Smoothelin, a Novel Cytoskeletal Protein Specific for Smooth Muscle Cells

Frank T.L. van der Loop, Gert Schaart, Erika D.J. Timmer, Frans C.S. Ramaekers, and Guillaume J.J.M. van Eys

Department of Molecular Cell Biology & Genetics, Cardiovascular Research Institute Maastricht, University of Limburg, Maastricht, The Netherlands

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; Babai 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

Address all correspondence to G. van Eys, Department of Molecular Cell Biology & Genetics, University of Limburg, P.O.Box 616, 6200 MD Maastricht, The Netherlands. Tel.: 31 43 388 1355. Fax: 31 43 3670948. E-mail: vaneys@molcelb.rulimburg.nl

EMBL accession number: Z49989

et al., 1987; Takahashi et al., 1988; Nagai et al., 1989; Frid et al., 1992). However, they do occasionally occur in other cell types. α-Smooth muscle actin expression is also found in developing striated muscle cells and in pathologic cardiac muscle (Babai et al., 1990; McHugh et al., 1991; Ausma et al., 1995). Marker proteins, such as calponin, metavinculin, and isoforms of myosin, have also been found in pericytes, platelets, and endothelial cells (Turner and Burridge, 1989; Nagai et al., 1989; Borrione et al., 1990; Takeuchi et al., 1991). Taken together it can be stated that expression of most of these marker proteins is observed in rather early, i.e., proliferative stages of differentiation and so far only smooth muscle myosin heavy chain isoforms may function as markers for the contractile phenotype of smooth muscle (Owens, 1995).

Here we describe the cloning of a cDNA coding for a smooth muscle–specific protein, which has been characterized and designated smoothelin. The protein is expressed in cells of smooth muscle tissues investigated, but only when these cells are fully differentiated and are part of contractile tissue. Smoothelin forms a filamentous network, if transfected into COS7 or smooth muscle cells. However, it does not belong to one of the known families of structural proteins. It may be a valuable addition to markers used for the assessment of the state of differentiation of smooth muscle cells.

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 *Oreochromus mossambicus*) 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/pancreatin: 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 explants 1-mm thick tissue slices were incubated as described for the cell cultures.

For immunohistochemistry tissues were mounted in Tissue-Tek (OCT-compound; Miles Inc. Elkhart, IN), and 3–5-μm thick sections were cut at −25°C and air-dried overnight at room temperature or immediately fixed with methanol (at −20°C for 5 min) followed by acetone (−20°C for 30 s) and air-dried for 3 h before use. Cells were grown on coverslips and also fixed in methanol/acetone.

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/Triton 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 antiserum (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 for 5 min with 0.5% Triton X-100 (BDH Chemicals Ltd., Poole, UK) in PBS (137 mM NaCl, 13 mM $Na_2HPO_4.2H_2O$, 3 mM KH_2PO_4, pH 7.4; Merck, Darmstadt, Germany), followed by a PBS washing step for 5 min. Methanol/acetone 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-Ig-TR/goat anti–mouse-IgG1-TR; SBA), or horseradish peroxidase (peroxidase-conjugated rabbit anti–mouse; DAKO A/S, Copenhagen, Denmark). The secondary antibodies were applied for 30 min at room temperature. Peroxidase activity was detected with 4-amino-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 glycerin-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 neuramidase (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.

For one-dimensional SDS-PAGE using a Mini Protean II Electrophoresis Cell (Bio-Rad Laboratories) 7.5–10% polyacrylamide slab gels containing 0.1% SDS (Laemmli, 1970) were used. Two-dimensional nonequilibrium gel electrophoresis (pH gradient 3.5–10) was performed according to O'Farrell (1975). After electrophoretic separation, the proteins were stained with Page Blue 83 (BDH Chemicals Ltd.) or subjected to Western blotting as described elsewhere (Schaart et al., 1991). Culture supernatant of monoclonal antibody R4A directed against smoothelin was used in a 1:5 dilution and immunodetection with horseradish peroxidase–conjugated rabbit anti–mouse-Ig (DAKO A/S) was performed according to standard procedures (Schaart et al., 1991).

