Smoothelin, a Novel Cytoskeletal Protein Specific for Smooth Muscle Cells

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Abstract. The characterization of a novel 59-kD cytoskeletal protein is described. It is exclusively observed in smooth muscle cells by Northern blotting and immunohistochemical analysis and therefore designated "smoothelin." A human smooth muscle cDNA library was screened with the monoclonal antibody R4A, and a full-size cDNA of the protein was selected. The cDNA was sequenced and appeared to contain a 1,113-bp open reading frame. Based on the cDNA sequence, the calculated molecular weight of the polypeptide was 40 kD and it was demonstrated to contain two N-glycosylation sites. Computer assisted analysis at the protein level revealed a 56-amino acid domain with homologies of ~40% with a sequence bordering the actin-binding domains of dystrophin, utrophin, β-spectrin and α-actinin. In situ hybridization demonstrated that human smoothelin is encoded by a single copy gene which is located on chromosome 22. Immunohistochemistry and Western blotting revealed synthesis of smoothelin in smooth muscle of species evolutionarily as far apart as human and teleost. Northern blotting indicated that sequence as well as size of the mRNA (~1,500 bases) are conserved among vertebrates. Cell fractionation studies and differential centrifugation showed that the protein cannot be extracted with Triton X-100, which indicates that it is a part of the cytoskeleton. Transfection of the human cDNA into smooth muscle cells and COS7 cells produced a protein of 59 kD, which assembled into a filamentous network. However, in rat heart-derived myoblasts association with stress fibers was most prominent. Smoothelin was not detected in primary or long term smooth muscle cell cultures. Also, transcription of smoothelin mRNA was almost instantly halted in smooth muscle tissue explants. We conclude that smoothelin is a new cytoskeletal protein that is only found in contractile smooth muscle cells and does not belong to one of the classes of structural proteins presently known.

Smooth muscle cells are of mesodermal origin and are present in the wall of almost the entire gastrointestinal tract, essential parts of the cardiovascular system, the respiratory system, the urinary tract, and in the reproductive system. Differences in size, function, and biochemistry between smooth muscle cells in different organs have been observed and may reflect differences in origin and state of differentiation (Gabbiani et al., 1981; Haeberle et al., 1992; Owens, 1995).

Smooth muscle cells can be divided into two phenotypes: a proliferative (synthetic) and a contractile phenotype. Proliferative smooth muscle cells can differentiate to the contractile phenotype. The differentiation process is, contrary to that in striated muscle cells, reversible, a phenomenon referred to as modulation. As deduced from embryological studies, smooth muscle differentiation is characterized by the onset of expression of a particular set of proteins, some of which are characteristic for the different phenotypes (Campbell and Chamley-Campbell, 1981; Skalli et al., 1986; Campbell et al., 1989; Owens, 1995). For the understanding of smooth muscle differentiation, it is important to distinguish proteins that are characteristic for a given stage of smooth muscle differentiation.

Contractile, cytoskeletal, and cytoskeleton-associated proteins have been described as differentiation markers for smooth muscle cells but their value as such is not well defined (Takahashi et al., 1988; Gunning et al., 1990; Babi et al., 1990; Nanaev et al., 1991; Duband et al., 1993; Owens, 1995). At present, marker proteins such as smooth muscle myosin, α-smooth muscle actin, (meta)vinculin, SM22, calponin, and h-caldesmon, are considered to be smooth muscle specific and some have been suggested to be phenotype-specific (Glukhova et al., 1986; Lees-Miller.
Materials and Methods

Tissue Samples and Cell Culture

Normal adult human tissues, obtained at autopsy, and tissues from a variety of animals (pig, dog, cattle, rabbit, rat, mouse, the toad Xenopus laevis, and the fish Salmo salar) were immediately frozen in liquid nitrogen and stored at −80°C until use. Smooth muscle cells were obtained from human vena, bovine aorta, embryonic chicken gizzard, human uterine, and mammary artery by enzymatic dispersion (collagenase/pancytatin: Life Technologies, Gaithersburg, MD). Embryonic chicken gizzard cells were cultured for 72 h at high density on gelatin-coated coverslips. Smooth muscle cells cultured for at least five passages are considered to be “long-term cultures.” The rat heart-derived myoblast cell line H9C2(2-1) (Kimes and Brandt, 1976) and COS7 cells (Gluzman, 1981) were purchased from Amer. Type Culture Collection (Rockville, MD). Cells were cultured in Dulbecco’s Modified Essential Medium supplemented with 15% FCS (Life Technologies). For the myoma tissue experiments, if transfected into COS7 or smooth muscle cells.

