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Expression of the smoothelin gene is mediated by alternative promoters

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Abstract

Objective: Two major isoforms of smoothelin have been reported, a 59-kDa smoothelin-A in visceral smooth muscle cells and a 110-kDa smoothelin-B in vascular smooth muscle cells. The present study was undertaken to investigate the expression of these smoothelin isoforms in different smooth muscle tissues and to determine how they are generated. **Methods:** Western blotting with a new, well-defined, smoothelin antibody was used to confirm the existence of two major smoothelin isoforms. Northern blotting, RT-PCR, primer extension and 5′RACE were applied to analyse the expression of these isoforms in human and mouse. Promoter reporter assays were carried out to establish the existence of a dual promoter system governing the expression pattern of the gene. **Results:** Antibody C6G confirmed the existence of two smoothelin proteins. Northern blotting showed that in vascular tissues a larger smoothelin transcript is generated than in visceral tissue. The cDNA of this larger smoothelin-B was cloned. Computer analysis of the open reading frame suggests an α-helical structure of 130 amino acids at the amino terminus of smoothelin-B. The smoothelin gene was cloned and sequenced. It comprises about 25 kb and contains 21 exons. The translational start of smoothelin-B is located in exon 2, whereas transcription and translation of the previously described smoothelin-A starts inside exon 10. Smoothelin-A and -B were demonstrated to be generated by two physically separated promoters. Splice variants within the calponin homology domain at the 3′ end of the gene were found for both isoforms. **Conclusions:** Two major smoothelin isoforms are generated from a single gene by a dual promoter system in a tissue specific manner. Further variation in the smoothelin proteins is achieved by alternative splicing in the calponin homology domain.

Keywords: Contractile apparatus; Gene expression; Smooth muscle

1. Introduction

Smooth muscle cells (SMCs) are characterized by the expression of SMC-specific marker proteins such as calponin, SM22α, α-smooth muscle actin (α-SMA), smooth muscle myosin heavy chain isoforms (SM-MHC) and smoothelin. Despite the shared expression of these proteins, SMCs are heterogeneous in many respects. This heterogeneity is reflected in differences in morphology, physiology and origin of SMCs [1–3]. Accordingly, SMCs can be divided into sub-populations with a proliferative or a contractile phenotype [3–5]. Moreover, SMCs of the visceral organs, digestive tract and the reproductive system differ from the SMCs found in the blood vessels [6,7]. Some of these variations are related to differences in embryological origin. SMCs lining the digestive tract and

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ducts associated with the gut, as well as SMCs around the bladder, trachea and bronchi are all derived from the lateral mesoderm [8]. In contrast, the majority of the vascular SMCs are considered to be either locally recruited from the primordium, pro-epicardial organ or neural crest after invasion by endothelial cells, or from circulating progenitor cells [9,10]. Also, *trans*-differentiation of endothelial cells to SMCs has been reported [11].

SMC specific proteins can be used as markers of development or differentiation if their expression is specific for a particular phenotype or stage of SMCs or if isoforms are characteristic of specific SMC lineages. Smoothelin has both properties. It is a marker protein for the end-stage of SMC differentiation since it has been found only in contractile SMCs [12,13]. Furthermore, the two adult isoforms of smoothelin are expressed in a tissue specific manner: a 59 kDa isoform, smoothelin-A, in visceral and urogenital tissues, and a 110 kDa isoform, smoothelin-B, in vascular tissues (previously described as 94 kDa protein) [14,15]. Transient synthesis of a third smoothelin isoform has been detected in embryonic striated muscle cells in chicken [16].

SMCs can also be grouped according to the nature of their contraction. Tonic and phasic SMCs can be distinguished. Tonic SMCs, which are primarily found in blood vessels, have a slow rate of force activation and relaxation, lower maximum speeds of shortening and good force maintenance. Phasic SMCs, in contrast, show a high rate of force activation and relaxation, high maximum speeds of shortening and poor force maintenance [17]. The latter are mainly found in SMCs in the gastrointestinal tract. These physiological differences in distinct SMC populations are reflected in proteins that make up the cyto-architectural structures of the SMCs [3,18]. Studies in chicken embryos revealed that during development, smoothelin-B is not only found in vascular but also in visceral SMCs of the digestive tract. When the embryonic visceral SMCs change their contractile profile from tonic to phasic (around hatching), smoothelin-B is replaced by the smoothelin-A isoform [16]. This expression pattern not only makes smoothelin a valuable marker for SMC differentiation, but is also indicative of a function of smoothelin in SMC contraction.

Previously, we have demonstrated that smoothelin is encoded by a single copy gene, located on chromosome 22q12.3 and we and others have provided a rough map of the structure of the gene [19–21]. In this study, Northern blotting proved that there are two transcripts mediating the generation of the two major smoothelin isoforms. The cloning and characterization of vascular SMC-specific smoothelin-B cDNA and the smoothelin gene are described. Alternative splicing was found to introduce variations in the calponin homology domain (CH-domain) of the smoothelins, which probably mediates actin binding. Promoter reporter assays show that alternative promoters are used to produce smoothelin-A or smoothelin-B mRNA.

