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A novel isoform of the smooth muscle cell differentiation marker smoothelin

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Abstract Studies on smooth muscle cell differentiation and those on vascular development in mouse and humans have long been hampered by the lack of suitable markers. Here we describe a novel, large isoform of smoothelin, a structural protein of differentiated, contractile smooth muscle cells. The protein, which is highly conserved in mouse and humans, shows homology with other cytoskeleton-associated smooth muscle cell proteins and contains an actinin-type actin-binding domain. Northern blot analysis from various mouse organs identified short and long smoothelin mRNA forms, which exhibit distinct tissue expression patterns. The short form is highly expressed in visceral muscle tissues such as intestine and stomach and is not detectable in brain, while the long mRNA form is expressed in all vascularized organs. These results may provide new tools and approaches to study both smooth muscle cell differentiation and proliferative vascular disease.

Key words Smooth muscle cell differentiation · Proliferative vascular disease · Actinin-type actin-binding domain · Smooth muscle cell marker · Cytoskeleton-associated protein

Abbreviations *PCR* Polymerase chain reaction · *RACE* Rapid amplification of cDNA ends · *SDS* Sodium dodecyl sulfate · *VSMC* Vascular smooth muscle cell

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Introduction

Vascular smooth muscle cells (VSMCs) show a variety of differentiation states, with the two most distinctive ones being the contractile (differentiated) and the synthetic (proliferative) phenotype [1, 2]. Several proteins have been suggested as specific markers for mammalian SMCs, such as α -smooth muscle actin, smooth muscle myosin heavy chain isoforms 1 and 2, calponin, metavinculin, and SM22- α . However, most of these genes are also expressed in other tissues during development and disease [3–7]. Additional differentiation markers for VSMC are needed to

dissect regulatory mechanisms in SMCs during development and vascular disease.

Recently a cytoskeleton-associated protein of 59 kDa has been identified in various species and named smoothelin, as it is expressed specifically in differentiated SMC [8, 9]. Smoothelin is encoded by a single copy gene on human chromosome 22q12 [10]. Its function and regulation in various adult organs and in embryonic development are still unknown. Western blot analysis using an anti-smoothelin monoclonal antibody identified a cross-reacting VSMC-specific protein with an apparent molecular weight of about 95 kDa [11, 12]. Here we describe the cloning and characterization of a novel isoform of smoothelin in mouse and human with a calculated molecular weight of approximately 100 kDa. The protein shows homology with an actinin-type actin-binding domain at its C-terminus and is highly conserved in mouse and human. The two smoothelin isoforms are distinct with respect to patterns of tissue-specific expression.

Materials and methods

Cloning of mouse smoothelin

A part of the presumptive mouse smoothelin cDNA sequence was deduced from overlapping expressed sequence tags (GenBank) with homology to the known parts of the human smoothelin cDNA (EMBL accession number Z49989). Various primer pairs were designed to screen mouse cDNA pools [Marathon-Ready cDNA: mouse heart and 7-day embryo (Clontech Laboratories, Palo Alto, Calif.) and oligo dT-primed reverse transcribed RNA from mouse intestine, heart and 13-day embryo].

Rapid amplification of cDNA ends (RACE) was performed with sequence information of new smoothelin parts to obtain novel 5' and 3' sequences. For cDNA amplifications a polymerase mix consisting of KlenTaq and a proofreading polymerase (Clontech), was used, following the manufacturer's instructions for polymerase chain reaction (PCR) and cycling conditions with respect to the primers' melting and annealing temperatures. For final full-length cDNA amplification we used 5'-ACGTTGCTGAACCGGCTGGGCTCT-3' for the large and 5'-AGGGGGCAGTATGAAGACTAC-3' for the small isoform as forward (upstream) primers and for both isoforms 5'-GTCAAAACACCTCTCCCCTTT-3' and 5'-CACTCTGCTCACACCGCCTGCGCTGCG-3' as reverse (downstream) primers. PCR and RACE products were cloned directly into the PCR 2.1-TOPO vector (Invitrogen, Carlsbad, Calif.) according to the manufacturer's protocol and checked by enzymatic digestion and internal primer pair PCR. Positive clones were double-strand sequenced with the dye terminator method on an automated DNA sequencer (Applied Biosystems, Foster City, Calif.).