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 (Altschul 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&S, 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 0.1× SSC/0.1% SDS. Probes were ^{32}P-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 10^6 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 phalloidine 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 pI 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

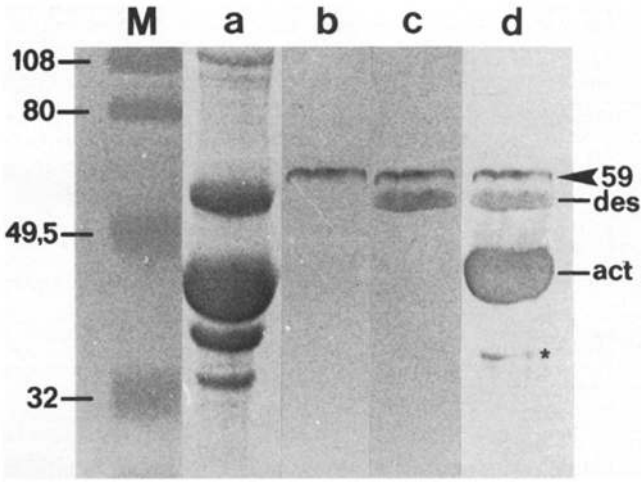

Figure 1. Western blot showing the immunoreaction of a human colon smooth muscle preparation after subsequent incubations of the same blot with monoclonal antibodies R4A (lane *b*), anti-desmin RD301 (lane *c*), and anti-smooth muscle actin sm-1 (lane *d*). Lane *a* shows the Coomassie Brilliant blue staining of the preparation. Marker lane is indicated by *M*. Asterisk indicates a degradation product of actin.

