pooled from each of the various organs from 16 rats; v\text{max} is reported in micrometers per second (±SD).

B) Statistical Analysis

Statistical analysis of the replicated measurements on the pooled samples was performed as follows. Differences between samples of total SMMHC cDNA, (+) insert isoform cDNA and proteins, (−) insert isoform cDNA, and v\text{max} were tested with one-way ANOVAs, followed by tests for normal and rank correlations. Differences between human and rat (+) insert protein expression were tested by a two-way ANOVA.

C) Results

A) Sequencing Human SMMHC Insert cDNA

The region of human SMMHC mRNA hypothetically coding for the seven-amino acid insert was identified by cDNA sequence comparisons with other species. Amplification of that region from human bladder cDNA was performed, and two distinct products were obtained (Fig. 1A). Sequencing of the clones derived from these amplicons confirmed that the two products differed by 21 nucleotides. Comparisons with other species’ sequences showed that the cDNA amplified was indeed the region coding for the inserted surface loop (Fig. 1B). Interestingly, the human and rat nucleotide sequences coding

### Fig. 1: A: PCR amplification products from cDNA derived from human bladder RNA. The primers were designed to amplify both the (+) and (−) insert smooth muscle myosin heavy chain (SMMHC) isoforms. Results presented in duplicate. B: Nucleotide sequence encoding for the (+) and (−) insert amino acid sequence in human, rat, mouse, chicken, and rabbit mRNA. The 21-bp sequences are identical in the human and rat but differ by 1 bp in the mouse, 2 bp in the rabbit, and 3 bp in the chicken.
because the primers targeted a region not subject to alternative splicing. SMMHC cDNA was found in all tissues studied (Fig. 2). Tissues containing very low amounts of total SMMHC (<2 × 10⁻⁵ ng/μg RNA) were not analyzed any further because such a low content was presumably a reflection of the ubiquitous vascular smooth muscle and not of the organ itself. The (+) and (−)insert SMMHC isoform analysis was therefore pursued only for the bladder, aorta, small intestine, stomach, uterus, and trachea. Relative quantification was performed by normalizing the content of each isoform with respect to total SMMHC, a good marker of total smooth muscle content in each tissue, and with respect to a calibrator organ. The calibrator organ for a given isoform was chosen to be the tissue that contains the greatest amount of that isoform. The small intestine is the organ that contains the greatest amount of (+)insert isoform, so it was set to 100% (Fig. 3). All other organs express significantly less (+)insert cDNA than the small intestine [stomach expresses 58 ± 4% (+)insert cDNA with respect to the small intestine and normalized to total SMMHC, trachea 19 ± 3%, bladder 10 ± 2%, uterus 5 ± 2%, and aorta 0 ± 0%; P < 0.05; Fig. 3]. Statistically significant differences are also found between the stomach and all other organs and between the trachea and aorta or uterus (P < 0.05; Fig. 3). The organ that contains the greatest amount of (−)insert isoform is the uterus, which was set to 100% (Fig. 4). Thus, with respect to the uterus and normalized to total SMMHC, the stomach (99 ± 15%) and aorta (98 ± 13%) express significantly more of the (−)insert isoform than the bladder (56 ± 13%) and small intestine (53 ± 11%) (P < 0.05; Fig. 4). The trachea expresses an intermediate level of (−)insert isoform (72 ± 9%). Because comparisons among different sets of primers is invalid and because the reference organs are not the same for the (+) and (−)insert isoforms, Figs. 3 and 4 should not be directly compared.