For sequence assembling, editing, and alignments we used the Lasergene software programs EditSeq, SeqMan, and MegAlign (DNASTAR, Madison, Wis.). Databank searches for nucleotide and protein homologies were performed with BLAST, PROFILE, and PROSITE [13].

Cloning of human smoothelin

Primer design for human smoothelin cDNA was based on the published form of smoothelin [8]; 5' and 3' RACE and full-length amplification (upstream primer: 5'-AGAATCCAGGGGACGGTTGCTGA-3', downstream primer: 5'-CCCACATACACACGCAGCGTTTTGAT-3') were performed with human fetus Marathon-Ready cDNA (Clontech). The cDNAs were subcloned, checked, and sequenced as described above.

RNA isolation and northern blot analysis

Total RNA was prepared from various organs from the C57BL6 strain mouse (postnatal day 18), and from the head and neck portion of a 13-day mouse embryo. RNA was isolated by adsorption to a silica gel based membrane in a high-salt buffer system according to the manufacturer's instruction (Qiagen, Hilden, Germany). Total RNA was denatured [in 50% formamide, 2.2 M formaldehyde, 0.1 M (*N*-2-morpholino)propane sulfonic acid pH 7, 40 mM sodium acetate, 5 mM EDTA pH 8] and separated in a 1.2% agarose-formaldehyde gel. The RNA was then transferred to a positively charged nylon membrane (Gene Screen Plus, Du Pont NEN) with a semidry electroblotting unit and immobilized by UV crosslinking. Prehybridization was carried out in 1% bovine serum albumin, 1 mM EDTA, 0.25 M NaHPO₄ pH 7.2, 7% sodium dodecyl sulfate (SDS) for 1 h at 65°C [14]. Overnight hybridization was carried out in the same buffer except for the addition of probe and 50 µg/ml herring sperm DNA, which were denatured simultaneously by boiling for 5 min. One fragment of the mouse smoothelin (digested with *Sac*II and *Bbs*I) was generated as probe, covering 241 bp of the C-terminal region from nucleotide position 2648–2889. The DNA probe was gel-purified and then labeled with [α -³²P]dCTP using a multiprime DNA labeling system (Amersham, Buckinghamshire, UK). The washes were performed as follows: twice with 2×SSC+0.1% SDS for 30 min at room temperature, once with 1×SSC+0.1% SDS for 15 min at 59°C, followed by a wash at 65°C with 1×SSC+0.1% SDS for 15 min and a final wash at 65°C with 0.5×SSC + 0.1% SDS for 20 min. The blot was stripped by boiling in 0.5% SDS and reprobed with rat GAPDH as described above.

Results

Identification of novel smoothelin isoforms

In order to identify differentiation markers for VSMCs we investigated the origin of the vascular 95-kDa protein detected with the anti-smoothelin antibody R4A [11, 12]. We screened various mouse and human cDNA pools for longer smoothelin with PCR techniques. Primers were designed based on sequence information from mouse expressed sequence tags, which showed homology to the human smoothelin cDNA. In addition, we performed 3'-RACE reactions. However, we failed to isolate alternatively spliced isoforms encoding larger open reading frames that could account for the 95-kDa protein.

To evaluate the possibility of an upstream transcriptional start we performed 5'-RACE reactions using cDNA pools from various tissues. RACE products of up to 1.5 kb were obtained, indicating a novel upstream transcriptional start in mouse and human. Cloning and subsequent sequencing of these RACE products revealed an extended, upstream open reading frame. This novel open reading frame encodes a protein with a calculated molecular weight of 100.4 kDa and can thus account for the observed VSMC-specific protein with an apparent molecular weight of about 95 kDa. Figure 1 presents the complete mouse cDNA sequence and the protein translation of the large isoform. The C-terminal domain contains a region with homology to the actinin-type actin-binding domain (see also Fig. 3). The complete cDNA sequences of the mouse and human large isoform have been deposited (EMBL accession numbers AJ 010305 and AJ 010306).

