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Smoothelin Contains a Novel Actin Cytoskeleton Localization Sequence With Similarity to Troponin T

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Abstract Smoothelin, a cytoskeletal protein, exists in a large isoform specifically expressed in vascular smooth muscle cells, and a small visceral isoform generated by a downstream transcriptional start site. Using fusions to the green fluorescent protein, we could show that both smoothelin isoforms are localized at actin containing filaments and mapped two domains that are each sufficient for localization at the actin cytoskeleton. The first domain is located in the vascular-specific, N-terminal half of smoothelin and the second in the common, C-terminal half. The second domain shares clear sequence similarity with a domain of troponin T involved in actin filament association. These results suggest that the tissue-specific expression of smoothelin isoforms might contribute to the different contractile properties of smooth muscle cells. J. Cell. Biochem. 85: 403–409, 2002. © 2002 Wiley-Liss, Inc.

Key words: actin; smoothelin; troponin T; cytoskeleton; muscle contraction; smooth muscle

Smooth muscle cells (SMC) play an essential role in the maintenance of homeostasis in vertebrates through changes in their contraction-relaxation cycle. The features of these cells differ markedly between tissues and species; moreover, there are often regional differences within a given tissue [Kuriyama et al., 1998]. Smooth muscles are responsible for the long-lasting movements characteristic of enteric organs and may display a highly contractile type with fast contraction velocities or a less contractile type with slow contraction [Arner and Malmqvist, 1998]. The regulation of Ca^{2+}-dependent contraction and relaxation in smooth muscle is much less understood than in striated muscles [Geeves, 1999].

Two independently working proteins, caldesmon and calponin, are involved in regulation of contraction [Czurylo et al., 1997]. Both caldesmon and calponin inhibit the actin regulated ATPase activity of myosin in a Ca^{2+}-calmodulin dependent manner [Marston et al., 1994; Winder and Walsh, 1996]. The mechanism by which caldesmon controls actin-tropomyosin interaction in smooth muscle is comparable to the troponin complex in skeletal and cardiac muscle [Marston et al., 1998]. Additional proteins influence the inhibitory action of caldesmon like the S100 protein [Polyakov et al., 1998] or PAK1, which can phosphorylate caldesmon [Foster et al., 2000].

Nearly all proteins involved in SMC force generation are expressed as alternatively spliced variants. SMC specific isoforms of muscle and nonmuscle proteins are responsible for SMC contraction. There are SMC specific isoforms of tropomyosin, actin, MHC, MLC, MLCK, caldesmon, calponin, S100, Ca^{2+}-calmodulin dependent kinase II (for review see [Sobue et al., 1999]). Expression levels and isoform expression patterns of these proteins and additional unidentified ones contribute to the special contractile properties of a given SMC.

Smoothelin is a recently identified cytoskeletal protein which is specifically expressed in differentiated SMC [van der Loop et al., 1996]. Smoothelin is expressed as a large vascular specific and as a small visceral specific isoform [van Eys et al., 1997; Krämer et al., 1999], resulting from alternative transcriptional start

Abbreviations: α-SMA, alpha smooth muscle actin; GFP, green fluorescent protein; MHC, myosin heavy chain; SFS, spectrin family similarity; SM, smooth muscle; TnT, troponin T; VSMC, vascular smooth muscle cell

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sites [Krämer et al., 2001]. In addition, we recently identified two novel variants with different C-terminal spectrin family similarity (SFS) domains generated by alternative splicing [Krämer et al., 2001]. Smoothelin was shown to colocalize with the actin crosslinking protein filamin [Wehrens et al., 1997] and with α-SMA [Christen et al., 1999].

