Expression of Smooth Muscle Myosin Heavy Chain Isoforms in Asthma and their Molecular Mechanics

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To the student who lost himself at the library and found this thesis.

To patients with asthma.

To the rats I killed for Science.

Acknowledgments:

First and foremost, I would like to thank my supervisor Dr. Anne-Marie Lauzon. She accepted me into her laboratory in spite of the fact that I had almost never handled a laboratory pipet. She gave me invaluable guidance and was an excellent supervisor. I particularly appreciated her extraordinary availability, her kindness and her support in my work.

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Table of content:

Abstract:

Two smooth muscle (SM) myosin heavy chain isoforms, generated by alternative mRNA splicing, differ by the presence (SM-B) or absence (SM-A) of a 7 amino acid insert in the motor domain. The rate of actin filament propulsion (v_{max}) of SM-B, as measured in the *in vitro* motility assay, is 2-fold greater than that of SM-A. I investigated the expression and function of these isoforms in healthy SM and in asthma.

First, I determined the sequence of the SM-B isoform in human SM and quantified its expression at the mRNA and protein levels in several human organs. The SM-B isoform was mostly expressed in rapidly contracting phasic SM. I then purified myosin from multiple rat organs and found a rank correlation between SM-B content and v_{max}.

I then quantified the expression of SM-B and several other contractile protein genes in endobronchial biopsies from normal and asthmatic subjects. SM-B, myosin light chain kinase (MLCK), which is responsible for myosin activation, and transgelin, a ubiquitously expressed actin binding protein but whose function is unknown, were overexpressed in the asthmatic biopsies. The increased SM-B expression and myosin activation, due to the increased MLCK expression, both contribute to the increased rate of shortening of the asthmatic airway SM. In addition, I showed that beyond its enzymatic effects, MLCK mechanically enhances v_{max}. The binding of SM22 to actin, however, did not alter v_{max}.

Finally, I addressed the mechanisms behind the unique capacity of SM to maintain force at low energy cost, namely the latch-state. This property is mostly observed in SM-A containing, tonic muscle. Using a laser trap, I measured the binding force of unphosphorylated (non-active) SM-A and SM-B myosin isoforms and found that they can both attach to actin and maintain force. I also measured v_{max} at different MgADP concentrations and found that SM-A has a greater affinity for MgADP. Because MgADP must be released before myosin can detach from actin, these results suggest that the SM-A isoform remains attached longer to actin, allowing it to get into the latch-state. These findings explain the greater propensity of tonic muscle to get into the latch-state.

Résumé:

La chaîne lourde de myosine du muscle lisse existe sous deux isoformes qui sont générés par épissage alternatif de l'ARNm, et qui sont seulement différents par la présence (SM-B), ou l'absence (SM-A) de 7 acides aminés dans la région de la tête de la molécule. SM-B peut propulser des filaments d'actine (vmax) dans un essai de motilité *in vitro* deux fois plus vite que SM-A. J'ai donc étudié l'expression et la fonction de ces isoformes dans les tissues de muscle lisses sains et dans l'asthme.

J'ai tout d'abord identifié la séquence de l'isoforme SM-B chez l'Humain, puis j'ai quantifié son expression dans différents organes humains au niveau de l'ARNm et des protéines. J'ai observé que l'isoforme SM-B est principalement exprimé dans les muscles lisses rapides dits phasiques. J'ai ensuite purifié la myosine de plusieurs organes de rats et j'ai trouvé que vmax augmentait de façon croissante et corrélative avec le contenu du muscle lisse en SM-B.

De plus, j'ai mesuré l'expression de gènes codant pour SM-B ainsi que pour d'autres protéines contractiles du muscle lisse dans des biopsies endobronchiales de patients normaux et asthmatiques. Le SM-B, la kinase des chaînes légères de myosine (MLCK), ainsi que la transgeline (SM-22), sont surexprimées dans les biopsies des patients asthmatiques. La MLCK ayant pour rôle d'activer la myosine, l'augmentation de SM-B combinée avec celle de la MLCK contribuent à augmenter la vitesse de contraction du muscle lisse décrite chez les asthmatiques. De plus, j'ai aussi montré que la MLCK pouvait accélérer v_{max} par un mécanisme supplémentaire et indépendant de la phosphorylation des chaînes légères de myosine. Nous avons aussi trouvé que la SM-22 ne change pas v_{max}, malgré qu'elle s'attache à l'actine; son rôle exact reste donc inconnu.

Enfin, vu que le muscle lisse a la capacité unique de maintenir une force élevée tout en consommant peu d'ATP (propriété appelée 'latch-state'), j'ai étudié les propriétés mécaniques permettant de comprendre le latch-state au niveau moléculaire. Ce phénomène est surtout observé dans les muscles lisses lents, dit toniques, qui contiennent surtout l'isoforme de myosine SM-A. J'ai utilisé une trappe au laser pour mesurer les forces d'attachement des isoformes de myosine SM-A et SM-B non phosphorylés (non-

activés) avec des filaments d'actine. J'ai trouvé que les deux isoformes de myosine non phosphorylée peuvent s'attacher aux filaments d'actine et qu'ils peuvent maintenir une force similaire. J'ai aussi mesuré v_{max} lors de concentrations croissantes d'ADP, et j'ai ainsi montré que SM-A a une plus grande affinité pour l'ADP. Vu que la relâche d'ADP est le facteur limitant avant que la myosine ne se détache de l'actine, mes résultats suggèrent que l'isoforme SM-A reste attache plus longtemps, lui laissant plus d'opportunité pour être dephosphorylé alors qu'il est encore attaché à l'actine. Ces résultats expliquent la plus grande tendance du muscle lisse tonique à entrer en état de latch-state.

Contribution of authors

Chapter 2 consists of a manuscript that has been published and is reproduced with permission from the publisher: "(+)Insert (SMB) Smooth Muscle Myosin Heavy Chain Isoform Expression in Human Tissues ", by Renaud Léguillette, Fulvio R. Gil, Nedjma Zitouni, Stéphane Lajoie-Kadoch, Apolinary Sobieszek, Anne-Marie Lauzon, from American Journal of Physiology, Cell physiology (2005) vol. 289, 5:pC1277-1285.

This study was performed by the first author (Renaud Léguillette) under the supervision of Dr. Anne-Marie Lauzon (Department of Physiology, Meakins-Christie Laboratories, McGill University). Fulvio R. Gil was a Masters student under the supervision of Dr. Lauzon. He generated the PCR products to sequence most of the smooth muscle myosin heavy chain SM-B isoform and assembled the sequence of the gene. Dr. Nedjma Zitouni is the research assistant in Dr Lauzon's laboratory and purified the functional myosin from the tissues. She also performed the Western blots to assess the SM-B content in the human tissues. Stéphane Lajoie-Kadoch is a PhD student from Dr. Hamid's group, at the Meakins-Christie Laboratories, who tought me the PCR, real-time PCR, cloning and sequencing techniques. Dr. Apolinary Sobieszek is a renowned biochemist from the Institute for Biomedical Aging Research, Austrian Academy of Sciences, who helped us troubleshoot the myosin purification technique from small organs. I (R. Léguillette) performed (+)insert myosin isoform sequencing in humans, real-time PCR in human tissues, Western blot on rat tissues, *in* vitro motility assays of rat tissues, and manuscript redaction.

Chapter 3 consists of the manuscript "Overexpression and function of the myosin SM-B isoform, SM22, and MLCK in mild asthmatic endobronchial biopsies" by R. Léguillette, M. Laviolette, C. Bergeron, N. Zitouni, P. Kogut, J. Solway, A.-M. Lauzon, which has been submitted to the American Journal of Respiratory and Critical Care Medicine. This study was performed by the first author (Renaud Léguillette) under the supervision of Dr. Anne-Marie Lauzon (Department of Physiology, Meakins-Christie Laboratories, McGill University). Dr. Laviolette, a pneumologist from the Centre de

Recherche de l'Hôpital Laval at Université Laval, obtained endobronchial biopsies from asthmatic and normal patients. Dr. Bergeron was a fellow at the Meakins-Christie Laboratories and tought me the immuno-histochemistry technique. She also performed the immuno-histochemistry analysis for SM22. Dr. Nedjma Zitouni purified myosin from the rat tissues studied and performed Western blots analysis. P. Kogut, a technician in Dr. Sloway's laboratory, in the Department of Medicine at The University of Chicago, generated recombinant SM22. Dr. Solway edited the manuscript. I performed real-time PCR analysis of contractile protein genes in endobronchial biopsies, adsorption tests for immuno-histochemistry, immuno-histochemistry of MLCK and SM-B, *in vitro* motility assays with rat tissues, with MLCK, and with SM22, SM-22 cosedimentations and immunoprecipitations and Western blot analysis, and manuscript redaction.

Chapter 4 consists of the manuscript "Tonic and phasic smooth muscle myosin affinity for adenosine diphosphate and unbinding force from actin ", by Renaud Léguillette, Nedjma B. Zitouni, Karuthapillai Govindaraju, Anne-Marie Lauzon. The manuscript is under final editorial preparation for submission to the Journal of Cell Science. This study was performed by the first author (Renaud Léguillette) under the supervision of Dr. Anne-Marie Lauzon (Department of Physiology, Meakins-Christie Laboratories, McGill University). Dr. Nedjma Zitouni purified myosin from the tissues and performed Western blot analysis. Dr. Karuthapillai Govindaraju, a research associate at the Meakins-Christie Laboratories, helped with the troubleshooting of the myosin purifications. I performed the laser trap alignment and stiffness calibration, dichroic mirror testing for imaging, , laser trap and *in vitro* motility assay measurements, and manuscript redaction.

Chapter 1: Introduction:

Smooth muscle is found in the wall of all hollow organs of the body and performs a variety of functions. Its contraction can result in content propulsion, increase in fluid pressure and opening or closing of sphincters. Smooth muscle has been identified in the early 1900s, when individual cells have been viewed after maceration of fixed tissues. These muscle cells were called smooth because, in contrast to skeletal muscle, no intracellular transverse divisions could be seen. Despite efforts from several groups, the exact intracellular organization and mechanics of the smooth muscle contractile apparatus are still poorly understood.

Structurally, smooth muscle cells can be divided into the cytoskeleton and the contractile apparatus. The cytoskeleton is mainly composed of microtubules, cytoplasmic actin, dense bodies, vimentin and desmin. Vimentin and desmin are also called intermediate filaments because of their diameter of 10nm, which is intermediate between the thick filaments (14-18nm) and thin filaments (7-8nm) from the contractile apparatus. The cytoskeleton is thought to resist compressive forces and therefore to have a critical role in cellular pre-stress and mechanotransduction (Wang, Butler et al. 1993; Stamenovic, Mijailovich et al. 2002; Wang, Tolic-Norrelykke et al. 2002). The interactions between compressive forces generated by the contractile apparatus and the cytoskeleton have been studied in a tensegrity model where the stiffness of the cytoskeleton structure increases proportionally to the stress applied to the cells (Wang, Butler et al. 1993).

The contractile apparatus is composed of thick and thin filaments. Thin filaments are made of α and γ smooth muscle actin, the regulatory proteins tropomyosin, caldesmon and calponin, and potentially, SM22. Calponin is also found in the cytoskeleton (North, Gimona et al. 1994). Thick filaments are assemblies of myosin molecules but their exact structure and distribution is still unknown and subject to controversy. The contraction generated by smooth muscle cells results from the interactions between regulatory proteins, myosin and actin filaments. Of all the proteins participating in the contraction of smooth muscle cells, only myosin is a molecular motor that can generate force and motion.

A smooth muscle myosin knockout mouse has been generated in which only the expression of non-muscle myosin was possible (Morano, Chai et al. 2000). These mice showed abnormalities in gastrointestinal tract contraction and ductus arteriosus closure, dilated cardiomyopathy, enlarged thin-walled bladder, as well as neonatal mortality. Interestingly, their smooth muscle tissues could still contract when stimulated with KCl, but the rapid first phase of contraction, observed in wild type animals, had disappeared. Thus, this model showed that smooth muscle myosin is necessary for vital functions. In this sense, altered smooth muscle mechanics may lead to health-related issues. For example, smooth muscle mechanics is altered in asthma (Ma, Cheng et al. 2002).

This thesis will therefore focus on the expression of smooth muscle contractile proteins in humans, their molecular mechanics, and their potential role in airway smooth muscle in the context of asthma. My hypothesis is that contractile protein expression is altered in asthma, leading to abnormalities in airway smooth muscle function.

I- EXPRESSION AND FUNCTION OF SMOOTH MUSCLE MYOSIN HEAVY AND LIGHT CHAIN ISOFORMS:

The shortening velocity of smooth muscle from different organs can vary by as much as 20-fold (Malmqvist and Arner 1991; Murphy, Walker et al. 1997). Interestingly, the molecular basis behind this phenomenon has not yet been established. Because myosin isoform content has been shown to have a direct impact on striated muscle performance (Lutz and Lieber 2002), it is reasonable to assume that the same may be true for smooth muscle. This section will focus on the different smooth muscle myosin heavy and light chain isoforms and their contractile role in the different types of smooth muscle.

A- Tonic and phasic smooth muscle:

Smooth muscle contractility is heterogeneous even from cell to cell within one organ, e.g. blood vessels. Despite this heterogeneity, smooth muscle has been classified, based on electrophysiological properties, as either tonic or phasic (Somlyo and Somlyo 1968; Kroeger and Stephens 1975; Kuriyama, Mishima et al. 1975; Himpens, Matthijs et al. 1988; Somlyo and Somlyo 1990; Somlyo and Somlyo 1994). When activated by KCl depolarization, phasic smooth muscle contracts rapidly, showing an initial transient peak in force that quickly declines (Somlyo and Somlyo 1968). Conversely, tonic smooth muscle contracts much more slowly and maintains tension for long periods of time (Somlyo and Somlyo 1968). Phasic and tonic smooth muscles also differ in their membrane activity. Phasic smooth muscle can generate action potentials. Phasic smooth muscle cells have a large number of gap junctions that allow intercellular propagation of electrical potential waves generated by pacemakers. This is why they are also called single unit muscles (Kannan and Daniel 1980; Daniel, Kannan et al. 1986). To the contrary, tonic muscle does not respond to electrical stimulation with action potentials, but does respond to pharmacological or humoral agents (Somlyo and Somlyo 1994). Tonic smooth muscle cells have very few gap junctions (2.7 ±0.3 per 100 cells) (Daniel,

Kannan et al. 1986) but have more nerve contacts, thus functioning as distinct units with individual neuronal control. Tonic smooth muscle is therefore called multi-unit.

Parallel to these functional differences tonic and phasic smooth muscles have a distinct distribution among organs. Typically, phasic smooth muscle is found in the rapidly contracting organs like the intestine, the bladder, the portal vein, and small arterioles. Tonic smooth muscle is found in slowly contracting, tone-maintaining organs like the uterus, large veins and arteries, the fundus region of the stomach, and sphincters. Evidence is accumulating to suggest that the content in regulatory and contractile proteins between phasic and tonic smooth muscle also differs (Szymanski, Chacko et al. 1998). The next sections will focus on the expression and function of the different smooth muscle myosin heavy and light chain isoforms.

B- Myosin:

Molecular motors, such as myosin, kinesin and dynein, constitute a superfamily of mechanoenzymes that can transform the chemical energy liberated from ATP hydrolysis into mechanical work. Sequence analysis allowed to differentiate 11 classes of motor proteins (Sellers and Goodson 1995). Among those motor proteins, myosin molecules represent several classes, which differ in their structure and function. Comparative sequence analysis of most of the myosin classes showed that the motor domain has many well conserved regions with basic structural elements, namely an actin- and a nucleotide-binding sites (Cheney, Riley et al. 1993; Goodson and Spudich 1993; Spudich 1994).

All vertebrate cells contain non-muscle (or cellular) myosin, which plays a role in cell division, cell shape, cell motility, intracellular movement and cell secretion. This non-muscle myosin is a class II myosin. The two isoforms of non-muscle myosin heavy chains (NMMHC-A and NMMHC-B) are products of two different genes, have a tissue-specific expression, and have also been found in humans (Katsuragawa, Yanagisawa et

al. 1989; Simons, Wang et al. 1991). The nomenclature is unfortunate because smooth muscle myosin heavy chain isoforms (described later in this thesis) were also named as A and B.

Skeletal, cardiac, and smooth muscle cells share the same basic molecular structure, suggesting that they diverged from a common ancestor (Nagai, Larson et al. 1988). Skeletal, cardiac, and smooth muscle myosin are different isoforms of myosin class II. They are all hexamers composed of two heavy chains (~200 kDa) noncovalently associated with two pairs of light chains of 17 and 20 kDa, respectively. The three-dimensional structure of skeletal myosin has been described by crystallography and is a good model for all myosins (Rayment, Rypniewski et al. 1993). The light chains are attached to the "IQ motifs" (described in section C-3) which are well conserved between myosin classes (Espreafico, Cheney et al. 1992; Xie, Harrison et al. 1994). The four known isoforms of smooth muscle myosin heavy chains are generated by alternative splicing of a single gene located on chromosome 16p13.13-13.12 in humans (Deng, Liu et al. 1993) .

C- Structure of smooth muscle myosin heavy and light chain isoforms:

There are four isoforms of smooth muscle myosin heavy chain described in the literature that show variations at the N- and the C- terminal portions of the protein, as well as two isoforms of each of the regulatory (LC_{20}) and essential (LC_{17}) light chains. The catalytic portion (or head) of the myosin heavy chain is at the N-terminus and the elongated coiled-coil α-helix (or tail) is at the C-terminus (Figure 1). The head is mostly responsible for the enzymatic activity and binding to actin, whereas the tail is involved in self-association and filament formation (Figure 1) (Rovner, Fagnant et al. 2002). It has also been suggested that the tail has a direct effect on cross-bridge cycling (Martin, Bhatti et al. 1997). The function of the various myosin heavy and light chain isoforms will be discussed in detail in this chapter.

Figure: 1

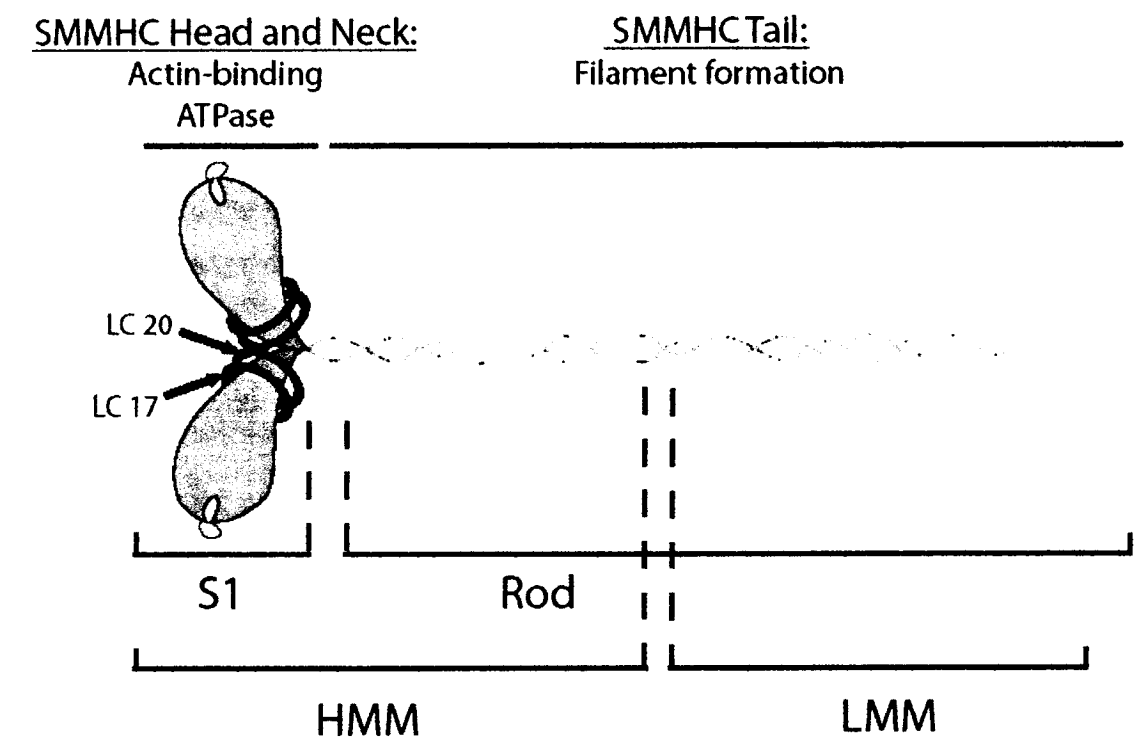

Figure 1: Schematic illustrating the structure of the smooth muscle myosin heavy and light chains: The functional regions and the fragments obtained by two distinct proteolytic cleavages are shown. SMMHC: Smooth muscle myosin heavy chain, LC_{20}: Regulatory light chain (20 kDa), LC_{17}: Essential light chain (17 kDa), HMM: Heavy meromyosin, LMM: Light meromyosin.

1- Carboxy-terminus smooth muscle myosin heavy chain isoforms: discovery, nomenclature and structure:

Separation of the 200kDa and the 204kDa proteins on SDS-polyacrylamide gels allowed the identification of the tail isoforms in turkey gizzard (Kawamoto and Adelstein 1987) and in swine carotid and stomach smooth muscle (Rovner, Thompson et al. 1986; Eddinger and Murphy 1988). The longer tail isoform was called SM1 and the shorter one was called SM2 (Figure 2) (Eddinger and Murphy 1988). Their distinct amino acid sequences have been determined using cDNA clones (Figure 2) (Babij and Periasamy 1989; Nagai, Kuro-o et al. 1989). Their sequence is identical through amino acid 1928 but from this point, SM1 contains a sequence of 43 amino acids whereas SM2 contains a different sequence of only 9 amino acids (Figure 2). The shorter sequence of SM2 results from the insertion of a 39 nucleotide exon that encodes a stop codon at the beginning of its sequence (Figure 2) (Babij and Periasamy 1989; Nagai, Kuro-o et al. 1989). Of note, the SM1 (204kDa) isoform has a serine residue in an acidic environment which can be phosphorylated *in vitro* and in intact cells by casein kinase II (Kelley and Adelstein 1990). The amino acid sequence of this phosphorylation site is well conserved among smooth muscle SM1 and non-muscle myosin from a variety of tissues and species (Babij and Periasamy 1989; Shohet, Conti et al. 1989; Murakami, Healy-Louie et al. 1990; Saez, Myers et al. 1990) which suggests that it probably has an important function. The role of this phosphorylation is not known, but it does not affect myosin conformation (extended 6S vs folded 10S), filament formation, ATPase activity, nor velocity of actin filament propulsion (v_{max}) in the *in vitro* motility assay (Kelley and Adelstein 1990).

Figure: 2

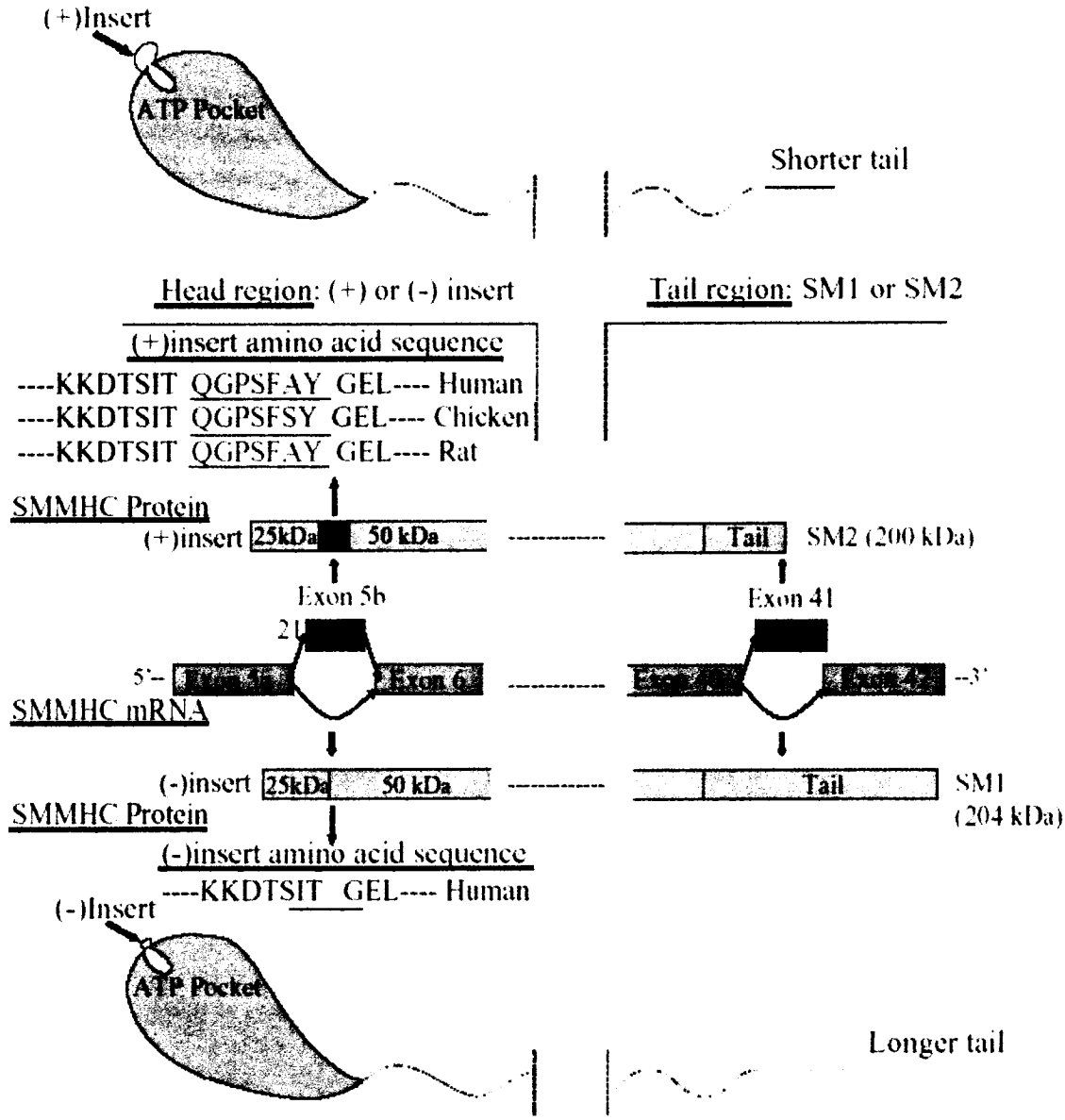

Figure 2: Schematic illustrating the alternative splicing of the mRNA that occurs in SMMHC at the 5' and 3' ends. Inclusion of exon 5b generates the (+)insert isoform, whereas inclusion of exon 41, encoding a stop codon, generates the shorter (200kDa) SM2 isoform. The 7 amino acid insert of the N-terminus is shown in red and is underlined. The amino acid sequence of the insert is identical in human and rat but differs by one amino acid in the chicken. From (Low, Leguillette et al. 2006)

Myosin molecules are assembled as dimers and there is controversy in the literature regarding whether or not, SM1 and SM2 form homodimers or heterodimers (Kelley, Sellers et al. 1992; Tsao and Eddinger 1993). Kelley and coworkers showed, by immunoprecipitating SM1 from bovine aortic smooth muscle, that only homodimers were present (Kelley, Sellers et al. 1992). However, others have reported that porcine and rabbit SM1 and SM2 light meromyosin (LMM; Figure 1) could cross-link either as homodimers or heterodimers (Tsao and Eddinger 1993). The fact that some smooth muscle tissues have a non-stoichiometric SM1/SM2 ratio is consistent with both views. More recently, baculovirus-transfected insect cells were used to produce pure populations of SM1 or SM2 (Rovner, Fagnant et al. 2002). The cross-linking of SM1 with SM2 was then asessed, and although SM1 and SM2 heterodimers could be formed *in vitro*, cells were found to preferentially contain homodimers of each isoform (Rovner, Fagnant et al. 2002).

2- Amino-terminus smooth muscle myosin heavy chain isoforms: discovery, nomenclature and structure:

Because of its important catalytic function, extensive research has addressed the role of individual sections of the myosin head. There are two flexible surface loops in the head region (Figures 1 and 3). Loop-1 is located above the nucleotide binding site at the junction of the 25-50 kDa proteolytic domains, whereas loop-2 is the actin binding site. There are two myosin heavy chain isoforms that differ by an insert in loop 1 (Figures 2 and 3).

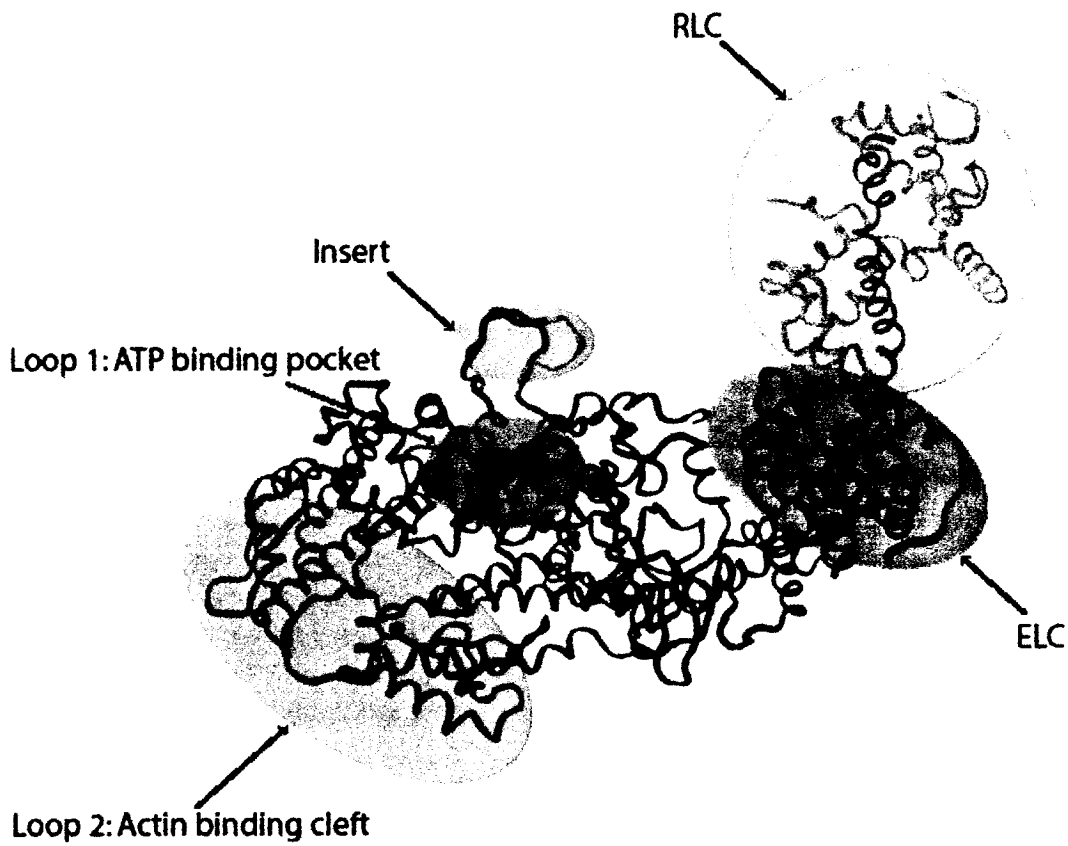

Figure 3: Model of the (+) and (-)insert N-terminus SMMHC isoforms. The insert is shown in red. The (-)insert loop is shown in blue like the rest of the heavy chain. The regulatory light chain (LC_{20}) is shown in yellow and the essential light chain (LC_{17}) is shown in pink. The model was generated using the coordinates of Rayment (Rayment, Rypniewski et al. 1993) using Insight 2, v. 95.0 (MSI). Modified from (Low, Leguillette et al. 2006).

Sequence divergence in the N-terminal region of the smooth muscle myosin heavy chain was first observed in rabbit and chicken tissues (Hamada, Yanagisawa et al. 1990; Babij, Kelly et al. 1991). Both studies identified a short 21 base pair exon that was inserted or not, between exon 5a and exon 6 (Figures 2 and 3). Based on the crystal structure of the skeletal myosin head, this region is part of loop 1 (Figure 3). Interestingly, several species share the same alternative splicing site, and the work presented in Chapter 2 of this thesis reveals the sequence of the insert in human myosin (Figure 2) (Leguillette, Gil et al. 2005).

The nomenclature is still not standardized in the field of myosin isoforms. The N-terminal inserted smooth muscle myosin heavy chain II is referred to as SM-B or (+)insert whereas the non-inserted isoform is referred to as SM-A or (-)insert. As mentioned at the beginning of the section on myosin, an isoform of vertebrate non-muscle myosin heavy chain is also called the B isoform, i.e. non-muscle myosin IIB. This non-muscle myosin isoform also has an insert in loop 1 but of 10 amino acids (Takahashi, Kawamoto et al. 1992). Non-muscle myosin IIB should not be mistaken with the smooth muscle SM-B isoform.

3- Ligth chain isoforms: nomenclature and structure:

LC_{20} and LC_{17} noncovalently bind to the "IQ motif" of the heavy chain, which has the consensus sequence is IQXXXRGXXRXXY (or W) (Espreafico, Cheney et al. 1992; Xie, Harrison et al. 1994). LC_{20} can be phosphorylated and is responsible for smooth muscle myosin activation (Figure 3). The existence of two isoforms of the LC_{20} has been reported in vascular smooth muscle. These isoforms originate from separate genes and differ by an 11 amino acid substitution (Mougios and Barany 1986); However, there is no known functional difference between these two isoforms. LC_{17}, on the other hand, is known to enhance the molecular mechanical properties of the myosin heavy chain (VanBuren, Waller et al. 1994) but how it exerts this function remains obscure (VanBuren, Waller et al. 1994). Two isoforms of the LC_{17} (LC_{17a} and LC_{17b}) are also generated by alternative mRNA splicing from a single gene, and differ by the substitution of 5 of the last 9 amino acids in their carboxyl terminus (Nabeshima, Nabeshima et al.

1987; Hasegawa, Ueno et al. 1988; Helper, Lash et al. 1988; Lenz, Lohse et al. 1989; Lash, Helper et al. 1990; Hasegawa and Morita 1992). LC_{17a} has a more acidic isoelectric point (pI = 4.13) than LC_{17b} (pI = 4.19) (Helper, Lash et al. 1988). The carboxyl terminal sequence of LC_{17b} (in gizzard) is identical to that of the non-muscle LC_{17} (Nabeshima, Nabeshima et al. 1987; Hasegawa and Morita 1992). Both LC_{17a} and LC_{17b} can bind to SM-1 or SM-2 and SM-A and SM-B (Figure 3).

D- Expression and function of the carboxyl terminal myosin isoforms:

1- Expression in normal tissues and in diseases:

The content of SM1 and SM2 is tissue specific (Cavaille, Janmot et al. 1986; Mohammad and Sparrow 1988). Depending on the species, the SM1/SM2 protein ratio can be as low as 0.6 to as high as 4 in tonic and phasic tissues (Mohammad and Sparrow 1988; Sparrow, Mohammad et al. 1988). Pig carotid artery (Rovner, Thompson et al. 1986) and bovine aorta smooth muscle (Kelley, Sellers et al. 1992) contain as much SM1 as SM2. Furthermore, there is a good correlation between mRNA and protein content for SM1 and SM2, suggesting that little post-transcriptional regulation occurs (Meer and Eddinger 1996).

The expression of SM1 and SM2 changes with the proliferative state of smooth muscle cells. The SM1/SM2 ratio increases when smooth muscle cells are actively dividing, as shown in embryonic development of the vascular system, in arteriosclerotic vessels, or in cell culture (Rovner, Murphy et al. 1986; Kawamoto and Adelstein 1987; Kuro-o, Nagai et al. 1991; Babij, Kawamoto et al. 1992). The SM1/SM2 ratio shifts during maturation, as observed in pig airway smooth muscle where the ratio is 0.95 in adults and 2.1 in neonates (Mohammad and Sparrow 1988). Human airway may be an exception as the same group did not find any difference between adult and human infant bronchial and tracheal tissue, where the SM1/SM2 ratio was 0.69 (Mohammad and Sparrow 1989). Interestingly, other investigators found a similar SM1/SM2 ratio (0.72)

in trachea and bronchus of adult asthmatics, suggesting that the expression in these isoforms is not altered in asthma (De Marzo, Di Blasi et al. 1989). The distribution of SM1 and SM2 in tissues can however be complex in other diseases. For example, SM2 is significantly decreased in arteries from hypertensive rats, whereas the SM1/SM2 ratio does not change in the portal vein from the same animals (Sparrow, Mitchell et al. 1990). The rabbit model of bladder obstruction further increased the complexity by revealing that, in addition to differences in SM1/SM2 content between organs (Cher, Abernathy et al. 1996), there are also regional differences in the expression of these isoforms within one tissue (Mannikarottu, Hypolite et al. 2005). Indeed, it seems that even cells from one region can show heterogeneity in their SM1 and SM2 content (Meer and Eddinger 1996). Lastly, the SM1/SM2 ratio also appears to be at least partially under hormonal control in reproductive organs. The uterus is frequently studied because of the quick and dramatic changes occurring in weight, force, and velocity of contraction after a few days of estrogen treatment or during pregnancy (Hewett, Martin et al. 1993). An increased SM1/SM2 ratio has been reported in rat uterine smooth muscle treated with estrogen (Hewett, Martin et al. 1993) as well as during pregnancy (Sparrow, Mohammad et al. 1988; Morano, Erb et al. 1993).

2- Function of the carboxyl terminus isoforms:

The function of SM1 and SM2 is not clear. The tissue-specific expression of SM1 and SM2 as well as the presence of a phosphorylation site by casein kinase in SM1 but not in SM2 suggest that these two isoforms function differently. However, no differences in their molecular mechanics have been reported so far *in vitro* (Kelley, Sellers et al. 1992; Rovner, Fagnant et al. 2002). Ikebe and coworkers used proteolysis to remove the SM1 tailpiece from chicken gizzard myosin and found that it enhances myosin filament formation (Ikebe, Hewett et al. 1991). This was later confirmed using the baculovirus and insect cell system to express three different smooth muscle myosin rods that either had the same tail as SM1 or SM2, or that were lacking the non-helical C-terminal tail (Rovner, Fagnant et al. 2002). The tendency to form filaments followed the following order: No tail> SM1> SM2. Furthermore, electron microscopy images of paracrystals of the rods of SM1 and SM2 homodimers showed that they have a very different

conformation (Rovner, Fagnant et al. 2002). Altogether these results suggest that, although there are no differences observable in their molecular mechanics *in vitro*, the myosin filaments assembly may differ between the SM1 and SM2 isoforms *in vivo*, which may confer functional differences at the whole muscle level (Rovner, Fagnant et al. 2002).