Fig. 1 Complete cDNA and protein sequence of the large mouse smoothelin isoform. The complete mouse smoothelin cDNA sequence with protein translation of the large isoform is shown. *Underlined* In-frame start and stop codons. The open reading frame encodes a protein with a calculated molecular weight of 100.4 kDa. The upstream sequence contains an in-frame stop codon (TGA; *asterisk*). *Dotted lines* ATG at codon 543 that corresponds to the previously described start of human smoothelin [8]; *dashed lines* sequence homology to the actinin-type actin-binding domain. *Boxes 1–3* Three short stretches of amino acids with some homology to calponin h1 and h2; *box 1* a sequence similar to a part of the calponin homology domain of calponin h2; *boxes 2, 3* sequences with homology to the repeat motifs 2 and 3 of calponin h1. *Double underlined* Four possible N-glycosylation sites

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1  AC GTTGC TGA ACC GCG C TGG G C T C C T G G G C T G C G G C T C C T C C C G G T C G G T C T G C C G G T C T A C T G C A T C T A C C G G G T C C C G A C C A A A C T A A C A C G A A A C T 100
101 GCGCCGGAACGCGCGGCCGACTGAGAGAAATTCAGTGCCACAGGACCTGGCGGGAGCTGACATCTAGGAATATAGGACCAGCAAG ATG GCA 197
1 GAC GAG GCT TTA GCT GGG CTG GAT GAA GGA GCC CTC CGA AAA CTG CTG GAG GTC ACT GCA GAT CTG GCA GAA CGG 2
2 D E A L A G L D E G A L R K L L E V T A D L A E A R 27
3 CGG CGG ATC CGC TCA GCC ATC CGG GAG CTG CAG CGA CAG GAG CTG GAA CGA GAA GAG GAG GCT CTG GCG TCC AAA 347
28 R R I R S A I R E L Q R Q E L E R E E E A L A S K 52
348 CGT TTC CGT GCT GAG CGG CAA GAC AAC AAG GAG AAC TGG CTA CAC TCT CAA CAG CGA GAA GCT GAA CAG CAG GCC 422
53 R F R A E R Q D N K E N W L H S Q Q R E A E Q Q A 77
423 GCC CTC GCA CGA TTG GCA GGG AGA CTG GAG TCC ATG AAT GAT GTC GAG GAG TTG ACC ACA CTG CTT CGG AGT GCC 497
78 A L A R L A G R L E S M N D V E E L T T L R L R S A 102
498 GGT GAA TAT GAG GAG CGC AAG CTG ATC AGA GCT GCC ATC CGC CGA GTG CGA GCT CAG GAG ATT AAG GCT GCC ACC 572
103 G E Y E E R K L I R A A I R R V R A Q E E I K A A T 127
573 TTG GCT GGG AGA CTG TGC AGT AGA CTT CCC AGT AGT GGC CCC CGA GAG GAC AGC AGG AGG CAG GCA GCA CAC ACA 647
128 L A G R L C S R L L P S S G P R E D S R R Q A A H T 152
648 TTG GAC CCT GGT AAG GTG CCA GAG CCA GAG CAG CAG GAA CAG CAG ACA GAA GTA CTC GAG TCA ACC CCA ACC CCA 722
153 L D P G K V P E Q P E Q Q T E Q Q T E V L E S T P T P 177
723 GAG GAC ACC AGC CAG GAT GTG ACC ACA GTG ACA CTC CTG CTG AGG GCC CCG CTT GGG GGC AGG CCG AGC TCA CCT 797
178 E D T S Q D V T T V T L L L R A P P G G R P C S S F 202