Antibodies

Antibodies used in this study were (1) the mouse monoclonal antibody R4A directed against smoothelin. To generate the antibody mice were immunized with the residue of a chicken gizzard preparation, extracted with high salt/Tritton X-100. Fusion procedure and cloning of the hybridomas were performed according to standard protocols (Köhler and Milstein, 1975). The monoclonal antibody R4A was selected on the basis of its specific reactivity pattern with a selection of human cardiac, skeletal, and smooth muscle tissues. R4A is an antibody of the IgG1 subclass (Mouse mAb Isotyping kit: Life Technologies); (2) monoclonal antibody E7 to β-tubulin (Chu et al., 1987); (3) polyclonal rabbit antisera (pDes) to chicken gizzard desmin (Ramaekers et al., 1985); (4) affinity-purified polyclonal rabbit antibodies (pVim) raised against bovine lens vimentin (Ramaekers et al., 1983); (5) monoclonal antibody RD301 to desmin (Schaart et al., 1991); (6) affinity-purified polyclonal rabbit antibodies (pker) raised against human skin keratins (Ramaekers et al., 1983); and (7) monoclonal antibody sm-1 to smooth muscle actin was purchased from Sigma ImmunoChemicals (St. Louis, MO) (Skalli et al., 1986). In addition, rhodamine-labeled phalloidin (purchased from Molecular Probes Inc., Eugene, OR) was used to stain actin stress-fibers.

Immunohistochemistry

For the characterization of antibody R4A, immunofluorescence as well as immunoperoxidase staining has been performed on a variety of tissues of different species. In all cases 3–5-μm-thick cryostat sections were used. The sections were pretreated with 5 min with 0.5% Triton X-100 (BDH Chemicals Ltd., Poole, UK) in PBS (137 mM NaCl, 13 mM Na2HPO4, 2H2O, 3 mM KH2PO4, pH 7.4; Merck, Darmstadt, Germany), followed by a PBS washing step for 5 min. Methanol/acetoene fixed sections were used without Triton X-100 pretreatment.

Tissue sections or fixed cells were incubated with the primary antibody for 30 min at room temperature, washed with PBS, and incubated with a secondary antibody conjugated to either fluorescein isothiocyanate (FITC) (goat anti-mouse-IgG-FITC, Southern Biotechnology Associates Inc., Birmingham, AL), or Texas red (TR) (goat anti-rabbit-IgG-TR/goat anti-mouse-IgG1-TR, SBA), or horseradish peroxidase (peroxidase-conjugated rabbit anti-mouse–IgG: DAKO A/S, Copenhagen, Denmark). The secondary antibodies were applied for 30 min at room temperature. Peroxidase activity was detected with 4-amin-9-ethylcarbazole (AEC; Aldrich Chemical Company, St. Louis, MO) as described previously (Schaart et al., 1991). After three washing steps with PBS, the fluorescently stained tissues were mounted in Mowiol (HOECHST, Frankfurt, FRG) or in Kaiser’s glycercin-gelatin (Merck) for AEC-stained sections.

Protein Gel Electrophoresis and Western blotting

Cultured cells (~10^6) or about 40 cryostat sections (each 20-μm thick) of fresh frozen tissues were collected, washed with 1 ml PBS, and centrifuged for 5 min at 12,000 g. After centrifugation, the pellet was subjected to a Triton X-100 extraction step and after a PBS wash suspended in 1% Triton X-100, 5 mM ethylenediaminotetraacetic acid disodium salt dihydrate (EDTA; Merck), 0.4 mM phenylmethylsulfonyl fluoride (PMSF; Merck) in PBS, pH 7.4 (Schaart et al., 1991), and extracted for 5 min on ice. After centrifugation for 5 min at 12,000 g, the pellet was washed in 1 ml PBS. After a final centrifugation step (5 min, 12,000 g), the cytoskeletal preparation was dissolved by boiling for 4 min in sample buffer (Laemmli, 1970), containing 2.3% sodium dodecylsulfate (SDS) and 5% β-mercaptoethanol (BioRad Laboratories, Richmond, CA). Removal of N-glycosylation residues was achieved by treatment of smooth muscle homogenates by neuraminidase (50 U/ml) (Sigma) in the presence of leupeptin (1 μg/ml) and PMSF (20 mM) for 5 h at 37°C under conditions as advised by the manufacturer.