2. Methods

2.1. Tissue samples and cell culture

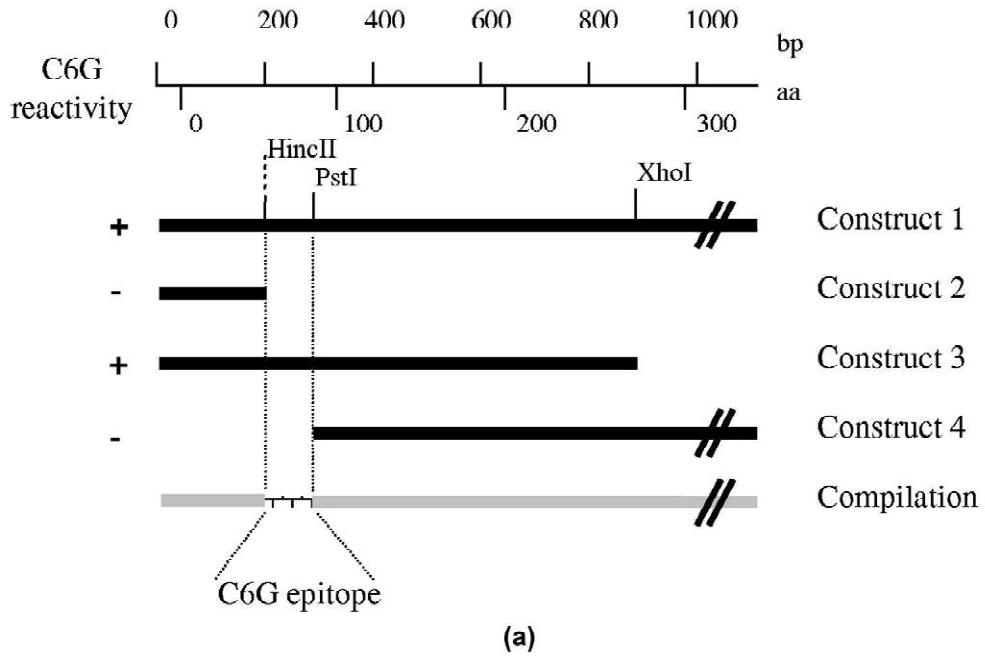
Human primary SMCs were obtained from iliac artery by enzymatic dispersion (collagenase/elastinase/soybean trypsin inhibitor: Worthington Biochem., Lakewood, NJ, USA). Cells were cultured in Opti-MEM I Reduced Serum Medium supplemented with 15% fetal calf serum (Life Technologies, Paisley, UK). Human tissues (umbilical cord/iliac/uterine artery, aorta, uterus, gut, colon, ileum, stomach, skeletal muscle, liver, brain, heart) and mouse tissues (uterus, prostate, lung, oesophagus, stomach, colon, iliac artery, carotid artery, aorta and heart) were isolated, immediately frozen and stored at $-80\,^{\circ}C$ until use. The use of human and animal tissues was approved by the ethical board of the university. Cells and tissues were screened for the presence of smoothelin by indirect fluorescent antibody technique. Procedures were carried out as described previously [12].

2.2. Antibody generation

To generate a mouse monoclonal antibody directed against human smoothelin, mice were immunized with recombinant human smoothelin-A. The fusion procedure and cloning of the hybridomas were performed according to standard protocols [22]. Isotyping of the monoclonal antibody was done with the Mouse Mab Isotyping kit according to the manufacturer's instructions (Life Technologies). Smooth muscle specificity of the monoclonal antibody was determined by screening cells and tissues by Western blotting and indirect fluorescence staining as described previously [12]. The epitope recognized by the antibody was mapped using deletion expression constructs of the smoothelin-A cDNA. Several fragments were cloned in frame in the pRSET vector and proteins were produced in *E. coli* of the M15 strain (Fig. 1a) and analyzed on Western blot as described in the next section.

2.3. Protein gel electrophoresis and Western blotting

Cryostat sections (20 per sample, 10 μm) of freshly frozen tissues were collected, washed with phosphate buffered saline (PBS) and centrifuged for 5 min at $12\,000\times g$. After centrifugation, the pellet was subjected to a Triton X-100 extraction step. Cells were resuspended in 1% Triton X-100, 5 mM ethylenediaminotetraacetic acid disodium salt dihydrate (Merck, Darmstadt, Germany), 0.4 mM phenylmethylsulfonyl fluoride (Merck) in PBS, pH 7.4 and extracted for 5 min on ice. After centrifugation for 5 min at $12\,000\times g$, the pellet was washed in 1 ml PBS. After a final centrifugation step (5 min, $12\,000\times g$), the preparation was dissolved by boiling for 4 min in sample buffer [23], with 2.3% sodium dodecylsulfate (SDS) and



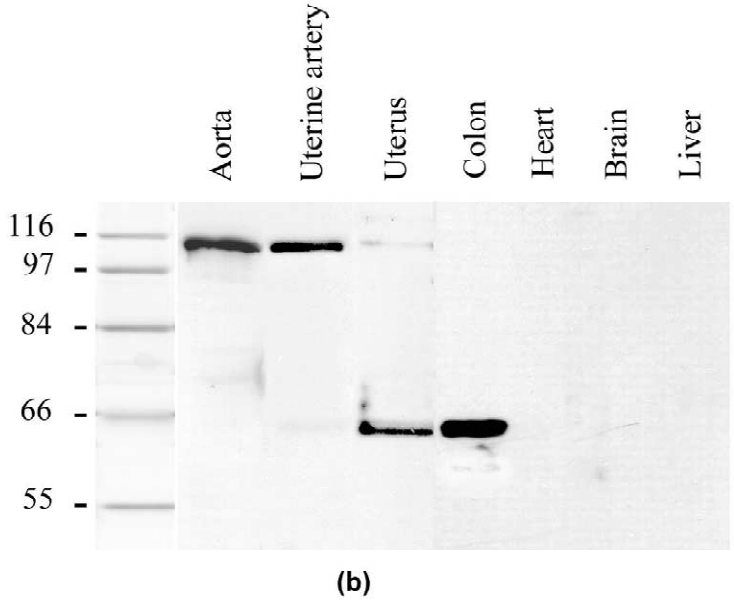


Fig. 1. (a) Epitope mapping of the new antibody C6G. Deletion constructs of smoothelin-A cDNA were transfected in *E. coli* M15 and expression of the protein fragments was induced. C6G reactivity of protein products is indicated. The epitope recognized by C6G is located between amino acids 56 and 86 of the smoothelin-A protein (512–542 in smoothelin-B). (b) Western blots of smooth muscle tissues with antibody C6G directed against smoothelin. Vascular tissues show a prominent band at 110 kDa, smoothelin-B, whereas visceral smooth muscle displays the 59 kDa smoothelin-A. Non-smooth muscle tissues do not express smoothelins.

5% β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA, USA).

For SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on a Mini Protean II Electrophoresis Cell (Bio-Rad Laboratories), 7.5–10% polyacrylamide slab gels containing 0.1% SDS were used [23]. After electrophoretic separation, the proteins were stained with Page Blue 83 (BDH Chemicals, Poole, UK) or subjected to Western blotting on a nitrocellulose membrane (Schleicher & Schüll, BA85: Dassel, Germany). To estimate the amount of protein transferred, blots were stained with Ponceau red. The monoclonal antibody C6G, directed against

smoothelin, was used at a 1:5 dilution. Antigen–antibody interaction was visualized by chemiluminescence (ECL kit, Amersham, Buckinghamshire, UK).

2.4. Smoothelin expression analysis by Northern blot and RT-PCR

Total RNA of vascular tissues (umbilical cord artery, aorta, primary cell cultures of iliac artery) and non-vascular tissues (gut, colon, stomach, skeletal muscle) was extracted with LiCl [24]. Ten micrograms of total RNA was separated on a 1% agarose formaldehyde denaturing gel [24]. RNA was transferred to nitrocellulose (S&S BA83) and hybridized to smoothelin cDNA probes according to standard procedures. Probes were ^{32}P-labeled by random priming (Life Technologies) [25]. Filters were washed in decreasing SSC (NaCl/NaCitrate) concentrations with a final concentration of 0.1× SSC/0.1% SDS.