798 GCT TCC CCC CAC AAT TCA CCC ACC AGT GCC TCT CCA GAG CCT CTG CTG GAG CCT GCT GGA GCC CAG TGT CCT GCT 872
203 A S P H N S P T S A S P E L L E P A G A C Q C P A 227
873 GTG GAG GCT CCA GTC AGC TCT GAG CCA CTT CCA CAC CCT TCA GAA GCT CCT AGC CCT GAG CCC CCC ATG TCG CCG 947
228 V E A P V S S E P L P H P S E A P S P E P M S P 252
948 GTA CCG TCC AGC TCT CGG GCG CGG GTC ATC AGC AAG CCC CTG CCT GGC CCC ACA GAG CCC TCA GAT ACC TTG GAC 1022
253 V P S S S R G R V I S K P L P G P T E P S D T L D 277
1023 TCC ATC AGA GGC TTC TCC AAC ACT AAG AGA GCA GAC CCG TCT GAA ACG AAA TCC TGC CAA CGT TCA CTA TCT GTG 1097
278 S I R G F S N T K R A D P S E T K S C Q R S L S V 302
1098 CTC AGT CCC CGA CAA CCA ACC CCA AAT CGA GAG CCA ACC TCA CTT GCA GGA CCG TCC CAG TTC CGT GCA GTT GGC 1172
303 L S P R Q P T P N R E P T S L A G P S Q F R R V G 327
1173 TCT GTG AGA GAC AGA GTC CAA AAG TTC ACA TCT GAT TCT CCC GTG GTT GCC AGC CTC GAG GAT GGC CCA CCC CGA 1247
328 S V R D R V Q K F T S D S P V V A R L Q D G P P R 352
1248 ACA GCC CTT GCT TCA CCG ACC KCC ACA AGG CTC CCG GSC CCT TCC CTC ATC AGC ACC ACC CCT GCC TCC TCC TCC 1322
353 T A L A S P T P T R L P G P S L I S T T P A A S S 377
1323 TCC AGC AAC TCC TCC TCT CCG AGT CCC AGT GAC ACT TCC TCC CAC AAG AAG CAG AGA GAA CTT GCT CAT TCC TCT 1397
378 S S N S S S P S P S D T S S H K K Q Q R E L A H S L 402
1398 GCC GAG CTT CAG AGC TGC CCT CAA GAG GAG GGC CCT GGG GGG CGG GGC TTG GCT CTC AGG TCC CTT GAA AAC AGA 1472
403 A E L Q S C P Q 2 E E G P G R G L A L R S E N R 427
1473 GCA GGG GGG CCC AAG CCC TGC TCA GAA GAG CCC AGT ACC CCA CCG CCC GTG GCC GTT GGC ACT GGG GAG CCA GGG 1547
428 A G G P K P C S E E P S T P P P V A V G T G E F G 452
1548 GGC AGT ATG AAG ACT ACG TTT ACC ATT GAG ATC AAG GAT GGC CGT GGT GAG GCC TCA GGC ACC ACA GGC CGG GTG CTG CTG 1622
453 G S M K T T F T I E I K D G R G Q A S T G R V L L 477
1623 CCC ACA GGC AAC CAG AGA GCA GAA TTG ACT TTG GGA TTG CGG GCA CCC CCA ACC CTT CTC AGC ACC AGG AGT GGG 1697
478 P T G N Q R A E L T L G L R A P P T L L S S C S G 502
1698 GGC AAG AAC ACC ATC ACC CAC ATC AGC AAC CCT GGG ACT GTG ACC CGA CTG GGC AGT GTC ACT CAC GTC ACT ACC 1772
503 Q K N T I T H I S N P G T V T R L G S V T H V T T 527
1773 TTC AGC CAT GCC TCC CCT GGT AAC CGA GGA GGC TGC AAC TTT AAG ATG GAG CCA GAT CCT CCA GAG CCC CCC TCC 1847
528 F S H A S P G N R G G C N F M E P D P A E P P S 552
1848 ACC ACA GTG GAA GCA GCT AAT GGC GCA GAG CAG GCT CGA GTG GAC AAA GGC CCA GAG GGG CGG AGT CCC CTG AGT 1922
553 T T V E A A N G A E Q A R P V D K G P E G R S P L S 577