Furthermore, it has been suggested that the C-terminal portion of the myosin heavy chain can alter the function of the N-terminus. Indeed, the proteolytic removal of the SM1 tailpiece increases the ATPase activity at low Mg^{2+} concentrations, although the maximum ATPase activity is not different from intact myosin (Ikebe, Hewett et al. 1991). Others used peptides that mimick the tail regions of SM1 or SM2 to show that they can interact with the hinge region (S2-LMM region) of neighboring myosin molecules in thick filaments (Cai, Ferguson et al. 1995). The SM1 peptide permanently decreased the velocity of shortening by 60% and enhanced the Ca^{2+} sensitivity of smooth muscle fibers, while the SM2 peptide had effects of lower magnitude that were reversible (Cai, Ferguson et al. 1995). As a control, the investigators used an antibody that could bind myosin rods in the same region as the SM1 peptide and found that the antibody binding can also decrease the velocity of shortening. This suggests that perturbations of the structure in the S2-LMM region affects contractility (Cai, Ferguson et al. 1995).

The possible relationship between smooth muscle tissue shortening velocity and SM1/SM2 content is still controversial. Studies using uterine smooth muscle from rats reported a small positive correlation between SM1 content and the rate of shortening (Sparrow, Mohammad et al. 1988; Hewett, Martin et al. 1993), but this relationship was not maintained in many other smooth muscle tissues (Schildmeyer and Seidel 1989; Packer, Griffith et al. 1991; Upadhya, Samuel et al. 1993; Sherwood and Eddinger 2002).

E- Expression and function of the amino terminal myosin isoforms:

1- Expression in normal tissues:

Differences in myosin SM-A and SM-B expression were first described at the mRNA level in rat and rabbit visceral and vascular smooth muscle tissues. Phasic smooth muscle from the bladder and the intestine were reported to contain a large proportion of mRNA encoding for SM-B (White, Martin et al. 1993; DiSanto, Cox et al. 1997). Conversely, the SM-B mRNA was shown to be absent in large arteries (aorta) but it was found in small distal arteries (White, Martin et al. 1993; DiSanto, Cox et al. 1997). It is now well accepted that SM-A and SM-B are differentially expressed in a tissue-specific manner. Subsequent studies using a 7 amino acid insert-specific antibody corroborated the mRNA results and confirmed that tonic smooth muscle contains less SM-B than phasic smooth muscle (White, Zhou et al. 1998; Low, Mitchell et al. 1999). The stomach shows a mixed expression of myosin isoforms, with an increased content in SM-B going down from the fundus to the antrum, which parallels the secretory vs propulsive roles of these regions, respectively (Parisi and Eddinger 2002). Large tonic arteries contain little SM-B while it is expressed in smaller resistance phasic blood vessels and in the portal vein (DiSanto, Cox et al. 1997; Low, Mitchell et al. 1999; Sherwood and Eddinger 2002; Stiebellehner, Frid et al. 2003).

2- Expression of the SM-B isoform in diseases:

Interestingly, the expression of the SM-B isoform is altered in diseases. This has been shown for both visceral and vascular smooth muscles. First, in models of lung hypertension, the vascular smooth muscle of the venous and arterial microvasculature expresses the SM-B isoform, whereas it is rare in normotensive lungs (Stiebellehner 1998; Jones, Steudel et al. 1999). Furthermore, in rat spontaneous systemic hypertension, cardiac pre-capillary arterioles contain less SM-B than the normotensive controls (Wetzel, Lutsch et al. 1998). Studies of the expression of SM-B in visceral tissue in disease states have focused on the bladder smooth muscle after urinary obstruction

(reviewed in (Chacko, DiSanto et al. 1997; Chacko, DiSanto et al. 1999)), whereas studies on other organs are rare (Lofgren, Fagher et al. 2002; Gil, Zitouni et al. 2006). Urinary bladder hypertrophy caused by urethral obstruction decreases the bladder SM-B expression to 50% (Sjuve, Haase et al. 1996; Chacko, DiSanto et al. 1999; DiSanto, Stein et al. 2003). The changes in SM-B expression are due to shifts in existing cells, showing the ability of smooth muscle cells to adapt to their environmental conditions (DiSanto, Stein et al. 2003). In this sense, treatment of rabbits with Tanedan, which returns the hypertrophied bladder detrusor smooth muscle contractility to normal, also reverses the myosin isoform content to normal (Gomes, Disanto et al. 2000). Furthermore, SM-B expression decreases much more at the base of the bladder than at the dome (Mannikarottu, Hypolite et al. 2005), which correlates with the dome and the base of hypertrophied bladder being subjected to different expansion pressures (Schroder, Uvelius et al. 2002; Mannikarottu, Hypolite et al. 2005). Similarly to bladder obstruction, intestinal obstruction induces a decrease in rate of shortening concomitant with a decrease in SM-B myosin isoform expression (Lofgren, Fagher et al. 2002). Although both myosin isoforms are expressed in rat airways, we recently showed that the SM-B isoform expression is increased in a rat model of airway hyperresponsiveness (Gil, Zitouni et al. 2006). Furthermore,using primers specifically designed for human SM myosin, I demonstrated unequivocally the presence of the SM-B isoform in various human tissues (Leguillette, Gil et al. 2005) (Chapter 2) and I showed that its expression is increased in asthmatic bronchial biopsies ((Leguillette, Laviolette et al. 2006) (see chapter 3).

3- Regulation of the SM-B isoform expression:

The control of smooth muscle myosin heavy chain isoform expression is still poorly understood, but hormonal regulation in normal smooth muscle tissues has been reported (Calovini, Haase et al. 1995; Haase and Morano 1996; White 1997; Lofgren, Fagher et al. 2002). In the uterus, a tonic organ, estradiol decreases the SM-B content to undectectable levels and alters its distribution in the various smooth muscle layers (Calovini, Haase et al. 1995; White 1997). To the contrary, testosterone can increase the expression of the SM-B isoform in the uterus (Calovini, Haase et al. 1995). Visceral and

vascular smooth muscle expression of the SM-B isoform is also modulated by thyroxine. Exposure to thyroxine increased by 25% the SM-B mRNA levels for which baseline expression was quite high, and increased by 70% the SM-B levels in the aorta, for which baseline expression was low (Lofgren, Fagher et al. 2002). Multiple other stimuli, yet to be studied, most certainly also alter myosin isoform expression. Major advances in the field will certainly come from a better understanding of the precise mechanisms that control the alternative splicing of the myosin mRNA.

4- Function of the SM-B isoform *in vitro*:

The first study to investigate the function of the inserted and non-inserted myosin isoforms was performed in avian smooth muscle (Kelley, Takahashi et al. 1993). Kelley and collaborators purified myosin from gizzard,and aorta and found a positive correlation between the SM-B content and v_{max} as measured in the *in vitro* motility assay. They found that gizzard myosin, which contained mostly the SM-B isoform had a 2.5-fold faster v_{max} and has an ATPase activity 2-fold greater than aorta, which contained mostly the SM-A isoform. In addition, they ruled out the role of the light chain isoforms in inducing these differences in motility and activity by repeating the measurements after exchanging the light chains between gizzard and aorta myosins. This suggests, as proposed before (Spudich 1994), that the 7 amino acids in loop 1 are critical for the function of myosin. Similar results were later obtained using the baculovirus/insect cell system to express myosin protein constructs that only differed by the presence or absence of the seven amino acid insert (Rovner, Freyzon et al. 1997). Changes in ATPase activity were coupled to changes in v_{max}, suggesting that alterations in the amino acid sequence of the loop can affect the rate-limiting steps common to both the ATPase activity and the unloaded shortening velocity.

So how does the 7 amino acid insert alter the cross-bridge cycle? This has been dissected out at the molecular level using a laser trap assay with SM-A and SM-B myosin constructs (Lauzon, Trybus et al. 1998). Although the two isoforms generate similar unitary force and displacement, the presence of the insert decreases the

attachment time to actin following the power stroke by shortening both the time of MgADP release and the time of MgATP binding (Lauzon, Tyska et al. 1998). This is in agreement with the three-fold difference in MgADP dissociation rate constant between the two isoforms measured using enzymatic assays (Sweeney, Rosenfeld et al. 1998). The rate of MgADP release had been shown to be sufficiently slow to be the limiting step of the unloaded shortening velocity (Siemankowski, Wiseman et al. 1985; Spudich 1994). It had further been hypothesized that loop1 controls the rate of MgADP release, whereas loop 2 controls the inorganic phosphate release and thus, the ATPase activity (Spudich 1994). This view has however been challenged by the results of the functional studies, where addition of the insert changed both the ATPase activity and v_{max} (Kelley, Takahashi et al. 1993; Rovner, Freyzon et al. 1997), while alterations in the actin binding loop affected myosin's regulatory properties (Rovner, Freyzon et al. 1995). It should, however, be kept in mind that the biochemical and molecular mechanics measurements may in fact underestimate the effect of the insert *in vivo*. Indeed, isolated single smooth muscle cells containing 95% of SM-B can contract three times faster than cells containing 38% of SM-B (Eddinger and Meer 2001). This is much faster than what *in vitro* studies would have predicted, and suggests that other factors, in addition to the insert, may also contribute to differences in the rate of shortening of smooth muscle cells.

5- *In vivo* function, knockout mice studies:

Targeted deletion of exon 5B of the smooth muscle myosin gene allowed the generation of an SM-B knockout mouse (Babu, Loukianov et al. 2001). Unfortunately, it was later discovered that concomitant with the (+)insert deletion, the expression of other contractile proteins was also altered, rendering impossible the attribution, solely to the insert, of alterations found in their smooth muscle mechanics (Babu, Pyne et al. 2004). An increased expression of LC_{17b} (2-fold) and calponin (1.4-fold) was observed in the aorta, whereas the expression of caldesmon was decreased (50% in the aorta and 30% in the bladder) (Babu, Pyne et al. 2004). This decrease in caldesmon expression suggests that it may be co-expressed with the SM-B isoform in the intact animal and potentially alters its function. Nevertheless, mechanical data from tonic and phasic smooth muscle

from these knockout mice unequivocally show that the velocity of shortening is reduced (up to 3.1-fold in the bladder). This suggests that, similarly to the *in vitro* data, the presence of the SM-B isoform accelerates the acto-myosin interactions *in vivo* (Babu, Loukianov et al. 2001). However, a decrease in velocity of shortening was also observed in the knockout mouse aorta, which was surprising given that the aorta expresses little SM-B isoform (Babu, Pyne et al. 2004). This may potentially be attributable to the increased expression of LC_{17} and/or to the decreased expression of caldesmon (Babu, Pyne et al. 2004).

The results of the studies addressing the effect of the SM-B isoform suppression on force measurements are unclear (Babu, Loukianov et al. 2001; Karagiannis, Babu et al. 2003; Babu, Pyne et al. 2004). Depending on the activation system and on the type of smooth muscle studied, the maximal tension may be increased, decreased or unaltered in the knockout mice tissues (Babu, Loukianov et al. 2001; Karagiannis, Babu et al. 2003; Babu, Pyne et al. 2004). Furthermore, upon addition of exogenous MgADP, the knockout smooth muscle develops a greater increase in force than in stiffness, suggesting that more myosin heads are attached to actin, thus slowing the cross-bridge cycle (Karagiannis, Babu et al. 2003). It was suggested that in the absence of the SM-B isoform, there could either be an additional step in the cross-bridge cycle immediately prior to the MgADP release, or that the ADP off-rate is slower, prolonging the attachment time of myosin to actin. A slower MgADP off-rate is supported by molecular mechanics studies ((Lauzon, Tyska et al. 1998); section 4 above).

Our group has also measured pulmonary mechanics in these knockout mice (Tuck, Maghni et al. 2004). After a bolus of methacholine, the time to reach maximal airway resistance was prolonged by 18% in the knockout animals (Tuck, Maghni et al. 2004). However, the actual maximal airway resistance was not altered, potentially because this knockout was generated in a hyporesponsive background mice strain, making it difficult to reduce airway responsiveness any further.

F- Expression and function of the myosin light chains isoforms:

There is no known pattern of expression nor functional difference between the two LC_{20} isoforms. To the contrary, many studies focused on the expression and function of the LC_{17} isoforms and many controversies are found in the literature concerning their role in smooth muscle contraction. We will therefore concentrate on the distribution and function of the LC_{17} isoforms in smooth muscle.

1- Expression in smooth muscle tissues:

It has been reported that the LC_{17a}/LC_{17b} ratio tends to be greater in phasic than in tonic smooth muscle (Helper, Lash et al. 1988; Malmqvist and Arner 1991; Fuglsang, Khromov et al. 1993). However, exceptions have also been reported (Sobieszek and Jertschin 1986). Certain phasic smooth muscles only contain one LC_{17} isoform. For example, porcine jejunum and stomach and chicken gizzard contain only the LC_{17a} isoform (Helper, Lash et al. 1988; Kelley, Takahashi et al. 1993). Similarly to the myosin heavy chain isoforms, the expression of the essential light chains varies during hormonal changes; LC_{17a} increases in uterus during pregnancy (from 46% to 65%) and decreases post-partum (Morano, Erb et al. 1993). However, the relationship between LC_{17} isoforms and myosin heavy chain isoform expression is unclear. Indeed, we (Gil, Zitouni et al. 2006) and others (Mannikarottu, Hypolite et al. 2005) reported uncorrelated changes in LC_{17a} content and the SM-B SMMHC isoform in airway hyperresponsiveness and in bladder hypertrophy, respectively. The expression of essential light chain isoforms is also well correlated with mRNA levels in rabbit tissues, suggesting that there is little post-transcriptional regulation (Eddinger, Korwek et al. 2000).

2- Function of the essential light chains isoforms:

The interest for the LC_{17} isoforms started when a correlation was first described with the kinetics properties of smooth muscle. The ATPase activity declines with increasing LC_{17b} content in multiple bovine tissues (Helper, Lash et al. 1988) and in different segments of the porcine aorta (Hasegawa and Morita 1992). Investigators also

reported in the same study that myosin with LC_{17b} has a good affinity for filamentous actin, while LC_{17a} cannot bind actin (Hasegawa and Morita 1992). Maximal muscle strip shortening velocity showed the same positive correlation with LC_{17a} content as the ATPase activity (Malmqvist and Arner 1991; Hasegawa and Morita 1992).This correlation was also maintained in the pregnant rat model (Morano, Erb et al. 1993). These studies were however performed at the whole tissue level and other parameters could have biased the results. Indeed, as mentioned in section E- 4), the molecular mechanics of myosin molecules with different LC_{17} isoforms is not different, as measured in the *in vitro* motility assay (Kelley, Takahashi et al. 1993; Rovner, Freyzon et al. 1997). Furthermore, studies performed at the single cell level, found no correlation between the LC_{17} isoform expression levels and the unloaded shortening velocity (Eddinger, Korwek et al. 2000; Sherwood and Eddinger 2002). Altogether these results suggest that LC_{17} isoforms are not the major contributor to the differences in smooth muscle mechanics of contraction.

II- THE LATCH STATE:

It has been approximately 40 years since were obtained the first electron microscopy images of the smooth muscle cell contractile apparatus. In spite of that, the intracellular organization and dynamics of the thick and thin filaments during contraction are still speculative and subject to controversy. *In vitro* and *in vivo* biochemical and biomechanical studies have improved our understanding of the function of the smooth muscle contractile molecules. However, more work needs to be done to shine light on the mechanisms behind the high efficiency of smooth muscle. Indeed, the most outstanding physiological feature of smooth muscle is its ability to maintain force for long periods of time at low energy cost. This unique property of smooth muscle is called the latch state and will be the focus of this section. However, before elaborating on the latch state, a thorough review of the activation system of smooth muscle myosin is required.

A- Smooth muscle activation system:

A transient increase in myoplasmic Ca^{2+} concentration is observed after electrical stimulation of smooth muscle, which is attributable to Ca^{2+} release from the sarcoplasmic reticulum (Deth and Casteels 1977). When Ca^{2+}-chelating agents are used, contraction is inhibited in single smooth muscle cells and muscle strips (Fay 1977). Even if the intracellular Ca^{2+} rises instantaneously, there is a 200 ms delay (compared to less than 2ms in skeletal muscle) before the onset of active force development (Fay 1977). The release of Ca^{2+} induces phosphorylation of LC_{20} (Ratz and Murphy 1987). The delay in contraction is thus, due to the activation of the LC_{20} and to the attachment of myosin to actin. However, it is still unclear if intracellular activation of myosin results in reorganization of the thick filaments, which would also contribute to this delay. It has been shown that MLCK activity parallels both the ATPase activity and tension development (Gorecka, Aksoy et al. 1976; Sherry, Gorecka et al. 1978), and that MLCK phosphorylation of the LC_{20} is both necessary and sufficient for the ATPase activation

and contraction (Sobieszek and Bremel 1975). Furthermore, MLCK was found in many smooth muscle tissues, supporting again its essential role in smooth muscle activation (Chacko, Conti et al. 1977; Small and Sobieszek 1977; Small and Sobieszek 1977). When contraction is initiated in single cells or smooth muscle strips, the Ca^{2+}-calmodulin complex activates MLCK, which phosphorylates myosin LC_{20} (Driska, Damon et al. 1978; Cassidy, Hoar et al. 1979; Hoar, Kerrick et al. 1979).

It is believed that LC_{20} phosphorylation is not the only regulatory mechanism of contraction in smooth muscle. Contrary to skeletal muscle, the smooth muscle ATPase activity is independent of Ca^{2+} (it is only indirectly dependent on Ca^{2+} because of its activation of MLCK) (Stull, Silver et al. 1983; Stull, Kamm et al. 1988). Indeed, several studies have described the development of contraction in the absence of any increase in Ca^{2+} which led to the discovery of a dephosphorylation enzyme, namely phosphatase (MLCP), which is again Ca^{2+}-independent (Etter, Eto et al. 2001). Thus, LC_{20} phosphorylation results from the competitive effects of MLCK and MLCP. Inhibition of MLCP results in an augmented contractile response, a process known as Ca^{2+} sensitization (Nishimura, Kolber et al. 1988; Kitazawa, Kobayashi et al. 1989; Somlyo and Somlyo 1994). For example, protein kinase C (PKC), a kinase that inhibits phosphatase activity, leads to slow Ca^{2+}-independent contraction or Ca^{2+} sensitization (Morgan and Morgan 1984; Rasmussen, Takuwa et al. 1987; Rembold and Murphy 1988).

An actin-linked regulatory system similar to that observed in skeletal muscle had also been proposed in smooth muscle, where Ca^{2+} would interact with leiotonin (potentially an actin regulatory protein) and tropomyosin to regulate the state of thin filaments (Mikawa, Nonomura et al. 1978; Haeberle 1999). This would allow actin, in presence of Ca^{2+}, to trigger myosin ATPase activity (Mikawa, Nonomura et al. 1978; Haeberle 1999). However, it is now believed that technical difficulties biased those early studies (Seidel 1979). Therefore, it is well accepted that activation of smooth muscle contraction is not regulated at the thin filament level, as in skeletal muscle, but rather at the level of myosin.

B- What is latch?

Once it was established that Ca^{2+} was initiating smooth muscle contraction via phosphorylation of the LC_{20} by a Ca^{2+}-calmodulin dependent kinase, the relationship between myosin phosphorylation and muscle contraction generated a lot of interest. Early studies reported that maximum smooth muscle tension can be produced even when only a small percentage of the LC_{20} is phosphorylated (Driska and Hartshorne 1975). However, it is Dillon and coworkers who first demonstrated the correlation between LC_{20} phosphorylation levels and rate of shortening and the intriguing relationship between LC_{20} phosphorylation levels and force development (Figure 4) (Dillon, Aksoy et al. 1981).

Figure 4:

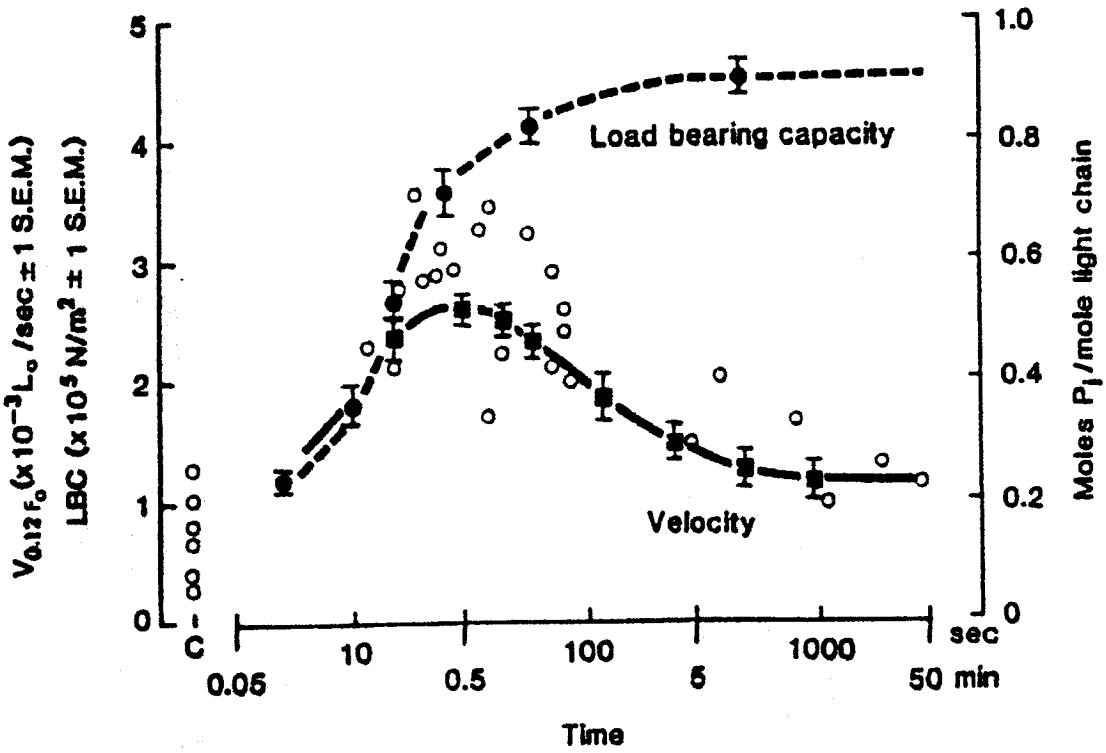

Figure 4: Load-bearing capacity (black circles), shortening velocity at an afterload of $0.12F_0$ (black squares) and fractional phosphorylation of tissue LC_{20} (open circles) as a function of time on a log scale after K^+ stimulation of a carotid media preparation. The mechanical data points are averaged from a series of five preparations (± 1 SEM), with extensions of the curves based on time course studies in other tissues. Each phosphorylation determination represents a single tissue, frozen at the indicated times (n=30), and C designates unstimulated tissues. From (Dillon, Aksoy et al. 1981).

Dillon and coworkers stimulated pig carotid arteries with KCl and measured the resulting LC_{20} phosphorylation level, velocity of shortening, as well as the load bearing capacity (Figure 4) (The load bearing capacity is defined as the total stress minus passive stress at optimal length at which the activated muscle yields when subjected to a quick stretch). Despite the fact that the readings of phosphorylation at such low levels were erroneous due to technical difficulties, the general aspect of the LC_{20} phoshorylation curve was later confirmed (Rembold, Wardle et al. 2004). LC_{20} phosphorylation, rate of shortening and load bearing capacity, increase over approximately 30 s. At this point, LC_{20} phosphorylation and the rate of shortening had reached a maximum and decreased back towards baseline at about 20 min after stimulation. Conversely, the load bearing capacity kept on increasing and reached a plateau at about 2 min, which was maintained for at least 50 min after stimulation. Dillon and coworkers have therefore shown that, even if LC_{20} phosphorylation is necessary for force development, the relationship between phosphorylation levels and load bearing capacity is not linear but hyperbolic. (Dillon, Aksoy et al. 1981; Dillon and Murphy 1982; Rembold and Murphy 1988; Rembold and Murphy 1988). In fact, force can be supra maximal even if LC_{20} phosphorylation levels are as low as 30%, providing a very non-linear force-phosphorylation relationship (Dillon, Aksoy et al. 1981; Rembold, Wardle et al. 2004). This high force maintenance state at a low LC_{20} phosphorylation level and low velocity of shortening has been termed the latch-state. This term was chosen to compare with the catch state, that was previously described in molluscan smooth muscle (Ishii, Mitsumori et al. 1989). However, the latch-state is different from the catch state, because cross-bridges are believed to still be actively cycling during latch, while they are not during catch (Ishii, Mitsumori et al. 1989).

The latch-state is specific to smooth muscle and is not found in skeletal muscle. However, this relationship is not the same in all smooth muscles and also varies with experimental conditions. While the function of the latch-state in tonic smooth muscle is most likely to maintain force for long periods of time, the latch-state has also been described in phasic smooth muscle (Fischer and Pfitzer 1989; Kwon, Melandri et al.

1992). Yet, the forces measured in phasic muscle were usually not maintained at peak value as measured in tonic smooth muscle (Himpens, Matthijs et al. 1988).

Since the experiments of Dillon in 1981 (Dillon, Aksoy et al. 1981), many theories have been suggested to explain the latch-state but to this day, the phenomenon remains unexplained. However, because the latch-state has also been described in skinned smooth muscle, and because during latch the ATPase activity is extremely low, the latch state is believed to be a property of the contractile apparatus itself (Guth and Junge 1982; Chatterjee and Murphy 1983). Investigators have proposed two main theories that involve the contractile apparatus. The first one, suggested by Dillon and coworkers (Dillon, Aksoy et al. 1981) states that dephosphorylation of LC_{20} while myosin is attached to actin creates non-cycling cross-bridges that are capable of maintaining force, but which create a load on the other cycling cross-bridges and thereby decrease velocity. The second theory suggests that dephosphorylated myosins can reattach to actin and maintain force (Siegman, Butler et al. 1985; Himpens, Matthijs et al. 1988; Somlyo, Goldman et al. 1988; Vyas, Mooers et al. 1992; Vyas, Mooers et al. 1994).

1- Theory 1: Dephosphorylation of myosin while attached to actin creates latch-bridges:

Hai and Murphy proposed a model to explain the latch state (Hai and Murphy 1988; Hai and Murphy 1988). The basis of this model is that myosin is activated by phosphorylation of its LC_{20}, but dephosphorylation of myosin molecules that are attached to actin induces the formation of latch bridges. Thus, the model proposes that dephosphorylated myosins can maintain force and detach very slowly from actin. The existence of non-cycling attached cross-bridges had been suggested even before the discovery of latch, to explain the high resistance to stretch at rest of certain smooth muscle at intermediate intracellular Ca^{2+} levels (Siegman, Butler et al. 1976). However, the contribution of these slowly cycling or non-cycling latch bridges to the decrease in

rate of shortening remains controversial. Molecular mechanics demonstrated that unphosphorylated myosin exerts a load on faster cycling phosphorylated myosin (Warshaw, Yamakawa et al. 1989; Warshaw, Desrosiers et al. 1990), whereas energetic measurements showed the opposite (Butler, Siegman et al. 1986). Nevertheless, there are two populations of myosin molecules in the model of Hai and Murphy: phosphorylated and dephosphorylated myosin. Each one could be attached, or not, to actin but it was postulated that latch bridges could only be generated by dephosphorylation of attached cross-bridges. The assumptions of the model were as follows: 1) Ca^{2+}-dependent MLCK activation of myosin was the only regulatory mechanism (MLCP activity was not regulated but constitutive), 2) myosin molecules can cycle independently from each other, and 3) unphosphorylated myosin cannot attach to actin (Hai and Murphy 1988). The fit of the model to data was very good provided that basal values of phosphorylation were considered as experimental artifacts (Hai and Murphy 1988). The model even allowed predictions of MgATP consumption (Hai and Murphy 1988). Because in this model cross-bridge cycling is determined only by the ratio of MLCK to MLCP activity, the authors concluded that myosin phosphorylation, by a Ca^{2+}-dependent regulatory mechanism, is both necessary and sufficient to obtain latch.

This model was later refined by the addition of regulated MLCP (Rembold and Murphy 1990; Walker, Wingard et al. 1994; Etter, Eto et al. 2001). Lastly, because their model did not fit well the newer and more precise LC_{20} phosphorylation data, Rembold and coworkers improved it by incorporating cooperative activation of dephosphorylated myosin by phosphorylated myosin and actin filaments (Rembold, Wardle et al. 2004).

2- Theory 2: Reattachment of dephosphorylated myosin to actin and force maintenance:

Multiple models of the latch-state proposed that detached dephosphorylated myosin is capable of reattaching to actin and thus contributes to force maintenance. The first model suggested that dephosphorylated myosin can reattach to actin and cycle under the control of a yet to be identified Ca^{2+}-dependant regulatory mechanism, other than

phosphorylation of the LC_{20} (Siegman, Butler et al. 1985). Then, many models suggested the existence of cooperativity between dephosphorylated myosin and other contractile proteins. Several models proposed cooperativity between phosphorylated and dephosphorylated myosin (Somlyo, Goldman et al. 1988; Vyas, Mooers et al. 1992; Vyas, Mooers et al. 1994). One of these models suggested that the rate of attachment of dephosphorylated myosin was dependent on the number of attached phosphorylated cross-bridges (Vyas, Mooers et al. 1992; Vyas, Mooers et al. 1994). Another model based on the cooperativity principle between phosphorylated and unphosphorylated myosin was also proposed to explain the complex relationship between Ca^{2+} and LC_{20} phosphorylation levels and the existence of the latch state in phasic smooth muscle (Himpens, Matthijs et al. 1988). However, none of these models proposed precise molecular mechanisms to explain how phosphorylated and dephosphorylated myosin molecules cooperate.

One possibility is that cooperativity between dephosphorylated myosin and phosphorylated myosin occurs through communication across the myosin thick filament (Butler, Siegman et al. 1986). However, such a communication system between myosin molecules is unlikely because there is no difference in the relationship of v_{max} versus percentage of phosphorylated myosin in the *in vitro* motility assay between filamentous and monomeric myosin molecules (Warshaw and Trybus 1991). A better understanding of myosin filaments assembly *in vivo* may suggest further cooperativity mechanisms through the thick filaments. Nevertheless, communication mechanisms via the actin filament-associated proteins have also been suggested (Somlyo, Goldman et al. 1988; Vyas, Mooers et al. 1992; Rembold, Wardle et al. 2004) and are discussed below.

The function of caldesmon, a thin filament regulatory protein, has been studied in the context of the latch-state. Caldesmon has an amino acid sequence that is very similar to the actin-binding sequence of MLCK, and is closely associated with actin in a ratio of 1 caldesmon to14 actin. Caldesmon inhibits the ATPase activity of actomyosin by reducing the transition from weak to strong binding, probably by competition for the actin binding sites (Chalovich, Cornelius et al. 1987; Horiuchi, Samuel et al. 1991; Marston and Redwood 1992). Furthermore, caldesmon can also bind specifically to

smooth muscle myosin, thus cross-linking thick and thin filaments (Marston, Pinter et al. 1992). When put in the *in vitro* motility assay at high concentration, caldesmon can slow the velocity of actin filaments, probably by exerting a drag effect (Haeberle, Trybus et al. 1992; Horiuchi and Chacko 1995). In the view of these data, it has been proposed that caldesmon may be responsible for force maintenance in latch (Marston 1989; Walsh and Sutherland 1989). However, to date force maintenance in presence of caldesmon has never been measured. It is nonetheless difficult to conceptualize that caldesmon cross-linking may be regulated at the same time by a system that also controls active shortening. Further studies are thus necessary to elucidate a possible role of caldesmon in the latch-state.

Most recently, Rembold and coworkers refined the Hai & Murphy model (Hai and Murphy 1988) by incorporating a cooperativity parameter that is a function of myosin phosphorylation (Rembold, Wardle et al. 2004). In this new model, the number of myosin binding sites on the actin filament is a critical parameter. Moreover, the number of myosin binding sites yielding the best fit to the experimental data, is seven. Coincidently, tropomyosin, another actin regulatory protein, is linked to seven actin monomers, and increases cross-bridge cycling rate *in vitro* (Haeberle, Trybus et al. 1992), suggesting that it may play a role in cooperativity (Rembold, Wardle et al. 2004).

Although all these cooperativity models propose attractive mechanisms to explain the latch-state, they also imply the existence of a system that monitors the number of phosphorylated myosin heads and their phosphorylation status. Such a monitoring system has not been shown to date (Rembold and Murphy 1993). Furthermore, many theories of the latch-state were generated from muscle strip level measurements and extrapolation to the molecular level. However, there are no molecular level data available that show any mechanism to explain the latch state. In chapter 4 of this thesis, I report mechanics measurements addressing at the molecular level, various theories of the latch-state.

3- The role of the affinity of smooth muscle myosin for MgADP in the latch-state:

Whereas the latch state was first described in vascular tonic smooth muscle (Dillon, Aksoy et al. 1981), it became apparent that depending on the experimental conditions and methods of stimulation, it could also be reproduced in phasic smooth muscle (Siegman, Butler et al. 1985; Himpens, Matthijs et al. 1988; Fischer and Pfitzer 1989; Kwon, Melandri et al. 1992). It is however well accepted that tonic smooth muscle has a greater propensity to enter into latch than phasic smooth muscle. One of the mechanisms suggested to explain the different susceptibility of tonic and phasic muscle to enter into latch is their different affinity for MgADP. Indeed, it has been shown that MgADP decreases the rate of relaxation of tonic smooth muscle much more than for phasic smooth muscle (Fuglsang, Khromov et al. 1993). This behavior has been suggested to be due to a greater affinity of tonic smooth muscle for MgADP. Because MgADP release from myosin is necessary for its detachment from actin, such a high affinity for MgADP would allow tonic muscle myosin to remain attached to actin longer and thereby, have more chances of getting dephosphorylated while attached, i.e getting into latch. It has even been suggested that dephosphorylation simply reduces the rate of MgADP release from cross-bridges and thus slows myosin detachment (Hai and Murphy 1988). However, none of these mechanisms have been tested at the molecular level. The role of the affinity of different myosin isoforms for MgADP is addressed in Chapter 4 of this thesis.

III- MECHANICS OF AIRWAY SMOOTH MUSCLE IN AIRWAY HYPERRESPONSIVENESS AND ASTHMA:

Asthma was already described in 1776 as a recurrent respiratory difficulty without fever, with crises that were called paroxysm (Lieutaud and D'artois 1776). Asthma was also described as frequently occurring in people who worked in a dusty environment, and post-mortem macroscopic abnormalities of the lungs were also reported (Lieutaud and D'artois 1776). More than two centuries later, asthma is now described as a chronic inflammatory disease of the bronchi, clinically characterized by airway obstruction, enhanced bronchial responsiveness, and airway inflammation (Holgate 1999; Busse and Lemanske 2001). Although the role of airway smooth muscle in asthma has been challenged, and despite the fact that the synthetic and secretory function of smooth muscle may play a role in the regulation of local inflammation, it is now well accepted that airway smooth muscle is the key effector of asthma because of its ability to contract. The following section will focus on the mechanics of smooth muscle in airway hyperresponsiveness and asthma.

A- Function of airway smooth muscle:

Airway smooth muscle has a common embryologic origin with gastrointestinal smooth muscle. Whereas smooth muscle has an obvious function in the gastro-intestinal system, it is not evident in the lung. If airway smooth muscle was removed, airway tone and diameter might be altered slightly, but these changes do not have obvious physiologic consequences. Conversely, smooth muscle plays a critical role in respiratory diseases such as asthma. Therefore, this led researchers to suggest that airway smooth muscle is a vestigial remnant tissue that has no essential physiologic function but that can lead to serious medical problems (Seow and Fredberg 2001).

Historically, possible roles for airway smooth muscle have been proposed, but availability of new data has invalidated those theories. The first role of airway smooth muscle that comes to mind is protection from noxious particles by bronchoconstriction. Although many particles and gases induce smooth muscle contraction (Nadel 1973; Wright, Sun et al. 1999), a local reduction in one region of the lung would lead to a more peripheral deposition in non-constricted regions, thereby negating any beneficial effect of bronchoconstriction (Macklem, Hogg et al. 1973). It was recently suggested that airway smooth muscle may assist exhalation by a peristalsis mechanism using bronchial rhythmic constriction (Kondo, Kobayashi et al. 2003). However, gas exchange also occurs normally even when bronchi are fully relaxed, meaning that this function would be far from essential to respiration. Similarly, it does not seem that airway peristalsis helps to clear mucus significantly compared to the efficiency of ciliated epithelium and coughing. Peristaltic contractions have been observed in rabbit and pig fetal lung explants, and it was suggested that perhaps, they play a role in the differentiation and branching of airways (Schittny, Miserocchi et al. 2000). Another role of peristaltic airway contractions was suggested by physiologists who noted an enlargement of venous and lymphatic vessels around constricted airways. They hypothesized that peristaltic airway contractions play a role in blood and lymph return, but the absence of valves in these vessels and the rhythmic variations in interstitial pressure due to ventilation, invalidated this hypothesis (Albelda, Hansen-Flaschen et al. 1986). The role of airway resistance in ventilation/perfusion matching is far less important than the regional lung distensibility, thus decreasing the interest for airway smooth muscle in this matter (West 2000). In this sense, the distribution of ventilation was not affected by relaxation after administration of isoproterenol (Siegler, Fukuchi et al. 1976). The observation that airway narrowing occurs during coughing, which increases velocity of air movement and mucus removal (Mead, Turner et al. 1967), suggests that airway smooth muscle may be activated during coughing, but there is no direct evidence to support this role. To the contrary, it was suggested that bronchodilators may actually improve the effectiveness of coughing by increasing expiratory flow rates (McCool and Leith 1987). Recent developments in treatments like thermoplasty, which destroys airway smooth muscle, were shown to be promising in avoiding the negative effects of bronchoconstriction during asthma crises

(Cox, Miller et al. 2004). Altogether, these results support a vestigial role of airway smooth muscle (Seow and Fredberg 2001).

B- Altered smooth muscle mass in asthma: Static alterations:

The increase in smooth muscle thickness in asthma has been described in a morphometric study performed in 1922, when investigators compared the thickness of the airway smooth muscle layer in 5 cases of fatal asthma, and in 4 patients who died from other causes (Huber and Koessler 1922). They had already addressed the issue of finding a good reference measurement to compare airway layers thickness and used the external diameter of the airway as a reference. Indeed, this was an issue because the internal and external diameters of the airways were decreased in constricted bronchi, and asthmatic airways were constricted more often and with a greater magnitude than normal airways. It was later shown, however, that the basal membrane perimeter is a better reference because it is quite constant even at different lung volumes or when smooth muscle is contracted (James, Hogg et al. 1988; James, Pare et al. 1988). Another advantage of using the basal membrane perimeter as a reference is that smooth muscle area from asthmatic and control groups from different studies can be compared by using the square root of the airway smooth muscle area versus the airway size (basal membrane perimeter) or the value of the intercept of the airway smooth muscle area versus the airway size (James, Pare et al. 1989). Many morphometric studies have shown that the airway smooth muscle layer is either thickened, of greater area, or of increased mass in asthmatic airways (Dunnill, Massarella et al. 1969; Heard and Hossain 1973; Saetta, Di Stefano et al. 1991; Carroll, Elliot et al. 1993; Ebina, Takahashi et al. 1993; Benayoun, Druilhe et al. 2003; Woodruff, Dolganov et al. 2004). It is now well accepted that airway smooth muscle mass is increased in fatal asthma, but the increase is not as significant in non-fatal asthma (Carroll, Elliot et al. 1993; Kuwano, Bosken et al. 1993). Indeed, using a special technique that allowed to distinguish smooth muscle cells from their matrix, Thomson and coworkers found that airway smooth muscle was not increased in these cases (Thomson, Bramley et al. 1996).