Alternative splicing was investigated by reverse transcription-polymerase chain reaction (RT-PCR). Five micrograms of total vascular RNA extracted from mouse tissues were subjected to oligo(d)T primed reverse transcription. Sets of primers that covered the smoothelin-A cDNA were used for PCR. Length of the PCR-generated fragments was compared with those generated by PCR of the smoothelin-A cDNA. Expression of detected alternative splice variants (all located in the CH-domain) was further investigated by RT-PCR with the following primers: forward 5′GTCGACATCCAGAACTTCCTCC; reverse 5′CGCAGGTGGTTGTACAGCGA. The PCR conditions applied were: 2 min initial denaturation at 94 °C, 30 s at 94 °C, 30 s at 54 °C, 30 s at 72 °C (35 cycles) with a final extension of 5 min using an MWG AG Biotech Primus 96plus thermal cycler. PCR products were analyzed on a 2% agarose gel.

2.5. Cloning of the human cDNA for smoothelin-B

RNA was isolated from human iliac artery smooth muscle by Trizol extraction according to the manufacturer's instructions. Concentration and quality of RNA were evaluated by Northern blot analysis using a ^{32}P-labeled smoothelin-A cDNA [25].

Rapid amplification of cDNA ends (5′RACE) was applied to investigate the possibility of a 5′ extension of the smoothelin-A mRNA. Based on sequences of the 5′ part of the smoothelin-A cDNA, the following primers were used in a 5′RACE system (Life Technologies): 5′GCATGGCTGAAGCTGGTGAC and 5′GTGAATGTGGTCTTCATACTG. PCR fragments were generated and cloned. Colonies were screened with a genomic fragment containing the sequence from −200 to +60 of the smoothelin-A cDNA. Clones containing cDNA inserts were mapped by restriction digestion and sequenced. Sequence comparison and structural analysis of the putative protein were carried out using BLAST algorithms [26] and protein structure program PHD [27,28].

2.6. Cloning and analysis of the human smoothelin gene

Approximately 10^5 clones of a human placental cosmid genomic library (Stratagene, La Jolla, CA, USA) were screened with a ^{32}P-labeled smoothelin-A cDNA probe. Two positive clones were purified to homogeneity. One of the clones contained the complete coding region of the smoothelin gene. In addition, 6 kb 5′ flanking sequence was present. This clone was subcloned into pUC19 and exon-containing subclones were identified by colony hybridization with cDNA fragments. A complete restriction map of the cosmid clone was constructed and exons and their flanking regions were sequenced. Sequencing was carried out according to Sanger et al. [29] using an Applied Biosystems AmpliTaq cycling kit for automatic sequencing in an Applied Biosystems 310 (Perkin Elmer, Norwalk, USA). Searches for sequence homology were performed through GenBank/EMBL databases. Intronic sequences were screened with HEXON software which allows detection of potential additional exons [30].

2.7. Primer extension analysis

The smoothelin-A transcription start site was determined by primer extension using a primer extension system from Promega (Madison, WI, USA). Briefly, 10 μg RNA extracted from human ileum with Trizol (Life Technologies) according to the manufacturer's instructions was reverse transcribed with SuperscriptII Reverse Transcriptase (Life Technologies) for 1 h at 42 °C using the following [γ-^{32}P] end-labeled primer: 5′GTGAATGTGGTCTTCATACTG. Annealing of the primer was done at 60 °C for 1 h. Primer extension products were analyzed on a gel containing 8% acrylamide, 7 M urea and 1× TBE buffer. Labeled φX174 HinfI DNA markers were loaded on the gel to determine the size of the generated fragments.

2.8. Smoothelin promoter reporter assays

To investigate the putative promoters of the smoothelin gene, smoothelin reporter constructs were made based on transcription start sites as determined by 5′RACE and primer extension assays (Fig. 6). Two smoothelin-A promoter fragments containing 1613 or 370 bp of the 5′ flanking region, exon 10 and 60 bp of intron 10 were cloned into the pGL3-basic vector (Promega) using SstI and HindIII sites. The 1613 bp fragment was subcloned in the pβGal-basic vector (Clontech Laboratories, Palo Alto, CA, USA) using SphI sites.

Five smoothelin-B promoter fragments of increasing length were cloned in the pGL3-basic vector. The fragment

sizes varied from 84 to 3372 bp of the region upstream of the smoothelin-B transcription start site. The first smoothelin-B exon and 28 bp of the first intron were included. The promoter constructs were transfected into PAC1 [31], CHO and Hela cells using Fugene-6 (Roche, Indianapolis, IN, USA) according to the manufacturer's protocol. Analysis of luciferase expression was performed 2 days after transfection with a luciferase assay system according to the provided instructions (Promega) in a Biocounter M1500 luminometer (Lumac, Landgraaf, The Netherlands). Measurements were carried out for 10 s after a 2 s delay. Co-transfection of a TK-promoter driven renilla luciferase vector for internal normalization influenced the behavior of the smoothelin promoter constructs tested, as previously described for α-SMA promoter assays [32]. Instead, luciferase activity of a control pGL3-promoter vector containing an SV40 promoter upstream of the luciferase gene was used to correct for variations, after subtracting the baseline activity of the pGL3-basic vector. Variation in lysate recovery was corrected for by determining protein concentration of the samples. All constructs were transfected in duplicate in three independent experiments. Cells transfected with the β-galactosidase reporter construct were fixed with 0.25% glutaraldehyde after 52 h. Staining solution containing 0.1 mg X-Gal was applied for 18 h at 37 °C.

3. Results

3.1. Application of the novel anti-smoothelin monoclonal antibody C6G

A monoclonal antibody, C6G, was generated against recombinant human smoothelin-A. The antibody of the IgG1 subclass reacted specifically with smooth muscle tissue. The epitope of the antibody, which recognizes both smoothelin-A and smoothelin-B, is located within a stretch of 30 amino acids in exon 11 (amino acids 56–86 of smoothelin-A, 512–542 of smoothelin-B; see Fig. 1a). Western blotting showed that the principal product recognized by C6G in visceral smooth muscle tissues was the 59 kDa smoothelin-A, whereas in vascular tissues the major product identified was the 110 kDa smoothelin-B (Fig. 1b). In non-smooth muscle tissues like liver, brain or heart, no immunoreactive product was detected. C6G reacted with smooth muscle tissues of human, goat and rabbit but not with dog, pig and mouse. C6G reacted relatively strongly with smoothelin-B compared with the previously described R4A antibody.

