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Jochen Krämer · Ana M. Aguirre-Arteta
Corinna Thiel · C. Michael Gross · Rainer Dietz
M. Cristina Cardoso · Heinrich Leonhardt

A novel isoform of the smooth muscle cell differentiation marker smoothelin

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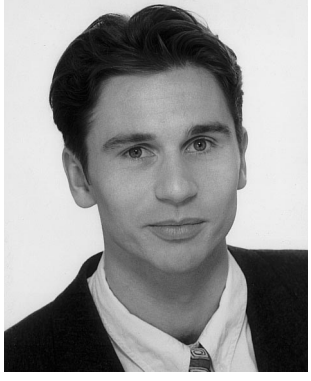
HEINRICH LEONHARDT studied biochemistry at the Free University of Berlin and earned his Ph.D. at the Max Planck Institute for Molecular Genetics in Berlin, Germany. He carried out his postdoctoral training at Harvard Medical School, Boston, USA, and now heads a research group at the Max Delbrück Center for Molecular Medicine in Berlin. His main research projects include differentiation and cell cycle regulation in muscle cells and the role and regulation of DNA methylation.



JOCHEN KRÄMER studied medicine at the University of Hamburg, Germany, and at the National Heart, Lung and Blood Institute, NIH, USA. He is currently working at the Max Delbrück Center in the "Cell Biology of Cardiovascular Disease" research group, part of a clinical trainee program for medical physicians (KAP) between MDC and the Franz Volhard Clinic, Berlin-Buch. His research interests include smooth muscle cell differentiation and prevention of restenosis after interventional therapy in cardiology.

J. Krämer · A.M. Aguirre-Arteta · C. Thiel · M.C. Cardoso · H. Leonhardt (✉)
Max Delbrück Center for Molecular Medicine,
Robert-Rössle-Strasse 10, D-13092 Berlin, Germany
e-mail: leonhardt@fvk-berlin.de,
Tel.: +49-30-94172341, Fax: +49-30-94172336

J. Krämer · C.M. Gross · R. Dietz
Franz Volhard Clinic, Max Delbrück Center for Molecular Medicine,
Humboldt University, Wiltbergstrasse 50, D-13125 Berlin, Germany

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

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'-ACGTTGCTGAACCGGCCTGGGCTCT-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'-CACTCTGCTCACAC-CGCCTGCGCTGCG-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 $NaHPO_4$ 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-^{32}]PdCTP 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).