(+)-Insert Protein Analysis in Human Tissues

The expression of the (+)insert SMMHC isoform was also determined at the protein level by Western blot analysis re-
peated six times on commercial samples pooled from several subjects. The (+)insert SMMHC isoform protein is expressed in all human tissues studied but is not detected in the brain (negative control) (Fig. 5A). The small intestine has the greatest (+)insert isoform protein expression and so was set to 100%. The results were therefore expressed with respect to the content in the small intestine and normalized to total SMMHC (Fig. 5B). All organs except the trachea (74.0 ± 16.7%) show statistically less (+)insert protein expression than the small intestine; the stomach expresses 47.7 ± 12.9% of the (+)insert protein in the small intestine, the bladder 42.7 ± 6.3%, the uterus 36.6 ± 8.4%, and the aorta 33.0 ± 8.3% (P < 0.05; Fig. 5B). Interestingly, the trachea also shows statistically more (+)insert than the aorta and the uterus (P < 0.05; Fig. 5B).

(+)-Insert Protein Analysis in Rat Tissues

The expression of the (+)insert SMMHC isoform was also determined at the protein level in samples pooled from four rats by Western blot analysis performed in triplicate (Fig. 6A). A semiquantitative analysis was performed as for human (+)insert protein (Fig. 6B). No significant differences in the pattern of expression were observed between the rat and human organs (P = 0.45).

(+)- and (-)-Insert SMMHC Rate of Movement

To assess at the molecular level the physiological significance of the differential expression of the (+)- and (-)-insert isoforms, we used the in vitro motility assay to measure $v_{\text{max}}$ for actin filaments propelled by myosin molecules purified from rat organs. Rat myosin was used as a model for human because the insert sequence is identical, the rest of the myosin sequence is 93% identical, and its expression of the (+)insert isoform is similar to human among organs (see Figs. 5 and 6). Measurements of $v_{\text{max}}$ were repeated five times with myosin pooled from 16 rat organs. $v_{\text{max}}$ was significantly different between all organs: small intestine (0.64 ± 0.01 μm/s), bladder (0.58 ± 0.03 μm/s), trachea (0.44 ± 0.01 μm/s), uterus (0.35 ± 0.01 μm/s), and aorta (0.23 ± 0.01 μm/s) (P < 0.05; Fig. 7A). We expected a linear regression between (+)-insert protein expression and $v_{\text{max}}$, but because we tested only a selected number of organs we have very few points, and thus the test for r = 0 is not significant. However, from the most tonic to the most phasic organ there is a rank correlation that is almost perfect between the (+)-insert protein expression and $v_{\text{max}}$, and a test with Kendall K is significant at the critical level of 0.05 (Fig. 7B).

DISCUSSION

In this study we demonstrated, for the first time, that the (+)-insert SMMHC isoform is expressed in human smooth muscle at the mRNA and protein levels. We also performed quantitative real-time PCR and semiquantitative protein analyses that showed differential expression of the (+)- and (-)-insert isoforms between human organs, suggesting a role for these isoforms in determining the rate of shortening of phasic and tonic smooth muscle. Furthermore, we showed that rat myosin is a good model of human myosin. Finally, using the in vitro motility assay, we observed a greater $v_{\text{max}}$ for myosin purified from phasic than from tonic rat smooth muscle, which correlated with the (+)-insert isoform expression.
Mapping Complete (+)Insert SMMHC on Human Genome

Using the cDNA sequence obtained for the complete (+)insert SMMHC isoform, we used the University of California-Santa Cruz’s BLAST tool (19) and identified 42 exons spanning 6.9 kb on the reverse strand of human chromosome 16. The 21-bp sequence encoding the seven-amino acid insert, starting and ending at nucleotides 15845014 and 15844994, constitutes a complete exon in itself. By analogy with previous publications in other species (3, 4) we called this exon 5b, because it is located between exons 5 and 6 (Fig. 8).

Exon 5b is flanked by the classic splice donor and acceptor sites AG and GT, respectively (Fig. 8). The expression of the (+) insert isoform results from alternative splicing of exon 5b (1, 46).