Fractionation of Smooth Muscle Tissues by Differential Centrifugation

All steps of the procedure described below were performed on ice and for centrifugation a T-60 rotor was used (Beckman Instrs., Fullerton, CA). Frozen tissue blocks of intestinal smooth muscle tissue (5 g wet weight human colon) were minced and homogenized in 15 ml SET buffer (8.5% sucrose, 5 mM Tris-HCl, pH 7.4, 2 mM EDTA) by a motor driven serrated teflon pestle at 1,000 rpm. (Braun Potter-S). Homogenates were passed through SET-soaked cheesecloth and pelleted at 1,000 g. Supernatants of the subsequent steps were centrifuged at 9,000, 20,000, and 110,000 g for 10, 15, and 60 min, respectively. Samples of the pellets (resuspended in SET buffer without EDTA) and the supernatants of different fractions were subjected to SDS-PAGE followed by Western blotting, as described above.
Construction and Screening of a Human Smooth Muscle cDNA Expression Library

A cDNA expression library was constructed with human colon smooth muscle derived poly-A+ RNA using the Lambda ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA). Total RNA was extracted from human colon smooth muscle by LiCl extraction (Auffray and Rougeon, 1980). Poly A+ RNA was purified by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). Synthesis of cDNA (by oligo(dT) priming) and packaging were performed as suggested by the manufacturer. After packaging the Uni-ZAP XR vector in phage (Gigapack II Packaging extract, Stratagene), clones containing cDNA inserts between 600 and 1,600 bp were selected by immunoscreening with antibody R4A. Inserts were subcloned and subjected to restriction analysis and sequencing after helper phage induced excision of plasmid vector pBluescript II pkS(-) (Stratagene). Sequencing was performed according to Sanger et al. (1975) using a T7 sequencing kit (Pharmacia, Uppsala, Sweden). Searches for sequence homology were performed through the CAOS/EMBL data base (release 1995). Sequence comparison and structural analysis of the putative protein were performed on a UNIX computer using BLAST, BLAST-X algorithms (Alschul et al., 1990), and protein structure program PHD (Rost and Sander, 1993, 1994).

To check whether the 1,554-bp clone contained a full-size cDNA, total RNA of human colon smooth muscle tissue was subjected to rapid amplification of cDNA ends (RACE) (5'RACE-kit, Life Technologies). The RACE products were amplified using Pwo polymerase (Boehringer Mannheim, Germany), cloned into pUC19 and sequenced as described above.

Northern Blot Analysis

Total RNA of various tissues and from a variety of vertebrates (human, bovine, dog, rat, and mouse) was extracted by LiCl as described previously (Auffray and Rougeon, 1980). 10 μg of total RNA was separated on a 2% agarose formaldehyde denaturing gel (Sambrook et al., 1989). RNA was transferred to nitrocellulose (S&K, Basel, Switzerland) and hybridized to the smoothelin-cDNA probe according to standard procedures (Church and Gilbert, 1984). Filters were washed in decreasing SSC (NaCl, Na-citrate) concentrations with a final concentration of 0.1x SSC/0.1% SDS. Probes were 32p-labeled by random priming using a kit with a DNA polymerase Klenow fragment (Life Technologies) according to Feinberg and Vogelstein (1983).

Transfection of Smoothelin cDNA into COS7 Cells

The full-size smoothelin cDNA was recloned in a pcDNA3 eukaryotic expression vector (Invitrogen, San Diego, CA) (this construct is further referred to as pcDNA3-SMO). Isolated plasmid was purified by CsCl gradient centrifugation (Sambrook et al., 1989). Transfection of exponentially growing human primary smooth muscle cells, HgC2(2-1) rat myoblasts, and COS7 cells was done with 5 μg purified DNA per 106 cells, using the DOTAP lipocarrier system as suggested by the manufacturer (Boehringer Mannheim, Germany). About 16 and 40 h after transfection cells were fixed with methanol/acetone or harvested after trypsinization. Fixed cells were incubated with rhodamine-labeled phallolidin and antibodies directed against smoothelin, vimentin, keratin, and tubulin as described above. Cells harvested after trypsinization were collected by centrifugation and prepared for Western blotting analysis as described above.