1923 GCA GAG GAG CTG ACG GCC ATT GAG GAC GAA GGA GTG CTG GAC AAG ATG CTG GAC CAG ACT ACG AAT TTT GAG GAG 1997
578 A E E L T A I E G D E G V L D K M L L D Q T T N F E E 602
1998 AGG AAG CTC ATC CGG GCT GCA CTG CGT GAG CTC CGA CAA AGA AAG AGA GAC CAG AGG GAC AAG GAA CGA GAA CGG 2072
603 R K L I R A A L R E L R Q R K R D Q Q R D K E R E R 627
2073 AGG CTA CGG GAG GCA CGG GCC CGG CCG GGC GAG AGC CGA AGC AAT GTG GGC CCG GAG ACC ACC ACC AGG CAC AGC 2147
628 R L R E A R A R P G E S R S N V A T E T T T R H S 652
2148 CAG CGG GCG GCT GAT GGC TCT ACT GTC GGC ACA GTT ACC AAA ACC GAG CGC CTC GPT CAC TCC AAT GAT GGC ACT 2222
653 Q R A A D G S T V G T V T K T E R L V H S N D G T 677
2223 CAG ACG GCC CGC ACC ACC ACA GTG GAG TCC AGT TTC ATG AGG CGC TTG GAG AAT GGC AGC AGC AGC ACC AGC ACC 2297
678 Q T A R T T T V E S S F M R R L E N G S S S S S T 702
2298 ACC ACC ACC ACG GTC CAA ACC AAG AAT TTT TCC TCT TCC TCT TCC TCG TCC TCG TCC AAA AAG ATG GGC AGT ATC 2372
703 T T T V Q T K N F S S S S S S S S K K M S I 727
2373 TTC GAC CGA GAG GAC CAG ACC AGC TCA CGT CCT GGC AGC CTG GCA GCC CTT GAA AGA CGC CAG GCA GAG AAG AAG 2447
728 F D R E D Q T S S R P G S L A A L E R R Q A E K K 752
2448 AAA GAG TTA ATG AAG GCA CAG AGT CTG CCC AAG ACT TCA GCA TCC CAA GCA CGC AAG GCC ATG ATT GAG AAG CTA 2522
753 K E L M K A Q S L L P K T S A S Q A R K A M I E K L 777
2523 GAG AAA GAA GGC TCT GCA GGT GGT CCT GGC ACA CCC CGT ACA GCT GTA CAG CGT TCT ACC AGC TTC GGA GTC CCC 2597
778 E K E G S A G G P G T P R T A V Q R S T S F G V P 802
2598 AAC CCC AAT AGC ATC AAG CAG ATG TTG CTG GAC TGG TGC CGA GCC AAG ACC CGC GGC TAC GAG CAC GTG GAC ATC 2672
803 N P N S I K Q M L L D W C R A K T R G Y E H V D I 827
2673 CAG AAC TTC TCC TCC AGC TGG AGT GAT AGG ATG GCC TTC TGT GCC CTG GTG CAC AAT TTC TTC CCT GAG GCT TTT 2747
828 D N F S S S S W S D R M A F C A L V H N F F P A E F 852
2748 CAC TAT GGG CAG CTG AGC CCA CAA AAC CGA CAG AAC TTT GAA ATG GCC TTC TCA TCC GCT GAG ATG CTG GTG 2822
853 D Y 3 G Q L S P Q N R R Q N F E M A F S S A E M L V 877
2823 CAC TGT GTA CCC TTG GTG GAG GTG GAG GAC ATG ATG ATC ATG GGC AAA AAG CCC GAC CCC AAG TGC GTC TTC ACC 2897
878 D C V P L V E V E D M M I M G K K P D P K C V F T 902
2898 CAC GTG CAA TCG CTG TAC AAC CAC CTG CGG CGC CAT GAG CTG CGT TTG CGC GGC AAG AAT GTC TAG CCACTCTGCTC 2974
903 V V Q S L Y N H L R R H E L R L R L R G K N V 923
2975 ACAACCGCTGCGTGGGGCTGTGCCACGCCCCAGCACCGGCACCTCCAGTGCCTGCCACTGCTGCCCGTCTGTCAAAACACCTCTCCC 3074
3075 CTTTGTACACACGACGCGTTTTGATAAATTTTGGTTTTTCACGAAAAAAAAAAAAAAAAAAAAAAAAA 3142