3.2. Cloning and characterization of human smoothelin-B cDNAs

Northern blotting analysis of cultured SMCs of human iliac artery, aorta and umbilical cord artery revealed a transcript of ~3000 nt hybridizing with the smoothelin-A cDNA, whereas in visceral smooth muscle tissues the major hybridizing transcript was only about 1600 nt (Fig. 2). The identification of two smoothelin isoforms on Northern and Western blots, while only one smoothelin gene had been found, suggested alternative splicing of the smoothelin gene [14,19]. An RT-PCR screen of RNA extracted from vascular tissues did not reveal an alternative splicing corresponding to the difference in size between the two smoothelin transcripts. Hence a 5′ extension of the smoothelin-A mRNA was investigated by a 5′ RACE procedure using RNA of a primary culture of human iliac artery SMCs. A discrete band of 1700 bp was generated in three independent experiments. This product was cloned and sequenced. Sequences of several clones were aligned and compared with the sequence of the smoothelin gene (see below). The 3000 nt transcript in iliac artery SMC cultures and vascular tissues hybridized with the 5′ RACE fragment. In visceral tissues, hybridization of the 5′RACE product with the 3000 nt (but not the 1600 nt) mRNA was only seen after long exposure (Fig. 2).

The smoothelin-B sequence contains a CH-domain as does smoothelin-A. The RT-PCR screen mentioned above revealed two alternative splicings in this CH-domain. These alternative splicings were found in smoothelin-A as well as smoothelin-B mRNA. The three variants are defined by the presence or absence of exon 20 (Fig. 3). Smoothelin-B1 (without exon 20) and -B2 (with exon 20) are the major transcripts in vascular SMC. The smoothelin-B1 cDNA has an open reading frame (ORF) of 2751 bp coding for a 917 amino acid protein. Computer calculations indicate a molecular weight of 100 kDa, which corroborates with the 110 kDa protein found on Western blots. Tertiary structure analysis suggests that the N-terminal 130 amino acids of the smoothelin-B isoforms adopt an α-helical configuration.

Smoothelin-B2 cDNA has an ORF of 2745 bp, coding for 915 amino acids producing a protein of 99 kDa. Smoothelin-B3 cDNA only contains the 5′ 70 bp of exon 20. The ORF of the A3/B3 variants continues through exon 21 causing the translational stop to be at the same position as in smoothelin-B1, with an insertion of 23 amino acids (Fig. 4). The homology of the CH-domain to the actin-binding domain of members of the spectrin family is 45%, irrespective of the splice variant. Alternative splicing of only the 5′ part of exon 20 inserts an extra 23 amino acids homologous to the CH-domain. Thus, the C-terminus of smoothelin-B3 contains a part of this domain in duplicate. Expression of the different alternative splice variants of smoothelin-B and -A showed no coherent tissue specificity (Fig. 5), although prostate, lung and oesophagus seemed to express less of the A1 variant compared to vascular tissues. All three splice variants were found in investigated smooth muscle tissues, with the exception of prostate that did not express the A3 variant. In most of the smooth muscle tissues tested, the expression

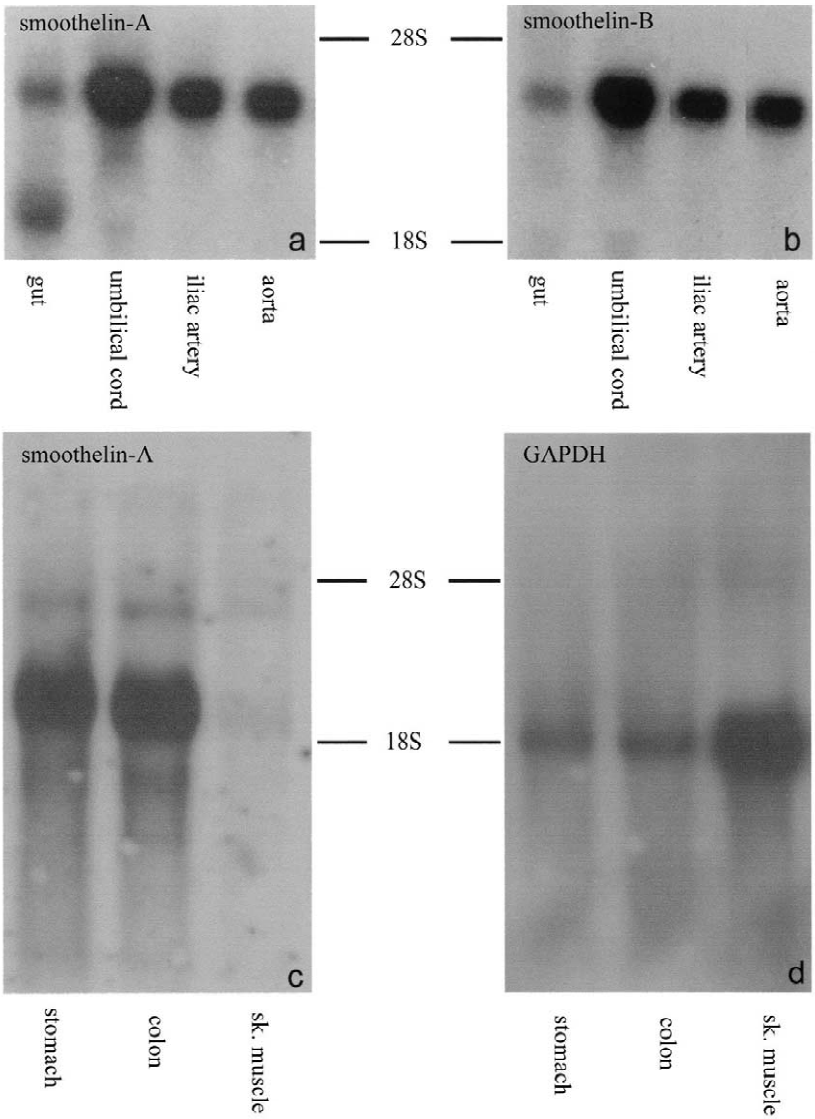


Fig. 2. Northern blot of smooth muscle tissue and artery-derived smooth muscle primary cell culture. Panels (a) and (b) display a Northern blot of human gut and cultured SMCs derived from human umbilical cord artery, human iliac artery and human aorta. Panel (a) shows these RNAs after hybridization with a probe containing a fragment of the smoothelin-A/B cDNA (exons 12–16 of the gene). Panel (b) (same blot) shows the result of hybridization with a probe containing a smoothelin-B-specific fragment (exons 1–7). Panels (c) and (d) display a Northern blot of human stomach, colon and skeletal muscle. Panel (c) has been hybridized with a smoothelin-A cDNA probe (long exposure) and panel (d) with a GAPDH cDNA probe. Notice the faint band for the vascular smoothelin-B transcript in the visceral samples, only visible after prolonged exposure.

level of the B3 and A3 variants was considerably lower compared to the other two variants. The human smoothelin-B1, B2 and B3 sequences have been deposited in GenBank under accession numbers Y13492, AY061971 and AY061972.