Altogether these results raise questions regarding the mechanisms explaining the increase in airway smooth muscle mass, notably if it results from hyperplasia or hypertrophy of smooth muscle cells. This last issue may be of importance, because studies in vascular remodeling, where smooth muscle mass is increased, suggest that the contractility of remodeled smooth muscle cells is not equivalent to that of normal tissues (Coflesky, Jones et al. 1987). Unfortunately, studies on hypertrophy and hyperplasia of airway smooth muscle in human tissues are rare and conclusions are controversial (Heard and Hossain 1973; Ebina, Takahashi et al. 1993; Benayoun, Druilhe et al. 2003; Woodruff, Dolganov et al. 2004). Nonetheless, one study suggested that hyperplasia is present in central airways, while hypertrophy may be more pronounced in peripheral airways (Ebina, Takahashi et al. 1993). Furthermore, Woodruff and coworkers recently used precise stereomorphometric measurements and also reported hyperplasia in airway smooth muscle of endobronchial biopsies of large airways in patients with mild asthma (Woodruff, Dolganov et al. 2004).

C- Altered smooth muscle mechanics in asthma: Force and rate of shortening:

Even if it is now well accepted that the smooth muscle mass is increased in asthma, its relative contribution to bronchoconstriction is still subject to controversy. Regardless of the mechanisms responsible for the increased smooth muscle mass, it has been suggested that it leads to altered smooth muscle mechanics and thus bronchoconstriction (James, Pare et al. 1989). Bronchoconstriction is the result of the contractile force generated by airway smooth muscle, and the impedance of the parenchymal tissues attached to the airways. The elastic recoil of the lung parenchyma and the stiffness of the airway wall limit the contraction of airway smooth muscle and explain the lower degree of contraction of smooth muscle observed *in vivo* than *in vitro* (Macklem 1987). Despite the fact that parenchymal tissue may also be an important restriction to bronchoconstriction in asthma, several investigators have generated models predicting

that the most powerful parameter to obtain exaggerated airway narrowing is an increase in smooth muscle mass ,because it leads to an increase in smooth muscle force (Lambert, Wiggs et al. 1993; Macklem 1996).

In addition to the increased force production secondary to the increased muscle mass, the question was also raised by many investigators, whether airway smooth muscle is intrinsically different in asthma, either by generating more force and/or by contracting faster. Another possibility is also that asthmatic airway smooth muscle may have different mechanics when subjected to variations in length.

1- Plasticity theory of smooth muscle contraction:

Skeletal muscle is subjected to the Frank-Starling Law, which means that in the limits of normal physiological conditions, the force it generates increases linearly with the pre-contraction fiber length. Skeletal muscle can thus only generate maximal force at a precise optimal length. Conversely, the relationship between force and length is not fixed in ASM. If given enough time to recover (of the order of minutes (Naghshin, Wang et al. 2003)), smooth muscle can generate the same maximal force after length perturbations (Pratusevich, Seow et al. 1995). For example, ASM is capable of a complete readjustment of its length-tension relationship when subjected to chronic shortening (Wang, Pare et al. 2001; Naghshin, Wang et al. 2003). This shift in length-tension relationship has been referred to as plasticity of smooth muscle contraction (Pratusevich, Seow et al. 1995). It has been proposed that smooth muscle plasticity relies on contractile filament rearrangements. Two main theories on the formation of contractile filaments have been suggested. The first possibility is that myosin filaments polymerization is labile and subject to rapid turnover (Seow, Pratusevich et al. 2000; Kuo, Wang et al. 2001; Herrera, Kuo et al. 2002). The second possibility is that actin filament length is dynamic and regulated (Gunst, Meiss et al. 1995). The two theories are discussed in the following paragraphs in light of the available data.

Because of the limitations in intracellular imaging techniques, there is no experimental evidence available characterizing the assembly and disassembly of myosin filaments in smooth muscle cells. However, experimental data indirectly suggest that the number of myosin filaments, as well as their structure can change with cell length or

during activation (Kuo, Wang et al. 2001; Smolensky and Ford 2005; Smolensky, Gilbert et al. 2006). The theory of dynamic myosin filaments has been used to explain the lack of relaxation of asthmatic ASM after deep inspirations. That is, because asthmatic ASM remains in a shortened state for long periods of time, it adapts to shorter lengths, thus generates more force at shorter lengths and is more difficult to stretch (Wang, Pare et al. 2001). However, this view has been challenged by recent results showing that asthmatic airways dilate when subjected to deep inspirations, but then re-constrict faster than those of normals (Brown, Scichilone et al. 2001; Jackson, Murphy et al. 2004).

The second theory to explain smooth muscle plasticity of contraction is based on dynamic actin remodeling and suggests that mechano-transduction processes regulate actin filament length. Variable actin length would allow for a better acto-myosin overlap at different cell lengths, thus optimizing force production (Dulin, Fernandes et al. 2003). The polymerizing end of the actin filaments is made up of capping proteins (HSP27, tropomodulin, CapZ) (Hart, Korshunova et al. 1997; Bamburg 1999; Yamboliev, Hedges et al. 2000). Regulation at the level of the capping proteins can thus lengthen or shorten actin filaments. The dynamic actin length theory has also been applied to asthma: It has been speculated that longer actin filaments in asthmatic ASM cells may contribute to their elastic behavior by allowing cells to generate more force even after being stretched (Dulin, Fernandes et al. 2003).

2- Force

It is interesting to note that, although there is conflicting information between different laboratories, it is generally agreed that isometric force generation is similar between normal and asthmatic airways (Roberts, Rodger et al. 1985; Goldie, Spina et al. 1986; Whicker, Armour et al. 1988; Cerrina, Labat et al. 1989; Bai 1991; Bjorck, Gustafsson et al. 1992). Some studies reported increased force generation by asthmatic smooth muscle (Schellenberg and Foster 1984; de Jongste, Mons et al. 1987; Bai 1990; Bramley, Thomson et al. 1994; Thomson, Bramley et al. 1996) but, none except for one (Bramley, Thomson et al. 1994) also evaluated the airway smooth muscle content. The difficulty of reporting force results relative to the optimal length and normalized to

volume or mass of the smooth muscle present in the tissue should however be taken into account.

3- Velocity of shortening:

The velocity of shortening is another mechanical property of airway smooth muscle that might be altered in asthma. Evidence that it may be important in asthma came from studies addressing the mechanics of airway smooth muscle when subjected to tidal stretches, i.e. in a dynamic environment. That is, tidal inflation and deep breaths have been shown to decrease airway resistance in normal lungs (Skloot, Permutt et al. 1995). However, this effect was not observed in asthmatic patients (Fish, Ankin et al. 1981; Skloot, Permutt et al. 1995). Recent studies using high-resolution CT scans demonstrated that central and peripheral airways from asthmatics do dilate during deep inspiration, but they quickly narrow back to their initial diameter values, whereas in non-asthmatics the airways remain dilated (Brown, Scichilone et al. 2001). Furthermore, applying tidal oscillatory stretches of only 2% of the muscle length *in vitro* decreases the force generated by activated airway smooth muscle by 50% in normal airways (Fredberg, Inouye et al. 1997). Altogether these findings suggest that a critical abnormality of asthmatic airways lies in the dynamic events that follow airway tidal stretching. It has been suggested that the degree of airway narrowing is an equilibrium state that depends on the rate of acto-myosin cross-bridges interaction vs the rate of stretch imposed to airway smooth muscle by tidal breathing (Fredberg, Inouye et al. 1999). A greater rate of smooth muscle shortening is likely to compensate for the disruption of cross-bridges induced by oscillatory stretches, thus generating more force under oscillations (Gunst 1983; Fredberg, Inouye et al. 1997; Solway and Fredberg 1997; Fredberg, Inouye et al. 1999). While numerous studies concentrated on force measurements in the context of asthma and airway hyperresponsiveness, fewer studies reported airway smooth muscle rate of shortening. However, the few studies that have addressed the question reported a greater rate of airway smooth muscle shortening in various animal models of asthma (Antonissen, Mitchell et al. 1979; Fan, Yang et al. 1997; Duguet, Biyah et al. 2000), in sensitized human bronchi (Mitchell, Ruhlmann et al. 1994), and even in asthmatic human single airway smooth muscle cells (Ma, Cheng et al. 2002). The general agreement is

thus that hyperresponsive airway smooth muscle exhibits increased rate of shortening. The cause for this phenomenon is not known yet, but it may be due to increased activation of myosin and/or to differences in the content of myosin isoforms with different mechanics or kinetics. Some studies have reported that myosin light chain phosphorylation levels are increased in hyperresponsive airways, secondary to increased expression of MLCK (Jiang, Rao et al. 1992; Jiang, Rao et al. 1992). In chapter 3, we demonstrate that the fast cycling SM-B myosin isoform expression is increased in asthmatic airway smooth muscle suggesting a molecular mechanism behing the increased rate of shortening observed in asthma.

IV- GOAL OF THIS THESIS

The goal of this thesis was to investigate the role of myosin isoforms in asthma and to understand their molecular mechanics, particularly in the latch-state.

In chapter 2, I determined the nucleotide sequence of the SM-B myosin isoform in human smooth muscle and studied its distribution in various tissues. I also compared v_{max} for rat myosin purified from multiple tonic and phasic organs.

In chapter 3, I studied the mRNA expression of smooth muscle myosin isoforms and other contractile proteins in endobronchial biopsies from asthmatic and normal patients. I then investigated the molecular mechanics of the contractile proteins for which the mRNA was upregulated in asthma.

In chapter 4, I used the *in vitro* motility assay and the laser trap to investigate, at the molecular level, fundamental mechanical properties of smooth muscle myosin, in order to gain more information about the mechanisms responsible for the latch-state.

Chapter 2:

(+)Insert Smooth Muscle Myosin Heavy Chain (SM-B) Isoform Expression in Human Tissues

I- Prologue:

Smooth muscle myosin heavy chain isoforms are generated by alternative splicing of a single gene. The SM-B and SM-A isoforms have distinct mechanics and differ by the presence or absence of a seven amino acid insert in the surface loop above the nucleotide catalytic site. These isoforms are expressed in a tissue-specific manner. That is, SM-B is found in greater content in phasic smooth muscle whereas SM-A is mostly found in tonic smooth muscle. In spite of numerous studies on myosin isoforms, the sequence of the human SM-B was unavailable in databanks. Thus, I sequenced the human SM-B isoform and investigated its expression at the mRNA and protein levels in a panel of several human tissues. To assess the functional significance of the differential myosin isoform expression among organs, I developed a protocol to purify myosin from small tissue samples and measured v_{max} for myosin from multiple rat organs.

(+)Insert Smooth Muscle Myosin Heavy Chain (SM-B) Isoform Expression in Human Tissues

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Running Head: (+)insert isoform expression in human smooth muscle

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II- Abstract

Two smooth muscle myosin heavy chain isoforms differ in their amino-terminus by the presence ((+)insert) or absence ((-)insert) of a 7 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 human 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 to all organs studied except for trachea, and are significantly greater in trachea compared to uterus and aorta. To assess the functional significance of this differential myosin isoform expression between organs, we measured the rate of actin filament movement (v_{max}) when propelled by myosin purified from rat organs, because the rat and human inserts are identical and their remaining sequences show 93% identity. v_{max} 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.

Key words: Phasic and tonic smooth muscle, real-time PCR, *in vitro* motility assay

III- Introduction

Smooth muscle is found in all hollow organs of the mammalian organism and its function ranges from tone maintenance to content propulsion. Many studies are pointing to the smooth muscle myosin heavy chain (SMMHC) as an important element contributing to these diverse contractile properties (see (Karagiannis and Brozovich 2003) for review). SMMHC is made up of a globular head, containing an ATPase site and an actin binding domain, and an alpha-helical tail to which regulatory and essential light chains are bound. SMMHC isoforms are generated by alternative splicing from a single gene (Eddinger and Murphy 1988; Nagai, Kuro-o et al. 1989; Babij 1993; White, Martin et al. 1993). Four SMMHC isoforms have been described in various animal species. The first two isoforms identified differ in the carboxyl-terminus by distinct sequences of 43 (SM1) or 9 (SM2) amino acids (Babij and Periasamy 1989; Nagai, Kuro-o et al. 1989). The next two isoforms differ in the amino-terminus by the presence ((+)insert) or absence ((-) insert) of a 7 amino acid insert in a surface loop above the ATPase site (Kelley, Takahashi et al. 1993; White, Martin et al. 1993). 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 using myosin constructs, the sole presence of the amino-terminal insert doubles the actin-activated ATPase activity and the rate of actin filament movement (v_{max}) in the *in vitro* motility assay (Kelley, Sellers et al. 1992; Rovner, Freyzon et al. 1997; Lauzon, Tyska et al. 1998). Because of these properties, there is considerable interest in the possibility that the selective expression of the (+) or (-)insert isoforms could contribute to the wide range of smooth muscle contractile properties.

The expression of the (+) and (-)insert SMMHC isoforms is tissue-specific (Babij 1993; Kelley, Takahashi et al. 1993; White, Martin et al. 1993; DiSanto, Cox et al. 1997; Eddinger and Meer 2001). 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 (Hamada, Yanagisawa et al. 1990; White,

Martin et al. 1993). 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 (Babij 1993; Morano, Erb et al. 1993; Haase and Morano 1996; Sjuve, Haase et al. 1996; Siegman, Butler et al. 1997; Wetzel, Lutsch et al. 1998; White, Zhou et al. 1998; Lofgren, Fagher et al. 2002; DiSanto, Stein et al. 2003). Significantly, positive correlations between mechanical performance and the presence of the (+)insert isoform are seen in an hypertrophied rat urinary bladder model (Sjuve, Haase et al. 1996), in an hypertrophied guinea-pig small intestine model (Lofgren, Fagher et al. 2002), and in a rat model of airway hyperresponsiveness (Lauzon 2001). A (+)insert SMMHC knockout mouse (Babu, Loukianov et al. 2001) confirmed the critical role of the insert in smooth muscle contractility. Notably, we showed alterations in the time-course of bronchoconstriction in these knockout mice (Tuck, Maghni et al. 2004). While 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 both at 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 (+)insert SMMHC isoform in humans, but also provides essential information for our understanding of human smooth muscle function in normal and pathological conditions.

IV- Methods:

Sequencing of the human SMMHC insert cDNA: To determine the sequence of the human SMMHC insert, the region hypothetically 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 20μl, was reversed transcribed using 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 #NM-002474): sense primer: 5'-CCGAAAACACCAAGAAGGTC-3' (642-661 nucleotides), antisense primer: 5'-GTTGGCTCCCACGATGTAAC-3' (849-868 nucleotides). These primers amplified both myosin isoforms ((+) and (-)insert). Note that we designed these primers to bind to two different exons, separated by two large introns (4kb and 3.5kb). 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 $MgCl_2$, 10X PCR buffer (Invitrogen), 0.2 mM of each dNTP, 2 units of Platinum Taq DNA polymerase (Invitrogen), 0.4 μM of each of the sense and antisense primers, and 1μl cDNA. The samples were amplified in a Programmable Thermal Controller (PTC-100, MJ Research Inc. Watertown, MA) for 40 cycles (45 s denaturation at 94°C, 45 s 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 by 4% 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 using a miniprep kit (Qiagen). Plasmids were commercially sequenced to verify their identity and integrity.

Sequencing of the Complete SMMHC cDNA: To determine the sequence of the complete SMMHC isoform, the cDNA generated as above was amplified in 4 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 hemi-

nested PCR to yield 4 overlapping segments. The primers were designed from human (-)insert SM1 SMMHC (Genbank accession #NM-002474). The PCR mixture consisted of 2 mM $MgSO_4$, 1X High Fidelity PCR buffer (Invitrogen), 0.2 mM of each dNTP mixture, 1 unit of High-Fidelity Platinum Taq (Invitrogen), 0.2 μM of each of the sense and antisense primers for the SMMHC gene, and 1 μl of the above cDNA strand. Amplification was performed for 35 cycles (1 min denaturation at 94°C, 1 min annealing at 55°C, 80 s of extension at 68°C per kb of amplification target) and 40 min final extension at 68°C. The PCR amplification products were resolved on 0.8% TAE-agarose gel electrophoresis and visualized by ethidium bromide. Fragments were cloned and commercially sequenced.

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 to 59 Caucasian subjects (depending on the organ) aged from 15 to 75 years, with the exception of fetal tissues that were pooled from 22 to 40 weeks old fetuses. The company also assessed the RNA by electrophoresis on denaturing gel and certified its quality and quantity. 1 μg of RNA from each of these tissues was reverse transcribed simultaneously to minimize variability, in a total reaction volume of 20μl. 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 due to the additive errors introduced in generating two reliable standard curves for two sets of primers (Vandenbroucke, Vandesompele et al. 2001). The (+) and (-)insert isoforms were therefore quantified in relative terms for the selected organs, using a previously validated algorithm (Livak and Schmittgen 2001; Liu and Saint 2002; Liu and Saint 2002) 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'-AGCAGCTACAGGCTGAAAGG-3' (2904-2923 nucleotides) and antisense primer: 5'- TGGAGGATGAGATCCTGGTC -3' (3036-3055 nucleotides). 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 OD was greater than 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 isoforms relative quantification, primers were designed using the insert sequence determined above and the human (-)insert SMMHC sequence (Genbank accession #NM-002474). The 21bp insert sequence itself served as the (+)insert sense primer. The (-)insert sense primer spanned the insert junction. The sequence of the primers was as follows: (+)insert isoform: sense primer 5'-CAAGGCCCATCTTTTGCCTAC-3' (703-723 nucleotides) and antisense primer 5'-GTTGGCTCCCACGATGTAAC-3' (849-868 nucleotides), (-)insert isoform: sense primer 5'- CAAGAAAGACACAAGTATCACGG-3' (700-722 nucleotides) and antisense primer: same as for (+)insert isoform. Note that to keep efficiencies close, the primers were designed to obtain amplicons of similar length (1bp difference) and the same antisense primer was used. PCR reactions were performed in a volume of 20 μl, containing 1 μl cDNA, 10 μL 2x QuantiTect SYBR® Green PCR (Qiagen), 7 μL of nuclease-free H_2O, 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 minutes at 95°C followed by an amplification of 45 cycles (denaturation at 95°C for 15s, annealing at 60°C for 20s and extension at 72°C for 20s) and 1 melting curve cycle. PCR reactions were performed in triplicate, i.e. 3 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 was confirmed by commercial sequencing. PCR reaction efficiencies were calculated for each reaction following a previously described method (Liu and Saint 2002; Liu and Saint 2002), and were used in the quantitative analysis that

was performed using LightCycler® software (v 3.5). The results are reported as mean ±SE.

(+)Insert and Total SMMHC Semi-Quantitative 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 to 49 Caucasian subjects (depending on the organ) aged from 16 to 65 years. Electrophoresis was done through 6% SDS polyacrylamide gel using 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 100μg or with 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, that specifically recognizes the 7 amino acid insert QGPSFAY (White, Zhou et al. 1998), or with the polyclonal BT-562 (Biomedical Technologies Inc.) that 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:

$$(+)\text{insert content} = \frac{\left(\frac{(+)\text{insert in target organ}}{(+)\text{insert in reference organ}}\right)}{\left(\frac{\text{Total myosin in target organ}}{\text{Total myosin in reference organ}}\right)} \quad (1)$$

The results are reported as mean ±SE.

Rat: Four Lewis female rats, ~16 weeks of age, were purchased from Harlan (IN, USA). All experiments were conducted in compliance with the animal ethics committee of McGill University. Animals were sacrificed with an overdose of sodium pentobarbital (100mg/kg, i.p.). The small intestine, stomach, trachea, bladder, uterus, and aorta were harvested and powdered in liquid nitrogen and homogenized at 4°C in sodium pyrophosphate extraction buffer (10mM sodium pyrophosphate, 0.3M KCl, 3mM $MgCl_2$, 2mM DTT, pH=6.8). Electrophoresis was performed as above except that 50 μg of total protein were loaded onto the gel. Organs from the 4 rats were pooled and measurements were repeated 3 times. The quantification was performed as described above for the human Western blots.

(+) and (-)Insert SMMHC In Vitro Rate of Movement:

To quantify at the molecular level the differences in mechanics between tonic and phasic smooth muscle, we measured using the *in vitro* motility assay the rate of actin filament movement (v_{max}) when propelled by smooth muscle myosin purified from rat organs. Sixteen Lewis rats were sacrificed 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 (90mM KCl, 2mM Na_2ATP) followed by filament formation at 50mM $MgCl_2$. Separation of myosin from actin was performed by ammonium sulfate fractionation. Myosin fractions were then dissolved and dialyzed against low salt buffer (40mM KCl, pH 6.7) to precipitate myosin filaments. The purified myosin was then thiophosphorylated with 0.6mM ATPγS, 7.5μM calmodulin, 5μM MLCK (kindly provided by Dr. J. R. Haeberle), 0.2mM $CaCl_2$ and 2mM $MgCl_2$. Unregulated actin was purified from chicken pectoralis acetone powder (Pardee and Spudich 1982), fluorescently labelled with tetramethylrhodamine isothiocyanate phalloidin (Sigma), and diluted in actin buffer: 25mM KCl, 25mM imidazole, 1mM EGTA, 4mM $MgCl_2$, 25mM DTT (pH 7.4) with an oxygen scavenger system: 0.1mg/ml glucose oxidase, 0.018mg/ml catalase, 2.3mg/ml glucose. The *in vitro* motility assay was performed as previously described (Warshaw, Desrosiers et al. 1990). Briefly, inactive myosin molecules were

removed by ultracentrifugation. Myosin was then diluted to a concentration of 0.25mg/ml in a myosin buffer: 300mM KCl, 25mM imidazole, 1mM EGTA, 4mM $MgCl_2$, 25mM DTT (pH 7.4). The myosin in solution was flushed through a chamber (Warshaw, Desrosiers et al. 1990) 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: 0.5% methylcellulose, 2mM MgATP, 25mM KCl, 25mM imidazole, 1mM EGTA, 4mM $MgCl_2$, 25mM DTT, and oxygen scavenger as above. Measurements were made at 30 °C. Actin movement was recorded by a SIT camera, digitized and analyzed (Scion-Image software). v_{max} measurements were repeated 5 times with myosin pooled from each of the various organs from 16 rats. v_{max} was reported in □m/sec ± SD.

Statistical Analysis:

Statistical analysis of the replicated measurements on the pooled samples was performed as follows: differences between tissues in total SMMHC cDNA, (+)insert isoform cDNA and proteins, (-)insert isoform cDNA, and v_{max} were tested using 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.

V- Results:

Sequencing the human SMMHC insert cDNA:

The region of human SMMHC mRNA hypothetically coding for the 7 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 for the insert are identical (Fig.1B). The human sequence differs from the mouse by 1 bp, from the rabbit by 2bp, and from the chicken by 3 base pairs (Fig.1B). To our knowledge, this is the first report of the (+)insert SMMHC isoform in human smooth muscle.

Sequencing the Complete (+)Insert SMMHC cDNA:

The sequence of the complete human (+)insert SMMHC (~7 kb) was accomplished by RT-PCR using human bladder RNA. Compilation of the sequences obtained for the complete (+)insert SMMHC transcript revealed two isoforms of 6882 bp and 6921 bp (see Supplementary Information S1). Comparison with the available human (-)insert isoform sequence (Genbank accession #NM-002474) revealed identical sequences, except for the 21 nucleotides coding for the 7 amino acid insert. This comparison also showed that the shorter mRNA product (6882 bp) generates the longer SM1 protein and the longer mRNA product (6921 bp) generates the shorter SM2 protein, due to the insertion of a short exon encoding a stop codon . Furthermore, there are no differences in the RNA encoding the carboxyl-terminal amino acids, i.e. the (+) and (-)insert SM1 and the (+) and (-)insert SM2 isoforms. These human (+)insert SM1 and (+)insert SM2 SMMHC cDNA sequences have been deposited to Genbank (Accession numbers: AY520816-AY520817).

Quantitative Real-Time PCR Analysis:

Quantitative real-time PCR was performed in triplicate on commercial human RNA pooled from several subjects. A panel of human organs were initially screened for their total SMMHC content. Total SMMHC includes the four isoforms, i.e. (+)insert SM1 and SM2, and (-)insert SM1 and SM2, 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 ($<2X10^{-5}$ 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 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, the trachea 19 ± 3%, the bladder 10 ± 2%, the uterus 5 ± 2%, and the 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 and 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 amongst different sets of primers is invalid and because the reference organs are not the same for the (+) and (-)insert isoforms, figures 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 repeated 6 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 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 4 rats by Western blot analysis performed in triplicate (Fig.6A). A semi-quantitative 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_{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_{max} were repeated 5 times with myosin pooled from 16 rat organs. v_{max} was significantly different between all organs: small intestine (0.64±0.01μm/s), bladder (0.58±0.03μm/s), trachea (0.44±0.01μm/s), uterus (0.35±0.01μm/s) and aorta (0.23±0.01μm/s) (Fig.7A, $p<0.05$). We expected a linear regression between (+)insert protein expression and v_{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_{max}, and a test with Kendall K is significant at the critical level of 0.05 (Fig.7B).

VI- 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 semi-quantitative 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_{max} for myosin purified from phasic than from tonic rat smooth muscle, which correlated with the (+)insert isoform expression.

Mapping the Complete (+)Insert SMMHC on the Human Genome:

Using the cDNA sequence obtained for the complete (+)insert SMMHC isoform, we used UCSC's BLAT tool (Kent, Sugnet et al. 2002) and identified 42 exons spanning 6.9 kb on the reverse strand of human chromosome 16. The 21 bp sequence encoding the 7 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 (Babu, Warshaw et al. 2000; Babu, Loukianov et al. 2001) we called this exon 5b, because it is located between exons 5 and 6 (Fig.8). Exon 5b is flanked by the classical splice donor and acceptor sites AG and GT, respectively (Fig.8). The expression of the (+) or (-)insert isoform results from alternative splicing of exon 5b (Babij 1993; White, Martin et al. 1993). (Note that this exon is also located on chromosome 16 in the mouse, while it is on chromosome 10 in the rat.) Also, our cDNA sequence demonstrates that the human 7 amino acid insert is identical to that of the rat and mouse (QGPSFAY), whereas it differs by one amino acid in the chicken (QGPSFSY) and rabbit (QGPSLAY) (Fig. 1B).

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 (Nagai, Kuro-o et al. 1989), 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 and Contractile Properties:

SMMHC was detected in all tissues studied. As real-time PCR is exquisitely sensitive (Bustin 2000), it is likely that myosin from the vasculature of an examined organ is also detected, thereby explaining the weak positive signal in non-contractile organs. This could explain why mRNA coding for the (+)insert SMMHC was found in all human tissues tested except for the bone marrow. It has been shown previously that the (+)insert SMMHC is the dominant isoform in rat and rabbit small vessels and arterioles (DiSanto, Cox et al. 1997; Wetzel, Lutsch et al. 1998; Jones, Steudel et al. 1999) and blood vessels could also explain the signal detected for this isoform in all human 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 to all other organs studied except for trachea, and were significantly greater in the trachea compared to uterus and 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 while 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 (Lofgren, Fagher et al. 2002). 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 (Eddinger and Meer 2001). The total RNA we purchased came from whole human stomach which probably explains why we observed a relatively high content of both (+) and (-)insert isoform mRNA. At the protein level, however, we cannot be sure of the exact region from which the samples were taken which may explain why we observed a lower content in (+)insert isoform. Although the urinary bladder is usually viewed as a phasic smooth muscle, it does not contract as frequently as the small intestine (Lofgren, Fagher et al. 2002). A lower amount of (+)insert isoform than in the small intestine was therefore anticipated. Conversely, the aorta (Rhee and Brozovich 2000) and uterus (Haase and Morano 1996 ; Morano, Koehlen et al. 1997) are well characterized tonic muscles, and we showed here that in humans they express almost exclusively the (-)insert isoform mRNA. The rabbit trachealis, on the other hand, has been referred to as either a tonic (Horiuti, Somlyo et al. 1989) or a phasic (Malmqvist and Arner 1991) smooth muscle. Topographical differences have also been reported in rat tracheal contractile properties; the segment closer to the main carina contracts more in response to an agonist (Florio, Styhler et al. 1996). Our results show that the human trachea contains mid-range levels of the (+)insert isoform mRNA and high levels of (+)insert protein, but again we cannot be sure of the exact segments from which our samples were taken. It is worth noting that a previous study reported that the (+)insert isoform mRNA was absent in human airways (Ma, Cheng et al. 2002). However, using primers specific to human smooth muscle myosin, we selectively quantified each isoform and demonstrated unequivocally that the (+)insert isoform mRNA is expressed in human airways and we confirmed its presence at the protein level.

While the regulation of the (+) and (-)insert isoforms by alternative splicing is still poorly understood, a reasonable correlation was observed between the mRNA and protein data (Figs. 3 & 5) suggesting minor post-transcriptional 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 v_{max}, 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 7 amino acid insert and not other potential inter-species variability in the SMMHC sequence.

Effects of Insert on Contractilty:

While 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 (Horiuti, Somlyo et al. 1989). 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, in the *in vitro* motility assay, v_{max} 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_{max}. Previous studies have also measured v_{max} in baculovirus expressed constructs differing only by the 7 amino-acid insert and similar results were reported (Rovner, Freyzon et al. 1997; Lauzon, Tyska et al. 1998). 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 (Fuglsang, Khromov et al. 1993; Khromov, Somlyo et al. 1995; Lauzon, Tyska et al. 1998; Sweeney, Rosenfeld et al. 1998) which is the rate limiting step in shortening velocity (Siemankowski, Wiseman et al. 1985). 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 (Fuglsang, Khromov et al. 1993; Khromov, Somlyo et al. 1995) 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 2-fold faster rate of MgADP release for (+)insert isoform constructs (Lauzon, Tyska et al. 1998). Furthermore, we recently found a greater affinity for MgADP of purified tonic calf aorta

myosin compared to phasic chicken gizzard myosin in the *in vitro* motility assay (Leguillette 2004). This is perhaps not surprising because the 7 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 (Kelley, Takahashi et al. 1993; Rovner, Freyzon et al. 1997; Lauzon, Tyska et al. 1998), 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 (Murphy, Walker et al. 1997), 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 the (+)insert SMMHC isoform expression has been studied in many models of pathology. Expression of the (+)insert SMMHC isoform decreases by 50% in the hypertrophied rat urinary bladder while the maximal shortening velocity and rate of force development decreases by 20% and 25%, respectively (Sjuve, Haase et al. 1996). 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 (Lofgren, Fagher et al. 2002). An 11-fold greater expression of the (+)insert SMMHC isoform is measured in the hyperresponsive rat of a genetic model of asthma (Lauzon 2001). The rate of contraction of explanted airways was also shown to be greater in airways of these hyperresponsive animals (Wang, Almirall et al. 1997). Other studies have also shown alterations in the (+)insert SMMHC isoform expression in pregnancy or in models of diseases, though the correlation to muscle function is not as clear (Morano, Erb et al. 1993; Siegman, Butler et al. 1997). 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.

ACKNOWLEDGEMENTS

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VII- Figures:

Fig. 1 A: PCR amplification products from cDNA derived from human bladder RNA. The primers were designed to amplify both the (+) and (-)insert SMMHC isoforms. Results presented in duplicate. **B: Nucleotide sequence encoding for the (+)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 chicken.

Figure 1

A

B

Nucleotides

SMB (+) Insert

Human: 609	GGCAAGAAAGACACAAGTATCACG	CAAGGCCCATCTTTTGCCTAC	GGAGAGCTGG 655
Rat:	----------GC--C------	--------------------	--G-------
Mouse:	----------GC--C------	-----T---------------	--G-------
Chicken:	--GC-A--G-----C--C-----T	-----T---------T-T---	--T-------
Rabbit:	--------G-----G--C------	-----T------C--------	--G-------

Amino Acids

SMB (+) Insert

Human: 204	G	K	K	D	T	S	I	T	Q	G	P	S	F	A	Y	G	E	L 222
Rat:	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-
Mouse:	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-
Chicken:	-	Q	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-
Rabbit:	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-

Fig. 2: Total SMMHC. Absolute quantification of human total SMMHC cDNA in a panel of organs (mean±SE). Measurements were performed in triplicate on commercial RNA pooled from 5-59 individuals. The inset shows the standard curve used.

Figure 2

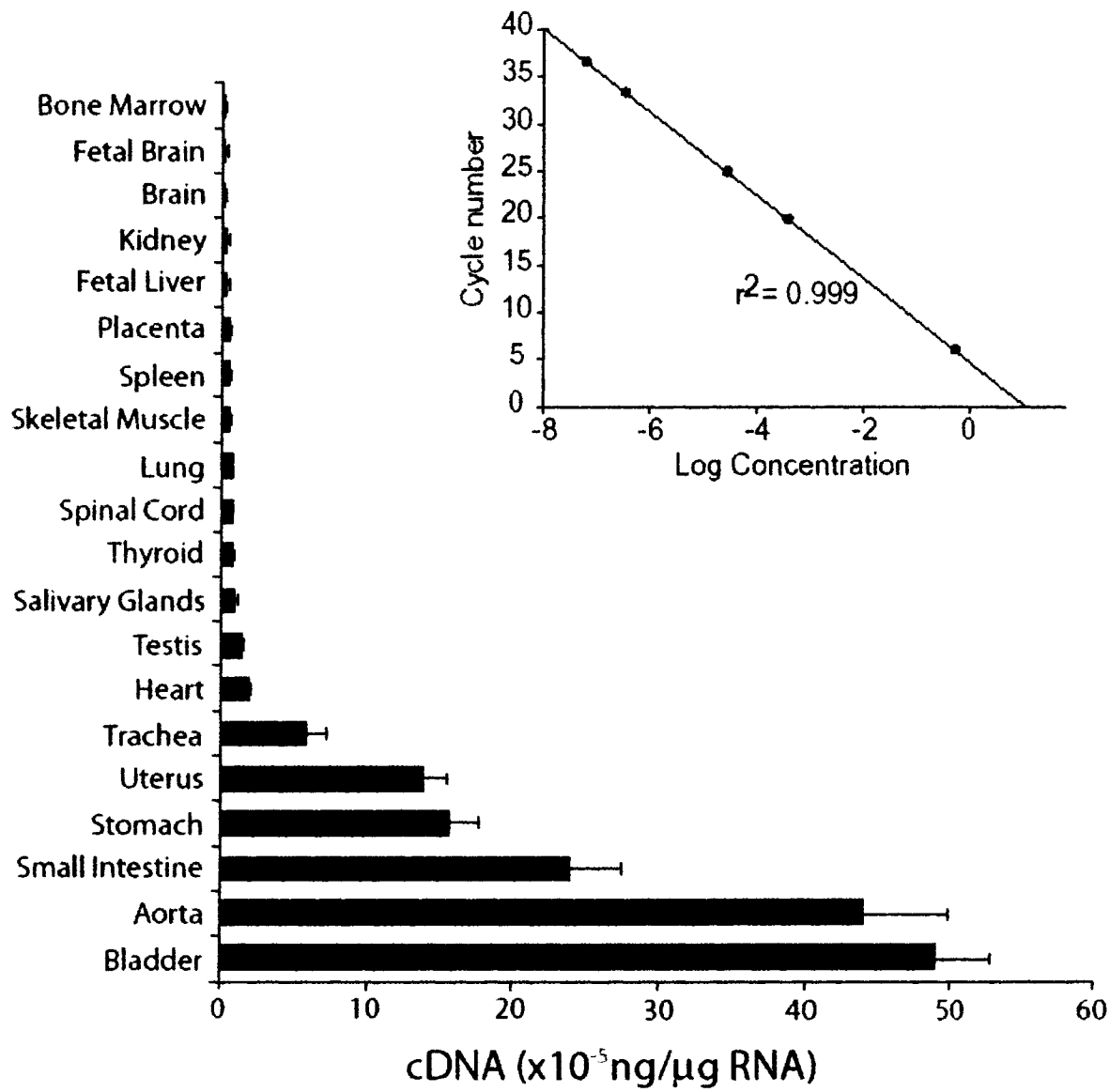


Fig. 3: (+)insert isoform cDNA analysis: Relative quantification of the human (+)insert SMMHC isoform with respect to the content in the calibrator organ, small intestine, and normalized to total SMMHC (mean±SE). Measurements were performed in triplicate on commercial RNA pooled from 5-59 individuals. Statistical differences were found between the small intestine and all other organs ($p<0.05$), between the stomach and all other organs ($p<0.05$), and between the trachea and uterus or aorta ($p<0.05$).

Figure 3

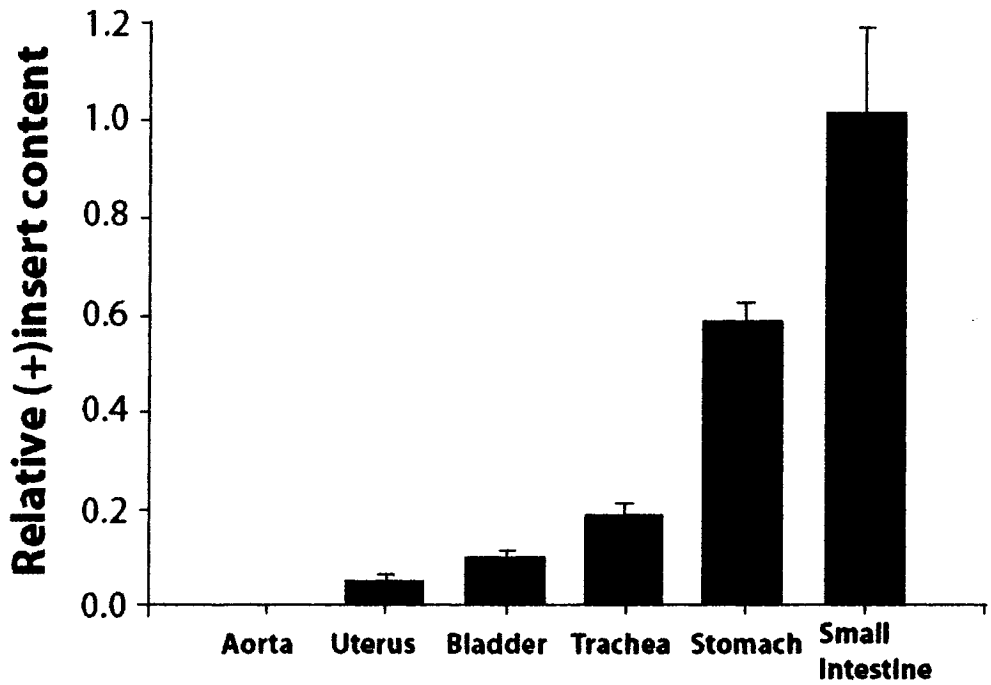


Fig. 4: (-)insert isoform cDNA analysis: Relative quantification of the human (-)insert SMMHC isoform with respect to the content in the calibrator organ, uterus, and normalized to total SMMHC (mean±SE). Measurements were performed in triplicate on commercial RNA pooled from 5-59 individuals. The uterus, stomach and aorta express statistically more (-)insert than the bladder and small intestine ($p<0.05$).