To study functional differences between the vascular and visceral specific smoothelin isoforms, we generated GFP fusion proteins to investigate their subcellular localization and to identify the domains responsible for association with the actin cytoskeleton. A deletion analysis showed that the SFS domain of smoothelin is neither necessary nor sufficient for binding to actin containing filaments. Instead, we mapped two novel domains that independently mediate association with actin filaments. The first domain is located in the vascular specific, N-terminal half, while the second one is located in the common C-terminal part and shares clear sequence similarity with the tail domain of troponin T (TnT). These results suggest that the cell type specific expression of smoothelin isoforms with different actin filament binding domains might in part account for the distinct contractile properties of different types of SM.

MATERIALS AND METHODS

Smoothelin Expression Constructs

For the functional characterization of smoothelin isoforms, cDNA fragments [Krämer et al., 1999, 2001] were fused in frame at the C-terminus of a red-shifted, humanized, enhanced green fluorescent protein (GFP) gene, and expressed under the control of the cytomegalovirus promoter in the backbone of the pEVRF1 vector [Matthias et al., 1989]. The constructs were created by an intermediate PCR cloning step and then subcloned with restriction enzymes into the above mentioned eukaryotic expression vector. Amino acids from smoothelin present in the fusion construct are indicated in Figure 2.

Cell Culture and Transfection

NIH 3T3 mouse fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C. The cells were transiently transfected with the various plasmid contructs using GenePorter reagent (GeneTherapy Systems). For microscopy, coverslips were placed in the culture dish before plating the cells. One or 2 days after transfection, cells were either fixed for subcellular localization studies or scraped for Western blot analysis. All fusion proteins showed the expected size (data not shown).

F-Actin Detection and Microscopy

All procedures were carried out at room temperature and all solutions were done in phosphate buffered saline (PBS), unless specified otherwise. Transfected cells grown on coverslips were removed 19 and 40 h post-transfection, washed with PBS, fixed with 3.7% formalin. Extraction of the cells before fixation was performed with 3% Triton X-100 or with 0.5% Triton X-100 in 10 mM Pipes, pH 6.8/100 mM NaCl/300 mM sucrose/3 mM $MgCl_2$ for 1 min on ice. After washing with PBS, cells were fixed with 3.7% formalin. For F-actin staining cells were permeabilized with 0.25% Triton X−100 for 10 min and blocked with 0.2% fish skin gelatine for 30 min. Cultures were then incubated for 30 min with Alexa-594 phalloidin (Molecular Probes) diluted 1:40 in PBS with 0.2% fish skin gelatine, washed, and mounted with Mowiol. Samples were screened on a Zeiss Axioplan 2 microscope and images were taken with a BioRad MRC1024 or a Zeiss LSM510 confocal systems. Images were assembled and annotated with Adobe Photoshop and Illustrator software.

RESULTS AND DISCUSSION

Smoothelin Is Localized at Actin Filaments

To investigate the subcellular localization of smoothelin isoforms, we generated fusion proteins with the GFP. The mouse cDNA of the large c2 (Lc2) and small c1 (Sc1) smoothelin isoforms [Krämer et al., 2001] were subcloned in frame downstream of the GFP sequence and the resulting constructs used to transfect mouse fibroblasts. Already 1 day after transfection, filamentous structures became visible in living cells. To further characterize the intracellular location of GFP fusion proteins, cells were fixed and stained with phalloidin that specifically labels cellular F-actin filaments. The direct comparison of the GFP signal with the phalloidin staining clearly shows that both, the short and the long, smoothelin fusion proteins are localized at cytoplasmic F-actin filaments (Fig. 1). These results are in good agreement