3.3. Cloning and analysis of the human smoothelin gene

A human cosmid library was screened with smoothelin-A cDNA probes and two cosmids containing the full size smoothelin gene were selected. Twenty-one exons have been positioned by hybridization with cDNA fragments and sequencing (Fig. 3). As such, the present map of the gene represents a refining of the previously reported one [20]. The structure of the human gene closely resembles the mouse gene structure [33]. The first two exons are separated from the other exons by a 5 kb intron. Exon 10 is the largest exon comprising 526 bp. In the middle of this exon the transcription start site of smoothelin-A is located (Fig. 3). The exon–intron boundaries match the GT-AG splice recognition sequence. Between exons 16 and 17, a 50 bp TAAA repeat has been found. The vascular specific part of the smoothelin-B cDNA sequence is coded for by the exons 1 through 9 and the 5′ half of exon 10 (Fig. 3). The 3′ half of exon 10 and the remaining 10 exons are coding for both smoothelin isoforms. The sequences of the exons have been deposited in GenBank under accession numbers AF115551–AF115570. Screening of intronic

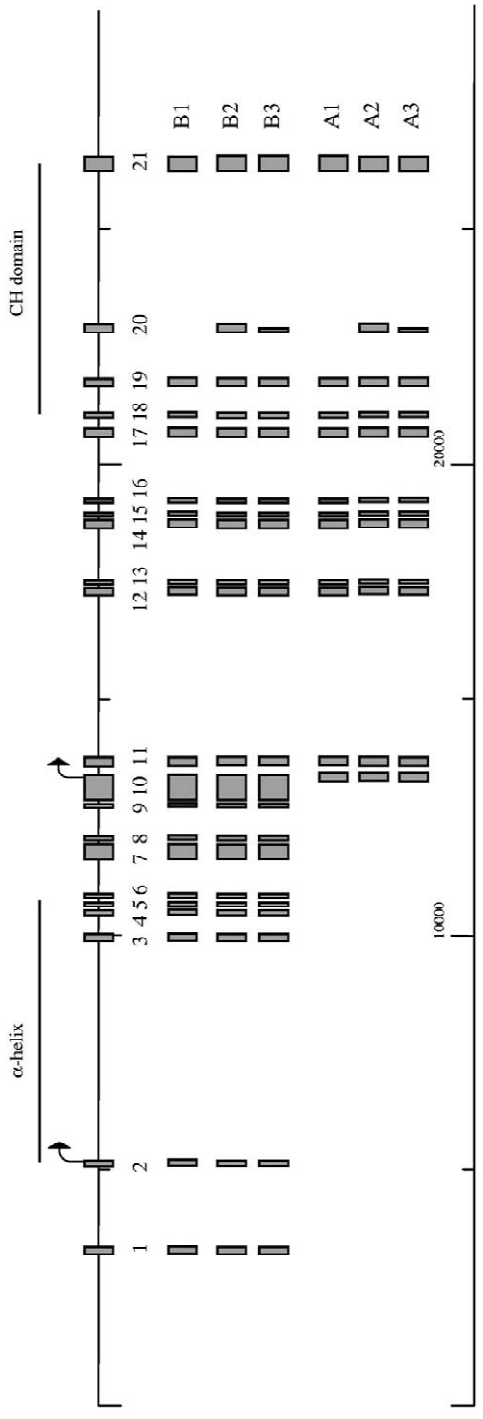


Fig. 3. Structure of the human smoothelin gene. The bottom line shows the size (in bp) of the gene. The upper line displays the position of the 21 exons. The smoothelin transcripts (mRNAs) are represented by gray boxes. Exons coding for the putative α-helical domain at the amino terminus of smoothelin-B and exons coding for the CH-domain are indicated. Translation initiation sites are depicted by arrows.

Fig. 4. CH-domains of the three alternative splice variants of the smoothelin isoforms (top lines) are compared with the consensus actin-binding CH-domain (bottom lines). Identical residues are shaded dark grey, similar residues are shaded light grey. Residues encoded by exon 20 are underlined.

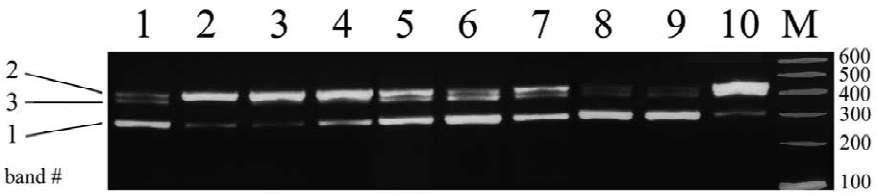


Fig. 5. RT-PCR results of various mouse tissues, showing the presence of three alternatively spliced smoothelin CH-domains in all smooth muscle tissues except prostate. Band 1 corresponds to smoothelin A1/B1 isoforms, band 2 to smoothelin A2/B2 and band 3 to smoothelin A3/B3, respectively. Lane 1, uterus; lane 2, prostate; lane 3, lung; lane 4, oesophagus; lane 5, stomach; lane 6, colon; lane 7, iliac artery; lane 8, carotid artery; lane 9, aorta; lane 10, heart; M, marker (bp).

sequences for internal exons with HEXON software revealed additional potential exons located in introns 11 and 19. These 'alternative' exons are conserved between mouse and human, contain correct splice sites and their addition to the mRNA would not disturb the reading frame. However, these exons have not been found in any mature smoothelin mRNAs so far.