Figure 4

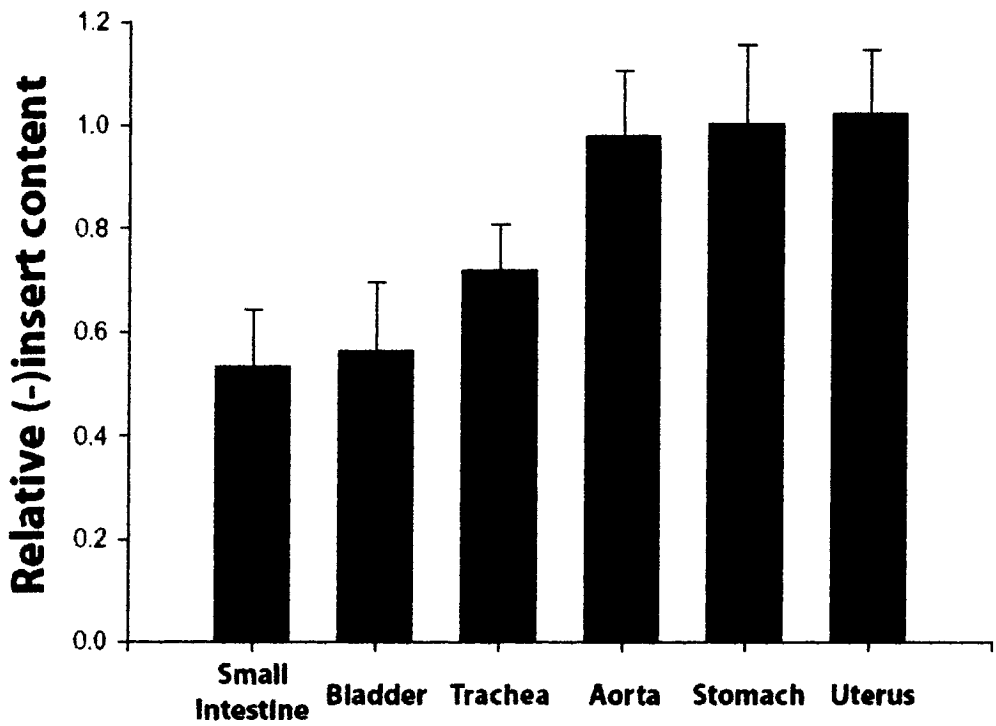


Fig.5 A: Western blot of human SMMHC and (+)insert protein: Western blot of total SMMHC and (+)insert isoform protein in the aorta, uterus, bladder, trachea, stomach, small intestine and brain. In these representative western blots,160 and 60 μg of total protein were loaded onto the gel for detection of the (+)insert SMMHC isoform and total SMMHC, respectively. **B: Human (+)insert isoform protein analysis:** Relative quantification of the human (+)insert SMMHC isoform with respect to the small intestine, and normalized to total SMMHC (mean±SE). The Western blots were repeated 6 times on commercial samples pooled from several subjects. All organs except the trachea express statistically less (+)insert protein than the small intestine and the aorta and uterus express statistically less (+)insert than the trachea ($p<0.05$).

Figure 5

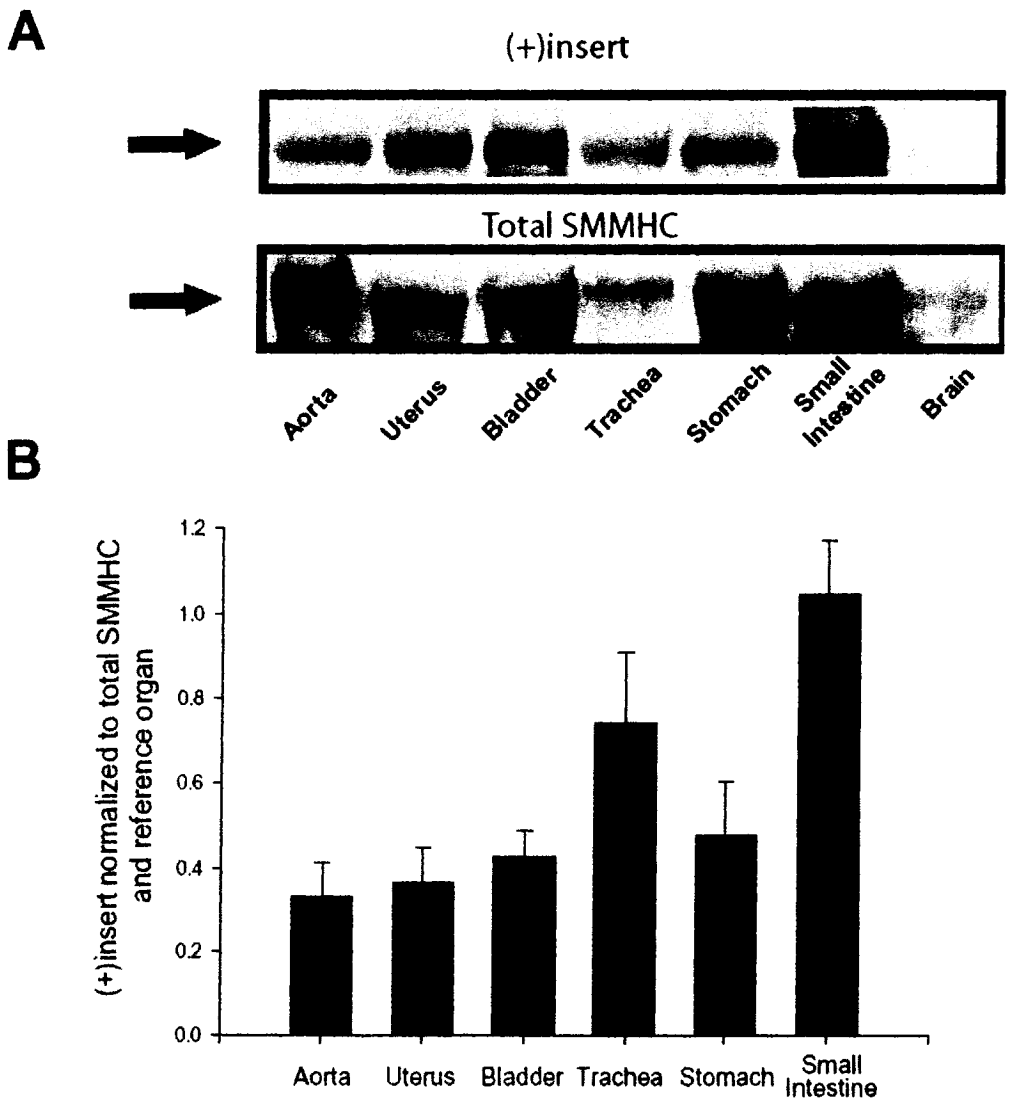


Fig. 6 A: Western blot of rat SMMHC and (+)insert protein: Western blot of total SMMHC and (+)insert isoform protein in the aorta, uterus, bladder, trachea, stomach and small intestine. 50 μg of total protein were loaded onto the gel. **B: Rat (+)insert isoform protein analysis:** Relative quantification of the rat (+)insert SMMHC isoform with respect to the small intestine, and normalized to total SMMHC (mean±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 to that in human p=0.45).

Figure 6

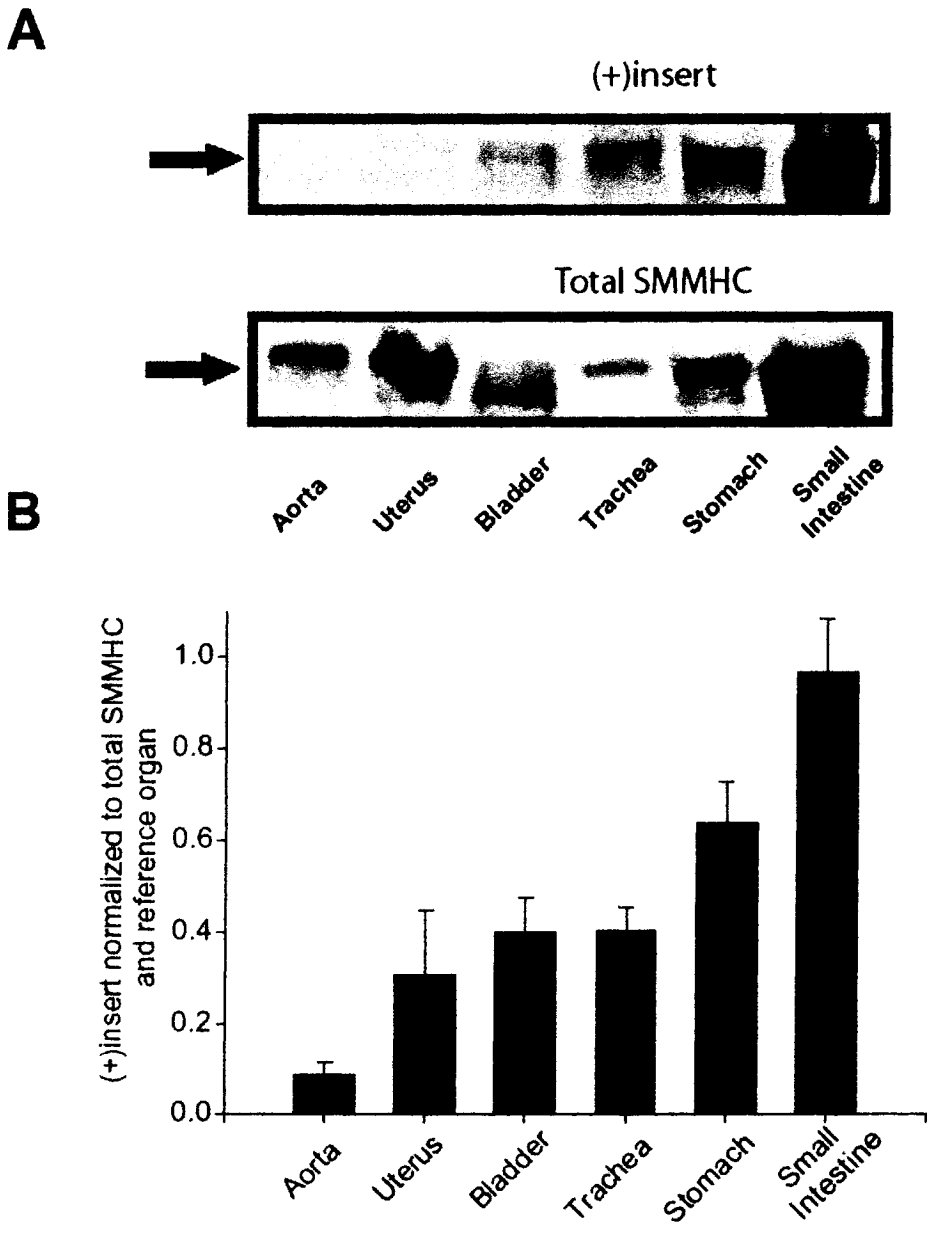


Fig.7 A: Rate of actin filament movement in the *in vitro* motility assay: Rate of actin filament movement (v_{max}) when propelled by myosin purified from rat aorta, uterus, bladder, trachea, and small intestine (mean±SD). v_{max} measurements were repeated 5 times with myosin pooled from 16 rat organs.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

Figure 7

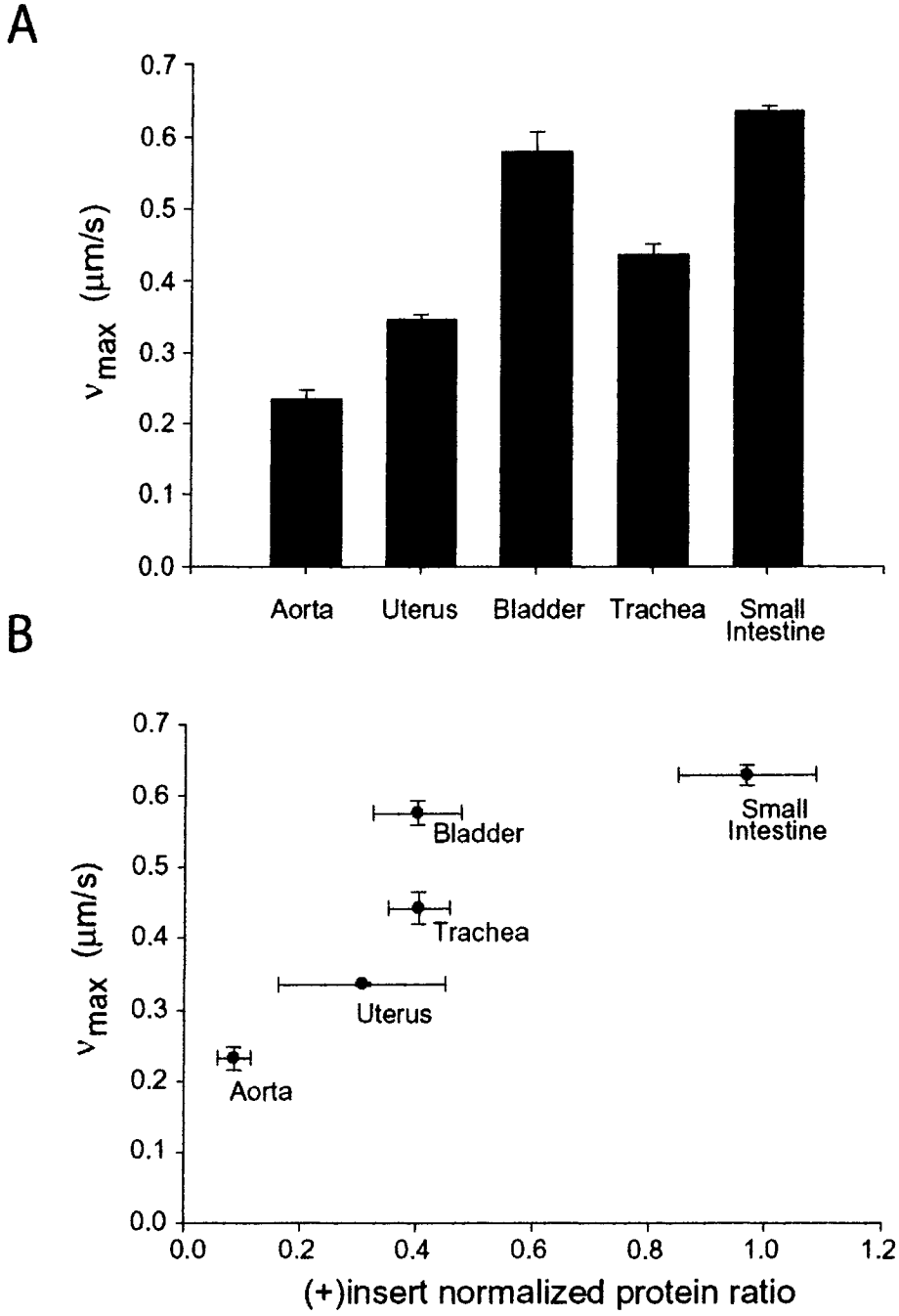
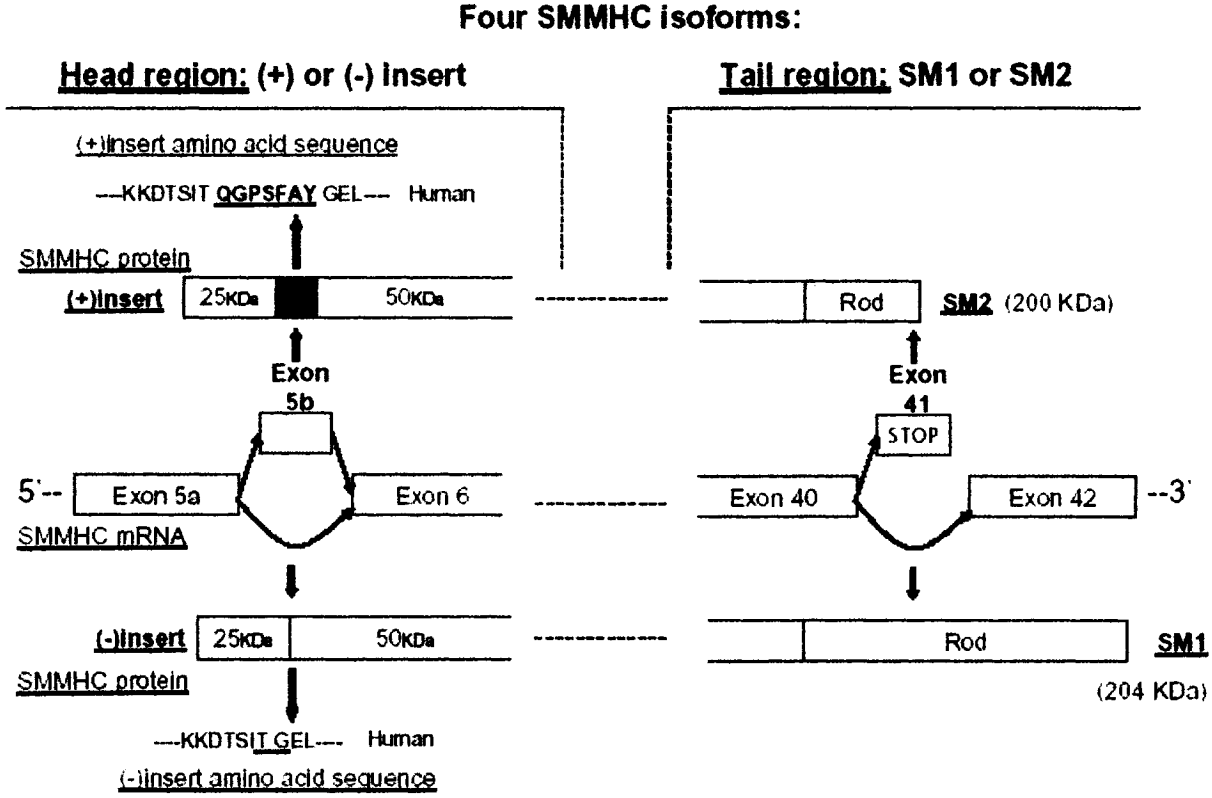


Fig. 8: Alternative splicing at the 5' and 3' ends of the SMMHC: Schematic illustrating the proposed mechanism for the alternative splicing of the mRNA that occurs in the human SMMHC at the 5' and 3' ends. Inclusion of exon 5b generates the (+)insert isoform, whereas inclusion of exon 41, encoding a stop codon, generates the shorter (200kDa SM2 isoform). The insert is shown in bold and underlined.

Figure 8



Chapter 3:

Myosin SM-B isoform, SM22, and MLCK are upregulated in mild asthmatic endobronchial biopsies

I- Prologue:

Asthma is characterized by airway obstruction, enhanced bronchial responsiveness, and airway inflammation. It is now well accepted that smooth muscle hypercontractility is a major contributor to the symptoms observed in asthma. Tidal breathing and deep inspiration have a bronchodilating effect in normal subjects but not in asthmatics. One hypothesis to explain this phenomenon is that airway smooth muscle from asthmatics contracts faster, thus catching back the dilatation induced by tidal stretch. An increase in airway smooth muscle rate of shortening has already been described in animal models of hyperresponsiveness and in human asthma. Thus, I investigated the expression of contractile protein genes in endobronchial biopsies from asthmatic and control subjects and tested their function in the *in vitro* motility assay.

Overexpression and function of the myosin SM-B isoform, SM22, and MLCK in mild asthmatic endobronchial biopsies

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II- Abstract:

Airway smooth muscle (SM) exhibits a greater velocity of shortening (Vmax) in asthmatics than in normals. This increase in Vmax is believed to counteract the relaxing effect of tidal breathing, thus maintaining asthmatic airways constricted. A greater Vmax can result from increased myosin activation. This has been reported in sensitized human airway SM and in models of asthma. A faster Vmax can also result from the expression of specific contractile protein genes that promote faster cross-bridge cycling. This possibility has never been addressed in asthma. We hypothesized that the expression of SM genes is altered in asthmatic airways, thus contributing to their increased Vmax. We quantified the expression of several SM genes in human airway endobronchial biopsies and found that the fast SM myosin heavy chain isoform (SM-B), transgelin (SM22), and myosin light chain kinase (MLCK), are increased in mild allergic asthmatics. Furthermore, we showed by immunohistochemistry that these genes are expressed at the protein level in both normals and asthmatics. To address the functional significance of this overexpression, we purified tracheal myosin from Fisher and Lewis rats, a model of innate airway hyperresponsiveness, and found a faster rate of actin filament propulsion (v_{max}) in the *in vitro* motility assay for myosin from the hyperresponsive rats. Moreover, we showed that, beyond its enzymatic effects, MLCK can mechanically increase v_{max}. Conversely, SM22 did not alter v_{max}. Our findings suggest that selective overexpression of SM genes in asthmatic airway leads to increased Vmax, thus contributing to the airway hyperresponsiveness observed and asthma.

III- Introduction:

The primary features of asthma are airway inflammation, bronchial hyperresponsiveness and intermittent airway obstruction. Altered airway smooth muscle (SM) function is considered to be an important contributor to airway hyperresponsiveness and asthma (Martin, Duguet et al. 2000). As suggested by mathematical models, airway narrowing results from a balance between the contraction produced by airway SM and the impedance of the surrounding tissues (Lambert, Wiggs et al. 1993; Macklem 1996). Asthmatic airway SM exhibits enhanced contractility (Bjorck, Gustafsson et al. 1992; Mitchell, Ruhlmann et al. 1994). This could be due to an increased SM mass and/or increased power of the contractile apparatus. Although still controversial, it is now believed that increased SM mass alone does not account for airway hyperresponsiveness (Eidelman, DiMaria et al. 1991; Thomson, Bramley et al. 1996; Thomson and Schellenberg 1998). Conversely, a greater velocity of airway SM shortening (Vmax) has been observed in many animal models of asthma (Antonissen, Mitchell et al. 1979; Fan, Yang et al. 1997; Duguet, Biyah et al. 2000), in sensitized human bronchi (Mitchell, Ruhlmann et al. 1994), and in single SM cells from asthmatic human airways (Ma, Cheng et al. 2002). A greater Vmax is likely to counteract the relaxing effect of tidal breathing and could therefore maintain asthmatic airway SM in a more constricted state (Gunst 1983; Solway and Fredberg 1997). While a greater force generating capacity would also contribute to an increased muscle power, several studies have reported that there was no difference in isometric force generation between asthmatic and control airway SM (Whicker, Armour et al. 1988; Bai 1991).

The contractile apparatus is responsible for muscle force and movement production. Vmax depends on the proteins involved and their level of activation. Myosin is the molecular motor that drives muscle contraction. Alternative splicing of the SM myosin heavy chain (SMMHC) gene generates four isoforms. Splicing in the 5' region results in the expression of two isoforms that differ by the presence (SM-B) or the absence (SM-A) of a seven amino acid insert in the surface loop above the nucleotide binding pocket (Babij 1993; White, Martin et al. 1993). The SM-B and SM-A isoforms are also referred

to as (+) and (-) insert isoforms, respectively. Splicing in the 3' region leads to the expression of two isoforms that differ by distinct sequences of 43 (SM-1) or 9 (SM-2) amino acids at the carboxy terminal (Eddinger and Murphy 1988; Nagai, Kuro-o et al. 1989). Although no differences in the molecular mechanics of SM-1 and SM-2 have been reported, SM-B propels actin filaments at two times the velocity (v_{max}) of SM-A, in the *in vitro* motility assay (Kelley, Takahashi et al. 1993; Rovner, Freyzon et al. 1997; Lauzon, Tyska et al. 1998). The expression and function of these SMMHC isoforms in airway SM hypercontractility has never been thoroughly addressed.

It is well established that smooth muscle contraction is mainly regulated by phosphorylation of the myosin regulatory light chains (LC_{20}) by myosin light chain kinase (MLCK) (Sobieszek 1977). Recently, it has been shown that MLCK can bind simultaneously to myosin and actin, and that this binding stimulates the ATPase activity, independently of the phosphorylation activity (Sellers and Pato 1984; Okagaki, Hayakawa et al. 1999; Ye, Kishi et al. 1999). Increased expression of MLCK has been described in models of asthma and in human airway SM (Jiang, Rao et al. 1992; Ammit, Armour et al. 2000; Benayoun, Druilhe et al. 2003) but its mechanical role in the enhancement of Vmax has never been addressed. Actin binding proteins are also known to alter SM cross-bridge kinetics. For example, caldesmon decreases both the ATPase activity and v_{max} (Okagaki, Higashi-Fujime et al. 1991; Haeberle, Trybus et al. 1992) whereas tropomyosin increases v_{max} (Okagaki, Higashi-Fujime et al. 1991). However, the expression of actin binding proteins has never been addressed in airway hyperresponsiveness and asthma.

In this study, we hypothesized that the expression of genes that code for contractile proteins is altered in asthmatic airway SM, thus contributing to their increased Vmax. We found that the expression of SM-B, transgelin (SM22) and MLCK is increased in endobronchial biopsies from humans affected with mild asthma. Furthermore, we found that the myosin purified from airways of the Fisher rats, an animal model of innate bronchial hyperresponsiveness that overexpresses SM-B in airway SM, has a greater v_{max} than myosin from controls. We also found that MLCK, in addition to its enzymatic

activity, can mechanically enhance v_{max}. Conversely, SM22 had no effect on cross-bridge cycling rate. Our findings suggest that the selective contractile protein gene expression in asthmatic airway SM leads to increased velocity of shortening, thus contributing to the airway hyperresponsiveness observed in asthma.

IV- Material and methods:

Selection of subjects for endobronchial biopsies:

Eight asthmatic subjects, whose diagnoses were made according to the definition of the American Thoracic Society and eight healthy subjects without allergy history, asthma, nor occupational exposure to sensitizing agents were recruited. The clinical characteristics of the subjects are provided in Table 1. Evaluation included a medical history, physical examination, skin prick tests to common allergens (Omega, Montreal, Canada), spirometry, and measurements of airway responsiveness to inhaled methacholine according to standardized procedures (ATS 1987). All subjects were nonsmokers and had not had respiratory infection within the last 2 months. Asthmatics were stable and were receiving a treatment of inhaled β2-agonist on demand, but no inhaled corticosteroid therapy. Control subjects had no systemic disease, were not receiving any medication, and had negative skin prick tests. The study was approved by the Laval Hospital and the Montreal Chest Institute Research Ethics Board of McGill University Health Center and all subjects provided written informed consent for their participation.

Bronchoscopy and endobronchial biopsy process:

Oxygen was administered at 5 L/min by a nasal catheter, and vital signs, electrocardiogram, and oxymetry data were monitored during the bronchoscopy. Local anesthesia of the airways was done with 2 or 4% lidocaine, up to a total dose of 400 mg. A flexible bronchoscope (Olympus OES 10 fiberscope; Olympus, Markham, Canada) and alligator forceps (Olympus FB-15C-1) were used for the procedure. Six to seven specimens were taken from the origins of sub-segmental, segmental or lobar bronchial carinae and kept in RNA-later® solution (Qiagen Inc., Mississauga, Canada) for mRNA extraction. In 1 control and 2 asthmatics, additional biopsies were taken and fixed in 4% paraformaldehyde for immunohistochemistry.

Relative quantification of contractile mRNA by real-time PCR analysis:

We quantified by real-time PCR the mRNA expression of myosin isoforms (SM-1, SM-2, SM-A, SM-B), actin isoforms (α, γ), SM myosin light chain kinase (MLCK), tropomyosin isoforms (α, β), SM caldesmon, and transgelin (SM22) in the endobronchial biopsies of five asthmatic and five healthy subjects. All primer sets spanned at least one intron. The sequence of the primers is shown in Table 2. The primer set called "Total SMMHC" amplifies the four SMMHC isoforms because it targets a region not subject to alternative splicing. The six to seven biopsies from each patient were pooled and homogenized in RNAlater® buffer and total RNA was extracted using a commercial kit (Mini Prep, Qiagen) following the manufacturer's recommendations. 1μg of RNA from each of these subjects was reverse transcribed simultaneously to minimize variability. PCR reactions were performed in a volume of 20μl, containing 1 μl cDNA, 10 μL 2x QuantiTect SYBR® Green PCR (Qiagen), 7 μL of nuclease-free H_2O, 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 minutes at 95°C followed by an amplification of 50 cycles (denaturation at 95°C for 15 s, annealing at 60°C for 20 s and extension at 72°C for 20s) and 1 melting curve cycle. PCR reactions were performed in triplicates, i.e. 3 repeats on the same RNA sample pooled from each subject. Each primer set generated only one PCR product. PCR reaction efficiencies were calculated for each reaction following a previously described method (Liu and Saint 2002; Liu and Saint 2002) and were used for relative quantification with REST© software (v2) (Pfaffl, Horgan et al. 2002). Results were expressed in fold increase in asthmatics compared to controls, using the ribosomal protein S9 as a reference gene and a correction was made for the differences in primer efficiency.

Immunohistochemistry on biopsies:

Protein expression of the SM-B SMMHC isoform, SM22, and MLCK was verified by immunohistochemistry in five asthmatic and five control subjects (some of the subjects were tested both at the mRNA and protein levels, see table 1). The paraformaldehyde-fixed biopsy specimens were dehydrated in alcohol, incubated in xylene and embedded in blocks of fresh molten paraffin wax. Sections of 5 μm were made with the cutting angle

in the axis of the bronchial mucosa. For general assessment of the tissue, three slides were deparaffinized, rehydrated and stained with hematoxylin-eosin. Only biopsies with smooth muscle bundles and preserved morphology were used. A peroxidase protocol for paraffin embedded sections was used for immunohistochemistry. For each target protein, all procedures were performed simultaneously and in the same conditions for asthmatics and normals. Slides were deparaffinized in xylene and in decreasing concentrations of alcohol. Tissues were permeabilized with Triton 0.2% and incubated with 5% peroxide except for MLCK, where they were initially incubated in citrate buffer (10 mM citric acid, pH= 6.0). After blocking with universal blocking solution for 30 min (Dako, Carpinteria, CA), slides were incubated overnight with the following anti-human primary antibodies against the SM-B SMMHC isoform (SM-B7, gift from Dr. A. Rovner, U. Vermont) (Low, Mitchell et al. 1999), SM22 (ab10135, ABCam), and MLCK (M7905, Sigma). Subsequently, the slides were incubated with biotinylated secondary antibody (E0453, Dako) and with ABC complex (Dako), and were then stained with DAB (diaminobenzidine)-chromogen (Dako) and counterstained with hematoxylin.

To control for non-specific binding, a competitive pre-adsorption test was performed. A synthetic peptide of the seven amino acid insert sequence (QGPSFAY), purified smooth muscle MLCK (generous gift from Dr. J. Heaberle), and recombinant SM-22 (see below) were incubated for 2 hours at room temperature with their respective antibodies at a molar ratio of 1:100. Incubation of slides with these antigen-antibody complexes were then performed, in the same conditions as described above.

Molecular mechanics:

We used the *in vitro* motility assay to quantify the rate of actin filament movement (v_{max}) when propelled by myosin molecules. The *in vitro* motility assay was described before (Lauzon, Tyska et al. 1998; Leguillette, Gil et al. 2005). The buffers were as follows: myosin buffer (300mM KCl, 25mM imidazole, 1mM EGTA, 4mM $MgCl_2$, 25mM DTT, pH 7.4); actin buffer (25mM KCl, 25mM imidazole, 1mM EGTA, 4mM $MgCl_2$, 25mM DTT, pH 7.4); and motility buffer (0.5% methylcellulose, 2mM MgATP, 25mM KCl, 25mM imidazole, 1mM EGTA, 4mM $MgCl_2$, 25mM DTT, pH 7.4) combined with an

oxygen scavenger (0.1mg/ml glucose oxidase, 0.018mg/ml catalase, 2.3mg/ml glucose). Myosin regulatory light chains were thiophosphorylated with 0.6mM ATPγS, 7.5μM calmodulin, 5μM MLCK, 0.2mM $CaCl_2$ and 2mM $MgCl_2$. Inactive myosin molecules were removed by ultracentrifugation (Lauzon, Tyska et al. 1998). Myosin (0.25mg/ml) was then perfused into a flow-through chamber (Warshaw, Desrosiers et al. 1990) and allowed to attach randomly onto the nitrocellulose-coated glass. Inactive myosin molecules were further inhibited by the addition of monomeric unlabelled actin dissolved in actin buffer. Actin fluorescently labeled with tetramethylrhodamine isothiocyanate phalloidin (TRITC P1951, Sigma) in actin buffer was then perfused into the chamber, followed by motility buffer. Actin movement was recorded by a SIT camera, digitized and analyzed (Scion-Image software). Measurements were made at 30 °C. v_{max} was reported in μm/sec ± SD. v_{max} was measured for three separate purifications from each rat strain. For each *in vitro* motility assay, the movement of a minimum of ninety filaments was analyzed.

Protein purification for the in vitro motility assay:

Tracheal SM myosin was purified from 8 Fisher and 8 Lewis rats, following our previously described protocol for small samples (Leguillette, Gil et al. 2005). All experiments were conducted in compliance with the animal ethics committee of McGill University. Chicken gizzard SM myosin was purified following a standard protocol (Sellers, Pato et al. 1981). Skeletal myosin was a generous gift from Dr. P. VanBuren (U. of Vermont). Unregulated actin was purified from chicken pectoralis acetone powder following the Pardee and Spudich protocol (Pardee and Spudich 1982). Recombinant SM22 was generated in *Escherichia coli* as previously described (Fu, Liu et al. 2000). Briefly, cDNA containing the full-length SM22 coding region was ligated in frame into *Bam*H I- and *Hin*d III-digested pQE-30 bacterial expression vector (Qiagen) containing an NH_2-terminal MRGHHHHHHGS "His" tag. Recombinant His-tagged SM22 was expressed in *Escherichia coli* and batch purified under native conditions using nickel [Ni-nitrilotriacetic acid (NTA)] beads (Qiagen). Transformed *E. coli* were grown to optical density of 0.6-1.0 at 600 nm, and then recombinant protein expression was induced for 6 h with 1 mM isopropyl-β-D-thiogalactopyranoside. Bacteria were lysed on ice for 30

min in buffer containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidiazole, and 1 mg/ml lysozyme followed by sonication on ice using a microtip. Lysate was centrifuged to remove cell debris, and then Ni-NTA beads were added and allowed to bind at 4°C for 30 minutes. Bound proteins and beads were washed 4 times with 50 mM sodium phosphate pH 8.0, 300 mM NaCl and 50 mM imidiazole. The retained proteins were eluted and stored in buffer containing 50 mM sodium phosphate pH 8.0, 300 mM NaCl, 250 mM imidazole, and protease (serine, cysteine, calpain, and metalloprotease) inhibitors (Roche). Two purifications of SM22 recombinant protein were done and tested in the *in vitro* motility assay.

Incubation, cosedimentation and immunoprecipitation of SM22 with actin for the in vitro motility assay:

SM22 was bound to unregulated actin following a previously described protocol (Fu, Liu et al. 2000) with slight modifications. Briefly, recombinant SM22 (3μM) was incubated in presence of unregulated monomeric actin (10μM) and tetramethylrhodamine isothiocyanate phalloidin (10μM) (Sigma) for 12 hours at 4°C in a buffer containing 12mM KPi (pH=6.8), 20mM imidazole, 24mM NaCl, 2mM $MgCl_2$, 1mM ATP, 1mM EGTA. To ascertain that only SM22-bound actin was used in the *in vitro* motility assay, cosedimentation of actin with SM22 was performed, and the pellet was washed twice and resuspended in actin buffer before use in the *in vitro* motility assay. For cosedimentation, the actin-SM22 solution was spun at 100,000g for 21 minutes. We further verified that the pellet contained mostly actin-bound SM22 using immunoprecipitation. The acto-SM22 mixture was incubated with an actin-specific antibody (#4700, Sigma) at 4°C. Protein-A sepharose beads (Amersham) were then incubated with the acto-SM22 solution for one hour before being spun down at 15000rpm for 2 minutes. The supernatant and the pellet were then boiled in Leammli buffer and loaded onto a gel for SDS-PAGE, followed by Western blotting (described below), with a primary antibody that targeted SM22 (ab10135, ABCam). To ascertain the sepharose beads were not non-specifically pulling down actin or SM22, control experiments were performed using different primary and secondary antibodies as shown in figure 8B.

MLCK incubation with skeletal myosin:

To bind MLCK to skeletal myosin, MLCK (1 μM) was incubated for 1 hour at 4°C with skeletal myosin (1 μM) in myosin buffer.

SDS-PAGE and Western Blotting of purified myosin:

The relative content of the SM-B myosin isoform with respect to total myosin in the Fisher and Lewis rat trachealis purifications was determined by Western blot analysis. Proteins were separated by SDS-PAGE using a 7% acrylamide gel. Equal volumes of myosin solutions were loaded onto a single gel. Protein concentration was estimated by a standard Bradford assay, and 100μg of proteins were loaded in each well. Proteins were transferred electrophoretically onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% nonfat milk and probed with the polyclonal SM-B6 antibody that specifically recognizes the 7 amino acid insert QGPSFAY (White, Martin et al. 1993; Low, Mitchell et al. 1999), or with the polyclonal BT-562 antibody (Biomedical Technologies Inc.) that recognizes all SMMHC isoforms. Antibody detection was done by Enhanced Chemiluminescence (Amersham) and quantification was performed with a Fluorchem 8500 imaging system using AlphaEase software (Alpha Innotech). Quantification was performed for three separate purifications, and loaded in duplicate for each rat strain, on six nitrocellulose membranes (Fig. 5B). The results are reported as mean ±SE.

Statistical Analysis:

Differences in mRNA expression between biopsies from asthmatics and controls were tested using Pair Wise Fixed Reallocation Randomisation Test© in REST© software (v 2). Differences in the SM-B tracheal protein content and in v_{max} between Fisher and Lewis rats, between actin bound SM22 and no SM22, and between myosin incubated with MLCK and no MLCK, were all tested using Student's t-tests. Significance was considered for values of $p<0.05$.