A: Sequence of smoothelin cDNA.

```
5'GGCACGAGCCCGCTGCCCGTGGCCGTCGCACTGCCGAGCCAGGGGGCAGTATGAAGACCACATTCACCATCGAGATCAAG  80
  GACGGCCGTGGCAGGCCTCCACAGGCCGGGTGCTGCTGCCCACAGGCAACCAGAGGGCAGAACTGACACTGGGGCTGCGG 160
  GCGCCCCGACCCTACTCAGCACCAGTAGTGGGGCAAGAGCACCATCACCCGTGTCAACAGCCCTGGGACCCTGGCTCGGC 240

                                                                        met glu    2
  TGGGCAGTGTCACTCATGTCACCAGCTTCAGCCATGCCCCCCCCAGTAGCCGAGGAGGCTGCAGCATCAAG ATG GAA  317

  pro glu pro ala glu pro leu ala ala ala val glu ala ala asn gly ala glu arg ala   22
  CCA GAG CCA GCA GAG CCT CTC GCT GCA GCA GTG GAA GCG GCC AAT GGG GCT GAG CGA GCC  377

  arg val asn lys ala pro glu gly arg arg leu ser ala glu glu leu met thr ile glu   42
  CGA GTG AAC AAA GCA CCA GAA GGG CGG CGT CTG AGC GCT GAG GAG CTG ATG ACT ATT GAG  437

  asp glu gly val leu asp lys met leu asp gln ser thr asp phe glu glu arg lys leu   62
  GAT GAA GGA GTC TTG GAC AAG ATG CTG GAT CAG AGC ACG GAC TTT GAA GAG CGG AAG CTC  497

  ile arg ala asp phe val ser ser asp lys gly arg glu thr ser gly thr arg ser gly   82
  ATC CGG GCT GAC TTC GTG AGC TCN GAC AAA GGA AGA GAG ACC AGC GGG ACA AGG AGC GGG  557

  asn gly gly cys arg arg his gly ala gly gln gly arg gly ala ala thr gln pro leu  102
  AAC GGC GGC TGC AGG AGG CAC GGG GCC GGC CAG GGG AGG GGC GCG GCA ACA CAG CCA CTG  617

  arg pro pro arg gly thr ala ala gly ser asp gly ser ala val ser thr val thr lys  122
  AGA CCA CCA CGA GGC ACA GCA GCG GGC AGC GAT GGC TCT GCT GTC AGC ACT GTT ACC AAG  677

  thr glu arg leu val his ser asn asp gly thr arg thr ala arg thr thr thr val glu  142
  ACT GAG CGG CTC GTC CAC TCC AAT GAT GGC ACA CGG ACG GCC CGC ACC ACC ACA GTG GAG  737

  ser ser phe val arg arg ser glu asn gly ser gly ser thr met met glu thr lys thr  162
  TCG AGT TTC GTG AGG CGC TCG GAG AAT GGC AGT GGC AGC ACC ATG ATG CAA ACC AAG ACC  797

  phe ser ser ser ser ser ser lys lys met gly ser ile phe asp arg glu asp gln arg  182
  TTC TCC TCT TCC TCC TCA TCC AAG AAG ATG GGC AGC ATC TTC GAC CGC GAG GAC CAG CGA  857

  ala thr gly arg his gly arg leu glu ser glu lys arg gln ala glu lys lys lys glu  202
  GCC ACG GGC CGC CAT GGC CGG CTC GAG AGT GAG AAA CGG CAG GCC GAG AAG AAG AAA GAG  917

  leu met lys ala gln ser leu pro lys thr ser ala ser gln ala arg lys ala met ile  222
  CTG ATG AAG GCG CAG AGT CTG CCC AAG ACC TCA GCC TCC CAG GCG CGC AAG GCC ATG ATT  977

  glu lys leu glu lys glu gly ala ala gly ser pro ala asp pro ala gln pro cys ser  242
  GAG AAG CTG GAG AAG GAG GGC GCG GCC GGC AGC CCT GCG GAC CCC GCG CAG CCG TGC AGC 1037

  asp pro pro ala ser gly ser pro thr pro thr ala ser ser arg ser ser trp thr gly  262
  GAT CCA CCA GCT TCG GGG TCC CCA ACG CCA ACA GCA TCA AGC AGA TCG TCG TGG ACT GGT 1097

  val glu pro arg leu gly ala tyr glu his val asp ile gln asn phe ser ser ser trp  282
  GTC GAG CCA AGA CTC GGG GCC TAC GAG CAC GTC GAC ATC CAG AAC TTC TCC TCC AGC TGG 1157

  ser asp gly met ala phe cys ala leu val his asn phe phe pro glu ala phe asp tyr  302
  AGT GAT GGG ATG GCC TTC TGT GCC CTG GTG CAC AAC TTC TTC CCT GAG GCC TTC GAC TAT 1217

  gly gln leu ser pro gln asn arg arg gln asn phe glu val ala phe ser ser ala glu  322
  GGG CAG CTT AGC CCT CAG AAC CGA CGC CAG AAC TTC GAG GTG GCC TTC TCA TCT GCG GAG 1277

  met leu val asp tyr val pro leu val glu val asp asp met met ile met gly lys lys  342
  ATG CTG GTG GAC TAT GTG CCC CTG GTG GAG GTG GAC GAC ATG ATG ATC ATG GGC AAG AAG 1337

  pro asp pro lys cys val phe thr tyr val gln ser leu tyr asn his leu arg arg his  362
  CCT GAC CCC AAG TGT GTC TTC ACC TAT GTG CAG TCG CTC TAC AAC CAC CTG CGA CGC CAC 1397

  glu leu ala ser arg gly lys asn val ***                                         373
  GAA CTG GCC TCG CGC GGC AAG AAT GTC TAG CCTGCCCGCCCGCATGGCCAGCCAGTGGCAACTGCCGCC 1466

  CCCACTCTCCGGGCACCGTCTCCTGCCTGTGCGTCCGCCCACCGCTGCCCTGTCTGTTGCGACACCCTCCCCCCCACAT 1545
  ACACACGCAGCGTTTTGATAAATTATTGGTTTTCAACG(AAAAAAAAAAAAAAAAA)                      1593
```

B: Amino acid homologies.

protein	*position*	*sequence*	*homology*
human smoothelin	270	**YEHVDIQNFSSSWSDGMAFCALVHNFFPEAFDYGQLS-PQNRRQNFEVAFSSAEMLV**	
human beta-spectrin	190	**YPHVNVTNFTSSWKDGLAFNALIHKHRPDLIDFDKLK-DSNARHNLEHAFNVAERQL**	46%
human alpha-actinin	180	**YKNVNIQNFHISWKDGLGFCALIHRHRPELIDYGKLR-KDDPLTNLNTAFDVAEKYL**	45%
human utrophin	166	**YSQVNVLNFTTSWTDGLAFNAVLHRHKPDLFSWDKVV-KMSPIERLEHAFSKAQTYL**	34%
human dystrophin	151	**YPQVNVINFTTSWSDGLALNALIHSHRPDLFDWNSVVCQQSATQRLEHAFNIARTQL**	35%