Production of Smoothelin Protein in E. coli

The smoothelin cDNA (1,593 bp clone) was excised from Bluescript II and inserted into the prokaryote expression vectors pQE9/10/11 (Quiagen, Kassel, Germany). Insertion in these vectors results in a fusion of the cDNA encoded protein to a 6-His peptide, allowing a single-step purification of the protein by binding to Ni-agarose. Protein was synthesized during overnight induction with 1 μM IPTG (Life Technologies). Proteins produced in E.coli were obtained by guanidine/urea extraction and purified according to the protocol of the manufacturer (Quiagen). After separation by SDS-PAGE, proteins were blotted onto nitrocellulose and identified by antibody R4A as described above.

In Situ Hybridization

Double target in situ hybridization on human metaphase preparations was performed as previously described (Voorter et al., 1995) combining a digoxigenin-labeled chromosome 22 centromeric probe with the biotin-labeled smoothelin cDNA probe. The applied concentration of both probes was 10 ng/μl hybridization mix. Hybridization and subsequent washes were performed according to standard procedures. The biotinylated probe was detected by FITC-conjugated avidin, and the digoxigenin-labeled probe by tetramethyl rhodamine isothiocyanate (TRITC), using an anti-digoxigenin monoclonal antibody in combination with TRITC-conjugated rabbit anti-mouse and swine anti-rabbit sera (DAKO A/S).

Results

Generation and Characterization of Antibody R4A

Balb/C mice were immunized with a chicken gizzard extract and hybridomas were produced. Antibody production was screened by immunofluorescence assays and by Western blotting on a variety of tissues. In immunofluorescence assays on sections of several tissues hybridoma clone R4A, producing an IgG1 type antibody, reacted exclusively with smooth muscle cells. Western blotting of one- and two-dimensional gels confirmed the microscopical observations. The immunoreactive protein was found to have an apparent molecular mass of 59 kD, migrated slightly slower than desmin (Fig. 1), and, as observed in two-dimensional gel electrophoresis, appeared to have a slightly basic pl between 8.0 and 8.5. Because of its restricted tissue distribution the 59-kD protein has been designated "smoothelin."

Molecular Cloning and Characterization of Smoothelin

A cDNA library was constructed from poly(A)+RNA isolated from human colon smooth muscle tissue. This library was screened with the monoclonal antibody R4A. Of the 25,000 plaques plated 14 were immunoreactive with R4A. The size of the cDNA inserts varied between 600 and 1,550 bp, indicating that the epitope of R4A is located at least 150 amino acid residues away from the carboxy terminus (the last 150 bp are noncoding; Fig. 2 A). All clones...
isolated contained sequences similar to the four largest
clones, as deduced from restriction patterns. The complete
amino acid sequence of homologous regions of human smooth-
elin, and members of the spectrin family, human β-spectrin,
and human dystrophin. Homologies are underlined by bold letters.

(b) Amino acid homologies.

| Protein         | Position | Sequence                      | Homology         |
|-----------------|----------|-------------------------------|------------------|
| Human smoothelin| 270      | YEEVDIQEGSWSWENIDGACPVDVEHFPFADQGLSE-PVREHRQYNFVAYFSAEBKLV |                |
| Human beta-spectrin| 190     | YEEVDIQEGSWSWENIDGACPVDVEHFPFADQGLSE-PVREHRQYNFVAYFSAEBKLV |                |
| Human alpha-actinin| 180     | YEEVDIQEGSWSWENIDGACPVDVEHFPFADQGLSE-PVREHRQYNFVAYFSAEBKLV |                |
| Human utrophin   | 166      | YEEVDIQEGSWSWENIDGACPVDVEHFPFADQGLSE-PVREHRQYNFVAYFSAEBKLV |                |
| Human dystrophin | 151      | YEEVDIQEGSWSWENIDGACPVDVEHFPFADQGLSE-PVREHRQYNFVAYFSAEBKLV |                |

Figure 2. (A) Nucleotide sequence of the smoothelin cDNA selected from a human colon smooth muscle cDNA library and the deduced amino acid sequence of the putative protein. The sequence with homologies to members of the spectrin super-family is boxed. Potential helical secondary structures are underlined. The polyadenylation signal is underlined. Notice the number of proline and proline-alanine residues in the area preceding the boxed sequence. (B) Amino acid sequence of homologous regions of human smoothelin, and members of the spectrin family, human β-spectrin, human α-actinin, human utrophin, and human dystrophin. Homologies are underlined by bold letters.
cells revealed a 59-kD, R4A reactive protein, the same size as the protein found in smooth muscle tissues such as colon (Fig. 3, lanes a and c). Transcription and translation of the full-size cDNA of smoothelin in a prokaryotic expression system gave an \(~60\)-kD, R4A immunoreactive molecule (Fig. 3, lane b). The calculated molecular weight of this product was \(~50\) kD because of the added 6His-tag and the translation of the 5' noncoding cDNA region. Further analysis shows that the putative protein contains two Asn-X-Ser sequences (residues 151 and 277), which are required for asparagine-linked N-glycosylation (Pless and Lennarz, 1977). Treatment of smooth muscle-derived smoothelin with neuramidase resulted in a reduction of the molecular mass by \(~10\) kD (Fig. 3, lanes g and h).