V- Results:

Contractile gene expression in asthmatic and control endobronchial biopsies:

We quantified, by real time PCR, the expression of SM-specific contractile genes in endobronchial biopsies from asthmatic and control subjects (Fig.1). The housekeeping gene ribosomal S9 unit was very stable and had similar levels in asthmatic and control biopsies (Asthmatic/control: +1.03, p= 0.78). Thus, ribosomal S9 was an adequate housekeeping gene. There was no significant difference between asthmatics and controls mRNA expression in the SMMHC SM-1 isoform, tropomyosin β, caldesmon, and α–actin. The expression of tropomyosin α (Asthmatic/control: 0.78, $p<0.03$) and γ–Actin (Asthmatic/control: 0.53, $p<0.01$) was significantly lower in asthmatics compared to controls. Conversely, the mRNA expression of total SMMHC, SM-2, SM-A, SM-B isoforms was significantly upregulated in asthmatics, along with significant upregulation of SM22 and MLCK. The increase in SM-A expression (Asthmatic/control: 1.63, $p<0.002$) and SM-2 (Asthmatic/control: 1.53, $p<0.005$) was of similar magnitude as that of total SMMHC (Asthmatic/control: 1.69, $p<0.001$). SM-B (Asthmatic/control: 2.41, $p<0.007$) showed the greatest upregulation among SMMHC isoforms, and was the only myosin isoform with a significant upregulation in asthmatics relatively to total SMMHC. SM22 (Asthmatic/control: 2.18, $p<0.005$) was also highly upregulated, whereas the upregulation of MLCK (Asthmatic/control: 1.21, $p<0.05$) was modest. Thus, these results show that the mRNA encoding for the SM-B SMMHC isoform, SM22, and MLCK is upregulated in mild asthmatic endobronchial SM.

SM-B isoform, SM22 and MLCK immunohistochemistry:

To confirm that SM-B, SM22 and MLCK are also expressed at the protein level in human airways, we performed immunohistochemistry on the endobronchial biopsies. The positive staining for the SM-B SMMHC isoform had a granular pattern (Figs. 2A and B). At high magnification the staining was intracellular and had an elongated spindle shape when the muscle bundle was cut longitudinally (Figs. 2A and B). Interestingly, the SM-B-positive cells were usually dispersed homogeneously in the SM tissue although they were seldom grouped (Figs.2A and B). The positive staining for SM22 was also granular

and intracellular (Figs. 3A and B). The SM22-positive cells were also dispersed homogeneously in the SM tissue (Figs. 3A and B). The staining for MLCK also confirmed its expression in the airway SM cells at the protein level (Figs. 4A and B). The absence of staining after competitive pre-adsorption (Figs. 2C, 3C, 4C) confirmed that the signal was specific in all cases.

Fisher and Lewis rat tracheal smooth muscle molecular mechanics and isoform expression:

To verify if overexpression of the SM-B isoform contributes to muscle hypercontractility, we measured the rate of actin filament propulsion (v_{max}) in the *in vitro* motility assay of purified Fisher and Lewis rat trachealis myosin. v_{max} was significantly faster for the myosin purified from Fisher rat tracheae (0.61 μm/s ±0.01) than from Lewis rat tracheae (0.47 μm/s ±0.01) ($p<0.001$; Figure 5A). To then verify that myosin purification did not alter the previously reported SM-B content in Fisher and Lewis rat trachealis (Gil, Zitouni et al. 2006), we performed Western blot analysis. The hyperresponsive Fisher rat trachealis had 1.8 ±0.2 fold more of the SM-B isoform ($p<0.001$) than the hyporesponsive Lewis rat (Fig. 5B). The difference between Fisher and Lewis rats is of the same magnitude as what we observed between the asthmatic and control biopsies (Fig. 1). Altogether, these data suggest that the overexpression of the SM-B isoform results in a faster myosin cycling velocity, thus a faster velocity of SM contraction.

Molecular mechanics of MLCK-bound skeletal myosin:

To verify if, in addition to its enzymatic role, MLCK could also mechanically enhance myosin function, we measured v_{max} in the *in vitro* motility assay for skeletal myosin before and after incubation with MLCK. The MLCK-bound skeletal myosin propelled actin filaments significantly faster (3.96 μm/s ±0.12) than the myosin free of MLCK (2.96 μm/s ±0.21) ($p\leq0.001$; Fig. 6). Because skeletal myosin is not activated by MLCK, this increase in velocity was not due to MLCK enzymatic activity. These data suggest that, in addition to phosphorylating SM LC_{20}, MLCK enhances the rate of SM shortening by a direct mechanical effect on myosin.

Molecular mechanics of SM22-bound actin filaments:

To verify if the over-expression of SM22 observed in the asthmatic subjects might contribute to muscle hypercontractility, the molecular mechanics of SM22-bound actin filaments, when propelled by myosin molecules, was assessed in the *in vitro* motility assay. No difference in v_{max} was observed when SM22 was completely absent (0.79μm/s ±0.01) or when it was bound to actin (0.79μm/s ±0.02) (Fig. 7). The binding of SM22 to actin was verified by immunoprecipitating the cosedimented actin-SM22 with an actin-specific antibody, and analyzing by Western blotting with an SM22-specific antibody (Fig. 8A). SM22 was mostly found in the pellet obtained after immunoprecipitation of actin, showing that actin was effectively bound to SM22 (Fig. 8A). Specificity of the immunoprecipitation procedure was verified by control experiments with antibodies against P44 MAP kinase and the chemokine CCR5 (Fig. 8B). These data show that, at the molecular level, SM22-bound actin does not directly alter cross-bridge cycling rate.

VI- Discussion:

In this study, we found that the expression of specific genes encoding for SM contractile proteins is upregulated in asthmatic bronchial biopsies. Significantly, we showed from a rat model of airway hyperresponsiveness that the upregulation of the SM-B myosin isoform leads to a faster rate of actin filament propulsion (v_{max}) in the *in vitro* motility assay. Because v_{max} correlates with unloaded shortening velocity (Sheetz, Block et al. 1986; Umemoto and Sellers 1990), a greater expression of the SM-B isoform contributes to the faster rate of airway SM shortening. We also found that SM22 is overexpressed in asthmatic bronchial biopsies. However, we did not elucidate its function at the molecular level. Finally, we demonstrated that in addition to its enzymatic properties, MLCK has a direct mechanical effect on myosin, also increasing its v_{max}, thereby contributing to the faster rate of asthmatic airway SM shortening.

Smooth muscle rate of shortening and airway hyperresponsiveness

There is ample evidence suggesting that airway diameter increases with each inspiration (see (Solway and Fredberg 1997) for a review). Also, if prevented from taking deep inspirations, normal humans respond similarly to asthmatic subjects when challenged with bronchoconstrictive agents (Skloot, Permutt et al. 1995; Moore, Verburgt et al. 1997). From those observations, Gunst (Gunst 1983) and Solway and Fredberg (Solway and Fredberg 1997) suggested that the kinetics of muscle contraction play a crucial role in bronchial hyperresponsiveness. That is, tidal breathing and especially deep inspirations putatively allow for detachment of cross-bridges. This must have a more pronounced relaxing impact on slowly contracting muscles than on fast ones, i.e. rapidly contracting muscle probably constricts to a greater extent between each breath or after deep inspirations (Gunst 1983; Solway and Fredberg 1997). Indeed, there is evidence that airway re-narrowing occurs faster in asthmatics than controls (Jackson, Murphy et al. 2004). Our results of alterations in expression of mRNA from the contractile machinery, along with our *in vitro* motility data, offer molecular mechanisms to explain the increased rate of airway SM shortening observed in airway hyperresponsiveness and asthma.

The role of SM-B in asthma: increased velocity of shortening of airway SM

The rate of shortening of airway SM is increased in human asthmatic SM cells (Ma, Cheng et al. 2002), human sensitized airways (Mitchell, Ruhlmann et al. 1994), and animal models of bronchial hyperresponsiveness (Antonissen, Mitchell et al. 1979; Fan, Yang et al. 1997; Duguet, Biyah et al. 2000; Blanc, Coirault et al. 2003). In the current study, we showed that the mRNA expression of SM-B doubles in airway SM from mild asthmatics compared to normals. Furthermore, we demonstrated that an increased SM-B isoform content in airway SM has a functional impact by measuring v_{max} for myosin purified from Fisher and Lewis rat trachealis muscle. We chose this animal model because fresh human airway SM is not readily available, and because the Fisher rats are hyperresponsive (Eidelman, DiMaria et al. 1991; Gil, Zitouni et al. 2006) and express more SM-B than the Lewis rats (Gil, Zitouni et al. 2006), as also shown in figure 5B. Interestingly, the difference in SM-B isoform expression between Fisher and Lewis rats is of the same magnitude as what we observed between asthmatic and control human biopsies.

Myosin is a mechano-enzyme: the breaking of MgATP leads to actin binding and a conformational change that results in the translational movement of actin. Structural differences between the SM-B and SM-A SMMHC isoforms explain the differences in their contractile kinetics. The 7 amino acid insert is strategically located to alter the in and out movement of the nucleotide (Kelley, Takahashi et al. 1993; White, Martin et al. 1993) (For review cf (Low, Leguillette et al. 2006)). Indeed, it has been shown that the presence of the insert increases the flexibility of the loop above the nucleotide binding pocket, and as a consequence, facilitates the binding of MgATP and the release of the hydrolysis product, MgADP (Sweeney, Rosenfeld et al. 1998). This was confirmed in our previous study(Lauzon, Tyska et al. 1998) in which we used a laser trap to demonstrate that the time of attachment of SM-B to actin is half that of SM-A, thereby explaining the 2-fold faster v_{max} for SM-B measured in the *in vitro* motility assay (Kelley, Takahashi et al. 1993; Rovner, Freyzon et al. 1997). In the same study, we also showed that there was no difference in molecular force generating capacity between these two isoforms (Lauzon, Tyska et al. 1998). It is interesting to note the striking similarities between the

molecular mechanical properties of this myosin isoform (Lauzon, Tyska et al. 1998) and the airway SM mechanical properties reported in asthma, namely, an increased rate of shortening yet no force enhancement (Bjorck, Gustafsson et al. 1992; Ma, Cheng et al. 2002).

The presence of the myosin SM-B isoform is also known to affect SM kinetics at the whole organ level. We previously purified myosin from multiple rat organs and showed a rank correlation in v_{max} from the slowest contracting tonic organ to the fastest contracting phasic organ (Leguillette, Gil et al. 2005). Furthermore, by measuring the pulmonary mechanics of the SM-B isoform knockout mouse (Babu, Loukianov et al. 2001), we showed the impact of the 7 amino acid insert at the whole animal level (Tuck, Maghni et al. 2004). We observed an 18% increase in the time to peak airway resistance in the knockout compared to the wild type mice (Tuck, Maghni et al. 2004). It is also noteworthy that the expression of the SM-B isoform has been shown to be altered in several models of disease. For example, bladder or intestinal obstruction causes a reduction in mRNA encoding for SM-B, with concurrent reductions in maximal shortening velocity (Sjuve, Haase et al. 1996; Lofgren, Fagher et al. 2002; DiSanto, Stein et al. 2003). Hormones (Calovini, Haase et al. 1995) as well as stages of development (Low, Mitchell et al. 1999) also alter the expression of the SM-B myosin isoform.

Immunohistochemistry showed intercellular heterogeneity in the distribution of SM-B in biopsies from both normals and asthmatics. Positive SM-B cells were not usually observed in clusters but individually dispersed. Similar results have been reported in swine stomach SM and rat lung airway SM (Low, Mitchell et al. 1999; Parisi and Eddinger 2002). Parisi and Eddinger (Parisi and Eddinger 2002) have previously suggested that such heterogeneity might be critical in conferring a wider range of shortening velocity to SM. For example, they suggested that cells expressing high levels of the SM-B isoform could, by contracting rapidly, stretch and activate SM-B-deprived neighborhood cells via mechano-transduction. Such a mechanism could explain how heterogeneity in cell phenotype would lead to amplification of the rate of SM cell activation.

Total SMMHC in asthma:

Although still controversial, it is rather widely accepted that airway SM mass is increased in asthma (Ebina, Takahashi et al. 1993; Lambert, Wiggs et al. 1993; James and Carroll 2000; Benayoun, Druilhe et al. 2003; Woodruff, Dolganov et al. 2004). Our results of a 1.7-fold upregulation of SMMHC mRNA (Fig.1) in the asthmatic biopsies may indirectly reflect an increase in airway SM mass. However, the mRNA data were normalized to a ubiquitously expressed ribosomal S9 gene, so our results do not allow for distinction between a greater number of SM cells expressing SMMHC (hyperplasia) and a similar number of SM cells each expressing more SMMCH (hypertrophy). Nevertheless, if more SMMHCs are present in the biopsies from asthmatics they potentially contribute to the hypercontractility of airway SM observed in asthma.

The role of MLCK in asthma: increased velocity of shortening of airway SM

Previous studies have reported significant increases of MLCK expression in asthma and in models of asthma. MLCK protein content was increased in human bronchial SM sensitized ex-vivo (Ammit, Armour et al. 2000) and in a dog model of allergic airway hyperresponsiveness (Jiang, Rao et al. 1992; Jiang, Rao et al. 1994). Our data show an upregulation of MLCK mRNA in asthmatics compared to controls. Our data agree with those of Ma and coworkers (Ma, Cheng et al. 2002) who also studied whole tissue biopsies from a similar subject population but using conventional RT-PCR. Others used laser capture to dissect out the SM portion of biopsies, along with mRNA amplification techniques, and did not find significant changes in MLCK expression between asthmatic and control bronchial biopsies (Woodruff, Dolganov et al. 2004). At the protein level, a morphometric analysis in patients with severe and moderate asthma, showed a significant increase in MLCK in the severe asthmatics (Benayoun, Druilhe et al. 2003). Discrepancies between studies may be due to differences in patient populations and quantification techniques.

Increased MLCK expression may accelerate SM shortening by two possible mechanisms. First, MLCK is the key activator of SM contraction by phosphorylation of the LC_{20},

thereby initiating the cross-bridge cycling (Kamm and Stull 1985). The maximal velocity of SM contraction is tightly dependent on the acto-myosin cross-bridge cycling rate (Sheetz, Block et al. 1986; Umemoto and Sellers 1990). Many studies have suggested that cross-bridge cycling rate is proportional to the amount of MLCK and the resulting LC_{20} phosphorylation levels (Gerthoffer and Murphy 1983; Stephens and Jiang 1997); however this direct link remains controversial (Gerthoffer 1987; Merkel, Gerthoffer et al. 1990; Mitchell, Seow et al. 2001). It has also been suggested that in addition to its enzymatic activity, MLCK may have mechanical effects on the cross-bridge cycling rate (Sato, Ye et al. 1995). This may contribute to the uncoupling between myosin phosphorylation levels and SM velocity of shortening, observed at the tissue level early in the contraction process (Mitchell, Seow et al. 2001). Indeed, it has been described in the skeletal muscle literature that MLCK binds to myosin via a telokin-like site, and that this attachment modifies the conformation of myosin filaments, moving them closer to the actin thin filaments (Levine, Kensler et al. 1996). In SM, it has been shown that MLCK also has actin-binding capabilities (Sellers and Pato 1984) and that this binding increases the myosin ATPase activity in a phosphorylation-independent manner (Ye, Kishi et al. 1999). In the current study, we verified if SM MLCK could also mechanically potentiate the cross-bridge cycling rate. We addressed this question, by incubating skeletal myosin with MLCK, because contrary to SM, skeletal myosin is not enzymatically activated by MLCK. We found a greater v_{max} for skeletal myosin in presence of SM MLCK. To our knowledge this enzymatic-independent increase in cross-bridge cycling rate by SM MLCK has never been described. Therefore, our data suggest that upregulation of MLCK in asthmatic airway SM biopsies increases cross-bridge cycling rate by both an enzymatic and a mechanical effects, thus contributing to the increased rate of shortening of airway SM.

The role of SM22 in asthma:

Although SM22 has been first purified from SM more than a decade ago (Shapland, Lowings et al. 1988), its function remains unknown. SM22 binds to filaments of actin in SM cells, both *in vitro* and *in vivo* (Shapland, Hsuan et al. 1993; Fu, Liu et al. 2000; Zhang, Kim et al. 2001), and is not essential for life and SM development (Zhang, Kim et

al. 2001). We hypothesized that SM22 would behave as several other actin-binding proteins and directly alter the cross-bridge cycling rate and/or ATPase activity (Haeberle 1994). Our results demonstrate no difference in v_{max} when SM22 is bound to actin. It is possible that SM22 has a role in actin filament remodeling. In this sense, actin elongation has been suggested to play a role in bronchial hyperresponsiveness (Dulin, Fernandes et al. 2003). It is also possible that the effect of SM22 involves multiple actin filaments, as suggested by the SM22 jellifying action of actin filaments, observed in specific ionic strength conditions (Shapland, Lowings et al. 1988). This would obviously be missed when performing measurements at the molecular level but should be investigated at the cellular level.

In summary, we have shown that the mRNA levels of SM-B, SM22, and MLCK are increased in mild asthmatic bronchial biopsies. Furthermore, we showed that upregulation of SM-B and MLCK increase v_{max}. This specific contractile protein gene upregulation most likely leads to increased SM velocity of shortening, thus contributing to the airway hyperresponsiveness observed in asthma.

Acknowledgments:

This study was supported by the following grants: Canadian Institute of Health Research (CIHR) # MGC-42667 and #MOP-79545, Réseau en Santé Respiratoire du Québec, Natural Sciences and Engineering Research Council # RGPIN 217457-00, and # EQPEQ 229606-00. RL is supported by CIHR and AML by the Fonds de Recherche en Santé du Québec. The authors thank Dr. Q. Hamid for the assessment of the morphology of the slides, Drs. S. Lajoie-Kaldoch and S. Létuvé for technical assistance.

VII- Tables and figures:

Table 1: Clinical characteristics of subjects:
Age of the subjects ranged from 24 to 47 year. FVC: forced vital capacity, FEV_1: pre-bronchodilator forced expiratory volume in 1 s (expressed in liter and % of predicted), PC_{20}: provocative concentration of methacholine inducing a 20% fall in FEV_1. Concentrations of methacholine up to 128 mg/ml were used. Allergens: Skin response read at 10 min if mean wheal diameter >3 mm). C: Cat, D: Dog, DPt: D. Pteronyssinus, Du: Dust, Rw: Ragweed.

Sex	FVC l (%)	FEV_1 l (%)	PC_{20} (mg/ml)	Allergens
Asthmatics				
F*	4.63 (119)	4.07 (126)	0.07	C, D, Du, DPt
F	4.18 (119)	3.18 (104)	2.41	C, Du, DPt
M	6.76 (115)	5.55 (116)	2.1	C, D, Du, DPt
F*	3.64 (113)	2.82 (107)	1.46	C, Du, Rw,
M	5.22 (111)	3.33 (85)	3.02	C, D, Du
Non asthmatics				
M*	6.24 (119)	5.15 (112)	>128	None
F	4.52 (116)	3.77 (116)	19.06	None
F	3.66 (111)	2.98 (104)	>128	None
F	4.05 (116)	3.17 (104)	139.04	None
F	3.23 (87)	2.64 (83)	84.16	None

*Biopsies also used for immunohistochemistry

Table 2: Primers sequences:

5'-GTGTGTGACAATGGCTCTGG-3'	Acin α2 Sens
5'-TTTGCTCTGTGCTTCGTCAC-3'	Acin α2 AS
5'-CAGCTCTCGCACTCTGTTCTT-3'	Acin γ1 Sens
5'-GCAAAACCAGCTTTGCACAT-3'	Acin γ1 AS
5'-GGCTTGTCTTCTTGGAGCTTT-3'	Caldesmon Sens
5'-GGCAGAGGAGGAAGAGAAGG-3'	Caldesmon AS
5'-ATGGAGAGATGCCTGTGGAC-3'	Linked Integrin kinase Sens
5'-TGCCAGAGTGTTTGTTCAGG-3'	Linked Integrin kinase AS
5'-TCATCCAGTTCCCTGAGGAC-3'	MLCK Sens
5'-TTCTCGCTGTTCTCCACCTT-3'	MLCK AS
5'-GAGCCAGGCCAGTAATGAAG-3'	Ribosomal S9 Sens
5'-TGAGTAACATTCCATATACATCATGC-3'	Ribosomal S9 AS
5'-AAGAATGATGGGCACTACCG-3'	SM22 Sens
5'-ATGACATGCTTTCCCTCCTG-3'	SM22 AS
5'-AGCAAGCTCAGGCGAGGA-3'	SMMHC SM-1 Sens
5'-ACAGTCTGCTGGGTTTTGCT-3'	SMMHC SM-1 AS
5'-CACAGGAAACTTCGCAGTGA-3'	SMMHC SM-2 Sens
5'-CCGTGGTGCAAAACTGTAGA-3'	SMMHC SM-2 AS
5'-CAAGAAAGACACAAGTATCACGG-3'	SMMHC SM-A Sens
5'-GTTGGCTCCCACGATGTAAC-3'	SMMHC SM-A and SM-B AS
5'-CAAGGCCCATCTTTTGCCTAC-3'	SMMHC SM-B Sens
5'-GACCAGGATCTCATCCTCCA-3'	Total SMMHC Sens
5'-AGCAGCTACAGGCTGAAAGG-3'	Total SMMHC AS
5'-GATCAAGGTCCTTTCCGACA-3'	Tropomyosin α Sens
5'-AGCAGGCAGAGTGGATGAAC-3'	Tropomyosin α AS
5'-ACTGGACAACGCACTCAATG-3'	Tropomyosin β Sens
5'-GGGTGGAAGGGGATAGGTAA-3'	Tropomyosin β AS

Figure 1: *Relative quantification of contractile genes by real-time PCR analysis:* Results are presented as fold increase in endobronchial biopsies from asthmatic compared to normal subjects. Quantification is normalized for S9 levels and corrected for primers efficiency. SMMHC: Smooth Muscle myosin heavy chain, SM-1, SM-2, SM-A, SM-B: Smooth muscle myosin heavy chain isoforms -1, -2, -A and -B, respectively, SM22: transgelin, MLCK: Myosin light chain kinase, S9: Ribosomal protein S9. †: Significant difference between asthmatics and normals. Total SMMHC: $p< 0.01$; SM-2: $p< 0.01$; SM-A: $p< 0.01$; SM-B $p< 0.01$; SM22: $p< 0.01$; MLCK: $p= 0.05$; Tropomyosin α: $p= 0.03$; γ actin: $p= 0.01$.

Figure 1:

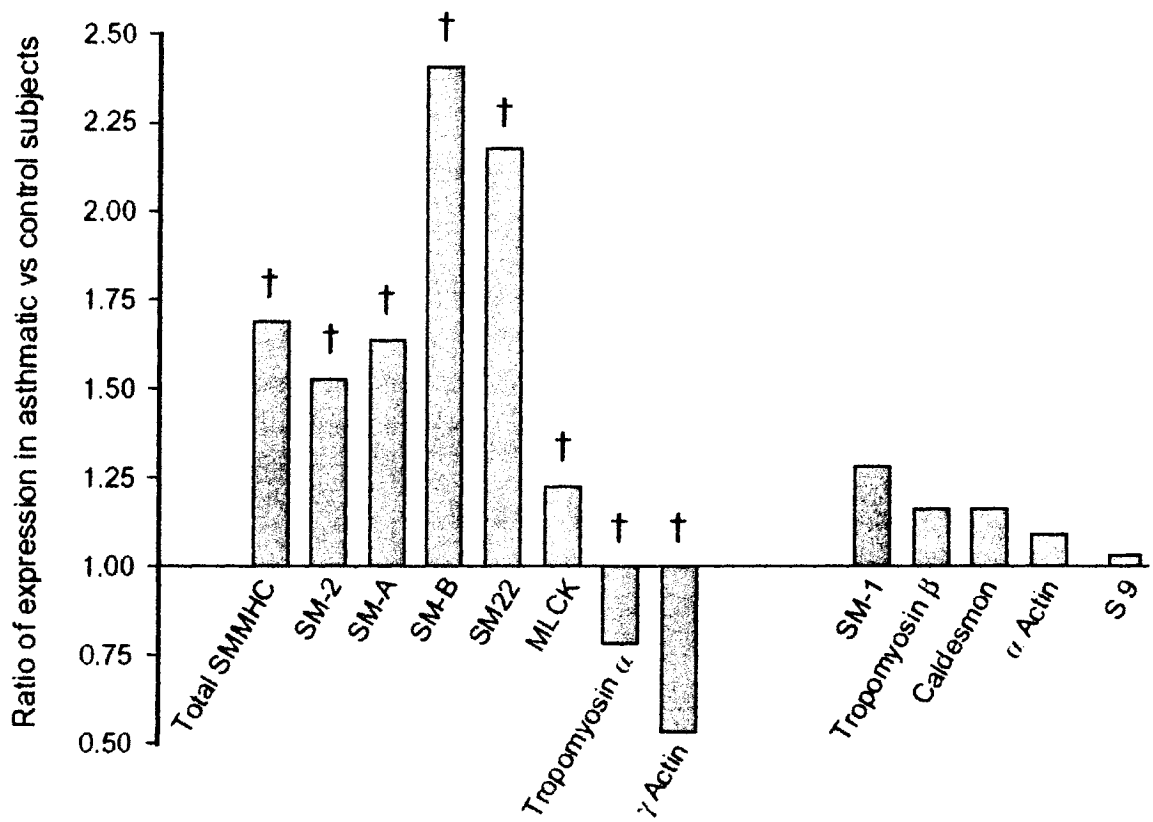


Figure 2: *Immunohistochemistry of the SM-B SMMHC isoform expressed in endobronchial biopsies from asthmatic and normal subjects:* Representative immunohistochemistry of the SM-B isoform expression in an endobronchial biopsy from an asthmatic subject (A) and from a normal subject (B). Representative immunohistochemistry from a normal subject after adsorption of the antibody with the QGPSFAY peptide (C). Brown staining is positive for the SM-B isoform.

Figure 2:

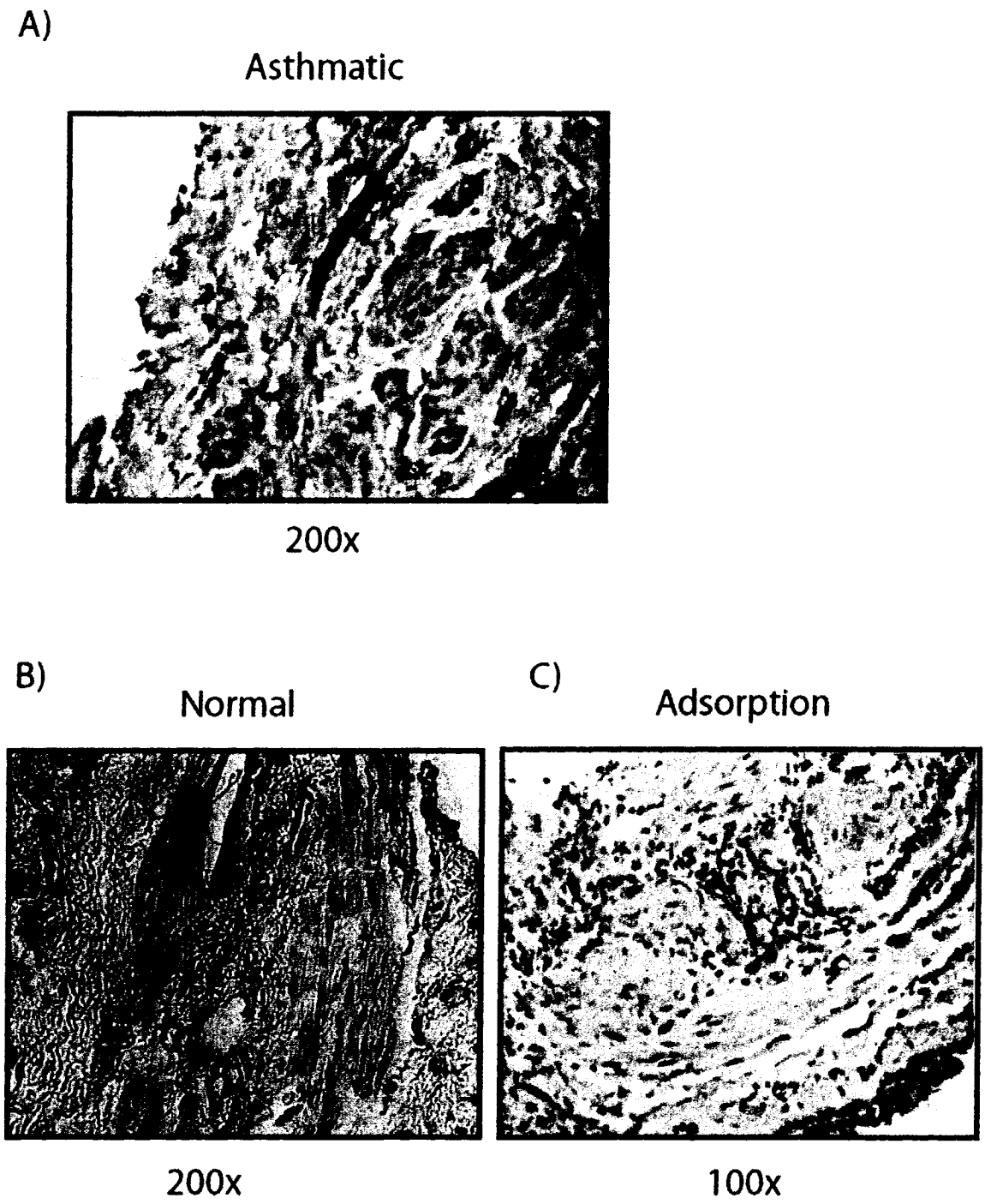


Figure 3: *Immunohistochemistry of SM22 expressed in endobronchial biopsies from asthmatic and normal subjects*: Representative immunohistochemistry of SM22 in an endobronchial biopsy from an asthmatic subject and enlarged view of the granular pattern (A). Representative immunohistochemistry of SM22 in an endobronchial biopsy from a normal subject (B). Representative immunohistochemistry of the SM-22 in an endobronchial biopy from a normal subject after adsorption of the antibody with recombinant SM-22 (C) . Brown staining is positive for SM22.

Figure 3:

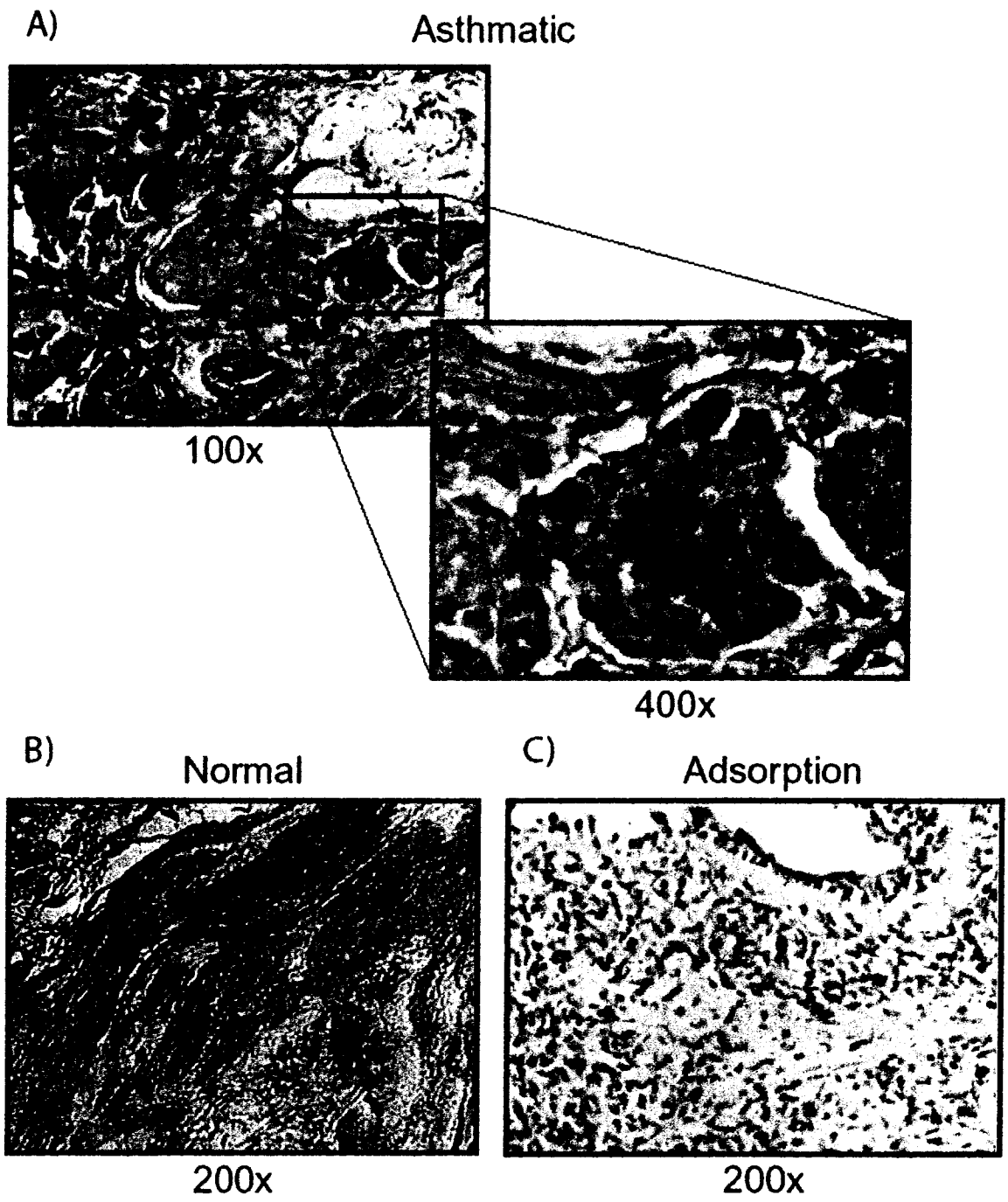


Figure 4: *Immunohistochemistry of MLCK expressed in endobronchial biopsies from asthmatic and normal subjects*: Representative immunohistochemistry of MLCK in an endobronchial biopsy from an asthmatic subject (A) and from a normal subject (B). Representative immunohistochemistry of MLCK in an endobronchial biopsy from a normal subject after adsorption of the antibody with purified MLCK. Brown staining is positive for MLCK.

Figure 4:

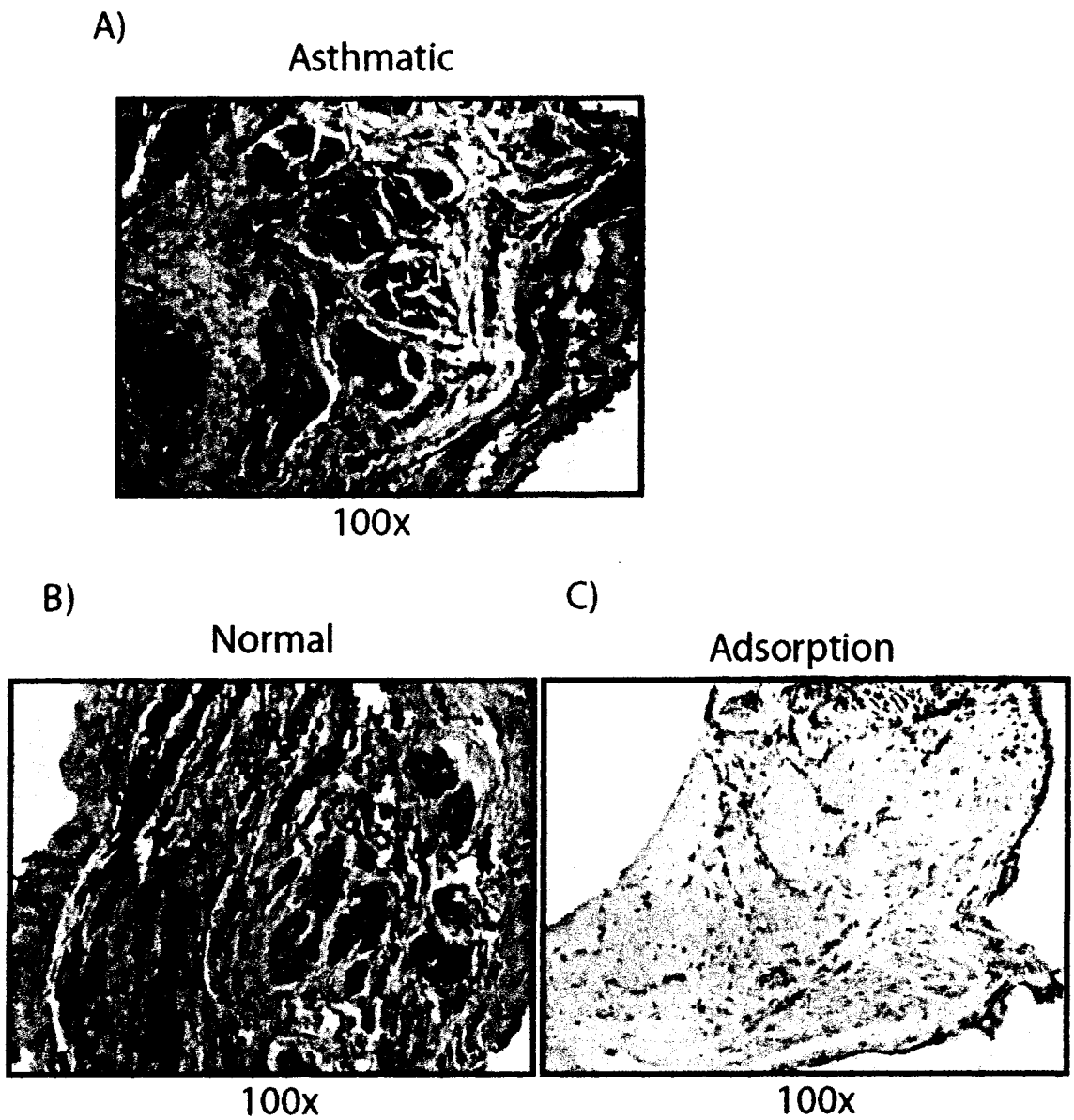


Figure 5: *Molecular mechanics and myosin isoform expression of the Fisher and Lewis rat trachealis muscle:* A: Scatter plot of actin filament propulsion velocity (v_{max}) measured in the *in vitro* motility assay for myosin purified from Fisher and Lewis rat tracheae. Horizontal bars: mean v_{max}. B: Representative Western blot analysis of myosin purified from eight Fisher and eight Lewis tracheae. Upper panel: SM-B isoform specific antibody, lower panel: Total smooth muscle myosin specific antibody.