(+)-Insert Isoform and Contractile Properties

SMMHC was detected in all tissues studied. Because real-time PCR is exquisitely sensitive (5), it is likely that myosin from the vasculature of an examined organ is also detected, thereby explaining the weak positive signal in noncontractile organs. This could explain why mRNA coding for the (+) insert SMMHC was found in all human tissues tested except for the bone marrow. It was shown previously that the (+)insert SMMHC is the dominant isoform in rat and rabbit small vessels and arterioles (6, 15, 45), and blood vessels could also explain the signal detected for this isoform in all human tissues (Fig. 7).

(+)-Insert Isoform

The tail of human SMMHC is also subject to alternative splicing, yielding the SM1 and SM2 isoforms. Our sequencing results confirmed that the SM1 and SM2 isoforms only differ in their tail region, with the rest of the sequence remaining the same. In agreement with a previous report (33), nucleotide sequence analysis also revealed that a 39-bp exon that is spliced into the myosin sequence encodes a stop codon that prematurely arrests translation, thus generating the SM2 isoform. Furthermore, this short exon (39 bp) is located on chromosome 16 between exons 40 and 42 (between nucleotides 15769099 and 15769137) and was called exon 41 (Fig. 8).

(+)-Insert Isoform

Fig. 7. A: rate of actin filament movement in the in vitro motility assay: rate of actin filament movement ($V_{max}$) when propelled by myosin purified from rat aorta, uterus, bladder, trachea, and small intestine (means ± SE). $V_{max}$ measurements were repeated 5 times with myosin pooled from 16 rat organs. Statistical differences were found between all organs ($P < 0.05$). B: $V_{max}$ vs. (+)-insert protein expression. A significant rank correlation was observed ($P < 0.05$).

Fig. 6. A: Western blot of total rat SMMHC and (+)-insert isoform protein in the aorta, uterus, bladder, trachea, stomach, and small intestine. Fifty micrograms of total protein was loaded onto the gel. B: rat (+)-insert isoform protein analysis: relative quantification of the rat (+)-insert SMMHC isoform with respect to the small intestine and normalized to total SMMHC (means ± SE). The Western blots were performed in triplicate on samples pooled from 4 rats. The pattern of expression of the (+)-insert isoform was not different from that in human ($P = 0.45$).

Mapping Complete (+)-Insert SMMHC on Human Genome

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tissues. Our data showed, as expected, that the greatest amount of SMMHC was found in contractile organs (Fig. 2). Correspondingly, only the organs that showed the greatest SMMHC mRNA expression were followed up at the protein level. The main purpose of this analysis was to verify whether or not the (+)insert isoform was also expressed at the protein level. This was confirmed, and a relative quantification of the (+)insert SMMHC isoform protein was carried out.

The (+)insert isoform mRNA and protein expression levels were significantly greater in the small intestine compared with all other organs studied except for the trachea and were significantly greater in the trachea compared with the uterus and the aorta. This myosin isoform distribution in human smooth muscle is in general agreement with the rate of shortening assigned to phasic and tonic smooth muscles in previous animal studies, i.e., rapidly contracting phasic muscle containing mostly the (+)insert isoform, with the (−)insert isoform being predominantly present in slowly contracting tonic muscle. Correspondingly, the small intestine is described in the guinea pig as a typical phasic muscle that contracts rapidly to propel its content (27). The rabbit stomach has two distinct regions in which the contractile function correlates with the myosin isoform content: the antrum, which contains mostly the (+)insert isoform, and the fundus, which contains mostly the (−)insert isoform (8). The total RNA we purchased came from different organs from the same animals. Our first study addressing the molecular mechanics of myosin isoform expression and by alternative splicing is still poorly understood, a reasonable correlation was observed between the mRNA and protein data (Figs. 3 and 5), suggesting minor posttranscriptional regulation. Furthermore, our quantitative mRNA data suggest that tonic smooth muscle (uterus, aorta) is characterized by its lack of (+)insert isoform. Together with our in vitro motility data that demonstrate a rank correlation between the (+)insert isoform expression and υmax, this organ’s specific (+)insert myosin isoform expression strongly suggests a role for this isoform in determining the rate of shortening of the whole muscle or even the whole organ. To our knowledge, this is the first study addressing the molecular mechanics of myosin purified from different organs from the same animals. Our conclusions therefore reflect the effects of the seven-amino acid insert and not other potential interspecies variability in the SMMHC sequence.