Sequence homology at the nucleic acid and protein level with members of the spectrin superfamily, dystrophin, utrophin, \(\beta\)-spectrin, and \(\alpha\)-actinin was found for the region between amino acid residues 270 and 331 of smoothelin (Fig. 2 B). Even if conserved mutations are not included, this region of smoothelin showed 39% homology with human and mouse dystrophin, 46% homology with human and mouse \(\beta\)-spectrin, and 34% for human utropin. Homologies with \(\alpha\)-actinin varied from 46% for human to 26% for \(D.\) discoideum. For the members of the spectrin family, this region is located directly following the suggested actin-binding site of these molecules.

The secondary structure of the putative protein was analyzed by PHD, a neural network program (Rost and Sander, 1993, 1994). Since no homologous sequences were available in the Swissprot databank, the expected accuracy of this method was estimated between 62 and 66%. Smoothelin has two regions with a potential helical domain: between amino acid residues 6 and 63, and between residues 179 and 229. In addition, in the region showing homology with the spectrin family proteins short stretches are found with helical potential (Fig. 2 A). However, even the rather large helical structures of smoothelin are considerably smaller than the ones found in, for instance, intermediate filament proteins. Proline residues are found next to the first helical region and in a proline-rich stretch (>35%) between residues 234 and 252 preceding the region homologous to the spectrin family.

In situ hybridization with a combination of a chromosome 22 centromeric probe and the 1,593-bp cDNA demonstrated that the gene coding for smoothelin is located on human chromosome 22 (Van der Loop, 1996).

**Expression of Smoothelin**

Northern blot analysis of RNA from several human tissues containing smooth muscle cells, such as colon, stomach, uterus, and aorta showed a band of \(~1,500\) bases after hybridization with the smoothelin cDNA probe (Fig. 4). No such hybridization signal was found with RNA isolated from brain, adipose tissue, cardiac and skeletal muscle, and intestinal epithelium (Fig. 4). Northern blot analysis of RNA of smooth muscle containing tissues derived from different species such as mouse, rat, dog, and bovine showed a strong hybridization signal (under stringent hybridization conditions) and revealed no visible difference in mRNA size between these species (Fig. 4).

The results of the Northern blot analysis were in agreement with the immunohistochemical screening of a number of different human tissues such as stomach, uterus, colon, prostate, aorta, and other arteries with monoclonal antibody R4A. Smooth muscle cells in all these tissues were positive whereas other tissue types, such as striated muscle, myoepithelium, myofibroblasts in Dupuytren's

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**Figure 3.** R4A immunostained Western blot of human colon smooth muscle extract (lane a), prokaryotic product based on the full-size cDNA in pQE9 (lane b), COS7 cells transfected with pcDNA3-SMO (lane c), and COS7 cells transfected with pcDNA3 (lane d). Lanes e and f are Coomassie Brilliant blue-stained proteins corresponding with lanes c and d, respectively. Lanes g and h show human smoothelin in colon tissue before and after a 5-h incubation with neuramidase. Marker lane is indicated by M.

**Figure 4.** Northern blot of a number of different tissues obtained from several species. Equal amounts of total RNA are applied to the gel. All RNAs hybridizing to the \(^{32}\)P-labeled smoothelin cDNA probe appear to be of the same size. RNAs were extracted from the following tissues: mouse stomach (a), rat uterus (b), rat stomach (c), rat skeletal muscle (d), dog stomach smooth muscle (e), dog intestinal smooth muscle (f), dog intestinal epithelium (g), bovine small intestinal smooth muscle (h), bovine skeletal muscle (i), bovine heart (j), human small intestinal smooth muscle (k), human uterus (l), and human intestinal epithelium (m).
Figure 5. Immunohistochemical screening of different species and tissues for the presence of smoothelin. Intestinal smooth muscle tissue of human (a), goat (b), Xenopus laevis (d), and chicken oesophagus (c) were all positive for R4A. In human large intestine (e), stomach (f), uterus (g), and prostate (h), smooth muscle cells showed a strong reaction with R4A (f and h show peroxidase staining). In rabbit (k) and cat (l) heart tissue only smooth muscle cells surrounding the blood vessels are visualized by R4A. Double-stained sections of human heart (i and j), chicken skeletal muscle (m and n) and human breast myoepithelial cells (o and p) incubated with R4A (i, m, and o) and either pDes (j) or sm-1 (n and p) show the absence of smoothelin in heart muscle, skeletal muscle, and myoepithelial cells as well as in vena (asterisk). Smoothelin is clearly present in smooth muscle cells of arterial vessels (k–m).