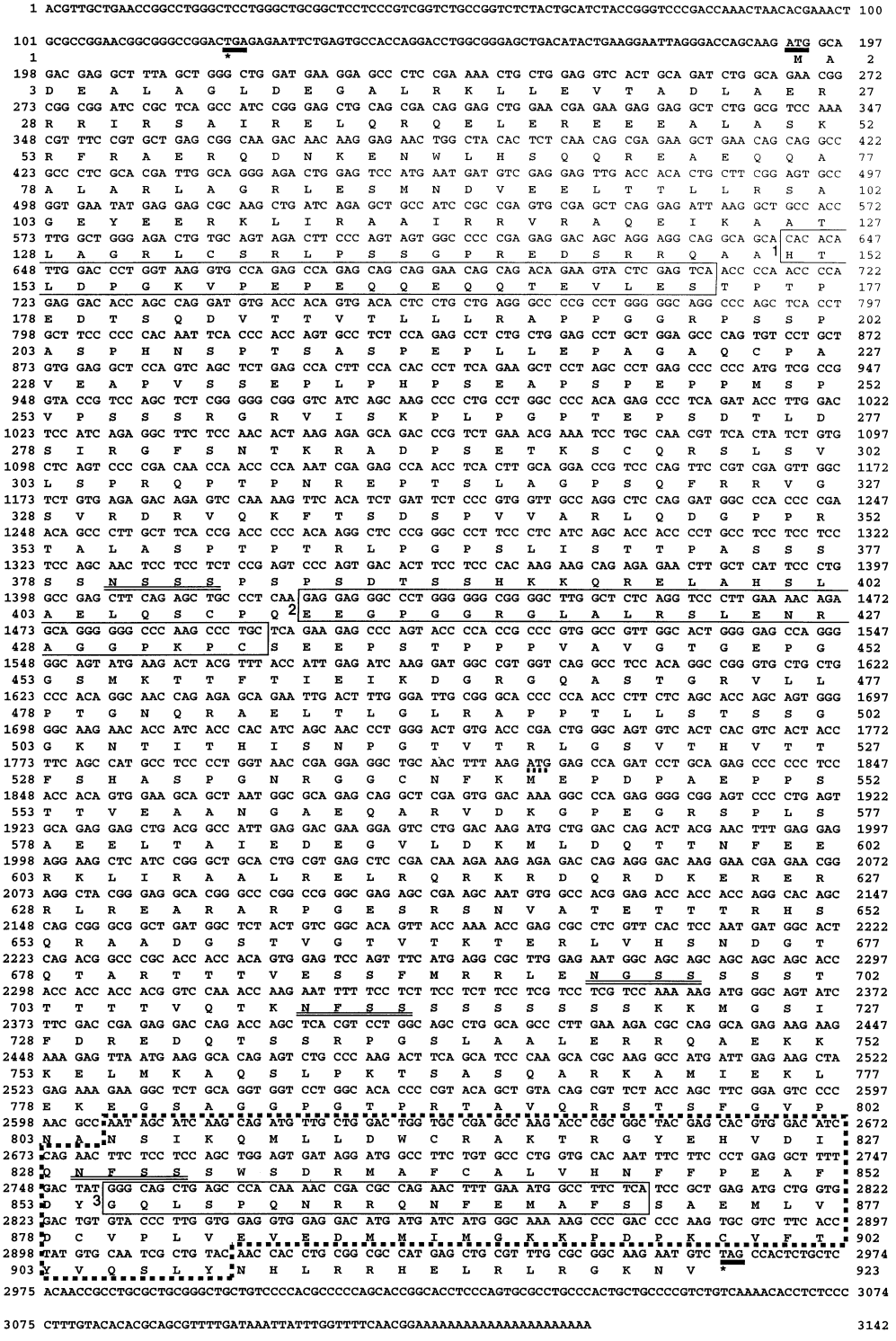


Fig. 1 Complete cDNA and protein sequence of the large mouse smoothelin isoform. The complete mouse smoothelin cDNA sequence with protein translation of the large isoform is shown. *Underlined* In-frame start and stop codons. The open reading frame encodes a protein with a calculated molecular weight 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