3.4. Smoothelin transcription start site mapping and promoter reporter assays

Primer extension of RNA extracted from human ileum with a smoothelin specific primer produced a fragment of 83 nt (Fig. 6a). Together with the 5′RACE results which revealed that in vascular tissues a much larger transcript is generated, this shows that the smoothelin-A and -B cDNA have completely different 5′ ends. These 5′ ends are separated by approximately 10 kb of genomic sequence, indicating that two different transcription start sites may be used. Sequences directly upstream of the putative transcription initiation sites have been screened for promoter/enhancer elements (Fig. 6b,c). The putative promoter regions of neither smoothelin-A nor smoothelin-B contain a TATA-box, but a number of promoter/enhancer elements have been identified. Amongst them are CArG(like) boxes, E-boxes, AP-2 and Sp-1 sites, a TGF-β-control element and GATA boxes. Comparison between human and mouse putative promoter sequences of smoothelin-B revealed a high degree (66%) of homology for about 700 bp upstream of the transcription initiation site. The putative smoothelin-A promoter, which also contains exons of the smoothelin-B-specific part, showed an even higher degree of conservation (73%). Intronic sequences were generally as conserved as exon sequences [33].

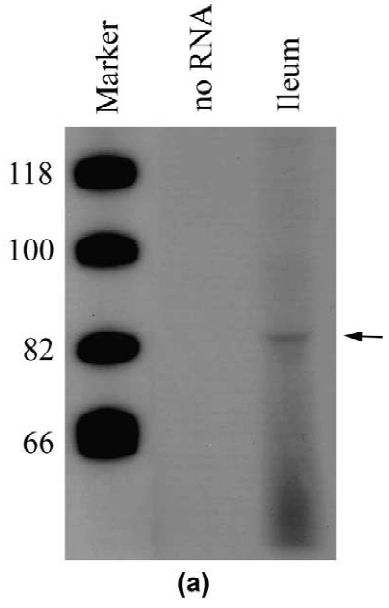
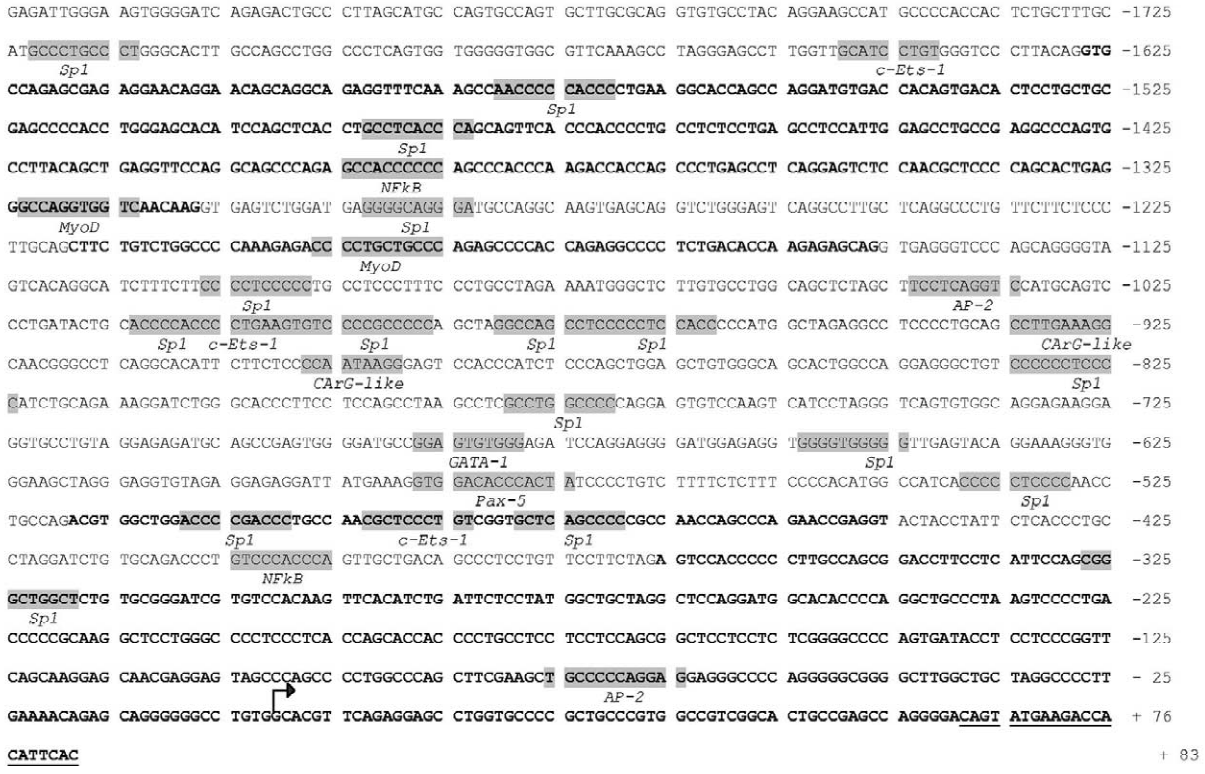


Fig. 6. Primer extension results and *cis*-acting elements present in the smoothelin-A or smoothelin-B promoter region. (a) Primer extension of RNA extracted from human ileum using a smoothelin specific primer (underlined in (b)) produced a product of 83 nt, showing that transcription starts at the site indicated in (b). (b) Smoothelin-A promoter region. *Cis*-acting elements are shaded. The transcription initiation site of smoothelin-A is depicted by an arrow. Exon sequences are bold. The sequence of the primer used for primer extension analysis is underlined. (c) Smoothelin-B promoter region with *cis*-acting elements indicated. Exon sequences are bold.

Promoter reporter assays indicated that the region upstream of the first smoothelin-B exon has promoter activity (Fig. 7a). A minimal promoter construct containing 84 bp of the region upstream of the transcription start site was able to drive luciferase expression, albeit at low levels. Increasing the size of the promoter fragments up to 750 bp resulted in a higher expression. Larger promoter fragments of 1097 and 1616 bp in contrast gave a lower luciferase activity. The highest activity was conferred by a construct containing 3372 bp of the promoter. Apart from the minimal promoter, all constructs that were tested showed considerable specificity for SMC.