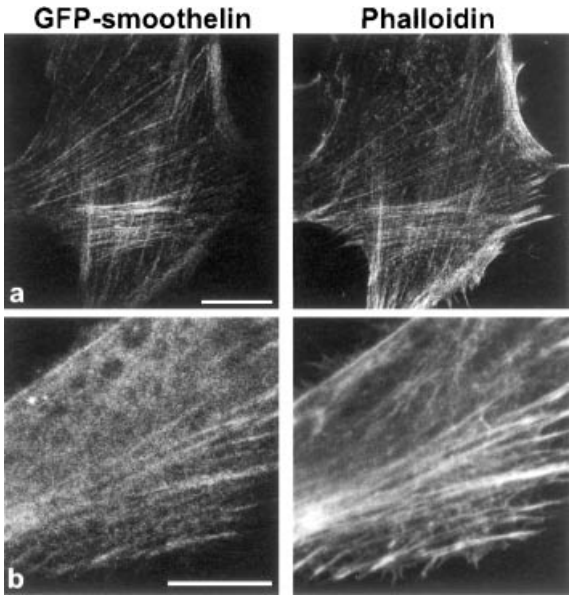


Fig. 1. Both smoothelin isoforms localize at actin containing filaments. Representative images of mouse fibroblasts, transiently transfected with full length large, Lc2 (**a**), and small, Sc1 (**b**), smoothelin isoforms. Each row shows the GFP-smoothelin signal (**left**) and corresponding phalloidin staining of actin filaments (**right**). Scale bars, 20 μm.

with the reported colocalization of smoothelin and α-SMA in primary porcine VSMC obtained by antibody staining [Christen et al., 1999].

Mapping Smoothelin Domains Involved in Binding to Actin Containing Filaments

To map the domain(s) responsible for this association with the actin filaments, we generated a set of deletions in these GFP-smoothelin fusion constructs. The structure of the constructs is outlined in Figure 2A. We could show before that smoothelin is expressed in two different splice variants (c1 and c2), resulting from alternative exon usage at the 3′ end [Krämer et al., 2001]. This splice site falls directly into the similarity region to the members of the spectrin family of actin binding proteins. Although this region results from different exons, the resulting protein isoforms have the same good similarity to the spectrin family [Krämer et al., 2001]. Therefore, we analyzed in parallel the different splicing variants and their ability to localize at the actin cytoskeleton. GFP-fusion proteins with the small isoform splice variants show the same localization at actin filaments. Expression of the SFS domain alone (c1- or c2-form) fused with the GFP showed that this domain is neither necessary nor sufficient for association with actin filaments. This holds true for both splice variants (SFSc1 and SFSc2). That is in agreement with recent crystallographic studies on the corresponding domain of utrophin, showing that this region does not play a central but rather a supporting role in actin binding [Keep et al., 1999; Moores et al., 2000].