Bars: (a, b, c, f, and h) 20 μm; (d, e, k, and l) 10 μm; (m and n) 6 μm; (g, i, and j) 5 μm; (o and p) 4 μm.
nodules, several epithelia, neural and connective tissues, were negative with R4A (Fig. 5). Analysis of different species evolutionary as distant as the teleost Oreochromis mossambicus and human showed that R4A reacted with smooth muscle cells of these species, but no reaction was found with mouse, hamster, and rat smooth muscle cells.

In smooth muscle tissue explants of uterine myoma, the smoothelin mRNA concentration dropped sharply after transfer of tissue into culture medium. Smoothelin mRNA was no longer detectable by Northern blotting 12 h after removal of the tissue from the patients (Fig. 6). However, immunohistochemical and Western blot analysis of these tissue explants revealed that smoothelin protein remained present in the tissue up to 5 d after explantation (data not shown). The reduction in smoothelin mRNA and protein cannot be attributed to degradation or necrosis of the tissue since GAPDH mRNA and proteins such as vimentin, desmin, and α-smooth muscle actin did not diminish. No smoothelin mRNA could be detected in (primary) cultures of cells derived from smooth muscle tissue (human myoma, human colon, and bovine aorta) or in long-term cultured human vascular smooth muscle cells.

Subcellular Localization and Organization of Smoothelin

After homogenization and differential centrifugation, smoothelin immunoreactivity was found in the 1,000 g and Triton X-100 pellet, the same fraction in which cytoskeletal proteins such as desmin and actin were present. Immunohistochemical analysis of several tissues revealed that smoothelin was abundantly present throughout smooth muscle tissues, but close observation of individual cells indicated an uneven distribution over the cytoplasm. Confocal laser scanning microscopy (CSLM) of tissue sections of arteries, myoma, and colon as well as primary embryonic chicken gizzard cells with doubled staining for either smoothelin/desmin or smoothelin/α-smooth muscle actin, indicated that smoothelin appeared to be organized in or be part of a filamentous structure (Fig. 7). From the superposed pictures it was obvious that desmin and smoothelin showed no colocalization in both human smooth muscle tissues or chicken primary cells (Fig. 7, b and d). Also, little or no colocalization has been observed between smoothelin and α-smooth muscle actin in tissues (Fig. 7 a). However, smoothelin colocalized with the stress fibers of the primary embryonic chicken cells (Fig. 7 c).

Since synthesis of smoothelin ceased in smooth muscle cell lines and in primary cultures of smooth muscle cells, transfection experiments were set up to reveal the cellular organization of smoothelin. Transfection of the smoothelin cDNA driven by a CMV promotor (pcDNA3-SMO) into human primary smooth muscle cells revealed a low level of smoothelin synthesis and a staining pattern that contained both filamentous and stress fiber-like elements (Fig. 8 e). However, the numbers of transfected cells were low and the filamentous pattern is difficult to see because of the small size of these cells. Transfection of the construct into rat heart-derived myoblasts resulted within 24 h in an abundant expression of smoothelin. Smoothelin appeared strongly associated with stress fibers (Fig. 8 d). However, a relatively weak filamentous pattern was observed in addition to the much stronger stained stress fibers. A clearly filamentous pattern was observed in transfected COS7 cells, 24 h after transfection. In ~5% of the cells, extensive filamentous networks reactive with R4A were found (Fig. 8 a). Observation of the cells after longer periods of transient smoothelin expression showed accumulation of R4A immunoreactive material in the cytoplasm, but no obvious changes in morphology of the cells were seen. Double-immunostaining of the transfected COS7 cells showed that smoothelin does not colocalize with vimentin (Fig. 8, b and c) or keratin intermediate filaments nor with F-actin. Networks formed by tubulin and smoothelin differed considerably, which was most obvious for mitotic cells containing spindles. These results do not elucidate to what extent differences in smoothelin patterns can be retraced to overproduction due to the CMV promotor or to intrinsic differences among the cell lines. As mentioned above, Western blotting of pcDNA3-SMO-transfected cells showed the synthesis of a 59-kD protein, reactive with R4A (Fig. 3). In cells transfected with the pcDNA3 plasmid, no R4A reactive material was found in immunohistochemical and Western blot assays.