**Effects of Insert on Contractility**

Although the kinetic properties of smooth muscle are partially determined by the regulatory pathways leading to phosphorylation of the regulatory light chains, the differences in contraction kinetics between tonic and phasic smooth muscle remain even after complete phosphorylation, achieved either with ATPγS or ATP plus okadaic acid (14). Thus it is likely that the acto-myosin complex plays an important role in conferring the kinetic differences between tonic and phasic smooth muscle. Indeed, in the present study we measured, with the in vitro motility assay, υmax when actin was propelled by fully...
phosphorylated myosin purified from phasic and tonic muscle. The phasic muscle contained up to 11 times more of the (+) insert isoform than the tonic muscle (Fig. 6) and propelled actin with an ~3-fold greater \( v_{\text{max}} \). Previous studies also measured \( v_{\text{max}} \) in baculovirus-expressed constructs differing only by the seven-amino acid insert, and similar results were reported (22, 36). Something intrinsic to the insert therefore alters the kinetics of muscle contraction, and evidence is accumulating to suggest that the insert affects the release of MgADP (11, 20, 22, 40), which is the rate-limiting step in shortening velocity (38). Tuning of the myosin molecule to alter the release of MgADP is therefore an attractive mechanism to explain the differences in smooth muscle rate of shortening. Indeed, Somlyo and coworkers (11, 20) showed, at the tissue level, a greater affinity for MgADP of tonic rabbit femoral artery than phasic rabbit bladder and guinea pig portal vein. Using the laser trap at saturating MgATP levels, we showed a twofold faster rate of MgADP release for (+) insert isoform constructs (22). Furthermore, we recently found (23) a greater affinity for MgADP of purified tonic calf aorta myosin compared with phasic chicken gizzard myosin in the in vitro motility assay. This is perhaps not surprising, because the seven-amino acid insert is strategically located in surface loop 1, right above the nucleotide binding pocket, potentially altering the MgADP binding site.

According to our in vitro motility measurements, as well as those from previous studies (18, 22, 36), the expression of the (+) insert SMMHC isoform can only explain a two- to three-fold difference in rate of shortening. Considering that, at the tissue level, greater differences in rate of shortening have been reported between tonic and phasic smooth muscles (32), other mechanisms must also contribute to the kinetics of the whole muscle. The three-dimensional arrangement of the myosin filaments and their interactions with regulatory and other contractile proteins are all likely to contribute to the rate of smooth muscle shortening. These factors will require further investigation.

(+) Insert SMMHC Isoform in Pathology

Alterations of (+) insert SMMHC isoform expression have been studied in many models of pathology. Expression of the (+) insert SMMHC isoform decreases by 50% in the hypertrophied rat urinary bladder, whereas the maximal shortening velocity and rate of force development decrease by 20% and 25%, respectively (39). The maximal shortening velocity of the guinea pig hypertrophic small intestine decreases by 50% and is accompanied by a significant decrease in (+) insert SMMHC isoform expression and an increased MgADP sensitivity (27). An 11-fold greater expression of the (+) insert SMMHC isoform is measured in the hyperresponsive rat of a genetic model of asthma (21). The rate of contraction of explanted airways was also shown to be greater in these hyperresponsive animals (43). Other studies have also shown alterations in (+) insert SMMHC isoform expression in pregnancy or in models of diseases, although the correlation to muscle function is not as clear (30, 37). The results of our study, demonstrating the presence of the (+) insert SMMHC isoform in human organs, are therefore timely in supporting the increasing interest in the investigation of the expression and function of the (+) insert SMMHC isoform in various models of human pathology.

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