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Structure and tissue-specific expression of smoothelin isoforms

The large smoothelin isoform is highly conserved in mouse and humans. Protein alignment shows the conservation of this gene between the two species, with 77.4% of the amino acids being identical, 15.4% similar, and only 7.2% showing no homology (Fig. 2). Interestingly, the

novel N-terminus and the actinin-type actin-binding domain at the C-terminus show the highest conservation and are nearly identical. This actinin-type actin-binding domain is conserved in the spectrin family of mouse and human cytoskeletal proteins (Fig. 3). In addition to this common C-terminal domain, the large smoothelin isoform has 542 additional amino acids at the N-terminus. Computer analyses on this N-terminal domain revealed two short re-

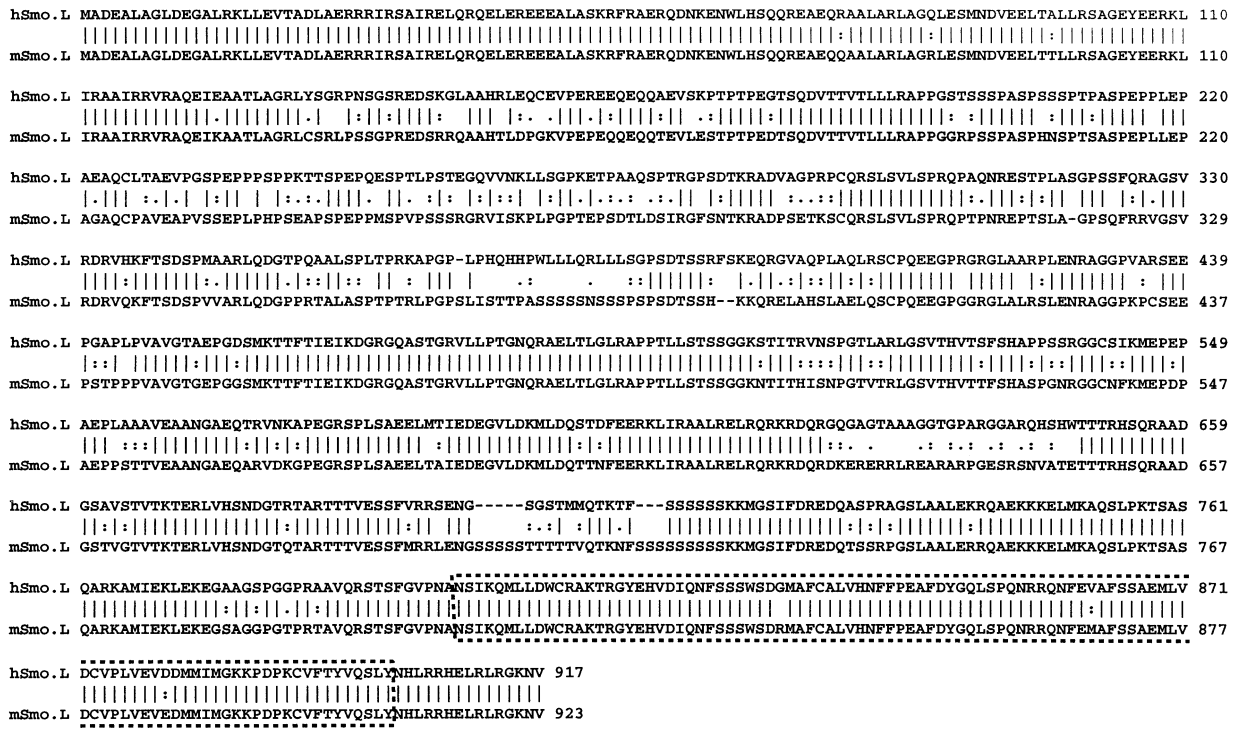


Fig. 2 Alignment of human and mouse smoothelin isoforms. The protein alignment of the large human (*hSmo.L*) and mouse (*mSmo.L*) smoothelin isoform was performed with the MegAlign software program (DNASTAR). Vertical line Amino acid identities; one, two dots conserved amino acids, depending on the degree of similarity; boxed with dashed lines homology to the actinin-type actin-binding domain

gions with homology to calponin (Fig. 1), a thin filament-associated smooth muscle protein with contraction regulatory properties [15, 16]. A third calponin homology domain is located in the common C-terminal domain.

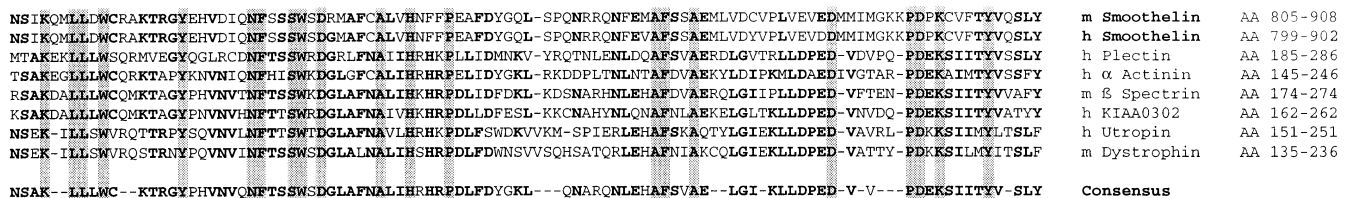
To test whether these rather different smoothelin isoforms show the same tissue-specific distribution, northern blot analyses were carried out with mouse RNA isolated from 13-day embryo and various organs from adult ani-