A smoothelin-A promoter fragment of 370 bp was not able to drive luciferase expression in any of the cell types tested. The minimal promoter for the smoothelin-A transcript therefore has to include elements more upstream of this region. A more extensive analysis of the smoothelin-A promoter to address this question is currently being performed. A larger smoothelin-A promoter fragment produced variable results in the vascular PAC1 SMCs that were used for the promoter assays. However, in the non-SMC cell lines tested, this 1613 bp fragment displayed no promoter activity at all (Fig. 7a). Because of the variation, the same fragment was cloned upstream of another reporter gene, β-galactosidase. In this assay, numerous positive



(b)

(c)

Fig. 6. (*continued*)

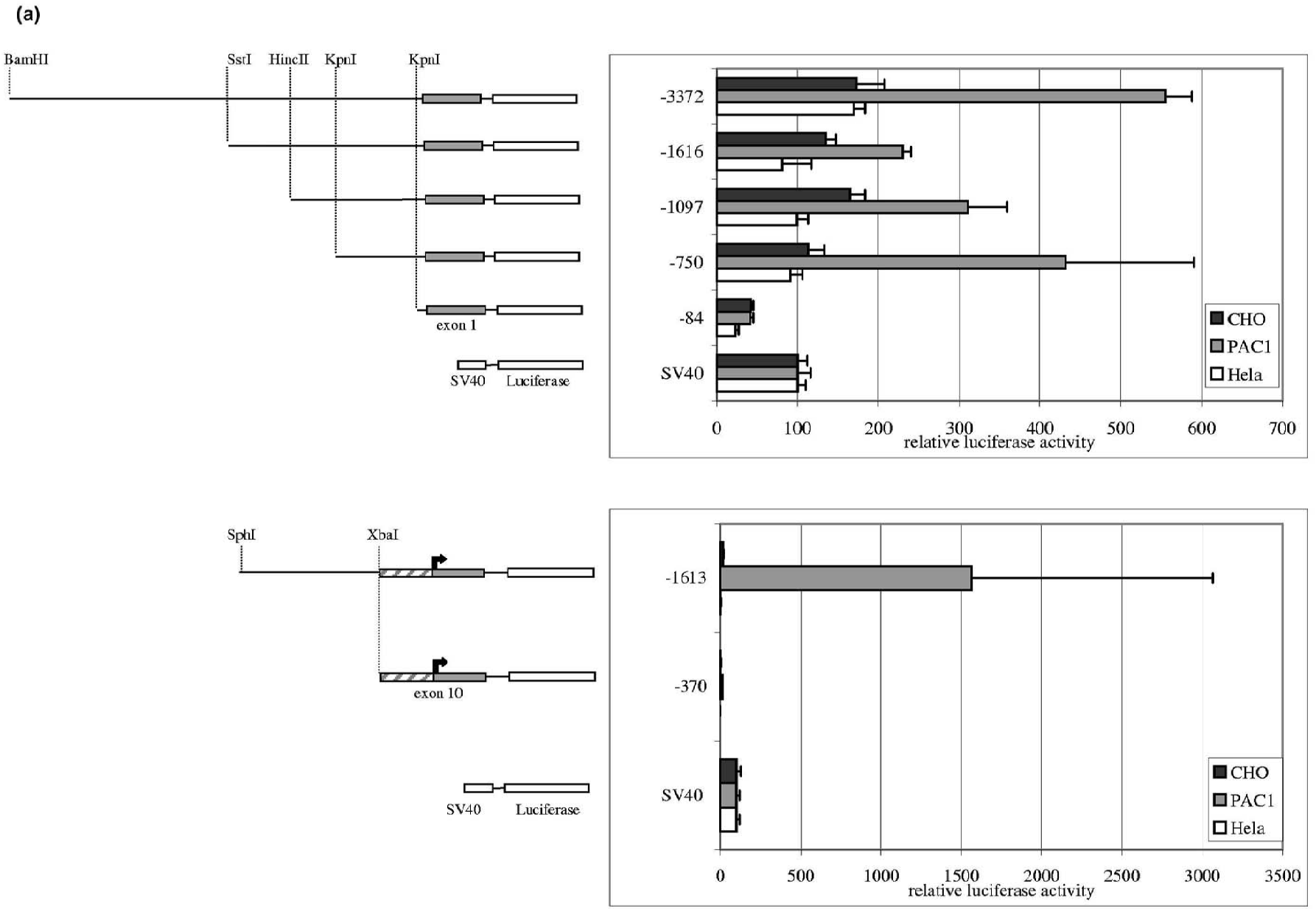
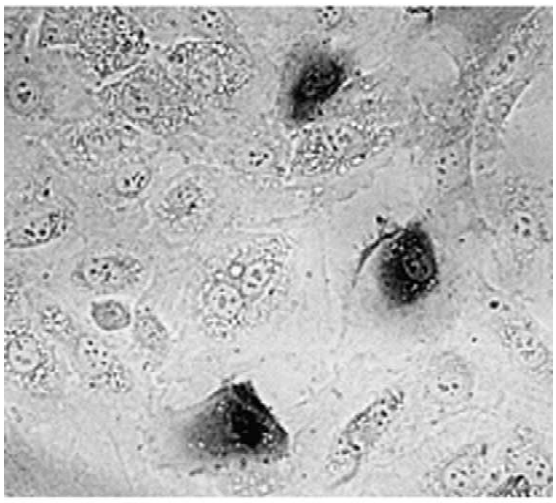


Fig. 7. (a) Luciferase promoter reporter assays. The relative promoter activity of several smoothelin-A and -B promoter constructs was determined in Chinese Hamster Ovary cells (CHO), rat Pulmonary Artery smooth muscle Cell line 1 (PAC1) and Hela cells. Activity of an SV40 early promoter was set at 100%. Reporter constructs tested are depicted next to the graphic representation of the results. (b) β-Galactosidase promoter reporter assay. PAC1 cells were transfected with a smoothelin-A promoter-driven β-galactosidase reporter gene. The picture shows positive cells after incubation in an X-gal solution.



(b)

Fig. 7. (*continued*)

cells were identified, confirming that this region of the smoothelin gene exerts promoter activity (Fig. 7b).

4. Discussion

The contractile apparatus of SMCs is constructed around SMC-specific isoforms of actin and myosin proteins. Smooth muscle-specific proteins such as calponin, SM22α and h-caldesmon are associated with α-SMA or myosin [18,34–36]. The function of these proteins is still unclear. Smoothelins are a recent addition to the group of actin-associated SMC-specific proteins. They share a number of characteristics with calponin and SM22α such as the presence of a CH-domain and transient expression in the primitive heart during embryogenesis [16]. However, smoothelin expression is restricted to contractile SMCs whereas calponin and SM22α are also expressed in less differentiated SMCs. As such, smoothelin has been used as a marker for the differentiated phenotype of SMCs [37,38].

Previously, we have characterized smoothelin as a structural protein, specific for contractile smooth muscle tissues. A smoothelin cDNA cloned from visceral smooth muscle tissue was demonstrated to code for the 59 kDa smoothelin-A [12]. However, Western blotting of smooth muscle tissues in several species revealed that two isoforms of smoothelin exist [14,15]. In addition to the 59 kDa smoothelin-A in visceral tissues, we found an immunoreactive product of 110 kDa in vascular tissues. Analysis of a variety of smooth muscle tissues indicated that this protein, designated smoothelin-B, is specific for contractile vascular SMCs in adults [14,37].