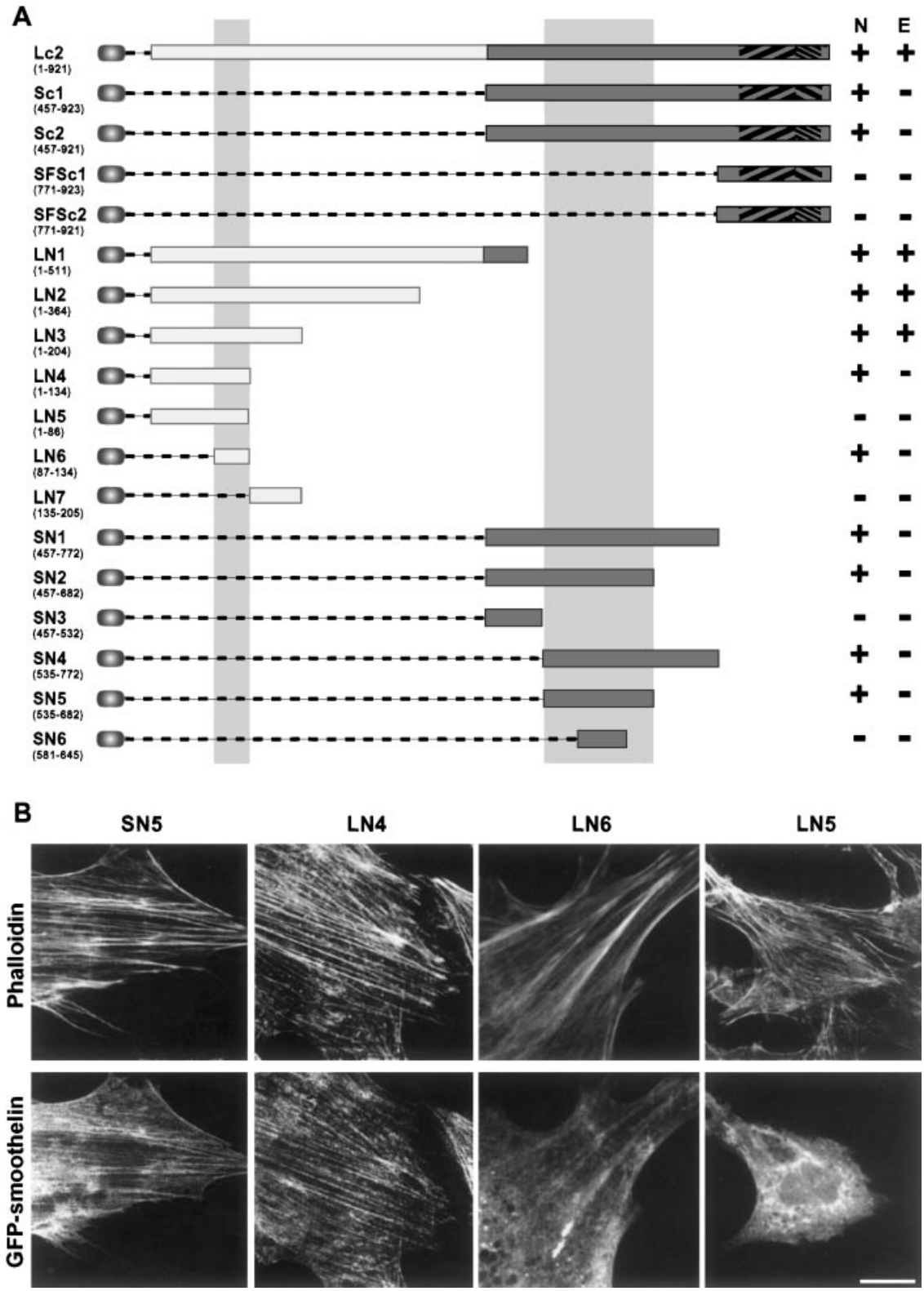
Since the C-terminus of smoothelin is not mediating the actin filament association, we dissected the N-terminus of the small smoothelin isoform. By further deletion of N-and C-terminal amino acids (constructs SN1-6), we could define a region between amino acids 535 and 682 (construct SN5), which is minimally necessary for actin filament localization (Fig. 2). Further N- and C-terminal deletions (construct SN6) resulted in a diffuse distribution of the fusion protein within the cytoplasm.

We then analyzed the unique large vascular specific N-terminus of the large isoform for additional sequences responsible for actin-filament association. The whole N-terminus alone is capable of directing the fusion protein to actin fibers. Further truncations of this large N-terminus to the first 134 amino acids still show a clear actin fiber localization. Removing the first 86 amino acids leaving only 48 amino acids between 87–134 still gives actin filament localization even though less pronounced. Representative examples of transiently transfected cells with the corresponding phalloidin stainings are shown (Fig. 2B).

In summary, we identified two separate domains that can by themselves independently mediate association with actin containing filaments. One domain is located in the large N-terminus of the vascular specific smoothelin isoform, and the second one is located in the common part, present in all smoothelin isoforms. These results show that the large vascular specific isoform contains two domains, which mediate association with actin containing filaments, whereas the small visceral specific isoform contains only one domain.

Binding of Smoothelin Deletion Mutants to Actin Filaments

To compare the strength of association of smoothelin mutants with actin filaments, we extracted transiently transfected cells with TritonX-100 before fixation and analyzed whether the GFP-smoothelin-fusion proteins remained associated with actin filaments.