Figure 5A:

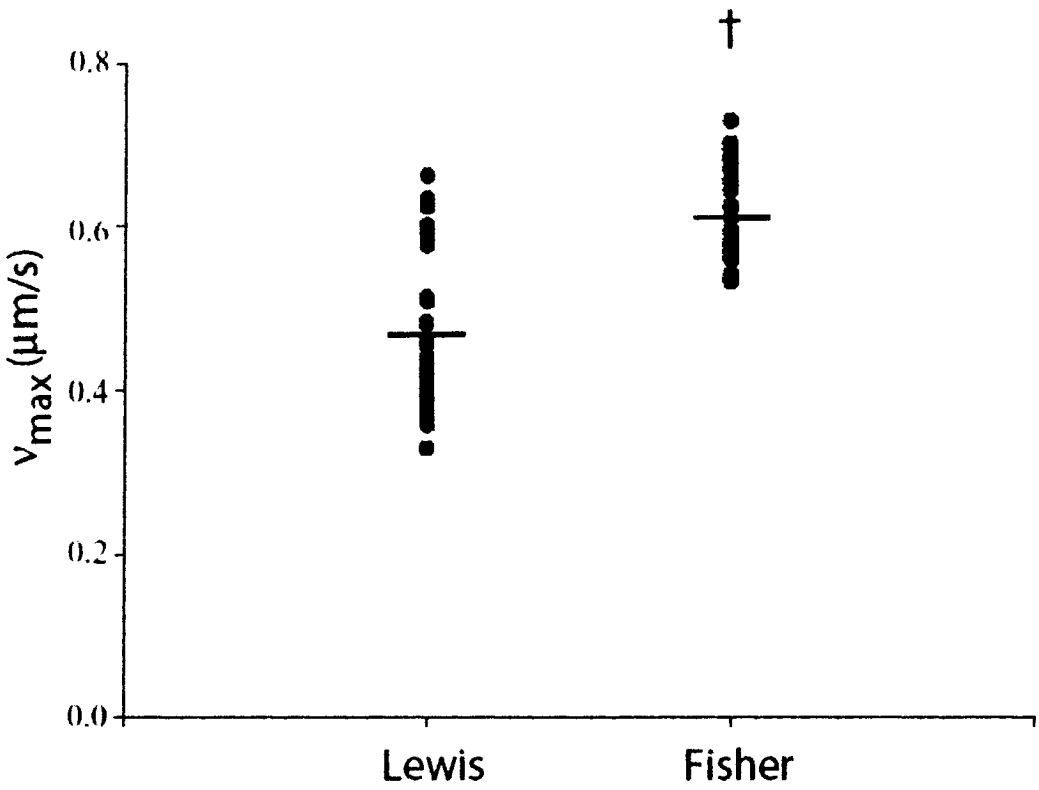


Figure 5B:

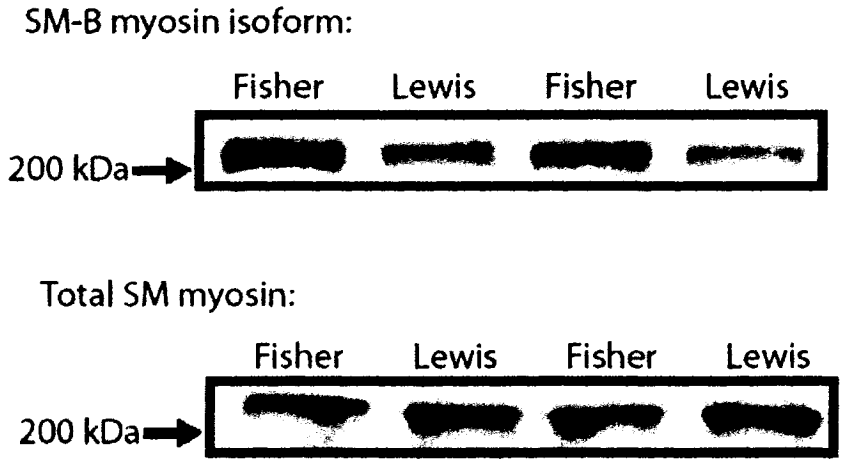


Figure 6: *Molecular mechanics of MLCK-bound skeletal myosin:* Scatter plot of actin filament propulsion velocity (v_{max}) measured in the *in vitro* motility assay after incubation of skeletal myosin with MLCK. Horizontal bar: mean v_{max}.

Figure 6:

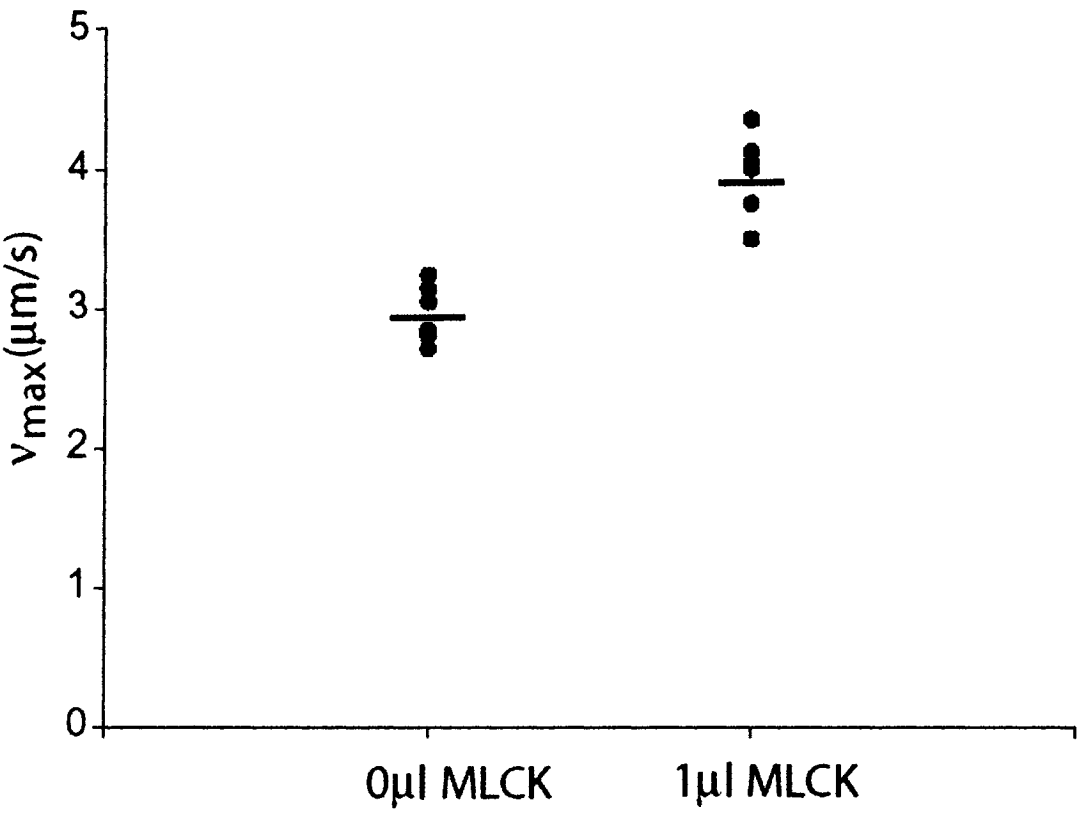


Figure 7: *Molecular mechanics of SM22-bound actin filaments:* Scatter plot of actin filament propulsion velocity (v_{max}) measured in the *in vitro* motility assay after incubation of actin with SM22. Horizontal bar: mean vmax.

Figure 7:

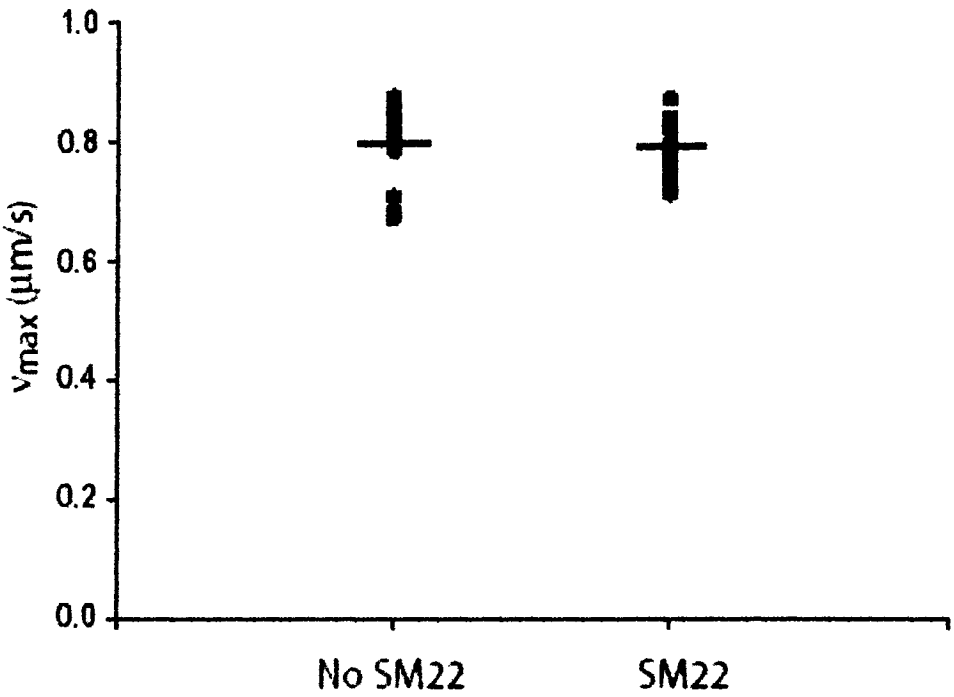


Figure 8: *Immunoprecipitation of cosedimented actin-SM22:* A: Western blot (performed with an anti-SM22 antibody) of actin immunoprecipitated (with an anti-actin antibody) without pre-incubation with SM22 (left panel) and with pre-incubation with SM22 (right panel; shown in duplicate). B: Western blot (performed with an anti-actin antibody) of actin immunoprecipitated with non-actin antibodies (P44 and CCR5; left panel) and actin specific antibodies (right panel). P: Pellet, S: Supernatant, IPP Ab: Antibodies used for immunoprecipitation.

Figure 8 A:

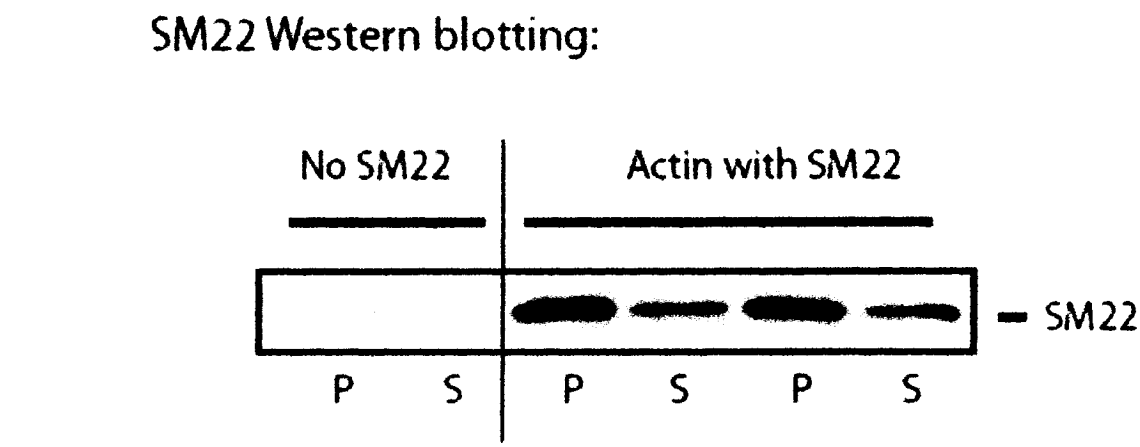
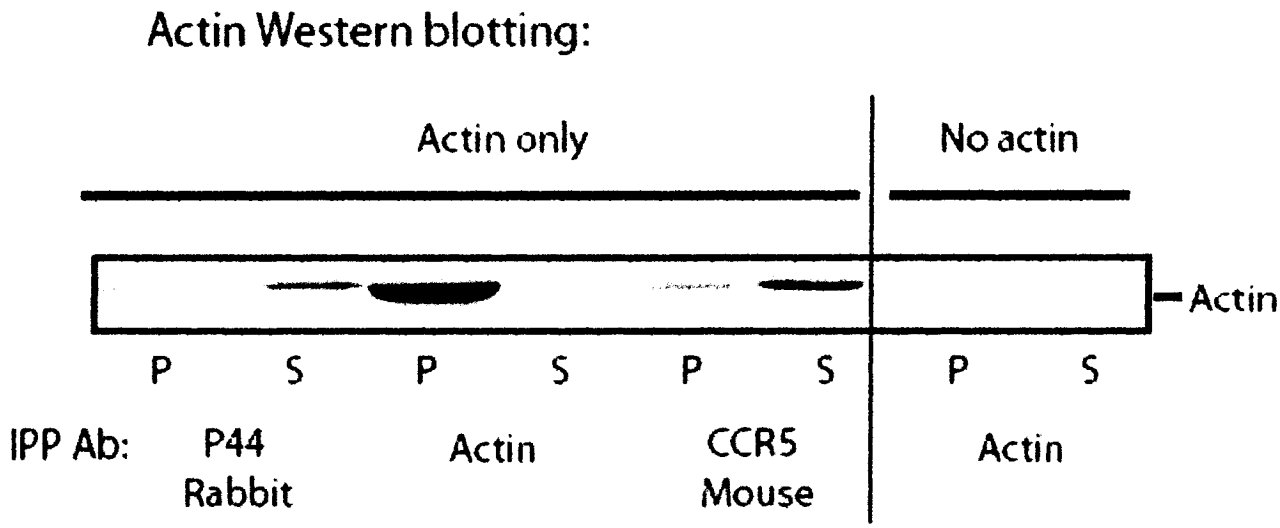


Figure 8B:



Chapter 4:

Tonic and phasic smooth muscle myosin affinity for adenosine diphosphate and unbinding force from actin.

I- Prologue:

A remarkable property of smooth muscle is its ability to maintain force for long periods of time at low levels of activation. This property, called the latch-state, is more prominent in tonic than phasic smooth muscle. Several models have been proposed to explain the latch phenomenon but they all remain speculative. To gain more information about the molecular mechanisms behind the latch-state, I measured the molecular mechanics of the different myosin isoforms at different activation levels and in presence of different concentrations of MgADP. I therefore tested the following two hypotheses: 1) unphosphorylated myosin can attach to actin and maintain force; 2) the SM-B myosin isoform has a greater affinity for MgADP than SM-A thereby, explaining the greater propensity of tonic muscle to enter into latch.

Tonic and phasic smooth muscle myosin affinity for adenosine diphosphate and unbinding force from actin.

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Running title: MgADP affinity and unbinding force.

Keywords: Velocity, force, phosphorylation, laser trap, in vitro motility, latch-state.

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II- Abstract:

Smooth muscle is unique in its ability to maintain force at low MgATP consumption. This property, called latch-state, is more prominent in tonic than phasic smooth muscle. Muscle strip studies have suggested that tonic muscle myosin has a greater affinity for MgADP and therefore remains attached to actin longer allowing for cross-bridge dephosphorylation and latch-bridge formation. A corollary hypothesis states that dephosphorylated myosin reattaches to actin and maintains force. These properties of myosin have been inferred from measurements made at the tissue level but have never been verified at the molecular level. Therefore, we investigated these mechanical properties of myosin directly at the molecular level. We used an in vitro motility assay to measure fluorescently labeled actin filament velocity (v_{max}) when propelled by myosin from phasic or tonic muscle, at increasing [MgADP]. A myosin mixture of 25% thiophosphorylated-75%unphosphorylated was used, to approximate in vivo steady-state conditions. The slope of v_{max} vs [MgADP] was significantly greater for myosin from tonic (-0.51±0.04) than phasic muscle (-0.15±0.04) demonstrating its greater affinity for MgADP. We then used a laser trap assay to measure the unbinding force from actin of unphosphorylated myosin from tonic and phasic muscle. Both myosin types attached to actin and their unbinding force (0.092pN ±0.022 for phasic and 0.084pN ±0.017 for tonic) was not significantly different. These results suggest that the greater affinity for MgADP of tonic muscle myosin and the reattachment of dephosphorylated myosin to actin could both contribute to the latch-state.

III- Introduction

Smooth muscle is involved in a broad range of physiological processes such as blood vessel tone maintenance and intestinal peristalsis. Two types of smooth muscle have originally been described based on their electrophysiological properties (Somlyo and Somlyo 1968). Tonic smooth muscle is defined as slowly contracting, multi-unit, tone maintaining, as found in most blood vessels, while phasic smooth muscle is defined as rapidly contracting, single unit, as in the intestine. Tonic and phasic smooth muscles also differ in their expression of amino-terminus myosin heavy chain and essential light chain (LC_{17}) isoforms. Tonic muscle expresses predominantly the (-)insert smooth muscle myosin heavy chain (SMMHC) and LC_{17b} isoforms whereas phasic muscle expresses mostly the (+)insert SMMHC and LC_{17a} isoforms (Malmqvist and Arner 1991; White, Zhou et al. 1998). It is generally agreed that the regulation of smooth muscle is mostly achieved by phosphorylation of the regulatory light chains (LC_{20}) (Sobieszek 1977). Interestingly, Murphy and coworkers have shown in carotid artery muscle strips that although the rate of shortening is correlated with LC_{20} phosphorylation, force can be maintained for long periods of time even after dephosphorylation of most cross-bridges (Dillon, Aksoy et al. 1981). This property, called latch-state, remains poorly understood. Many theories have been suggested to explain this unique ability of smooth muscle to maintain force at low energy cost. Murphy and coworkers have suggested that if cross-bridges were dephosphorylated (dePHOS) while attached to actin, they would remain attached and sustain force while no longer cycling or cycling slowly (Dillon, Aksoy et al. 1981). From work performed at the muscle strip level, Somlyo and coworkers more recently proposed that the affinity for adenosine diphosphate (MgADP) of tonic smooth muscle myosin, where latch is most evident, is higher than that of phasic smooth muscle (Fuglsang, Khromov et al. 1993). Because MgADP release from myosin is necessary for its detachment from actin (Spudich 1994), an attractive mechanism to explain the latch-state is that due to its greater affinity for MgADP, tonic muscle myosin remains attached longer to actin and gets dePHOS while attached (Hai and Murphy 1988), thus creating latch-bridges. Hai and Murphy even suggested that dephosphorylation may induce the latch-state simply by reducing the rate of MgADP release from cross-bridges (Hai and

Murphy 1988). However, these possibilities have never been investigated at the molecular level.

Alternative views of the latch-state also exist in which, instead of remaining attached after dephosphorylation, myosin would have the ability of reattaching to actin by a Ca^{2+}-dependent regulatory mechanism other than myosin LC_{20} phosphorylation (Siegman, Butler et al. 1985), or some cooperativity mechanism between phosphorylated (PHOS-) and dePHOS myosin (Himpens, Matthijs et al. 1988; Vyas, Mooers et al. 1992). However, the attachment of dePHOS myosin to actin has never been demonstrated at the molecular level. Indeed, biochemical studies report that unphosphorylated (unPHOS) myosin is in a bent conformation so it may not have the capacity to attach to actin (Trybus and Lowey 1984; Trybus 1989).

In the present study, we investigated fundamental mechanical properties of smooth muscle myosin in order to gain more information about the mechanisms responsible for the latch-state. We used the in vitro motility assay to quantify the rate of actin filament movement (v_{max}) when propelled by myosin from tonic and phasic smooth muscle, at increasing MgADP concentrations, and at different light chain phosphorylation levels. Furthermore, we used a laser trap to quantify the unbinding force (F_{unb}) from actin of unPHOS myosin tonic and phasic smooth muscle. Our results demonstrate that v_{max} decreases with increasing [MgADP] but that at low levels of phsophorylation, the decrease is more pronounced for myosin from tonic than phasic smooth muscle. These results suggest a greater affinity of tonic smooth muscle myosin for MgADP at low phosphorylation levels. Furthermore, we found that unPHOS myosin from both tonic and phasic smooth muscles can attach to actin and that their F_{unb} is not significantly different.

IV- Material and methods

Proteins:

Myosin was purified from bovine aorta (Megerman and Lowey 1981) and chicken gizzard (Ebashi 1976; Trybus 2000) as previously described. Thiophosphorylation was performed using Ca^{2+}, calmodulin (P2277 Sigma-Aldrich Canada Ltd, Ont.), myosin light chain kinase (generous gift from Dr J. Haeberle), and ATPγS (A1388 Sigma-Aldrich Canada Ltd, Ont.) (Trybus and Lowey 1984). Actin was purified from chicken pectoralis acetone powder (Pardee and Spudich 1982) and fluorescently labeled by incubation with tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (P1951 Sigma-Aldrich Canada Ltd, Ont.) (Warshaw, Desrosiers et al. 1990).

Western blot analysis of total and (+)insert SMMHC tissue content:

The total SMMHC and (+)insert isoform content of the gizzard and aorta after protein purification was verified by Western blot analysis. An equivalent amount of protein was loaded on the gel as assessed by a Bradford assay. Electrophoresis was done on 6% SDS polyacrylamide gel using a Laemmli buffer system. Proteins were electroblotted onto nitrocellulose membrane (Bio-Rad Laboratories Ltd, Mississauga, Ont.). Membranes were probed either with a polyclonal antibody that specifically recognizes the 7 amino acid insert QGPSFAY (Rovner, Freyzon et al. 1997)), or with a polyclonal SMMHC antibody that recognizes all SMMHC isoforms (Biomedical Technologies Inc., Stoughton, MA). Antibody detection was done by Enhanced Chemiluminescence (Amersham Biosciences, Piscataway, NJ) and quantification was performed with a Fluorchem 8500 imaging system using AlphaEase software (Alpha Innotech.

Buffers:

Myosin buffer (300mM KCl, 25mM imidazole, 1mM EGTA, 4mM MgCl2, 30mM dithiothreitol, pH adjusted to 7.4) and actin buffer (25mM KCl, 25mM imidazole, 1mM EGTA, 4mM MgCl2, 30mM dithiothreitol, with an oxygen scavenger system consisting of 0.1 mg/ml glucose oxidase, 0.018mg/ml catalase, 2.3mg/ml glucose, pH adjusted to 7.4) were used for both the in vitro motility assay and unbinding force experiments. The

motility assay buffer consisted of actin buffer to which MgATP (2mM) and increasing concentrations of MgADP (0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1mM) were added. The ionic strength was kept constant by adjusting KCl (Chang 1981). Methylcellulose (0.5%) was added to favor binding between actin and myosin. The range of MgADP concentrations (up to 1mM) was kept within physiological range. The laser trap unbinding assay buffer consisted of actin buffer to which MgATP (200μM) and methylcelullose (0.3%) were added.

In vitro motility assay:

The velocity of actin filament propulsion (v_{max}) by myosin molecules was measured in the in vitro motility assay, following a previously described protocol (Lauzon, Tyska et al. 1998) with slight modifications. Briefly, ultracentrifugation (Optima TLX ultracentrifuge and TLA-120.2 rotor, Beckman Coulter Inc., Fullerton, CA) of myosin ($500\mu g\,ml^{-1}$) with equimolar filamentous actin and 1mM MgATP in myosin buffer was performed to eliminate non-functional heads. unPHOS and PHOS myosins were mixed to obtain 100% unPHOS, 25% unPHOS, or 100% PHOS. The desired myosin mixture was perfused in a flow-through chamber (30μl) constructed from a nitrocellulose coated coverslip and a glass microscope slide. Incubation for 2 min allowed random attachment of myosin to the nitrocellulose and was followed by addition of bovine serum albumin (0.5mg/ml in actin buffer), unlabelled G-actin (10μM in actin buffer), MgATP (1mM in actin buffer), actin buffer (two washes), TRITC-labeled actin (10μM in actin buffer) incubated for 1 min, and motility assay buffer. For myosin MgADP affinity experiments, different MgADP concentrations were applied in random order. The flow-through chamber was then transferred to the stage of an inverted microscope (IX70, Olympus, Melville, NY) equipped with a high numerical aperture objective (100x magnification Ach 1.25n.a., Olympus, Melville, NY) and rhodamine epifluorescence. An image intensified video camera (VE1000SIT, Dage-MTI of MC Inc., Michigan City, IN) was used to visualize and record the actin filament movement (SVO-5800, Sony Corporation of America, New-York City, NY). The actin images were digitized (VG5 PCI RS170, Scion Corporation, Frederick, MD) and v_{max} was determined from the total path described by the filaments divided by the elapsed time using NIH tracking software (NIH

macro in Scion Image 4.02, Scion Corporation, Frederick, MD). All experiments were performed at 30°C.

Unbinding force measurements (F_{unb}) :

A single beam laser trap assay (Fig.1) was built using the Laser Tweezers® Workstation (Cell Robotics International Inc., Albuquerque, NM) combined to the motility assay to perform the unbinding force (F_{unb}) measurements. Before coating with nitrocellulose, coverslips were sprayed with 4.5μm polystyrene microspheres (Polybead®, Polysciences Inc., Warrington, PA) that served as pedestals. The trapping microspheres were made of 3μm polystyrene microspheres (Polybead®, Polysciences Inc., Warrington, PA) coated with N-ethylmaleimide (NEM)-modified skeletal myosin (generous gift from Dr. P. VanBuren) as previously described (Warshaw, Desrosiers et al. 1990; Lauzon, Tyska et al. 1998). The proteins and solutions were prepared as for the motility assay except that TRITC-labeled actin was mixed with microspheres ($13 \cdot 10^3$ microspheres/μl) in the unbinding assay buffer. One microsphere, visualized in bright field by a CCD camera (XC-75, Sony Corporation of America, New York City, NY), was captured in the trap and its position was recorded as above. An actin filament, visualized by fluorescence imaging as above, was attached to the microsphere and brought down in contact with myosin molecules randomly adherent to a pedestal (Fig. 1A). Approximately 10s were allowed for interaction between myosin and actin to occur. This established the baseline position of the microsphere in the trap and then, the trap was moved away from the pedestal at a constant velocity of $4 \mu m \cdot s^{-1}$. Despite pulling the trap away, the microsphere did not move until the force exerted by the trap on the microsphere was greater than that exerted by the myosin molecules on the actin filament (Fig. 1B). At this point, the microsphere sprang back to its unloaded baseline position in the center of the trap (Fig. 1C). The total unbinding force (F_{unb}) of the myosin molecules was calculated as

$$F_{unb} = k \cdot \Delta d \qquad (1)$$

where k= trap stiffness and Δd = maximal displacement of the center of mass of the trapped microsphere from its baseline position (Fig. 1). k was calibrated by applying a

viscous drag (or Stokes force) to a trapped microsphere, by moving it at a constant velocity (v) in 0.3% methylcellulose, and measuring the displacement (Δd) of the microsphere from the trap center (Fig. 2). The Stokes force (F_f) on the microsphere was calculated as

$$F_f = 6\pi\eta r v \quad (2)$$

where η= viscosity and r= microsphere radius. The viscosity of 0.3%methylcellulose was measured with a viscometer (DV-I at 60 rpm, Brookfield®, Middleboro, MA). Thus

$$k = F_f / \Delta d \quad (3)$$

The value of k used was the average of multiple measurements performed at many v. The reproducibility of our calibration and linearity of the trap stiffness are demonstrated in figure 2 where Δd is plotted as a function of v.

F_{unb} is reported as the average force per myosin molecule, calculated by dividing the measured F_{unb} by the number of myosin molecules estimated per actin filament length (VanBuren, Work et al. 1994), measured by imaging with the SIT camera. The concentration of myosin used was 0.1 mg/ml and the number of myosin molecules in contact with actin was estimated to be 24/μm of actin filament (VanBuren, Work et al. 1994).

Because F_{unb} measurements were performed on unPHOS myosin, the functioning of the myosin was assessed by measuring v_{max} of a 100% PHOS sample from the same purification. Furthermore, F_{unb} control experiments were performed by bringing actin filaments in contact with pedestals coated with bovine serum albumin (BSA) instead of myosin molecules. No attachment was observed with BSA.

Statistical analysis:

Differences in F_{unb} and v_{max} between myosin from tonic and phasic smooth muscles were tested using a Student's *t*-test and a Wilcoxon signed rank test, respectively. Differences in affinities were tested using a Student's *t*-test applied to the slopes of the linear regressions of the v_{max} vs MgADP for the myosins compared.

V- Results

Western blot analysis of total SMMHC and (+)insert isoform tissue content:

The content of total SMMHC and (+)insert isoform of the purified chicken gizzard (phasic) and bovine aorta (tonic) was quantified by Western blot analysis (Fig.7). Despite a greater total protein loading for the aortic smooth muscle, the (+)insert isoform was not detected. In contrast, a strong signal was observed for the (+)insert isoform in the chicken gizzard smooth muscle. These data suggest that, as previously reported for other tissues (White, Zhou et al. 1998), chicken gizzard and bovine aorta are essentially constituted of the (+) and (-)insert isoforms, respectively.

Affinity of tonic and phasic smooth muscle myosins for MgADP:

The rate of actin filament movement (v_{max}) when propelled by myosin from tonic and phasic smooth muscle was assessed using the in vitro motility assay. First, v_{max} for fully PHOS tonic and phasic smooth muscle myosin was measured. In accordance with the literature (Kelley, Takahashi et al. 1993; Rovner, Freyzon et al. 1997; Lauzon, Tyska et al. 1998), v_{max} for 100% PHOS myosin from phasic muscle (1.08 ±0.12μm/s; mean ±SD) is significantly faster ($p<0.001$) than for 100% PHOS myosin from tonic muscle (0.44 ±0.02μm/s) (Fig. 3). Next, v_{max} was measured for 25%PHOS-75%unPHOS myosin to mimic in vivo steady-state conditions (Dillon, Aksoy et al. 1981). We observed significantly slower ($p<0.001$) v_{max} for both myosin types compared to their respective 100% PHOS values (Fig. 3). Those results showed that as for the 100%PHOS myosin, 25% PHOS myosin from phasic muscle is also ~2-fold faster ($p<0.001$) than 25%PHOS myosin from tonic muscle (0.50±0.07μm/s for phasic vs 0.26±0.04μm/s for tonic) (Fig. 3).

To quantify the difference in affinity for MgADP between tonic and phasic muscle myosin, we measured v_{max} at increasing MgADP concentrations, at a phosphorylation level of 100%. v_{max} for both phasic and tonic smooth muscle myosin decreased with increasing MgADP (Fig. 4a). Linear regression of of v_{max} vs [MgADP] at 100%

phosphorylation level revealed that the slopes were almost similar with no statistical differences between phasic (-0.30±0.11, R^2=0.96) and tonic (-0.34±0.08, R^2=0.98) muscle myosin (Fig. 4a).

To test if the affinity of smooth muscle myosin for MgADP is affected by the level of phosphorylation, we measured v_{max} at increasing [MgADP] for 25% PHOS-75% unPHOS phasic muscle and tonic muscle myosin (Fig. 4b). Contrary to the slopes at 100%PHOS (Fig. 4a), the slopes at 25%PHOS-75% unPHOS were different between phasic and tonic muscle myosin. The slope for tonic muscle myosin was significantly greater (-0.52±0.10, R^2=0.96) than that of phasic muscle (-0.29±0.07, R^2=0.98). The greater slope of tonic muscle myosin suggests a greater affinity for MgADP at low phosphorylation level. Indeed, the concentrations of MgADP necessary to reach half of the initial v_{max} (i.e. K_I values) were 0.96mM for tonic (measured) and 1.74mM for phasic muscle (extrapolated). Furthermore, the slopes for phasic muscle myosin at 100% PHOS (-0.30±0.11, R^2=0.96) and at 25%PHOS-75% unPHOS (-0.29±0.07, R^2=0.98) were almost identical (Fig. 5a) whereas the slopes for tonic muscle myosin at 100% PHOS (-0.34±0.08, R^2=0.98) and at 25%PHOS-75%unPHOS (-0.52±0.10, R^2=0.96) were significantly different (Fig. 5 b; $P<0.001$). This later result suggests that the phosphorylation level affects tonic and phasic muscle myosin affinity for MgADP differently.

F_{unb} of unPHOS myosin from tonic and phasic smooth muscle:

To verify if unPHOS myosin can bind to actin and to quantify its attachment force, we used a single beam laser trap and measured the F_{unb} of unPHOS tonic and phasic muscle myosin. Table 1 shows F_{unb} per myosin molecule in contact with the actin filament. F_{unb} is not significantly different between tonic (0.084pN ±0.017) and phasic (0.092pN ±0.022) muscle myosin. These values are approximately 10-times smaller than the previously reported unitary force generated by PHOS myosin, i.e. 1pN (Lauzon, Tyska et al. 1998) and 0.6pN (VanBuren, Work et al. 1994; VanBuren, Guilford et al. 1995).

VI- Discussion

The major findings of this study are: 1) the affinity for MgADP of myosin from tonic muscle, at low phosphorylation conditions (25%PHOS-75% unPHOS), is significantly greater than that of myosin from phasic muscle; 2) the affinity for MgADP of myosin from tonic muscle is greater at low (25%PHOS-75%unPHOS) than at high (100%) phosphorylation levels; 3) unPHOS myosin from tonic and phasic smooth muscle can attach to actin; 4) the attachment forces to actin of unPHOS myosin from tonic and phasic smooth muscle are not statistically different.

Affinity of tonic and phasic smooth muscle myosin for MgADP:

Several studies have suggested that tonic and phasic smooth muscles have a different affinity for MgADP. Somlyo and coworkers showed that relaxation from rigor, induced by flash-photolysis of caged MgATP, is significantly impeded in tonic smooth muscle strips in presence of MgADP (Fuglsang, Khromov et al. 1993; Khromov, Somlyo et al. 1995). On the contrary, relaxation from rigor of phasic smooth muscle is much faster and barely affected by the addition of MgADP. Similarly, Lofgren and coworkers demonstrated that the rate of shortening, as measured by the quick-release technique, decreases more rapidly as a function of [MgADP] in tonic than phasic smooth muscle strips (Lofgren, Malmqvist et al. 2001). Force and stiffness measurements performed in the (+)insert isoform knockout mice bladder muscle strips also suggest a slower MgADP off-rate in the homozygous negative compared to homozygous positive or heterozygous mice (Karagiannis, Babu et al. 2003). All these studies, however, were performed at the muscle strip level and extrapolated to molecular level mechanisms.

To verify at the molecular level if tonic muscle myosin has a greater affinity for MgADP than phasic muscle myosin, we used the in vitro motility assay to assess the effect of [MgADP] on actin propulsion. Note that we chose a range of [MgADP] from 0 to 1mM because previous studies have demonstrated that intracellular free [MgADP] is in the range of 44 to 123μM (Krisanda and Paul 1983); (Askenasy and Koretsky 2002) but protein-bound MgADP including actin-bound MgADP may make it available in the proximity of the myosin heads (Butler and Davies 1980). Increasing [MgADP] in the

motility milieu should hinder the release of MgADP from the myosin nucleotide binding pocket. Because MgADP release is the rate-limiting step in the cross-bridge cycle (Spudich 1994), the greater the affinity for MgADP, the more pronounced will be the reduction in cycling rate, thus in v_{max}. We found that when myosin was fully thiophosphorylated, there was no difference in v_{max} between tonic and phasic smooth muscle myosin (Fig. 4a). These results are in agreement with those previously reported by Rovner and co-workers who performed v_{max} measurements of the (+) and (-)insert isoforms in the in vitro motility assay at increasing MgADP concentration, although they had the confounding effect of ionic strength correction by MgATP adjustments (Rovner, Freyzon et al. 1997). However, our results at 100%PHOS level, as well as those of Rovner (Rovner, Freyzon et al. 1997), disagree with muscle strip experiments (Fuglsang, Khromov et al. 1993; Khromov, Somlyo et al. 1995) but steady state phosphorylation levels are never that high.

To test if the affinity, of tonic muscle myosin is different from that of phasic muscle myosin at physiological phosphorylation conditions, we repeated the v_{max} measurements with 25%PHOS-75%unPHOS myosin. In those conditions, MgADP slowed v_{max} to a greater extent when propelled by myosin from tonic muscle than by phasic muscle myosin (Fig. 4b). These results confirm that in physiological phosphorylation levels, myosin from tonic muscle has a greater affinity for MgADP than phasic muscle myosin. It is important to note, however, that laser trap studies (Lauzon, Tyska et al. 1998) and fluorescence transients of the ADP analog 3'-deac-eda-ADP studies (Khromov, Webb et al. 2004) reported differences in MgADP release rates between phasic and tonic muscle myosin even at high thiophosphorylation levels, whereas we observed differences only at low phosphorylation levels (Figs. 4). It is possible that MgADP release rate is different in unloaded conditions vs when loaded. This was recently suggested in a molecular model (Baker, Brosseau et al. 2003).

Another interesting point regarding all of our v_{max} vs [MgADP] data is that better fits were obtained by linear regression than with logarithmic decays for both myosin types. This suggests that the behavior of smooth muscle myosin with increasing MgADP

concentration was not following a Michaelis-Menten kinetics model. Linear regression also yielded the best fits in other studies using the in vitro motility assay (Rovner, Freyzon et al. 1997) as well as the quick-release assay (Lofgren, Malmqvist et al. 2001).

Because our purification procedures yielded essentially pure myosin devoid of contaminating actin and actin regulatory proteins (data not shown), the mechanical results reported here reflect properties of the myosin molecules themselves. Phasic muscle myosin expresses mostly the myosin heavy chain that includes the 7 amino acid insert in the surface loop above the nucleotide binding pocket ((+)insert isoform) whereas tonic muscle mostly expresses the heavy chain without the insert ((-)insert) (White, Zhou et al. 1998). Western blot analysis of our purified myosin also showed that chicken gizzard had a much greater content of the (+)insert isoform than bovine aorta, in which the (+)insert isoform expression was below the level of detection (Fig. 7). It must be mentioned, however, that a potentially relevant variable is the LC_{17} isoform composition. In our study, we had no control over the light chain isoforms, so the purified myosin proteins were extracted with the same LC_{17} isoforms that they associated with in vivo. However, is has previously been demonstrated with recombinant myosin in the in vitro motility assay that the LC_{17} isoforms do not alter the myosin mechanical properties, thus attributing the 2-fold difference in v_{max} between the (+) and (-)insert isoforms to the insert itself (Rovner, Freyzon et al. 1997). Although the role of LC_{17} has not been addressed at low LC_{20} phosphorylation levels, a similar difference in v_{max} between phasic and tonic myosin were observed at 25%PHOS-75%unPHOS than at 100%PHOS level (Fig. 3), suggesting that the LC_{17} isoforms do not alter v_{max} at low LC_{20} phosphorylation levels either. Furthermore, the fact that the difference in v_{max} between phasic and tonic muscle myosin is maintained from 100%PHOS to 25%PHOS-75%dePHOS mixtures suggests that whatever is the mechanism responsible for slowing down v_{max} as a function of phosphorylation level, it is not different between tonic and phasic muscle. Our unbinding studies, as discussed below, suggest that because the force of attachment of unPHOS myosin is the same for tonic and phasic smooth muscle myosins, the reduction in v_{max} at low phsophorylation levels should be the same for phasic and tonic muscle myosin.