Here we present evidence that in humans, like in mice [39], this smoothelin-B isoform is translated from a 3000 nt mRNA whereas smoothelin-A is translated from a 1600 nt mRNA. The molecular weight of smoothelin-B as estimated from the cDNA open reading frame is 100 kDa which is in good agreement with the approximately 110 kDa MW observed in Western blotting analyses (small inter-species differences have been found, for instance the pig smoothelin-B is 115 kDa) [37]. Computer assisted structure analysis of the putative amino acid sequence revealed potential α-helical structures. The most prominent one is a 130 amino acid domain at the N-terminus of smoothelin-B. Such a domain may be involved in the formation of filamentous structures or may have a function in coiled-coil interactions with other molecules as described for many cytoskeletal and cytoskeleton-associated proteins [40]. At the C-terminus, a CH-domain with homology to the actin-binding domain of the spectrin family is present in smoothelin-B (as in smoothelin-A). Both smoothelins contain only one CH-domain, which binds directly to actin (unpublished data; Niessen/Gabbiani). In contrast, similar single CH-domains, found in other actin-associated proteins such as SM22α and calponin appear not to bind actin directly [41]. Alternative splicing of exon 20 can generate three different CH-domains in smoothelin-A and -B. Two of these alternative splicings in smoothelin-B have been described previously [21]. However, we show that a third alternative splicing can occur in all smooth muscle tissues except prostate and that these alternative splicings occur in both smoothelin-A and smoothelin-B mRNA. The lack of a tissue specific expression of any of these variants implies that these alternatively spliced actin-binding sites may have little physiological significance. Apparently, neither the α-helical structure at the N-terminal nor the alternative splicing products of the C-terminus of both smoothelins influence the subcellular distribution of smoothelin and more particularly its co-localization with actin. Therefore, our present data indicate that the smoothelin isoforms and their splice-variants perform, at least in part, similar functions in visceral and vascular SMCs. The influence of the alternative splicings on the affinity of the different smoothelin isoforms for actin is currently under investigation.

In situ hybridization and radiation hybrid analysis has shown that smoothelins are encoded by a single copy gene [19]. Consequently, three options were at hand to explain the occurrence of the smoothelin-B isoform in vascular smooth muscle tissue: (1) extensive post-translational modification of smoothelin-A; (2) alternative splicing; (3) alternative promoters.

If post-translational modification were responsible for the increase in molecular weight, the two isoforms of smoothelin would be translational products of the same mRNA. However, Northern blotting revealed two mRNAs both hybridizing to the smoothelin-A cDNA. A 1600 nt transcript was found in visceral tissues, whereas a 3000 nt mRNA was found in vascular SMCs. The sizes of these two mRNAs correspond with the two protein isoforms of smoothelin. The weak 3000 nt band observed in visceral tissues is most likely due to the presence of blood vessels,

although different expression ratios of the two major isoforms in various smooth muscle tissues cannot be completely ruled out. Thus, post-translational modification cannot be responsible for the increase in MW of the vascular isoform.

Alternative splicing was investigated by RT-PCR with primers enclosing the splice sites in the smoothelin-A mRNA. This revealed the presence of an additional exon (20) or a part of this exon in the mature vascular mRNA of both smoothelin-A and -B. Addition of this exon modifies the C-terminus of the proteins, making them two amino acids shorter. In chicken embryonic striated muscle cells and in some prostate tumor cell lines, corresponding alternatively spliced mRNAs have been found [16,42]. The minor decrease in the ORF caused by the alternative splicing of exon 20 dismisses this mechanism as the origin of smoothelins-A and -B. In conclusion, alternative splicing does not generate the smoothelin-A and -B isoforms but it provides diversity in the smoothelin CH-domain, encoded by exons 18–21.

Consequently, alternative promoters are the remaining option for the generation of the smoothelin-A and -B isoforms. This is not common, although a number of these promoter systems have been described [43]. Primer extension and 5′RACE experiments provided evidence for two independent transcription start sites. The sequences located 5′ of both transcription start sites contain a number of elements known to be involved in gene expression regulation in SMCs, such as conserved CArG elements and E-boxes. Because the transcription start site of smoothelin-A is located inside exon 10, promoter elements may be present in upstream exons. Transfections of promoter–reporter constructs showed that regions upstream of the transcription initiation sites of smoothelin-B as well as smoothelin-A exert promoter activity. Moreover, both smoothelin promoters were able to drive expression in a SMC-specific way, although activity of the 1613 bp smoothelin-A promoter fragment was variable in PAC1 cells (this cell line of vascular origin was used since no visceral SMC line was available). The tissue specificity of the smoothelin-A promoter and its observed activity in two reporter systems nevertheless convincingly demonstrate the ability of this sequence to drive smoothelin-A expression in SMC. The lack of activity of the 370 bp putative smoothelin-A promoter region may be explained by the fact that this region consists entirely of exon sequence. The larger 1613 bp smoothelin-A promoter construct contains, amongst other elements, two conserved CArG boxes close to each other, present in a region that has a high homology between mouse and human even though it is an intron. It is tempting to speculate that these elements may play a role in the regulation of smoothelin-A expression like they do in other smooth muscle restricted genes.

The sequence directly upstream of the smoothelin-B transcription start site clearly displays promoter activity as can be deduced from comparison with the SV40 positive control. The lower luciferase activity of the constructs containing 1097 and 1616 bp of the smoothelin-B promoter compared with the construct containing 750 bp indicates that two or more repressor elements may be present in the region between −750 and −1616 bp. Interestingly, this reduction of promoter activity only occurs in SMCs. Strong SMC-specific enhancers appear to be present between −1616 and −3372 bp of the smoothelin-B promoter as evidenced by a large increase in promoter activity in SMCs of the 3372 bp smoothelin-B promoter construct. Numerous *cis*-acting elements can be identified in this region. The functionality of these elements however remains to be determined by footprinting, gel mobility shift assays and mutational analysis.

The use of alternative promoters sets the smoothelins apart from the other described SMC-specific genes. It apparently enables SMCs to regulate smoothelin expression in a strict tissue specific fashion. Physically separated promoters provide a way to respond differentially to extracellular signals (e.g. hormones). Comparison of the two promoters may reveal important insight in gene regulation in different SMC populations.

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