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-

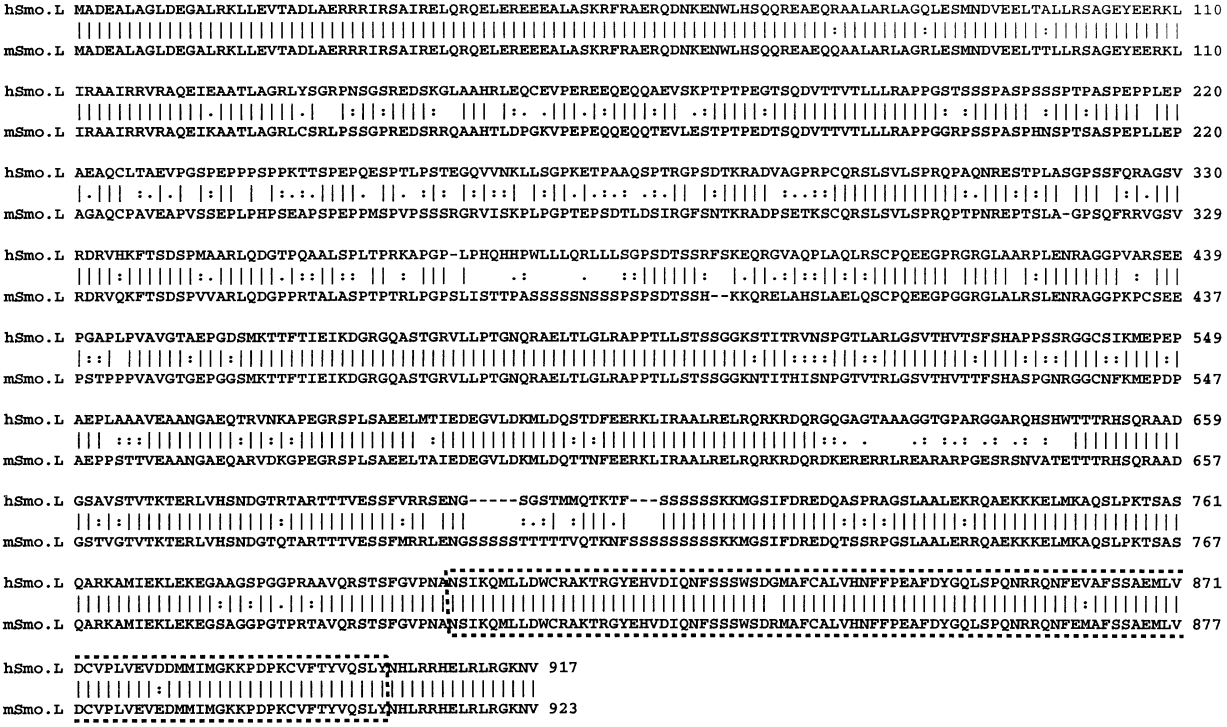


Fig. 2 Alignment of human and mouse smoothelin isoforms. The protein alignment of the large human (*hSmo.L*) and mouse (*mSmo.L*) smoothelin isoform was performed with the MegAlign software program (DNASTAR). *Vertical line* Amino acid identities; *one, two dots* conserved amino acids, depending on the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

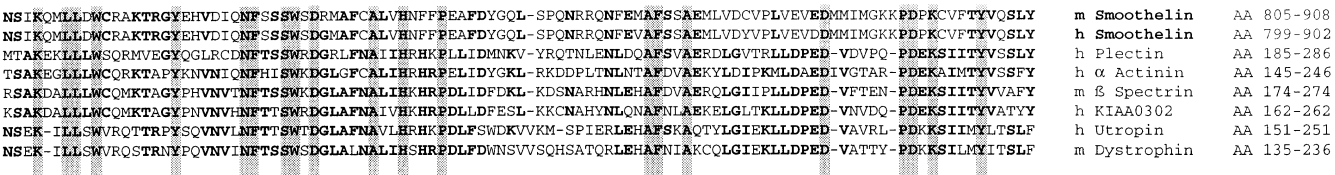
gions with homology to calponin (Fig. 1), a thin filament-associated smooth muscle protein with contraction regulatory properties [15, 16]. A third calponin homology domain is located in the common C-terminal domain.

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 animals. 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; *boldface* amino acids conserved in at least half of the sequences aligned. Sequences 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



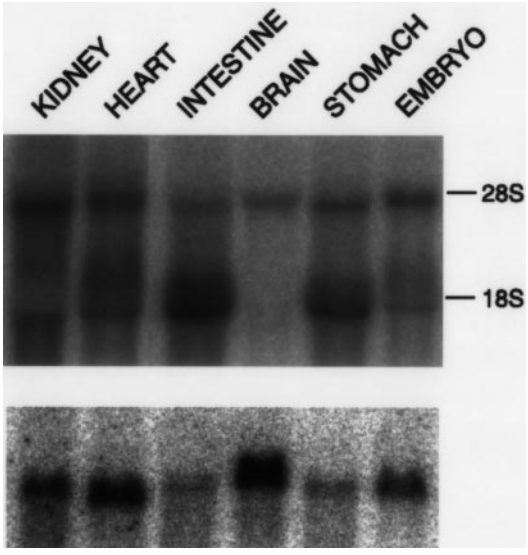


Fig. 4 Tissue-specific distribution of the long and short smoothelin transcripts. Northern blot analysis of mRNA from various mouse organs and 13-day mouse embryo. A PCR-amplified part of the mouse smoothelin cDNA (nucleotides 2648–2889, according to Fig. 1) was used as a probe. *Below* The same blot 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*)

ficult 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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