A
N
E
Lc2
(1-921)
Sc1
(457-923)
Sc2
(457-921)
SFSc1
(771-923)
SFSc2
(771-921)
LN1
(1-511)
LN2
(1-364)
LN3
(1-204)
LN4
(1-134)
LN5
(1-86)
LN6
(87-134)
LN7
(135-205)
SN1
(457-772)
SN2
(457-682)
SN3
(457-532)
SN4
(535-772)
SN5
(535-682)
SN6
(581-645)
B
SN5
LN4
LN6
LN5
Phalloidin
GFP-smoothelin

Interestingly, we could see a clear difference between the two smoothelin isoforms. The large vascular specific smoothelin isoform remains associated with the actin-filaments after extraction with 3% Triton X-100, whereas the short isoform does not (data summarized in Fig. 2A). There are at least two possibilities: that the presence of two domains is necessary for the tight association with the actin filaments or that the first domain is sufficient. Extraction of transiently transfected mouse fibroblasts with the different GFP-smoothelin deletion constructs showed that the 48 amino acids of LN6 are not sufficient for the tight actin filament association. Additional surrounding sequences (construct LN3) are necessary for the tight association to actin filaments. The fact that the constructs LN5 and LN7 do not localize shows that no additional separate domain exists which can mediate the localization by themselves. The presence of this additional, stronger actin filament binding domain in the large smoothelin isoform indicates functional differences between the tissue-specific smoothelin isoforms.

Smoothelin Homology to Troponin T

The mapping of smoothelin domains responsible for association with actin containing filaments made possible a more focused database search for similar sequences. Using just the short stretch from amino acid number 535–682, which is sufficient for association with actin filaments, we found a region that is very similar to a highly conserved region in TnT [Malnic et al., 1998; Bucher et al., 1999]. The alignment of similar TnT and smoothelin sequence shows that in a central stretch of 37 amino acids, 13 are identical and 10 are conserved (Fig. 3). This stretch (termed here "TnT-box") is located in a domain of TnT that is necessary for the interaction with tropomyosin (TM) [Oliveira et al., 2000]. In addition, this TnT domain is highly conserved in vertebrates and invertebrates and is involved in the activation of actomyosin ATPase, thereby playing an important role in heart and skeletal muscle contractile functions [Tobacman, 1996]. The association of smoothelin with actin-containing filaments may thus occur through binding to a filament bound tropomyosin-like protein and may—as in the case of TnT—have a regulatory role in muscle contraction. The corresponding sequence, the TnT box, is included in all known smoothelin isoforms and is, therefore, likely to be functionally important.

Database searches for sequences similar to the N-terminal domain yielded only similarity to smoothelin itself. Interestingly, this similarity falls into the second domain responsible for actin filament association and overlaps with the TnT-box (Fig. 4). This similar region contains 37 amino acids with a central stretch of 9 identical ones.

Three widely occurring actin-binding motifs are described to date: calponin homology domain, thymosin-β4 motif, and gelsolin homology domain. A fourth actin-binding motif is the ADF homology domain (reviewed in [Lappalainen et al., 1998]). Finally, actin-binding of MLCK is mediated by three repeats containing the consensus sequence DFRXXL [Smith et al., 1999]. The two domains of smoothelin mediating the association with the actin cytoskeleton (Fig. 2) show no discernible similarity to any of these known motifs and also BLAST searches of the GenBank database did not yield other sequences with significant similarity. These results indicate that the two smoothelin domains identified here contain a novel actin cytoskeleton localization motif.