Discussion

Although smooth muscle and striated muscle cells share some structural proteins, such as desmin, or synthesize closely related isoforms of proteins, as for actin and myosin, large differences have been demonstrated in the construction and physiology of their contractile apparatus. For example, structural proteins such as titin and nebulin, that are part of the contractile apparatus in striated muscle cells, are not found in smooth muscle cells, nor have structurally comparable molecules been identified as yet. However, a number of smooth muscle–specific proteins, such as calponin, SM-22, α-caldesmon, and metavinculin have been identified and characterized (Lees-Miller et al., 1987; Takahashi et al., 1988; Nagai et al., 1989; Frid et al., 1992; Owens, 1995). Several of these smooth muscle–specific proteins are associated with actin and myosin, i.e., the con-
tractile apparatus, but so far no structural proteins exclusively expressed in smooth muscle cells have been identified. A novel, smooth muscle-specific protein is presented which exhibits a filamentous organization and appears to be expressed in contractile type cells only. The protein has been designated smoothelin.

**Characterization of Smoothelin**

The nucleic acid and protein sequences of smoothelin have been elucidated and its secondary structure is predicted by computer analysis. The molecular weight as estimated from the cDNA open reading frame is 40 kD which is ~20 kD smaller than the size of the protein as detected by Western blotting after SDS-PAGE (Fig. 3). If the full-size, 1,593 bases, cDNA is ligated into a prokaryotic expression vector and translated in bacteria an ~60-kD protein is found on SDS-PAGE gels, which is ~10 kD larger than the calculated molecular weight (~50 kD). This increase of ~10 kD in apparent molecular weight is probably accounted for by a decreased SDS-binding capacity because of conformational properties of the alanine-proline sequences and the rigidity of the proline-rich region (Bersbers et al., 1983; Vaughan et al., 1993). Posttranslational additions have to account for the other 10-kD increase of molecular weight found in eukaryotic cells. The presence of two asparagine-linked N-glycosylation sites and the result of the neuramidase treatment are in agreement with this explanation.

Computer-assisted structure analysis of the putative amino acid sequence revealed potential helical structures but they are relatively small compared to those found in intermediate filament proteins such as desmin and vimentin (Quax-Jeukens et al., 1983). The proline residues are found on both sides of the first helical structure and show a major concentration in a proline-rich region in the center of the protein. This proline-rich region is located between the second potential helical domain of smoothelin and the region homologous to the spectrin family (see below). The helical domains may be involved in formation of filamentous structures by coiled-coil interaction as described for intermediate filament proteins. The observations on the primary embryonic chicken gizzard cells indicate a filamentous structure for smoothelin. Also, transfection of smoothelin cDNA results in the formation of filaments in COS7 cells and to a lesser extent in human primary smooth muscle cells. This is in agreement with the results of the CSLM and the fractionation studies of smooth muscle tissues, suggesting that smoothelin is part of a filamentous cytoskeletal/structural network. Nevertheless, there still is a possibility that the structures observed are the result of association of smoothelin with existing cytoskeletal or membrane-associated structures. Double staining immunofluorescence assays in transfected COS7 cells indicated that colocalization with intermediate filaments formed by desmin, keratin, and vimentin or with microtubules could be excluded, since staining patterns are largely different. Interaction of smoothelin with microfilaments needs further examination because of their colocalization in primary embryonic chicken cells and myoblast, and the presence of a potential actin-binding site in the protein sequence (see below). The results of these experiments should be interpreted with caution since (a) in the primary chicken gizzard cells the stress fibers are considered to be a culturing artifact, and (b) in the transfected cells the CMV promoter driven expression may result in such high cellular concent-

**Figure 7.** Confocal laser scanning microscopy of double-stained sections of human artery (a and b) and primary embryonic chicken gizzard cells (c and d). Cells and tissues were immunostained for smoothelin (red) and α-smooth muscle actin (a and c) or desmin (b and d) (green). In a–c, the position of the nucleus is indicated by blue staining (DAPI).
Figure 8. Immunofluorescence micrographs of COS7 cells transfected with the full-size human smoothelin cDNA. Transfected cells clearly show filamentous structures reactive with anti-smoothelin R4A (a). Double immunofluorescence staining of COS7 cells with R4A (b) and anti-vimentin (pVim) (c). Transfection of the smoothelin cDNA into rat heart-derived myoblasts showed colocalization with stress fibers (d) whereas transfection of rat smooth muscle cells showed a colocalization with stress fibers as well as filament formation (e). Bars: (a) 1 μm; (b and c) 3 μm; (d and e) 4 μm.
tations of smoothelin that associations may be formed which will not occur under physiological conditions.