mals. Smoothelin mRNA is already detectable even in 13-day embryos and can be amplified by reverse transcriptase PCR from mouse embryos as early as day 7 (data not shown). The different smoothelin isoforms were detected with a C-terminal probe that is present in the large and small isoform. The two forms are distributed differentially in organs of the adult animal. The small isoform is highly expressed in visceral organs such as intestine or stomach and is not detectable in brain, while the large isoform is expressed in vascularized organs (Fig. 4). Nonintestinal organs without contractile vessels such as liver seem to express far less smoothelin (data not shown).

Discussion

Smooth muscle cell differentiation and dedifferentiation are important features in the development of the vascular tree and in proliferative vascular diseases, such as atherosclerosis or restenosis after balloon angioplasty [17–20]. One specific marker to follow these complex processes seems to be smoothelin, a novel 59-kDa cytoskeletal protein specifically expressed in differentiated SMCs [8]. A monoclonal antibody against smoothelin also recognized a 95-kDa cytoskeletal protein that is expressed specifically in VSMCs [11]. The origin of the 95-kDa protein was dif-

Fig. 3 C-terminal protein sequences of mouse smoothelin isoforms compared to members of the spectrin family. The C-termini of mouse and human smoothelin were aligned with the actinin-type actin-binding domain found in the spectrin protein family. Gray background bars Amino acids conserved in all aligned sequences; bold-face amino acids conserved in at least half of the sequences aligned. Searches in this alignment were identified with BLAST database searches and downloaded from GenBank (accession numbers are: X15804 for α -actinin, U53204 for plectin, S66283 for β -spectrin, AB002300 for KIAA0302, X69086 for utrophin, and M68859 for dystrophin). *m, h* The origin from mouse and human, respectively



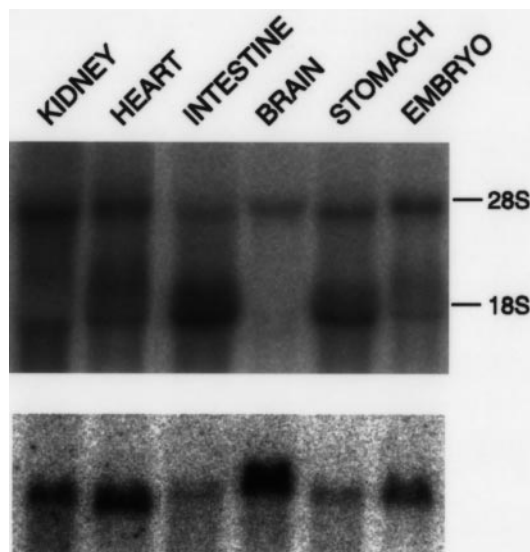


Fig. 4 Tissue-specific distribution of the long and short smoothelin transcripts. Northern blot analysis of mRNA from various mouse organs and 13-day mouse embryo. A PCR-amplified part of the mouse smoothelin cDNA (nucleotides 2648–2889, according to Fig. 1) was used as a probe. *Below* The same blot reprobed with GAPDH. Various amounts of total RNA were loaded to show the two isoforms in organs with varying abundance of the smoothelin mRNA (for comparison see signal intensity of the GAPDH reference, *below*)

difficult to explain as the 5'-end of the smoothelin human cDNA seemed to contain in-frame stop codons and longer transcripts were not described [8].

In this work we describe the cloning of a large smoothelin isoform with a calculated molecular weight of 100.4 kDa that is conserved in mouse and humans and can thus account for the 95-kDa protein recognized by monoclonal antibodies against smoothelin. Northern blot analyses showed that both short and long smoothelin transcripts are expressed *in vivo* and show different tissue-specific expression patterns.

In this work we analyzed many different 5'-RACE and internal PCR products but failed to identify a short form that could have been derived from the long smoothelin transcript by alternative splicing. These results suggest that the smoothelin gene contains at least two functional promoters that are differentially regulated. The comparison of these promoters with the smooth muscle myosin heavy-chain promoter should help to elucidate regulatory mechanisms controlling the differentiation of SMCs during development and disease.

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