The striking similarity of a central region of smoothelin involved in actin filament association, with a conserved domain of TnT, an anchoring factor of the contractile apparatus in striated muscle [Hinkle et al., 1999], suggests that smoothelin may play a similar role in the control of smooth muscle contraction. The tissue specific expression of smoothelin isoforms with

Fig. 2. Mapping of smoothelin domains involved in actin filament association. **A**: The structure of the different fusion constructs containing mouse smoothelin fragments fused in frame to the C-terminus of GFP is illustrated. Smoothelin amino acids included in each fusion construct are indicated below the name. The GFP at the N-terminus is shown as a cylinder, the unique large N-terminus is in light gray, the common C-terminus in dark gray, and the SFS-domains are hatched whereby alternative splice variants (C1 and C2) are represented with different patterns. The association of fusion proteins with actin filaments (+) or the lack thereof (–) was determined in transfected cells after extraction with 3% Triton 100 (E) and in nonextracted (N) cells. The two independent domains sufficient for association with actin filaments are highlighted with vertical shaded bars. **B**: Representative images of NIH-3T3 cells transiently expressing the GFP-Smoothelin fusion constructs indicated. The lower row shows the GFP-smoothelin signal and the upper row the corresponding phalloidin staining of actin filaments. Scale bar, 20 μm.