F_{unb} of unPHOS myosin from tonic and phasic smooth muscle:

Using a single beam laser trap assay, we directly measured the binding force of a population of unPHOS myosin from both tonic and phasic smooth muscle. We found that both tonic and phasic unPHOS smooth muscle myosin can bind to actin and that their attachment force is not different. The advantage of our assay over a single myosin molecule technique is that it measures the binding force from many myosin molecules at a time, thus increasing the signal to noise ratio, neglecting compliance in the system such as microsphere-actin linkage (Guilford, Dupuis et al. 1997; Mehta, Finer et al. 1997). Our system therefore allows detection of forces lower than those generated by cycling PHOS myosin molecules. The limitation of our assay was the precision of the actin length measurement by fluorescence imaging. Nevertheless, this effect was reduced by using long filaments attaching to many myosin molecules on a large pedestal, thereby increasing contact length and improving resolution and decreasing variation.

Using in vitro motility mixture assay data and mathematical modeling (Warshaw, Desrosiers et al. 1990) relating v_{max} to the unitary force generated by myosin, Harris and co-workers (Harris, Work et al. 1994) predicted an unPHOS to PHOS force ratio of 0.11 for gizzard smooth muscle myosin. Given that the unitary force generated by phasic muscle PHOS myosin has been reported to be between 0.6pN (VanBuren, Work et al. 1994; VanBuren, Guilford et al. 1995) and 1pN (Lauzon, Trybus et al. 1998), a value from 0.11pN to 0.06pN was expected for the unPHOS myosin binding force. Our results of 0.084pN to 0.092pN for tonic and phasic muscle myosin are thus in very good agreement with the literature.

Conclusion:

We demonstrated at the molecular level that myosin from tonic muscle has a greater affinity for MgADP than myosin from phasic muscle. Because myosin must release MgADP before it can detach from actin, this greater affinity for MgADP of tonic muscle

myosin may increase its chances of being dePHOS while attached to actin and therefore may explain its greater propensity to enter the latch state. Furthermore, we showed that unPHOS tonic and phasic muscle myosin can bind to actin and generate a load. Further studies will be required to elucidate if both the dephosphorylation of attached cross-bridges and the reattachment of dePHOS myosin contribute to the latch-state in presence of PHOS myosin and in a fully regulated system.

Acknowledgments:

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VII- Tables and Figures

Table 1: Comparison of F_{unb} generated by unPHOS myosin and F_{uni} generated by PHOS myosin:

Left: Average F_{unb} of unPHOS tonic and phasic myosin molecules as measured by the single beam laser trap assay. Right: For comparison purposes, F_{uni} and average F_{uni} for PHOS heavy meromyosin (HMM) as measured with the dual beam laser trap assay and microneedle, respectively.

	F_{unb}		F_{uni}	
	unPHOS Tonic SMMHC (pN ±SD)	**unPHOS** Phasic SMMHC (pN ±SD)	**PHOS** Tonic HMM construct (pN ±SE)	**PHOS** Phasic HMM construct (pN ±SE)
Average force per SMMHC molecule (n=number of events)	0.084 ±0.017 (n=8)	0.092 ±0.022 (n=6)	1.2 ±0.1* (n=8)	0.9 ±0.1* (n=7) 0.6 (0.4-0.8)# (Averaged F_{uni})

* Lauzon, A.M., *et al.* 1998. *J Muscle Res Cell Motil* 19:825-837.

VanBuren, P., *et al.* 1994. *Proc Natl Acad Sci U S A* 91:202-205.

Figure 1: A) A single beam laser trap is used to capture a 3μm diameter microsphere coated with NEM myosin. A TRITC-labelled actin filament is attached to the microsphere and brought in contact with unPHOS myosin randomly adhered to a 4.5μm pedestal on a coverslip. B) After allowing time for binding of unPHOS myosin to actin, the laser trap is moved away from the pedestal at constant velocity. Initially, the microsphere remains at the same position, now offset from the center of the trap. C) When the pulling force exerted by the trap exceeds the binding force of the unPHOS myosin molecules, the microsphere springs back into the trap center, its unloaded position. F_{unb} is equal to the product of Δd (maximal distance between the bead and the trap center) by the trap stiffness (calibrated using the Stokes force method (Dupuis, Guilford et al. 1997)).

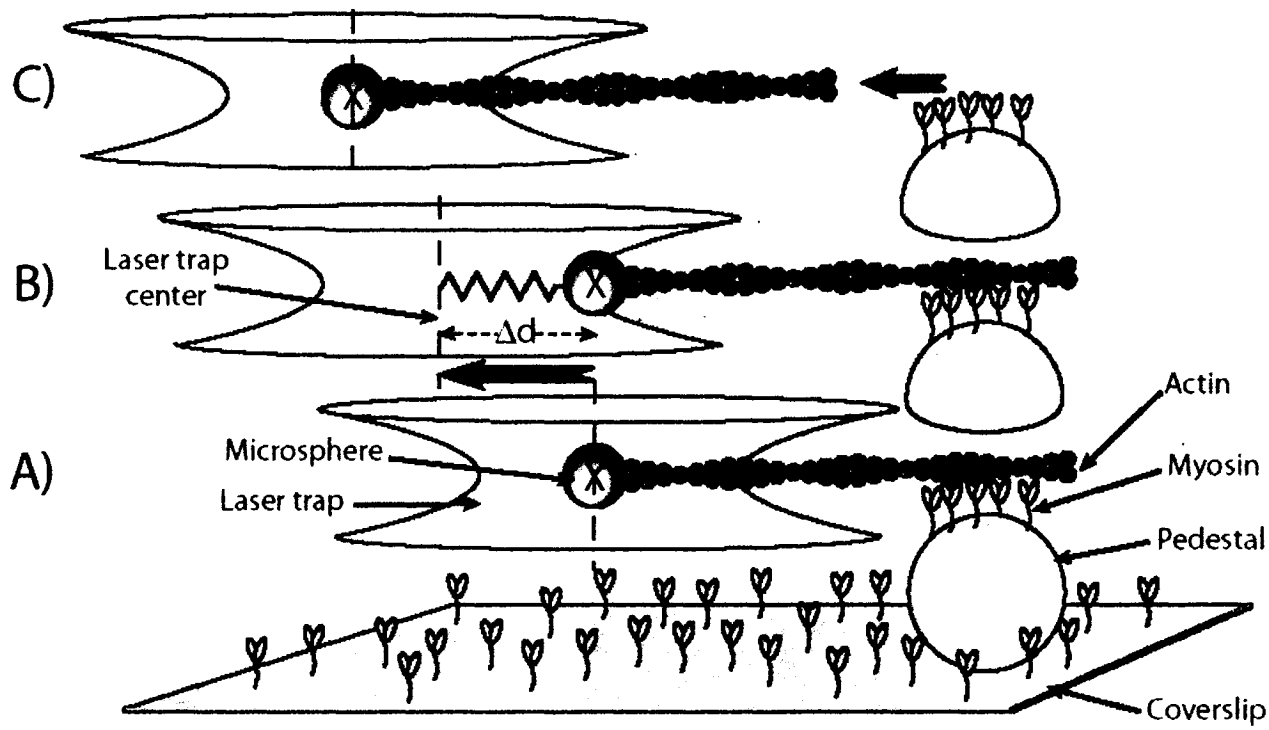


Figure 2 Trap calibration in 0.3% methylcellulose: The trapped microsphere was moved at increasing velocity (1.5- 50μm/s) in 0.3% methylcellulose and its displacement (Δd) with respect to the center of the trap was measured. Linear regression shows a very good fit (R^2= 0.92). These data demonstrated that the calibration is reproducible and that Δd is proportional to velocity (v).

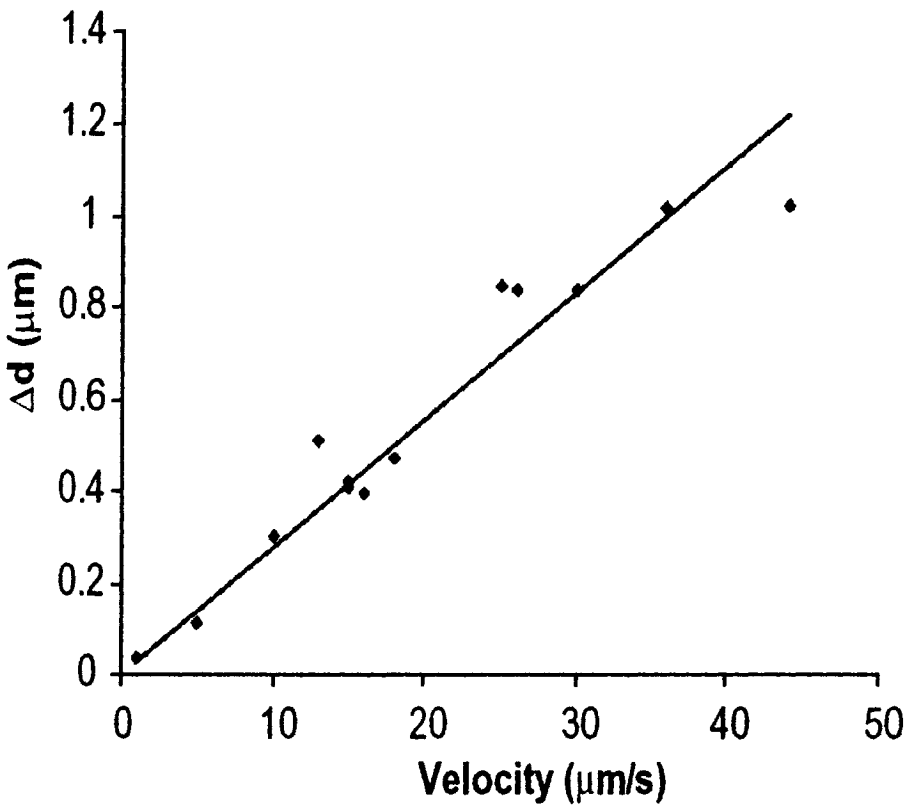


Figure 3: Baseline v_{max} for 100%PHOS and 25%PHOS tonic and phasic SM myosin as assessed by the in vitro motility assay, in presence of 2mM MgATP and absence of MgADP. (CG: chicken gizzard).

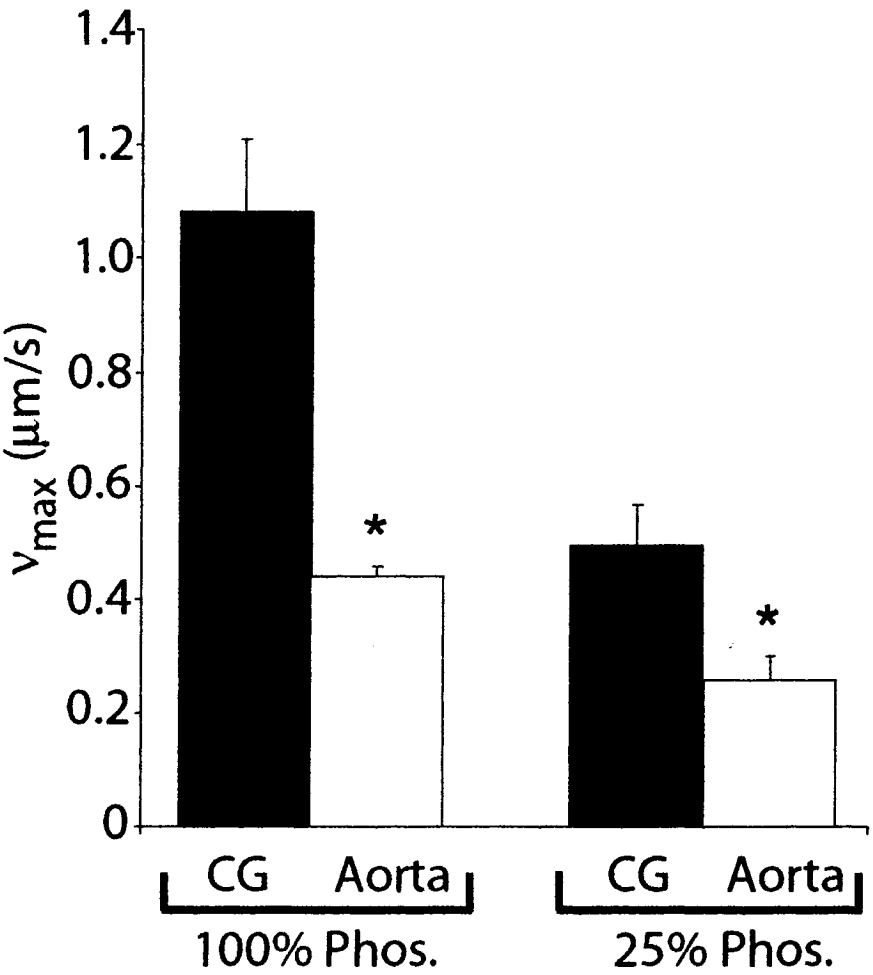
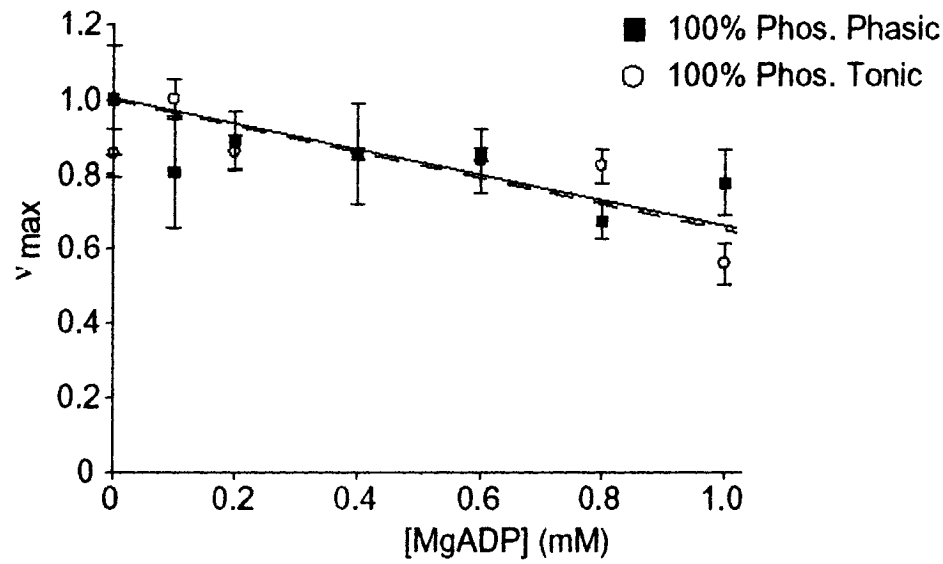


Figure 4: Relative v_{max} (normalized to v_{max} at 0mM MgADP) at increasing concentrations of MgADP for: A- 100%PHOS tonic (open circles) or phasic (filled squares) SM myosin and B- 25%PHOS-75%unPHOS tonic (open circles) or phasic (filled squares) SM myosin. The difference between the slopes of regression lines was not significant between 100%PHOS tonic and phasic myosin, but was significant between 25%PHOS-75%unPHOS tonic and phasic myosin ($p>0.0001$).

A



B

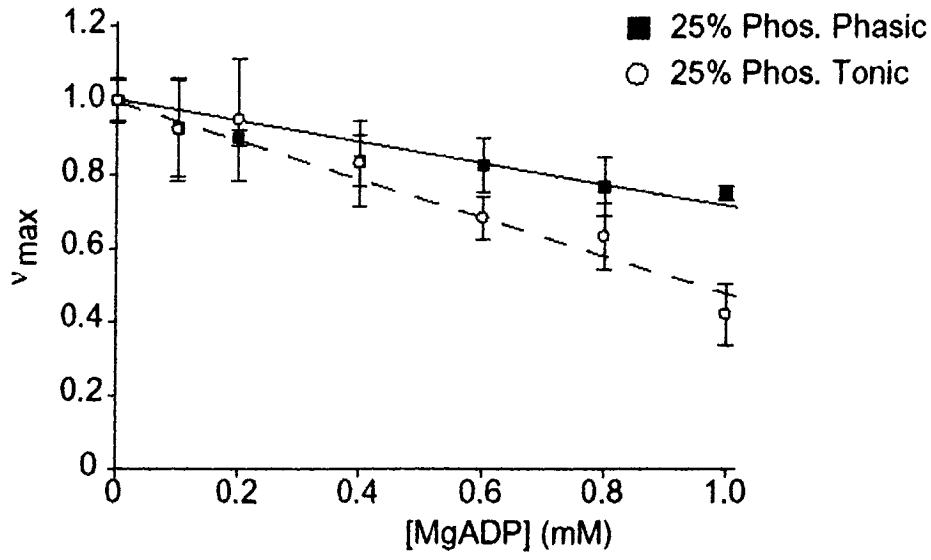
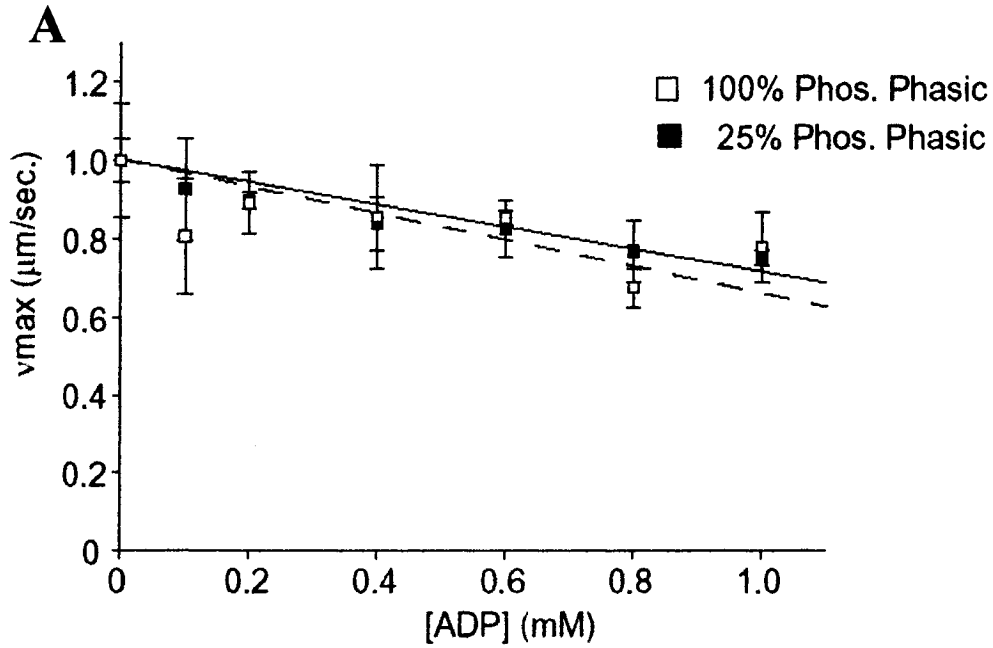


Figure 5: Relative v_{max} (normalized to v_{max} at 0mM MgADP) at increasing concentrations of MgADP for 100%PHOS or 25%PHOS-75%unPHOS for A) phasic SM myosin (Open squares= 100%PHOS, Filled squares= 25%PHOS-75%unPHOS) and B) tonic SM myosin (Open circles= 100%PHOS, Filled circles= 25%PHOS-75%unPHOS).The slopes of the regression lines were not significantly different between 100%PHOS and 25%PHOS-75%unPHOS phasic and tonic myosin.



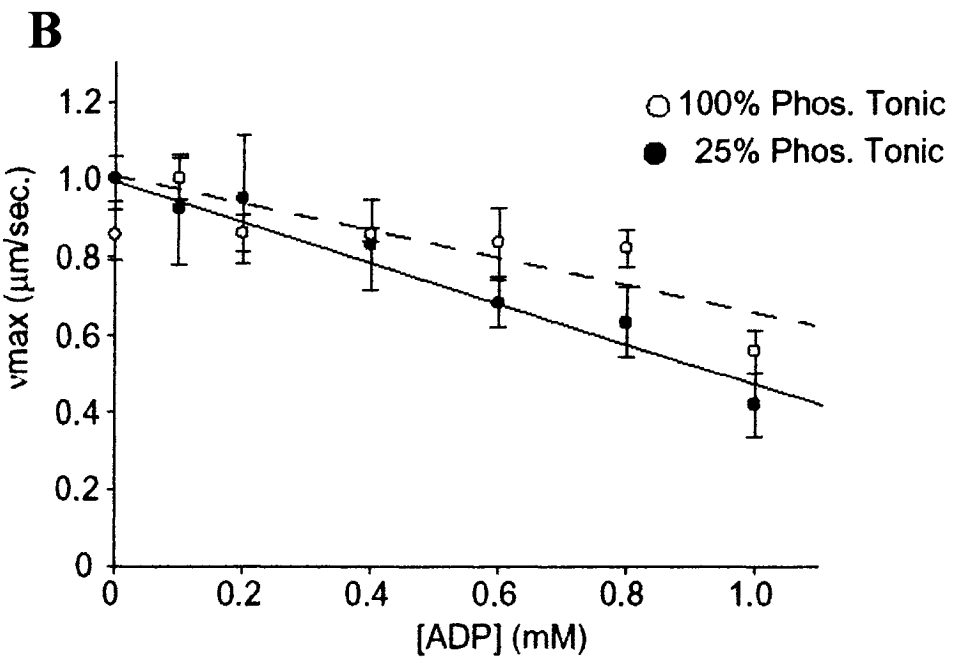


Figure 6: Representative microsphere center of mass (in x and y) with respect to the trap center during unbinding assay. The maximal displacement (Δd) from unloaded baseline position is measured and multiplied by the trap stiffness to obtain F_{unb}. Inset: Superimposed microsphere positions while pulling the trap towards the upper right direction: The microsphere remains at the same position while the trap is pulled away, showing a displacement with respect to the trap. When actin is released from myosin, the bead springs back to its unloaded baseline position.

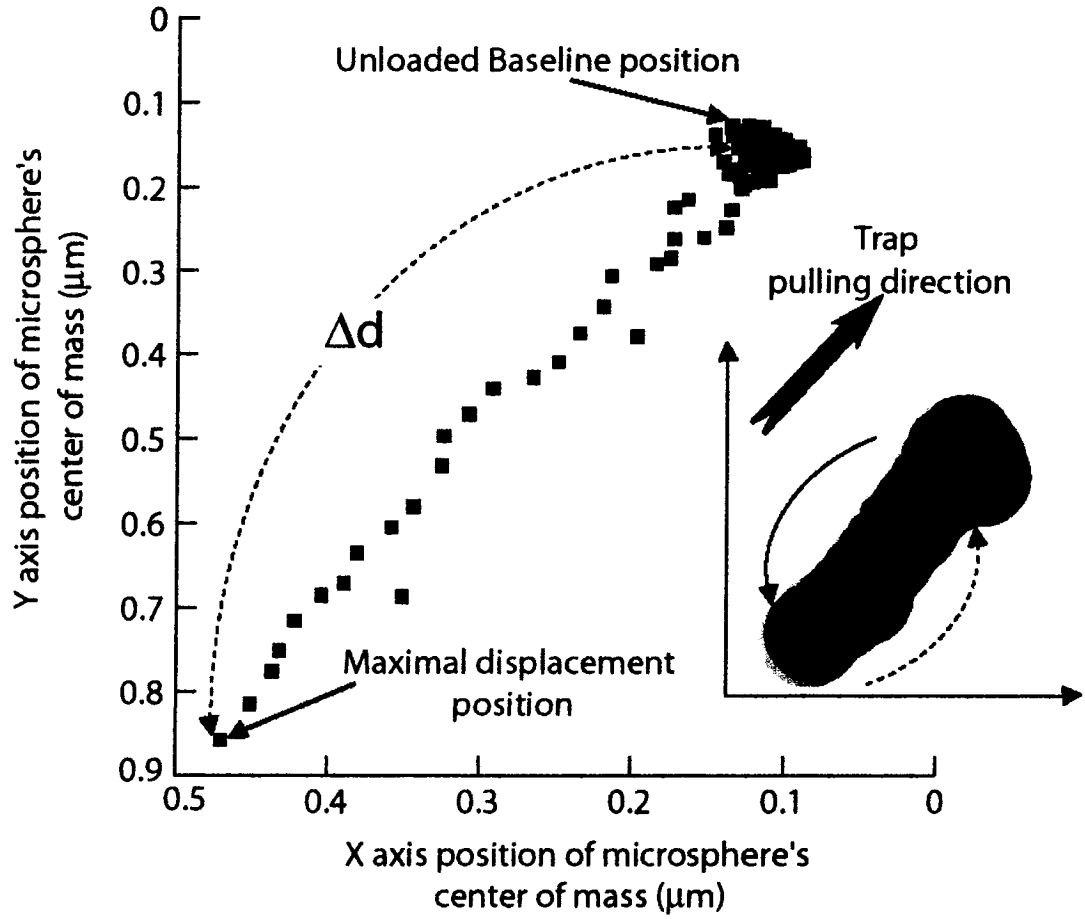
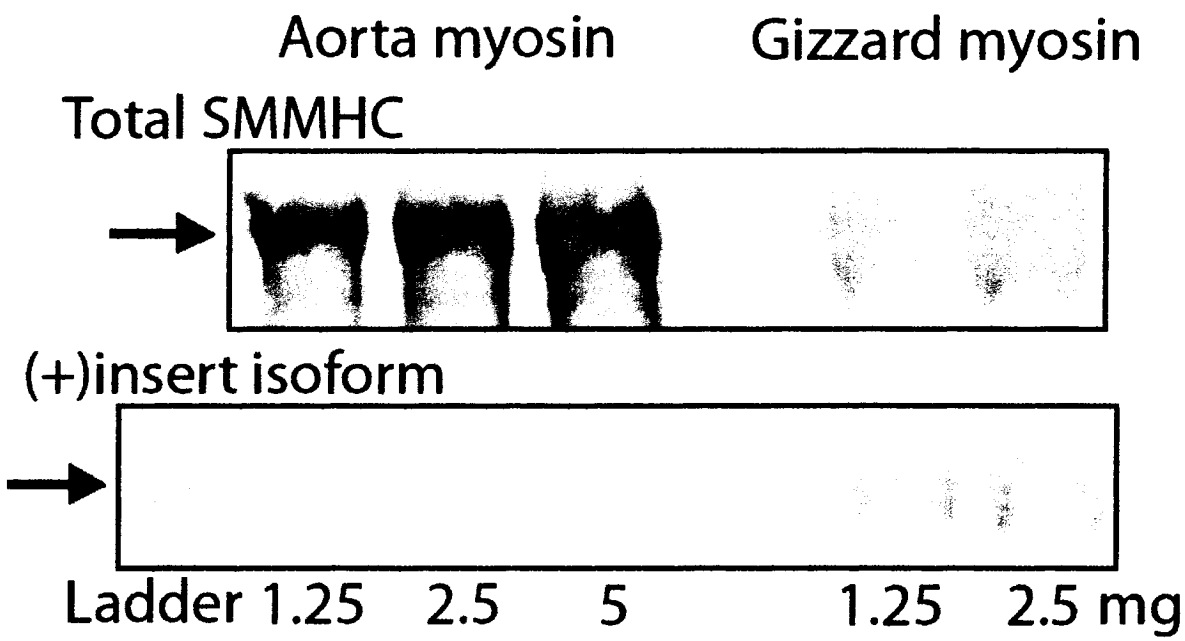


Figure 7: Total SMMHC and (+)insert isoform content of purified tonic (bovine aorta) and phasic (chicken gizzard) as assessed by Western blot analysis. Arrows indicate 200kDa.



Chapter 5: Discussion / Conclusions:

In this thesis, I studied the expression and function of the amino-terminus smooth muscle myosin heavy chain isoforms, as well as other contractile proteins, in normal tissues and in asthma. Up until my studies, the function of the amino terminus smooth muscle myosin heavy chain isoforms (SM-A and SM-B) had been addressed after purification from organs of different species, e.g. chicken gizzard and bovine aorta, or using artificial myosin constructs. My analysis of these myosin isoforms was the first to be performed in multiple organs from the same species. To do this in small animals like rats required the development of a very efficient myosin purification protocol. This was accomplished by extracting myosin in folding conditions which decreases its affinity for actin, allowing for a better yield. To our knowledge, this is the first report of functional myosin purification from such small tissue samples. Unfortunately, the efficiency of this purification protocol is still not sufficient to purify functional myosin from human bronchial biopsies and this will have to await for future development. However, it does open the door to the study of the function of myosin in several models of diseases.

The rank correlation among organs observed in the SM-B myosin isoform expression and the rate of actin filament propulsion in the *in vitro* motility assay (v_{max}) strongly suggests that the SM-B isoform is an important determinant of tissue shortening velocity. Indeed, we measured a greater SM-B expression in the rapidly contracting phasic muscles than in the slowly contracting tonic muscles. However, larger differences in rate of shortening between phasic and tonic smooth muscle are seen at the tissue level, than what we observed at the molecular level. This is, no doubt, due to our minimalist approach that is bound to either miss other important factors or overlook the ensemble effect. In particular, myosin filaments assembly *in vivo*, which is not addressed in the *in vitro* motility assay, may be dynamic and play an essential role in smooth muscle plasticity, as suggested in a recent model (Seow, Pratusevich et al. 2000; Herrera, Kuo et al. 2002). However, more than 50 years after smooth muscle cells were identified, data

showing the precise structure of thick filaments in the smooth muscle cells during contractions are still lacking and are the subject of intense research (Herrera, Kuo et al. 2002; Stephens 2002).

The greater expression of the SM-B isoform in the asthmatic bronchial biopsies offers a molecular mechanism to explain, at least in part, their airway smooth muscle hypercontractility. Because of the size of bronchial biopsies, proper quantification of the contractile protein genes could only be performed at the mRNA level. However, we observed a good correlation between the mRNA and protein measurements, at least in normal tissues, suggesting minor post-transcriptional regulation. Furthermore, because we quantified contractile proteins by referencing to a housekeeping gene ubiquitously expressed in cells, our results do not allow to distinguish hyperplasia from hypertrophy of airway smooth muscle cells in the endobronchial biopsies of the asthmatic subjects. To do such, precise stereomorphometric and cell counting techniques are necessary (Benayoun, Druilhe et al. 2003; Woodruff, Dolganov et al. 2004).

The increased expression of MLCK also contributes to the asthmatic airway smooth muscle hypercontractility by increasing myosin activation. To our knowledge, our study is the first report of an increased v_{max} induced by a mechanical action of MLCK on smooth muscle myosin.

To fully appreciate the role of those contractile proteins in airway hyperresponsiveness, mice genetically modified to lack or overexpress given contractile proteins will be required. Indeed, the time course to maximal airway resistance was significantly prolonged in the knockout mice lacking the SM-B isoform (see introduction) (Tuck, Maghni et al. 2004). Measuring the pulmonary mechanics in a SM-B knockout mouse constructed in a hyperresponsive mouse strain should more definitely elucidate the role of SM-B in asthma.

Many more questions remain regarding the sequence of the 7 amino acid insert. The effect of the flexibility and length of the surface loop on the mechanics of myosin have been investigated, but no study has addressed the role of the serine which is well conserved among species. Because serine is a phosphorylatable site, there is potential for additional regulation.

Finally, while the latch state was discovered in 1981, this thesis is the first effort that addresses its mechanism directly at the molecular level. It is no doubt because of the lack of technology that several molecularly based hypotheses were formulated but never tested. The limit of our approach is that our mechanical measurements were performed in a chemically static environment. To truly test the latch-state, mechanics measurements should be performed during active dephosphorylation to verify if indeed, force maintenance occurs in dynamic conditions. Nonetheless, our data support two previously postulated mechanisms for force maintenance: 1) reattachment of dephosphorylated myosin to actin and 2) high affinity of tonic muscle myosin (SM-A) for MgADP. The reattachment of dephosphorylated myosin suggests the addition of a new time constant in the Hai and Murphy model of the latch state since only detachment of myosin from actin is permitted so far in their model (fig. 1 in this section). We propose that K_7 is bidirectional and results from K_7' (detachment of dephosphorylated myosin from actin), and K_7" (reattachment of dephosphorylated myosin to actin) (fig. 1 in this section). Further investigation is needed to test our hypothesis and calculate the values of K_7" and K_7'. This theory represents an innovative addition to the Hai and Murphy model that would allow a non-exclusive approach of both the latch bridges formation during dephosphorylation and the cooperativity theories.

On the other hand, the high affinity of tonic muscle myosin offers in itself a mechanism to explain the greater propensity of tonic compared to phasic muscle to enter into latch.

Figure 1:

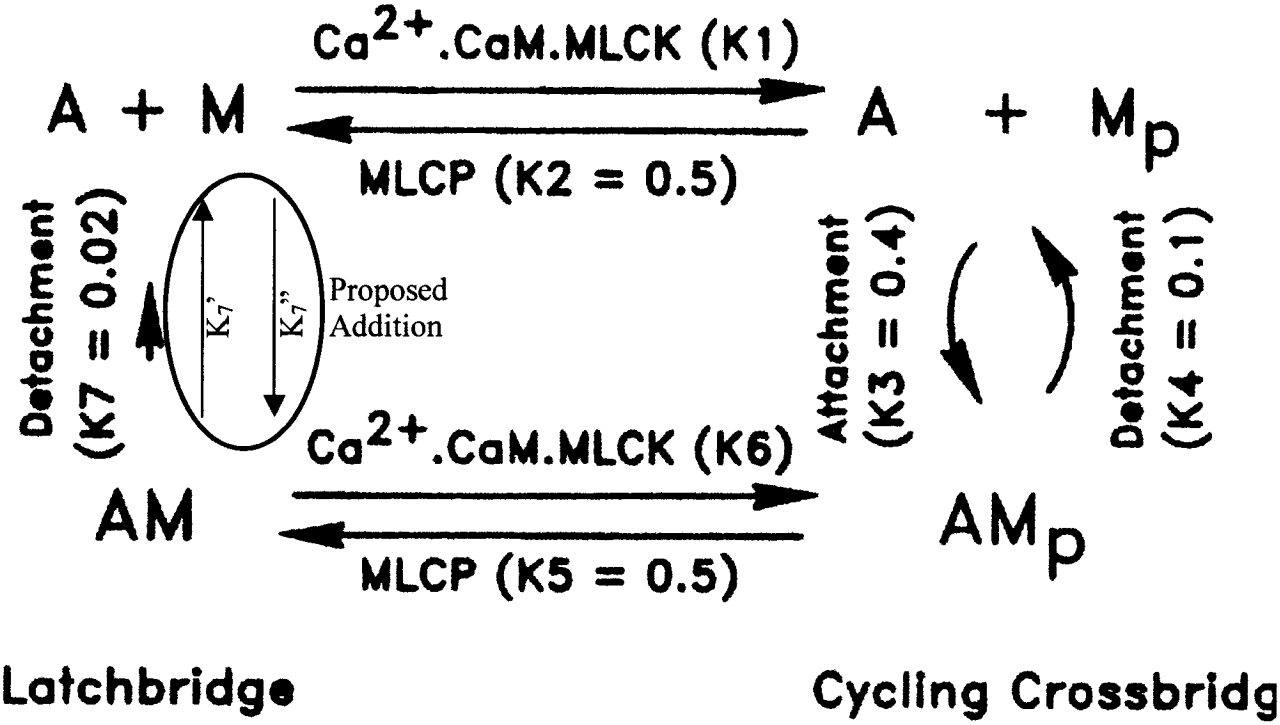


Figure 1: The four-state model: A, actin; myosin crossbridge states are M, detached, dephosphorylated; Mp, detached, phosphorylated; AMp, attached, phosphorylated; AM, attached, dephosphorylated (latchbridge). K1-K7 are first order rate constants resolved from experimental data obtained from the swine carotid media. K1 and K6 represent Ca^{2+}, calmodulin (CaM)-dependent myosin light chain kinase (MLCK) activity; K2 and K5 represent myosin light chain phjosphatase (MLCP) activity. K3 and K4 represent attachment and detachment of phosphorylated crossbriodgfes, and K7 represents latchbridges detachment. K_7' (detachment of dephosphorylated myosin from actin) and K_7" (reattachment of dephosphorylated myosin to actin) are additional rate constants we are proposing. Modified from (Hai and Murphy 1992).

Does the latch-state occur in asthma? This thesis does not answer that question. The original interest in the latch state in airway hyperresponsiveness and asthma came from the fact that asthmatic airways exhibit a deficient relaxation after deep inspirations. Our data, however, suggest that the latch-state should be more prominent in muscle containing a greater amount of the SM-A myosin isoform. To the contrary, we showed that asthmatic airway biopsies have an increased expression of the fast SM-B isoform, suggesting that the latch-state may not be an important mechanism in airway hyperresponsiveness and asthma. Conversely, our data are supportive of an increased rate of shortening in asthma. As previously suggested (see Introduction), a greater rate of shortening would allow asthmatic airway smooth muscle to rapidly contract between each breath thereby maintaining a permanently constricted state. The beneficial relaxation effect of tidal breath and deep inspiration would therefore be lost because the asthmatic airway smooth muscle would have time to catch back the constricted state between each breath.

In conclusion, understanding the fundamental properties of smooth muscle such as the mechanical role of the isoformic compositon and the latch-state phenomenon, is the first step towards a better understanding of the altered smooth muscle contractility observed in diseases such as asthma. Because asthma is an inflammatory disease, it will be very interesting in the future to study the role of inflammatory mediators in potentially altering the contractile protein expression and function. Lastly, airway remodeling should not be neglected, and future studies are necessary to determine if it may prevent the return of airway function to normal even after the alterations of the contractile proteins are reversed back to normal.

Statement of originality:

This thesis contributes to several novel findings on both the function of airway smooth muscle in asthma and the molecular mechanics of the latch-state.

First, the sequence of two of the four smooth muscle myosin heavy chain isoforms was not available in human databanks. I was the first to reveal the sequence of those isoforms (SM-B-SM1 and SM-B-SM2) in human tissues (Chapter 2). Furthermore, although the function of the myosin SM-B isoform had been extensively studied, no data were available comparing the molecular mechanics of smooth muscle myosin between different tissues of the same species. I developed a new myosin purification protocol that allows extraction of sufficient functional myosin from small samples such as rat organs to perform *in vitro* motility measurements. Thus, I showed that among organs of the same animals, v_{max} is commensurate with the expression levels of the SM-B myosin isoform.

In addition, I made a major contribution in the field of airway smooth muscle hypercontractility in asthma by showing that genes encoding for contractile proteins, namely the SM-B myosin isoform, SM22, and MLCK are upregulated in endobronchial biopsies from mild asthmatics. This result offers a molecular mechanism to support the hypothesis that the velocity of shortening of airway smooth muscle is a major contributor to airway hyperresponsiveness and asthma. Furthermore, I tested the functional significance in the *in vitro* motility assay of the overexpression of these contractile protein genes. I found that overexpression of SM-B and MLCK increases v_{max}. Significantly, I showed that MLCK directly potentiates the mechanics of myosin beyond its enzymatic effect.