Southern blotting and in situ hybridization (data not shown) indicate that no sequences with major homologies to the smoothelin cDNA are present in the human genome. However, a 56-amino acid sequence near the carboxy terminal domain of smoothelin shows homology with dystrophin, utrophin, b-spectrin, and a-actinin. In these members of the spectrin family this sequence is part of the amino-terminal domain, that has been demonstrated to bind to actin filaments (Winkelman et al., 1990; Baron et al., 1987; Dhermy, 1991; Winder et al., 1995). According to Karinch and coworkers (1990) a highly homologous 140-amino acid/16.5-kD fragment of this domain contains the actual-binding site(s) for actin. Other investigators have appointed three short sequence fragments within this 140-amino acid sequence as being involved in actin binding (Winder et al., 1995). However, among the members of the spectrin family the homology extends further towards the carboxy terminal for at least another 80-amino acid residues (Koening et al., 1988; Winkelman et al., 1990; Beggs et al., 1992; Tinsley et al., 1992). Smoothelin has a 56-amino acid sequence homology within this 80-amino acid domain (Fig. 2B). Taking into account the uncertainty of the actual sequences involved in actin binding by members of the spectrin family (Winder et al., 1995), the 56-amino acid sequence of smoothelin may possibly function as a (truncated) actin-binding domain. The association with stress fibers in the embryonic chicken gizzard cells, in the transfected heart-derived myoblasts and (to a lesser extent) in human primary smooth muscle cells appears to support the concept of an extended actin-binding sequence.

Expression of Smoothelin

Smoothelin is only expressed in fully differentiated smooth muscle cells. Expression has been found in smooth muscle tissue of all vertebrate species investigated. The apparently high level of evolutionary conservation indicates that smoothelin is an important constituent of smooth muscle cells. In addition, its expression appears to be more stringently regulated as compared to other smooth muscle-specific proteins. Smooth muscle cell components such as metavinculin, calponin, smooth muscle a-actin, and smooth muscle myosin isoforms have been observed in nonsmooth muscle cells in embryos, in culture, and in pathologically affected striated muscle tissue (Babai et al., 1990; McHugh et al., 1991; Ausma et al., 1995). Also, most of these proteins have been found in proliferative stages of smooth muscle cells, whereas smoothelin has been detected only in fully differentiated cells. Preliminary results in chicken embryos show that smoothelin appears relatively late during embryogenesis (around Hamburger/Hamilton stage 36) and has only been observed in smooth muscle tissue.

Several authors have suggested that smooth muscle cells can be divided into two phenotypes: the contractile and the proliferating/synthetic smooth muscle cell type (Campbell and Chamley-Campbell, 1981; Campbell et al., 1989). Phenotype switches between these stages of differentiation have been suggested, but variation amongst smooth muscle cells derived from different organs and the lack of reliable differentiation markers have hampered investigations in this matter. Cultured smooth muscle cells and smooth muscle cell lines belong by definition to the proliferating phenotype. Our results showed that smoothelin is not expressed in cultured smooth muscle cells. The embryonic chicken gizzard cells are the exception but they express smoothelin only during a limited period of time after dispersion of the tissue. The outcome from the experiments with the myoma explants too shows a shut-down of smoothelin transcription as soon as tissue integrity is disturbed. The rapid drop in smoothelin mRNA concentration after explantation indicates that expression of the smoothelin gene depends on factors, autocrine or bloodborne, or on continued (neural) stimulation. Smoothelin filaments can be detected 4 or 5 d after explantation, indicating a rather slow turnover of the protein once it has been incorporated into a filamentous structure. Breakdown of smoothelin appears to be faster in primary cell culture. The results of our observations suggest a relation between smoothelin expression and contractile potential. Screening of blood vessels of different size and structure indicates that smoothelin is only found in vessels with contractile properties (Van der Loop, 1996). Thus, different from other smooth muscle-specific proteins which appear to be expressed in both proliferative and contractile smooth muscle cells, smoothelin appears to be specific for contractile cells and may be a marker for this stage of differentiation.

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