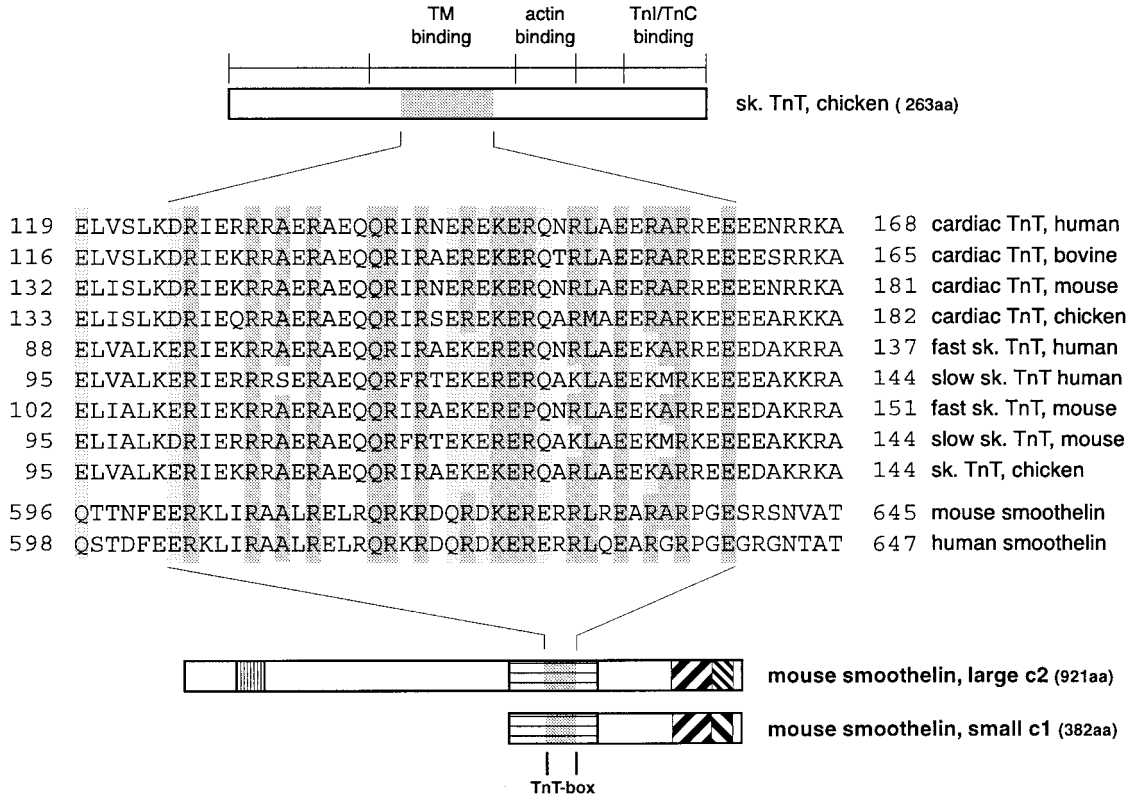


Fig. 3. Alignment of smoothelin with different vertebrate troponin T isoforms. The chicken skeletal TnT structure is schematically outlined at the top and the conserved region within the TM binding domain is indicated in gray. This region shows similarity to the C-terminal, common part of the large and short smoothelin isoforms, and falls into the domain mediating actin filament association as outlined at the bottom. The middle part shows the sequence alignment with different TnT isoforms where amino acids identical to smoothelin are highlighted in dark gray and similar amino acids in light gray. The sequences in this alignment were identified with BLAST database searches. GenBank accession numbers are: 4507627 for human cardiac TnT; P13789 for bovine cardiac TnT; P50752 for mouse cardiac TnT; P02642 for chicken cardiac TnT; AAF21629 for human fast skeletal TnT; P13805 for human slow skeletal TnT; AAF01502 for mouse fast skeletal TnT; AAC32546 for mouse slow skeletal TnT; AAA49102 for chicken skeletal TnT; AF064236 for mouse; and AF064238 for human smoothelin. The human smoothelin sequence in this region has been resequenced (GenBank AF064238).

different actin filament association domains may thus contribute to the different contractile properties of SMCs. The identification and mapping of these isoform-specific actin cytoskeleton localization sequences now allows a focused search for interacting factors. The presence of two independent binding domains suggests a regulatory role in the stabilization and/or crosslinking of components of actin containing filaments.

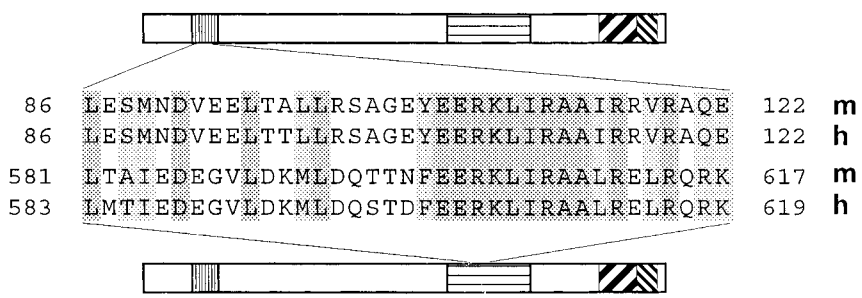


Fig. 4. Alignment of the two smoothelin domains responsible for actin filament association. The alignment of both actin filament binding domains of the large smoothelin isoform is shown with identical amino acids highlighted in dark gray and similar ones in light gray. The accession numbers of mouse (m) and human (h) smoothelin sequences are as in Figure 3.

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REFERENCES

Arner A, Malmqvist U. 1998. Cross-bridge cycling in smooth muscle: a short review. Acta Physiol Scand 164: 363–372.

Bucher EA, Dhoot GK, Emerson MM, Ober M, Emerson CP, Jr. 1999. Structure and evolution of the alternatively spliced fast troponin T isoform gene. J Biol Chem 274: 17661–17670.

Christen T, Bochaton-Piallat ML, Neuville P, Rensen S, Redard M, van Eys G, Gabbiani G. 1999. Cultured porcine coronary artery smooth muscle cells. A new model with advanced differentiation. Circ Res 85:99–107.

Czurylo EA, Kulikova N, Dbrowska R. 1997. Does calponin interact with caldesmon? J Biol Chem 272:32067–32070.

Foster DB, Shen LH, Kelly J, Thibault P, Van Eyk JE, Mak AS. 2000. Phosphorylation of caldesmon by p21-activated kinase. Implications for the Ca(2+) sensitivity of smooth muscle contraction. J Biol Chem 275:1959–1965.

Geeves R. 1999. Structural mechanism of muscle contraction. Annu Rev Biochem 68:687–728.

Hinkle A, Goranson A, Butters CA, Tobacman LS. 1999. Roles for the troponin tail domain in thin filament assembly and regulation. A deletional study of cardiac troponin T. J Biol Chem 274:7157–7164.