Lastly, the manuscript in Chapter 4 represents the first and only effort in the literature addressing the molecular mechanisms responsible for the latch-state. Until now, all studies of the latch state have proposed molecular mechanisms based on whole muscle mechanics measurements. In this manuscript, I tested two corollaries of that latch-state at

the molecular level. Using state-of-the-art techniques, I showed that non-phopshorylated myosin can attach to actin filaments and maintain force. Secondly, I showed that tonic muscle myosin has a greater affinity for MgADP than phasic myosin, and that this difference is enhanced by dephosphorylation.

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Appendix:

SUPPLEMENTARY S1

Human (+)insert SM1 SMMHC. Amino acid and nucleotide sequences are shown. The insert is bold-underlined. Sequences are cut at the 3'end stop codon.

```
   1  M  A  Q  K  G  Q  L  S  D  D  E  K  F  L  F  V  D  K  N  F  I  N  S  P  V  A  Q  A  D  W  A  A
K  R  L  V  W  V  P  S
   1
ATGGCGCAGAAGGGCCAACTCAGTGACGATGAGAAGTTCCTCTTTGTGGACAAAAACTTCATCAACAGCCCAGTGGCCCAGGCTGACTGGGCCGCCAAGAGAC
TCGTCTGGGTCCCCTCG

  41  E  K  Q  G  F  E  A  A  S  I  K  E  E  K  G  D  E  V  V  V  E  L  V  E  N  G  K  K  V  T  V  G
K  D  D  I  Q  K  M  N
 121
GAGAAGCAGGGCTTCGAGGCAGCCAGCATTAAGGAGGAGAAGGGGGATGAGGTGGTTGTGGAGCTGGTGGAGAATGGCAAGAAGGTCACGGTTGGGAAAGA
TGACATCCAGAAGATGAAC

  81  P  P  K  F  S  K  V  E  D  M  A  E  L  T  C  L  N  E  A  S  V  L  H  N  L  R  E  R  Y  F  S  G
L  I  Y  T  Y  S  G  L
 241
CCACCCAAGTTCTCCAAGGTGGAGGACATGGCGGAGCTGACGTGCCTCAACGAAGCCTCCGTGCTACACAACCTGAGGGAGCGGTACTTCTCAGGGCTAAT
ATATACGTACTCTGGCCTC

 121  F  C  V  V  V  N  P  Y  K  H  L  P  I  Y  S  E  K  I  V  D  M  Y  K  G  K  K  R  H  E  M  P  P
H  I  Y  A  I  A  D  T
 361
TTCTGCGTGGTGGTCAACCCCTATAAACACCTGCCCATCTACTCGGAGAAGATCGTCGACATGTACAAGGGCAAGAAGAGGCACGAGATGCCGCCTCACAT
CTACGCCATCGCAGACACG

 161  A  Y  R  S  M  L  Q  D  R  E  D  Q  S  I  L  C  T  G  E  S  G  A  G  K  T  E  N  T  K  K  V  I
Q  Y  L  A  V  V  A  S
 481
GCCTACCGGAGCATGCTTCAAGATCGGGAGGACCAGTCCATTCTATGCACAGGCGAGTCTGGAGCCGGGAAAACCGAAAACACCAAGAAGGTCATTCAGTA
CCTGGCCGTGGTGGCCTCC

 201  S  H  K  G  K  K  D  T  S  I  T  Q  G  P  S  F  A  Y  G  E  L  E  K  Q  L  L  Q  A  N  P  I  L
E  A  F  G  N  A  K  T
 601
TCCCACAAGGGCAAGAAAGACACAAGTATCACGCAAGGCCCATCTTTTGCCTACGGAGAGCTGGAAAAGCAGCTTCTACAAGCAAACCCGATTCTGGAGGC
TTTCGGCAACGCCAAAACA
```

241 V K N D N S S R F G K F I R I N F D V T G Y I V G A N I E T Y L
L E K S R A I R
721
GTGAAGAACGACAACTCCTCACGATTCGGCAAATTCATCCGCATCAACTTCGACGTCACGGGTTACATCGTGGGAGCCAACATTGAGACCTATCTGCTAGA
AAAATCACGGGCAATTCGC

281 Q A R D E R T F H I F Y Y M I A G A K E K M R S D L L L E G F N
N Y T F L S N G
841
CAAGCCAGAGACGAGAGGACATTCCACATCTTTTACTACATGATTGCTGGAGCCAAGGAGAAGATGAGAAGTGACTTGCTTTTGGAGGGCTTCAACAACTA
CACCTTCCTCTCCAATGGC

321 F V P I P A A Q D D E M F Q E T V E A M A I M G F S E E E Q L S
I L K V V S S V
961
TTTGTGCCCATCCCAGCAGCCCAGGATGATGAGATGTTCCAGGAAACCGTGGAGGCCATGGCAATCATGGGTTTCAGCGAGGAGGAGCAGCTATCCATATT
GAAGGTGGTATCATCGGTC

361 L Q L G N I V F K K E R N T D Q A S M P D N T A A Q K V C H L M
G I N V T D F T
1081
CTGCAGCTTGGAAATATCGTCTTCAAGAAGGAAAGAAACACAGACCAGGCGTCCATGCCAGATAACACAGCTGCTCAGAAAGTTTGCCACCTCATGGGAAT
TAATGTGACAGATTTCACC

401 R S I L T P R I K V G R D V V Q K A Q T K E Q A D F A V E A L A
K A T Y E R L F
1201
AGATCCATCCTCACTCCTCGTATCAAGGTTGGGCGAGATGTGGTACAGAAAGCTCAGACAAAAGAACAGGCTGACTTTGCTGTAGAGGCTTTGGCCAAGGC
AACATATGAGCGCCTTTTC

441 R W I L T R V N K A L D K T H R Q G A S F L G I L D I A G F E I
F E V N S F E Q
1321
CGCTGGATACTCACCCGCGTGAACAAAGCCCTGGACAAGACCCATCGGCAAGGGGCTTCCTTCCTGGGGATCCTGGATATAGCTGGATTTGAGATCTTTGA
GGTGAACTCCTTCGAGCAG

481 L C I N Y T N E K L Q Q L F N H T M F I L E Q E E Y Q R E G I E
W N F I D F G L
1441
CTGTGCATCAACTACACCAACGAGAAGCTGCAGCAGCTCTTCAACCACACCATGTTCATCCTGGAGCAGGAGGAGTACCAGCGCGAGGGCATCGAGTGGAA
CTTCATCGACTTTGGGCTG

521 D L Q P C I E L I E R P N N P P G V L A L L D E E C W F P K A T
D K S F V E K L
1561
GACCTACAGCCCTGCATCGAGCTCATCGAGCGACCGAACAACCCTCCAGGTGTGCTGGCCCTGCTGGACGAGGAATGCTGGTTCCCCAAAGCCACGGACAA
GTCTTTCGTGGAGAAGCTG

561 C T E Q G S H P K F Q K P K Q L K D K T E F S I I H Y A G K V D
Y N A S A W L T
1681
TGCACGGAGCAGGGCAGCCACCCCAAGTTCCAGAAGCCCAAGCAGCTCAAGGACAAGACTGAGTTCTCCATCATCCATTATGCTGGGAAGGTGGACTATAA
TGCGAGTGCCTGGCTGACC

601 K N M D P L N D N V T S L L N A S S D K F V A D L W K D V D R I
V G L D Q M A K
1801
AAGAATATGGACCCGCTGAATGACAACGTGACTTCCCTGCTCAATGCCTCCTCCGACAAGTTTGTGGCCGACCTGTGGAAGGACGTGGACCGCATCGTGGG
CCTGGACCAGATGGCCAAG

641 M T E S S L P S A S K T K K G M F R T V G Q L Y K E Q L G K L M
T T L R N T T P
1921
ATGACGGAGAGCTCGCTGCCCAGCGCCTCCAAGACCAAGAAGGGCATGTTCCGCACAGTGGGGCAGCTGTACAAGGAGCAGCTGGGCAAGCTGATGACCAC
GCTACGCAACACCACGCCC

681 N F V R C I I P N H E K R S G K L D A F L V L E Q L R C N G V L
E G I R I C R Q
2041
AACTTCGTGCGCTGCATCATCCCCAACCACGAGAAGAGGTCCGGCAAGCTGGATGCGTTCCTGGTGCTGGAGCAGCTGCGGTGCAATGGGGTGCTGGAAGG
CATTCGCATCTGCCGGCAG

721 G F P N R I V F Q E F R Q R Y E I L A A N A I P K G F M D G K Q
A C I L M I K A
2161
GGCTTCCCCAACCGGATCGTCTTCCAGGAGTTCCGCCAACGCTACGAGATCCTGGCGGCGAATGCCATCCCCAAAGGCTTCATGGACGGGAAGCAGGCCTG
CATTCTCATGATCAAAGCC

761 L E L D P N L Y R I G Q S K I F F R T G V L A H L E E E R D L K
I T D V I M A F

2281
CTGGAACTTGACCCCAACTTATACAGGATAGGGCAGAGCAAAATCTTCTTCCGAACTGGCGTCCTGGCCCACCTAGAGGAGGAGCGAGATTTGAAGATCAC
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801 Q A M C R G Y L A R K A F A K R Q Q Q L T A M K V I Q R N C A A
Y L K L R N W Q
2401
CAGGCGATGTGTCGTGGCTACTTGGCCAGAAAGGCTTTTGCCAAGAGGCAGCAGCAGCTGACCGCCATGAAGGTGATTCAGAGGAACTGCGCCGCCTACCT
CAAGCTGCGGAACTGGCAG

841 W W R L F T K V K P L L Q V T R Q E E E M Q A K E D E L Q K T K
E R Q Q K A E N
2521
TGGTGGAGGCTTTTCACCAAAGTGAAGCCACTGCTGCAGGTGACACGGCAGGAGGAGGAGATGCAGGCCAAGGAGGATGAACTGCAGAAGACCAAGGAGCG
GCAGCAGAAGGCAGAGAAT

881 E L K E L E Q K H S Q L T E E K N L L Q E Q L Q A E T E L Y A E
A E E M R V R L
2641
GAGCTTAAGGAGCTGGAACAGAAGCACTCGCAGCTGACCGAGGAGAAGAACCTGCTACAGGAACAGCTGCAGGCAGAGACAGAGCTGTATGCAGAGGCTGA
GGAGATGCGGGTGCGGCTG

921 A A K K Q E L E E I L H E M E A R L E E E E D R G Q Q L Q A E R
K K M A Q Q M L
2761
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GATGGCCCAGCAGATGCTG

961 D L E E Q L E E E E A A R Q K L Q L E K V T A E A K I K K L E D
E I L V M D D Q
2881
GACCTTGAAGAACAGCTGGAGGAGGAGGAAGCTGCCAGGCAGAAGCTGCAACTTGAGAAGGTCACGGCTGAGGCCAAGATCAAGAAACTGGAGGATGAGAT
CCTGGTCATGGATGATCAG

1001 N N K L S K E R K L L E E R I S D L T T N L A E E E E K A K N L
T K L K N K H E
3001
AACAATAAACTATCAAAAGAACGAAAACTCCTTGAGGAGAGGATTAGTGACTTAACGACAAATCTTGCAGAAGAGGAAGAAAAGGCCAAGAATCTTACCAA
GCTGAAAAACAAGCATGAA

1041 S M I S E L E V R L K K E E K S R Q E L E K L K R K L E G D A S
D F H E Q I A D
3121
TCTATGATTTCAGAACTGGAAGTGCGGCTAAAGAAGGAAGAGAAGAGCCGACAGGAGCTGGAGAAGCTGAAACGGAAGCTGGAGGGTGATGCCAGCGACTT
CCACGAGCAGATCGCTGAC

1081 L Q A Q I A E L K M Q L A K K E E E L Q A A L A R L D D E I A Q
K N N A L K K I
3241
CTCCAGGCGCAGATCGCAGAGCTCAAGATGCAGCTGGCCAAGAAGGAGGAGGAGCTGCAGGCGGCCCTGGCCAGGCTTGACGATGAAATCGCTCAGAAGAA
CAATGCCCTGAAGAAGATC

1121 R E L E G H I S D L Q E D L D S E R A A R N K A E K Q K R D L G
E E L E A L K T
3361
CGGGAGCTGGAGGGCCACATCTCAGACCTCCAGGAGGACCTGGACTCAGAGCGGGCCGCCAGGAACAAGGCTGAAAAGCAGAAGCGAGACCTCGGCGAGGA
GCTGGAGGCCCTAAAGACA

1161 E L E D T L D S T A T Q Q E L R A K R E Q E V T V L K K A L D E
E T R S H E A Q
3481
GAGCTGGAAGACACACTGGACAGCACAGCCACTCAGCAGGAGCTCAGGGCCAAGAGGGAGCAGGAGGTGACGGTGCTGAAGAAGGCCCTGGATGAAGAGAC
GCGGTCCCATGAGGCTCAG

1201 V Q E M R Q K H A Q A V E E L T E Q L E Q F K R A K A N L D K N
K Q T L E K E N
3601
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GACGCTGGAGAAAGAGAAC

1241 A D L A G E L R V L G Q A K Q E V E H K K K K L E A Q V Q E L Q
S K C S D G E R
3721
GCAGACCTGGCCGGGGAGCTGCGGGTCCTGGGCCAGGCCAAGCAGGAGGTGGAACATAAGAAGAAGAAGCTGGAGGCGCAGGTGCAGGAGCTGCAGTCCAA
GTGCAGCGATGGGGAGCGG

1281 A R A E L N D K V H K L Q N E V E S V T G M L N E A E G K A I K
L A K D V A S L
3841
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1321 S S Q L Q D T Q E L L Q E E T R Q K L N V S T K L R Q L E E E R
N S L Q D Q L D
3961
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CCTGCAAGACCAGCTGGAC

1361 E E M E A K Q N L E R H I S T L N I Q L S D S K K K L Q D F A S
T V E A L E E G
4081
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1401 K K R F Q K E I E N L T Q Q Y E E K A A A Y D K L E K T K N R L
Q Q E L D D L V
4201
AAGAAGAGGTTCCAGAAGGAGATCGAGAACCTCACCCAGCAGTACGAGGAGAAGGCGGCCGCTTATGATAAACTGGAAAAGACCAAGAACAGGCTTCAGCA
GGAGCTGGACGACCTGGTT

1441 V D L D N Q R Q L V S N L E K K Q R K F D Q L L A E E K N I S S
K Y A D E R D R
4321
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CGCGGATGAGAGGGACAGA

1481 A E A E A R E K E T K A L S L A R A L E E A L E A K E E L E R T
N K M L K A E M
4441
GCTGAGGCAGAAGCCAGGGAGAAGGAAACCAAGGCCCTGTCCCTGGCTCGGGCCCTTGAAGAGGCCTTGGAAGCCAAAGAGGAACTCGAGCGGACCAACAA
AATGCTCAAAGCCGAAATG

1521 E D L V S S K D D V G K N V H E L E K S K R A L E T Q M E E M K
T Q L E E L E D
4561
GAAGACCTGGTCAGCTCCAAGGATGACGTGGGCAAGAACGTCCATGAGCTGGAGAAGTCCAAGCGGGCCCTGGAGACCCAGATGGAGGAGATGAAGACGCA
GCTGGAAGAGCTGGAGGAC

1561 E L Q A T E D A K L R L E V N M Q A L K G Q F E R D L Q A R D E
Q N E E K R R Q

4681
GAGCTGCAAGCCACGGAGGACGCCAAACTGCGGCTGGAAGTCAACATGCAGGCGCTCAAGGGCCAGTTCGAAAGGGATCTCCAAGCCCGGGACGAGCAGAA
TGAGGAGAAGAGGAGGCAA

1601 L Q R Q L H E Y E T E L E D E R K Q R A L A A A A K K K L E G D
L K D L E L Q A
4801
CTGCAGAGACAGCTTCACGAGTATGAGACGGAACTGGAAGACGAGCGAAAGCAACGTGCCCTGGCAGCTGCAGCAAAGAAGAAGCTGGAAGGGGACCTGAA
AGACCTGGAGCTTCAGGCC

1641 D S A I K G R E E A I K Q L R K L Q A Q M K D F Q R E L E D A R
A S R D E I F A
4921
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CAGAGATGAGATCTTTGCC

1681 T A K E N E K K A K S L E A D L M Q L Q E D L A A A E R A R K Q
A D L E K E E L
5041
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CCTCGAGAAGGAGGAACTG

1721 A E E L A S S L S G R N A L Q D E K R R L E A R I A Q L E E E L
E E E Q G N M E
5161
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GGAGCAGGGCAACATGGAG

1761 A M S D R V R K A T Q Q A E Q L S N E L A T E R S T A Q K N E S
A R Q Q L E R Q
5281
GCCATGAGCGACCGGGTCCGCAAAGCCACACAGCAGGCCGAGCAGCTCAGCAACGAGCTGGCCACAGAGCGCAGCACGGCCCAGAAGAATGAGAGTGCCCG
GCAGCAGCTCGAGCGGCAG

1801 N K E L R S K L H E M E G A V K S K F K S T I A A L E A K I A Q
L E E Q V E Q E
5401
AACAAGGAGCTCCGGAGCAAGCTCCACGAGATGGAGGGGGCCGTCAAGTCCAAGTTCAAGTCCACCATCGCGGCGCTGGAGGCCAAGATTGCACAGCTGGA
GGAGCAGGTCGAGCAGGAG

1841 A R E K Q A A T K S L K Q K D K K L K E I L L Q V E D E R K M A
E Q Y K E Q A E
5521
GCCAGAGAGAAACAGGCGGCCACCAAGTCGCTGAAGCAGAAAGACAAGAAGCTGAAGGAAATCTTGCTGCAGGTGGAGGACGAGCGCAAGATGGCCGAGCA
GTACAAGGAGCAGGCAGAG

1881 K G N A R V K Q L K R Q L E E A E E E S Q R I N A N R R K L Q R
E L D E A T E S
5641
AAAGGCAATGCCAGGGTCAAGCAGCTCAAGAGGCAGCTGGAGGAGGCAGAGGAGGAGTCCCAGCGCATCAACGCCAACCGCAGGAAGCTGCAGCGGGAGCT
GGATGAGGCCACGGAGAGC

1921 N E A M G R E V N A L K S K L R R G N E T S F V P S R R S G G R
R V I E N A D G
5761
AACGAGGCCATGGGCCGCGAGGTGAACGCACTCAAGAGCAAGCTCAGGCGAGGAAACGAGACCTCTTTCGTTCCTTCTAGAAGGTCTGGAGGACGTAGAGT
TATTGAAAATGCAGATGGT

1961 S E E E T D T R D A D F N G T K A S E -
5881 TCTGAGGAGGAAACGGACACTCGAGACGCAGACTTCAATGGAACCAAGGCCAGTGAATAA

Supplementary S2

Human (+)insert SM2 SMMHC. Amino acid and nucleotide sequences are shown. The insert is bold-underlined. Sequences are cut at the 3'end stop codon.

```
   1  M  A  Q  K  G  Q  L  S  D  D  E  K  F  L  F  V  D  K  N  F  I  N  S  P  V  A  Q  A  D  W  A  A
K  R  L  V  W  V  P  S
   1
ATGGCGCAGAAGGGCCAACTCAGTGACGATGAGAAGTTCCTCTTTGTGGACAAAAACTTCATCAACAGCCCAGTGGCCCAGGCTGACTGGGCCGCCAAGAGAC
TCGTCTGGGTCCCCTCG

  41  E  K  Q  G  F  E  A  A  S  I  K  E  E  K  G  D  E  V  V  V  E  L  V  E  N  G  K  K  V  T  V  G
K  D  D  I  Q  K  M  N
 121
GAGAAGCAGGGCTTCGAGGCAGCCAGCATTAAGGAGGAGAAGGGGGATGAGGTGGTTGTGGAGCTGGTGGAGAATGGCAAGAAGGTCACGGTTGGGAAAGA
TGACATCCAGAAGATGAAC

  81  P  P  K  F  S  K  V  E  D  M  A  E  L  T  C  L  N  E  A  S  V  L  H  N  L  R  E  R  Y  F  S  G
L  I  Y  T  Y  S  G  L
 241
CCACCCAAGTTCTCCAAGGTGGAGGACATGGCGGAGCTGACGTGCCTCAACGAAGCCTCCGTGCTACACAACCTGAGGGAGCGGTACTTCTCAGGGCTAAT
ATATACGTACTCTGGCCTC

 121  F  C  V  V  V  N  P  Y  K  H  L  P  I  Y  S  E  K  I  V  D  M  Y  K  G  K  K  R  H  E  M  P  P
H  I  Y  A  I  A  D  T
 361
TTCTGCGTGGTGGTCAACCCCTATAAACACCTGCCCATCTACTCGGAGAAGATCGTCGACATGTACAAGGGCAAGAAGAGGCACGAGATGCCGCCTCACAT
CTACGCCATCGCAGACACG

 161  A  Y  R  S  M  L  Q  D  R  E  D  Q  S  I  L  C  T  G  E  S  G  A  G  K  T  E  N  T  K  K  V  I
Q  Y  L  A  V  V  A  S
 481
GCCTACCGGAGCATGCTTCAAGATCGGGAGGACCAGTCCATTCTATGCACAGGCGAGTCTGGAGCCGGGAAAACCGAAAACACCAAGAAGGTCATTCAGTA
CCTGGCCGTGGTGGCCTCC

 201  S  H  K  G  K  K  D  T  S  I  T  Q  G  P  S  F  A  Y  G  E  L  E  K  Q  L  L  Q  A  N  P  I  L
E  A  F  G  N  A  K  T
 601
TCCCACAAGGGCAAGAAAGACACAAGTATCACGCAAGGCCCATCTTTTGCCTACGGAGAGCTGGAAAAGCAGCTTCTACAAGCAAACCCGATTCTGGAGGC
TTTCGGCAACGCCAAAACA
```

241 V K N D N S S R F G K F I R I N F D V T G Y I V G A N I E T Y L
L E K S R A I R
721
GTGAAGAACGACAACTCCTCACGATTCGGCAAATTCATCCGCATCAACTTCGACGTCACGGGTTACATCGTGGGAGCCAACATTGAGACCTATCTGCTAGA
AAAATCACGGGCAATTCGC

281 Q A R D E R T F H I F Y Y M I A G A K E K M R S D L L L E G F N
N Y T F L S N G
841
CAAGCCAGAGACGAGAGGACATTCCACATCTTTTACTACATGATTGCTGGAGCCAAGGAGAAGATGAGAAGTGACTTGCTTTTGGAGGGCTTCAACAACTA
CACCTTCCTCTCCAATGGC

321 F V P I P A A Q D D E M F Q E T V E A M A I M G F S E E E Q L S
I L K V V S S V
961
TTTGTGCCCATCCCAGCAGCCCAGGATGATGAGATGTTCCAGGAAACCGTGGAGGCCATGGCAATCATGGGTTTCAGCGAGGAGGAGCAGCTATCCATATT
GAAGGTGGTATCATCGGTC

361 L Q L G N I V F K K E R N T D Q A S M P D N T A A Q K V C H L M
G I N V T D F T
1081
CTGCAGCTTGGAAATATCGTCTTCAAGAAGGAAAGAAACACAGACCAGGCGTCCATGCCAGATAACACAGCTGCTCAGAAAGTTTGCCACCTCATGGGAAT
TAATGTGACAGATTTCACC

401 R S I L T P R I K V G R D V V Q K A Q T K E Q A D F A V E A L A
K A T Y E R L F
1201
AGATCCATCCTCACTCCTCGTATCAAGGTTGGGCGAGATGTGGTACAGAAAGCTCAGACAAAAGAACAGGCTGACTTTGCTGTAGAGGCTTTGGCCAAGGC
AACATATGAGCGCCTTTTC

441 R W I L T R V N K A L D K T H R Q G A S F L G I L D I A G F E I
F E V N S F E Q
1321
CGCTGGATACTCACCCGCGTGAACAAAGCCCTGGACAAGACCCATCGGCAAGGGGCTTCCTTCCTGGGGATCCTGGATATAGCTGGATTTGAGATCTTTGA
GGTGAACTCCTTCGAGCAG

481 L C I N Y T N E K L Q Q L F N H T M F I L E Q E E Y Q R E G I E
W N F I D F G L
1441
CTGTGCATCAACTACACCAACGAGAAGCTGCAGCAGCTCTTCAACCACACCATGTTCATCCTGGAGCAGGAGGAGTACCAGCGCGAGGGCATCGAGTGGAA
CTTCATCGACTTTGGGCTG

521 D L Q P C I E L I E R P N N P P G V L A L L D E E C W F P K A T
D K S F V E K L
1561
GACCTACAGCCCTGCATCGAGCTCATCGAGCGACCGAACAACCCTCCAGGTGTGCTGGCCCTGCTGGACGAGGAATGCTGGTTCCCCAAAGCCACGGACAA
GTCTTTCGTGGAGAAGCTG

561 C T E Q G S H P K F Q K P K Q L K D K T E F S I I H Y A G K V D
Y N A S A W L T
1681
TGCACGGAGCAGGGCAGCCACCCCAAGTTCCAGAAGCCCAAGCAGCTCAAGGACAAGACTGAGTTCTCCATCATCCATTATGCTGGGAAGGTGGACTATAA
TGCGAGTGCCTGGCTGACC

601 K N M D P L N D N V T S L L N A S S D K F V A D L W K D V D R I
V G L D Q M A K
1801
AAGAATATGGACCCGCTGAATGACAACGTGACTTCCCTGCTCAATGCCTCCTCCGACAAGTTTGTGGCCGACCTGTGGAAGGACGTGGACCGCATCGTGGG
CCTGGACCAGATGGCCAAG

641 M T E S S L P S A S K T K K G M F R T V G Q L Y K E Q L G K L M
T T L R N T T P
1921
ATGACGGAGAGCTCGCTGCCCAGCGCCTCCAAGACCAAGAAGGGCATGTTCCGCACAGTGGGGCAGCTGTACAAGGAGCAGCTGGGCAAGCTGATGACCAC
GCTACGCAACACCACGCCC

681 N F V R C I I P N H E K R S G K L D A F L V L E Q L R C N G V L
E G I R I C R Q
2041
AACTTCGTGCGCTGCATCATCCCCAACCACGAGAAGAGGTCCGGCAAGCTGGATGCGTTCCTGGTGCTGGAGCAGCTGCGGTGCAATGGGGTGCTGGAAGG
CATTCGCATCTGCCGGCAG

721 G F P N R I V F Q E F R Q R Y E I L A A N A I P K G F M D G K Q
A C I L M I K A
2161
GGCTTCCCCAACCGGATCGTCTTCCAGGAGTTCCGCCAACGCTACGAGATCCTGGCGGCGAATGCCATCCCCAAAGGCTTCATGGACGGGAAGCAGGCCTG
CATTCTCATGATCAAAGCC

761 L E L D P N L Y R I G Q S K I F F R T G V L A H L E E E R D L K
I T D V I M A F

2281
CTGGAACTTGACCCCAACTTATACAGGATAGGGCAGAGCAAAATCTTCTTCCGAACTGGCGTCCTGGCCCACCTAGAGGAGGAGCGAGATTTGAAGATCAC
CGATGTCATCATGGCCTTC

801 Q A M C R G Y L A R K A F A K R Q Q Q L T A M K V I Q R N C A A
Y L K L R N W Q
2401
CAGGCGATGTGTCGTGGCTACTTGGCCAGAAAGGCTTTTGCCAAGAGGCAGCAGCAGCTGACCGCCATGAAGGTGATTCAGAGGAACTGCGCCGCCTACCT
CAAGCTGCGGAACTGGCAG

841 W W R L F T K V K P L L Q V T R Q E E E M Q A K E D E L Q K T K
E R Q Q K A E N
2521
TGGTGGAGGCTTTTCACCAAAGTGAAGCCACTGCTGCAGGTGACACGGCAGGAGGAGGAGATGCAGGCCAAGGAGGATGAACTGCAGAAGACCAAGGAGCG
GCAGCAGAAGGCAGAGAAT

881 E L K E L E Q K H S Q L T E E K N L L Q E Q L Q A E T E L Y A E
A E E M R V R L
2641
GAGCTTAAGGAGCTGGAACAGAAGCACTCGCAGCTGACCGAGGAGAAGAACCTGCTACAGGAACAGCTGCAGGCAGAGACAGAGCTGTATGCAGAGGCTGA
GGAGATGCGGGTGCGGCTG

921 A A K K Q E L E E I L H E M E A R L E E E E D R G Q Q L Q A E R
K K M A Q Q M L
2761
GCGGCCAAGAAGCAGGAGCTGGAGGAGATACTGCATGAGATGGAGGCCCGCCTGGAGGAGGAGGAAGACAGGGGCCAGCAGCTACAGGCTGAAAGGAAGAA
GATGGCCCAGCAGATGCTG

961 D L E E Q L E E E E A A R Q K L Q L E K V T A E A K I K K L E D
E I L V M D D Q
2881
GACCTTGAAGAACAGCTGGAGGAGGAGGAAGCTGCCAGGCAGAAGCTGCAACTTGAGAAGGTCACGGCTGAGGCCAAGATCAAGAAACTGGAGGATGAGAT
CCTGGTCATGGATGATCAG

1001 N N K L S K E R K L L E E R I S D L T T N L A E E E E K A K N L
T K L K N K H E
3001
AACAATAAACTATCAAAAGAACGAAAACTCCTTGAGGAGAGGATTAGTGACTTAACGACAAATCTTGCAGAAGAGGAAGAAAAGGCCAAGAATCTTACCAA
GCTGAAAAACAAGCATGAA

1041 S M I S E L E V R L K K E E K S R Q E L E K L K R K L E G D A S
D F H E Q I A D
3121
TCTATGATTTCAGAACTGGAAGTGCGGCTAAAGAAGGAAGAGAAGAGCCGACAGGAGCTGGAGAAGCTGAAACGGAAGCTGGAGGGTGATGCCAGCGACTT
CCACGAGCAGATCGCTGAC

1081 L Q A Q I A E L K M Q L A K K E E E L Q A A L A R L D D E I A Q
K N N A L K K I
3241
CTCCAGGCGCAGATCGCAGAGCTCAAGATGCAGCTGGCCAAGAAGGAGGAGGAGCTGCAGGCGGCCCTGGCCAGGCTTGACGATGAAATCGCTCAGAAGAA
CAATGCCCTGAAGAAGATC

1121 R E L E G H I S D L Q E D L D S E R A A R N K A E K Q K R D L G
E E L E A L K T
3361
CGGGAGCTGGAGGGCCACATCTCAGACCTCCAGGAGGACCTGGACTCAGAGCGGGCCGCCAGGAACAAGGCTGAAAAGCAGAAGCGAGACCTCGGCGAGGA
GCTGGAGGCCCTAAAGACA

1161 E L E D T L D S T A T Q Q E L R A K R E Q E V T V L K K A L D E
E T R S H E A Q
3481
GAGCTGGAAGACACACTGGACAGCACAGCCACTCAGCAGGAGCTCAGGGCCAAGAGGGAGCAGGAGGTGACGGTGCTGAAGAAGGCCCTGGATGAAGAGAC
GCGGTCCCATGAGGCTCAG

1201 V Q E M R Q K H A Q A V E E L T E Q L E Q F K R A K A N L D K N
K Q T L E K E N
3601
GTCCAGGAGATGAGGCAGAAACACGCACAGGCGGTGGAGGAGCTCACAGAGCAGCTTGAGCAGTTCAAGAGGGCCAAGGCGAACCTAGACAAGAATAAGCA
GACGCTGGAGAAAGAGAAC

1241 A D L A G E L R V L G Q A K Q E V E H K K K K L E A Q V Q E L Q
S K C S D G E R
3721
GCAGACCTGGCCGGGGAGCTGCGGGTCCTGGGCCAGGCCAAGCAGGAGGTGGAACATAAGAAGAAGAAGCTGGAGGCGCAGGTGCAGGAGCTGCAGTCCAA
GTGCAGCGATGGGGAGCGG

1281 A R A E L N D K V H K L Q N E V E S V T G M L N E A E G K A I K
L A K D V A S L
3841
GCCCGGGCGGAGCTCAATGACAAAGTCCACAAGCTGCAGAATGAAGTTGAGAGCGTCACAGGGATGCTTAACGAGGCCGAGGGGAAGGCCATTAAGCTGGC
CAAGGACGTGGCGTCCCTC

1321 S S Q L Q D T Q E L L Q E E T R Q K L N V S T K L R Q L E E E R
N S L Q D Q L D
3961
AGTTCCCAGCTCCAGGACACCCAGGAGCTGCTTCAAGAAGAAACCCGGCAGAAGCTCAACGTGTCTACGAAGCTGCGCCAGCTGGAGGAGGAGCGGAACAG
CCTGCAAGACCAGCTGGAC

1361 E E M E A K Q N L E R H I S T L N I Q L S D S K K K L Q D F A S
T V E A L E E G
4081
GAGGAGATGGAGGCCAAGCAGAACCTGGAGCGCCACATCTCCACTCTCAACATCCAGCTCTCCGACTCGAAGAAGAAGCTGCAGGACTTTGCCAGCACCGT
GGAAGCTCTGGAAGAGGGG

1401 K K R F Q K E I E N L T Q Q Y E E K A A A Y D K L E K T K N R L
Q Q E L D D L V
4201
AAGAAGAGGTTCCAGAAGGAGATCGAGAACCTCACCCAGCAGTACGAGGAGAAGGCGGCCGCTTATGATAAACTGGAAAAGACCAAGAACAGGCTTCAGCA
GGAGCTGGACGACCTGGTT

1441 V D L D N Q R Q L V S N L E K K Q R K F D Q L L A E E K N I S S
K Y A D E R D R
4321
GTTGATTTGGACAACCAGCGGCAACTCGTGTCCAACCTGGAAAAGAAGCAGAGGAAATTTGATCAGTTGTTAGCCGAGGAGAAAAACATCTCTTCCAAATA
CGCGGATGAGAGGGACAGA

1481 A E A E A R E K E T K A L S L A R A L E E A L E A K E E L E R T
N K M L K A E M
4441
GCTGAGGCAGAAGCCAGGGAGAAGGAAACCAAGGCCCTGTCCCTGGCTCGGGCCCTTGAAGAGGCCTTGGAAGCCAAAGAGGAACTCGAGCGGACCAACAA
AATGCTCAAAGCCGAAATG

1521 E D L V S S K D D V G K N V H E L E K S K R A L E T Q M E E M K
T Q L E E L E D
4561
GAAGACCTGGTCAGCTCCAAGGATGACGTGGGCAAGAACGTCCATGAGCTGGAGAAGTCCAAGCGGGCCCTGGAGACCCAGATGGAGGAGATGAAGACGCA
GCTGGAAGAGCTGGAGGAC

1561 E L Q A T E D A K L R L E V N M Q A L K G Q F E R D L Q A R D E
Q N E E K R R Q

4681
GAGCTGCAAGCCACGGAGGACGCCAAACTGCGGCTGGAAGTCAACATGCAGGCGCTCAAGGGCCAGTTCGAAAGGGATCTCCAAGCCCGGGACGAGCAGAA
TGAGGAGAAGAGGAGGCAA

1601 L Q R Q L H E Y E T E L E D E R K Q R A L A A A A K K K L E G D
L K D L E L Q A
4801
CTGCAGAGACAGCTTCACGAGTATGAGACGGAACTGGAAGACGAGCGAAAGCAACGTGCCCTGGCAGCTGCAGCAAAGAAGAAGCTGGAAGGGGACCTGAA
AGACCTGGAGCTTCAGGCC

1641 D S A I K G R E E A I K Q L R K L Q A Q M K D F Q R E L E D A R
A S R D E I F A
4921
GACTCTGCCATCAAGGGGAGGGAGGAAGCCATCAAGCAGCTACGCAAACTGCAGGCTCAGATGAAGGACTTTCAAAGAGAGCTGGAAGATGCCCGTGCCTC
CAGAGATGAGATCTTTGCC

1681 T A K E N E K K A K S L E A D L M Q L Q E D L A A A E R A R K Q
A D L E K E E L
5041
ACAGCCAAAGAGAATGAGAAGAAAGCCAAGAGCTTGGAAGCAGACCTCATGCAGCTACAAGAGGACCTCGCCGCCGCTGAGAGGGCTCGCAAACAAGCGGA
CCTCGAGAAGGAGGAACTG

1721 A E E L A S S L S G R N A L Q D E K R R L E A R I A Q L E E E L
E E E Q G N M E
5161
GCAGAGGAGCTGGCCAGTAGCCTGTCGGGAAGGAACGCACTCCAGGACGAGAAGCGCCGCCTGGAGGCCCGGATCGCCCAGCTGGAGGAGGAGCTGGAGGA
GGAGCAGGGCAACATGGAG

1761 A M S D R V R K A T Q Q A E Q L S N E L A T E R S T A Q K N E S
A R Q Q L E R Q
5281
GCCATGAGCGACCGGGTCCGCAAAGCCACACAGCAGGCCGAGCAGCTCAGCAACGAGCTGGCCACAGAGCGCAGCACGGCCCAGAAGAATGAGAGTGCCCG
GCAGCAGCTCGAGCGGCAG

1801 N K E L R S K L H E M E G A V K S K F K S T I A A L E A K I A Q
L E E Q V E Q E
5401
AACAAGGAGCTCCGGAGCAAGCTCCACGAGATGGAGGGGGCCGTCAAGTCCAAGTTCAAGTCCACCATCGCGGCGCTGGAGGCCAAGATTGCACAGCTGGA
GGAGCAGGTCGAGCAGGAG

1841 A R E K Q A A T K S L K Q K D K K L K E I L L Q V E D E R K M A
E Q Y K E Q A E
5521
GCCAGAGAGAAACAGGCGGCCACCAAGTCGCTGAAGCAGAAAGACAAGAAGCTGAAGGAAATCTTGCTGCAGGTGGAGGACGAGCGCAAGATGGCCGAGCA
GTACAAGGAGCAGGCAGAG

1881 K G N A R V K Q L K R Q L E E A E E E S Q R I N A N R R K L Q R
E L D E A T E S
5641
AAAGGCAATGCCAGGGTCAAGCAGCTCAAGAGGCAGCTGGAGGAGGCAGAGGAGGAGTCCCAGCGCATCAACGCCAACCGCAGGAAGCTGCAGCGGGAGCT
GGATGAGGCCACGGAGAGC

1921 N E A M G R E V N A L K S K L R G P P P Q E T S Q -
5761 AACGAGGCCATGGGCCGCGAGGTGAACGCACTCAAGAGCAAGCTCAGAGGGCCCCCCCCACAGGAAACTTCGCAGTGA