Keep NH, Norwood FL, Moores CA, Winder SJ, Kendrick-Jones J. 1999. The 2.0 A structure of the second calponin homology domain from the actin-binding region of the dystrophin homologue utrophin. J Mol Biol 285:1257–1264.

Krämer J, Aguirre-Arteta AM, Thiel C, Gross CM, Dietz R, Cardoso MC, Leonhardt H. 1999. A novel isoform of the smooth muscle cell differentiation marker smoothelin. J Mol Med 77:294–298.

Krämer J, Quensel C, Meding J, Cardoso MC, Leonhardt H. 2001. Identification and characterization of novel smoothelin isoforms in vascular smooth muscle. J Vasc Res 38:120–132.

Kuriyama H, Kitamura K, Itoh T, Inoue R. 1998. Physiological features of visceral smooth muscle cells, with special reference to receptors and ion channels. Physiol Rev 78:811–920.

Lappalainen P, Kessels MM, Cope MJ, Drubin DG. 1998. The ADF homology (ADF-H) domain: a highly exploited actin-binding module. Mol Biol Cell 9:1951–1959.

Malnic B, Farah CS, Reinach FC. 1998. Regulatory properties of the NH2- and COOH-terminal domains of troponin T. ATPase activation and binding to troponin I and troponin C. J Biol Chem 273:10594–10601.

Marston S, Burton D, Copeland O, Frase I, Gao Y, Hodgkinson J, Huber P, Levine B, el-Mezgueldi M, Notarianni G. 1998. Structural interactions between actin, tropomyosin, caldesmon and calcium binding protein and the regulation of smooth muscle thin filaments. Acta Physiol Scand 164:401–414.

Marston SB, Fraser ID, Huber PA. 1994. Smooth muscle caldesmon controls the strong binding interaction between actin-tropomyosin and myosin. J Biol Chem 269:32104–32109.

Matthias P, Müller MM, Schreiber E, Rusconi S, Schaffner W. 1989. Eukaryotic expression vectors for the analysis of mutant proteins. Nucleic Acids Res 17:6418.

Moores CA, Keep NH, Kendrick-Jones J. 2000. Structure of the utrophin actin-binding domain bound to F-actin reveals binding by an induced fit mechanism. J Mol Biol 297:465–480.

Oliveira DM, Nakaie CR, Sousa AD, Farah CS, Reinach FC. 2000. Mapping the domain of troponin T responsible for the activation of actomyosin ATPase activity. Identification of residues involved in binding to actin. J Biol Chem 275:27513–27519.

Polyakov AA, Huber PA, Marston SB, Gusev NB. 1998. Interaction of isoforms of S100 protein with smooth muscle caldesmon. FEBS Lett 422:235–239.

Smith L, Su X, Lin P, Zhi G, Stull JT. 1999. Identification of a novel actin binding motif in smooth muscle myosin light chain kinase. J Biol Chem 274:29433–29438.

Sobue K, Hayashi K, Nishida W. 1999. Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation [in process citation]. Mol Cell Biochem 190:105–118.

Tobacman LS. 1996. Thin filament-mediated regulation of cardiac contraction. Annu Rev Physiol 58:447–481.

van der Loop FT, Schaart G, Timmer ED, Ramaekers FC, van Eys GJ. 1996. Smoothelin, a novel cytoskeletal protein specific for smooth muscle cell. J Cell Biol 134: 401–411.

van Eys GJ, Voller MC, Timmer ED, Wehrens XH, Small JV, Schalken JA, Ramaekers FC, van der Loop FT. 1997. Smoothelin expression characteristics: development of a smooth muscle cell in vitro system and identification of a vascular variant. Cell Struct Funct 22:65–72.

Wehrens XH, Mies B, Gimona M, Ramaekers FC, Van Eys GJ, Small JV. 1997. Localization of smoothelin in avian smooth muscle and identification of a vascular specific isoform. FEBS Lett 405:315–320.

Winder SJ, Walsh MP. 1996. Calponin. Curr Top Cell Regul 34:33–61.