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 utropin, and human dystrophin. Homologies are indicated by bold letters.

isolated contained sequences similar to the four largest clones, as deduced from restriction patterns. The complete nucleotide sequence and deduced amino acid sequence are presented in Fig. 2 *A*. To check whether the largest clone contained a full-size cDNA, a 5′RACE reaction was performed. No additional sequence was found which indicated that the 1,593-bp clone contained the full-length cDNA. The 1,593-bp cDNA contains a 1,113-bp open reading frame that encodes a putative protein of 371 amino acids. Other open reading frames were much shorter. Smoothelin has a relatively high number of serine (11.5%), alanine (10%), arginine (8.4%), and methionine (3.5%) residues. The calculated molecular weight of the protein, as deduced from the largest open reading frame of smoothelin, is 40.406 kD with a pI of 8.5.

Western blotting of smoothelin cDNA transfected COS7

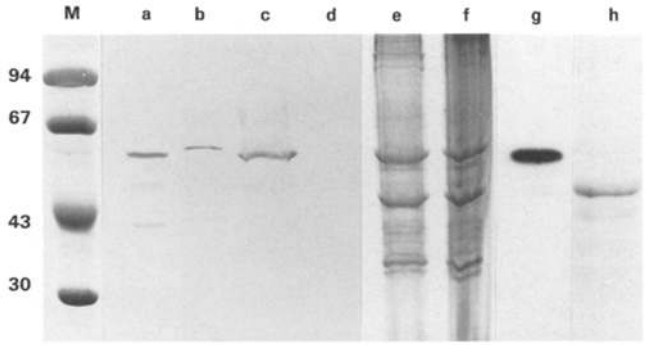

Figure 3. R4A immunostained Western blot of human colon smooth muscle extract (lane *a*), prokaryotic product based on the full-size cDNA in pQE9 (lane *b*), COS7 cells transfected with pcDNA3-SMO (lane *c*), and COS7 cells transfected with pcDNA3 (lane *d*). Lanes *e* and *f* are Coomassie Brilliant 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*.

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, β-spectrin, and α-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 β-spectrin, and 34% for human utropin. Homologies with α-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

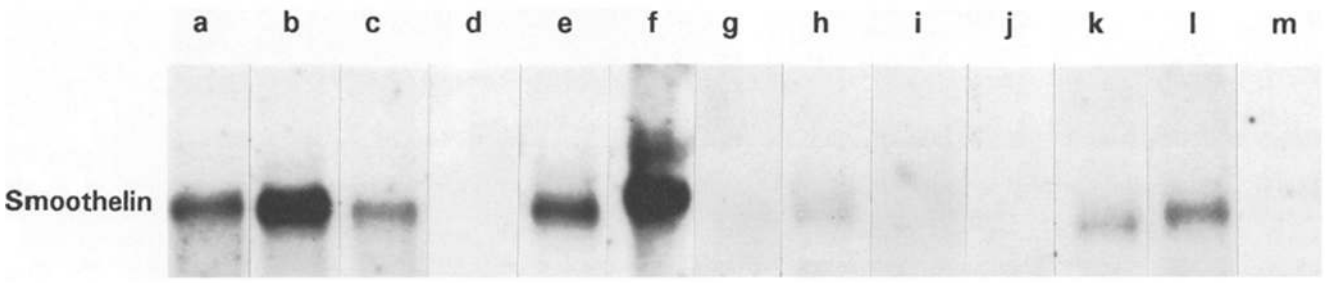

Figure 4. Northern blot of a number of different tissues obtained from several species. Equal amounts of total RNA are applied to the gel. All RNAs hybridizing to the ^{32}P-labeled smoothelin cDNA probe appear to be of the same size. RNAs were extracted from the following tissues: mouse stomach (*a*), 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*).

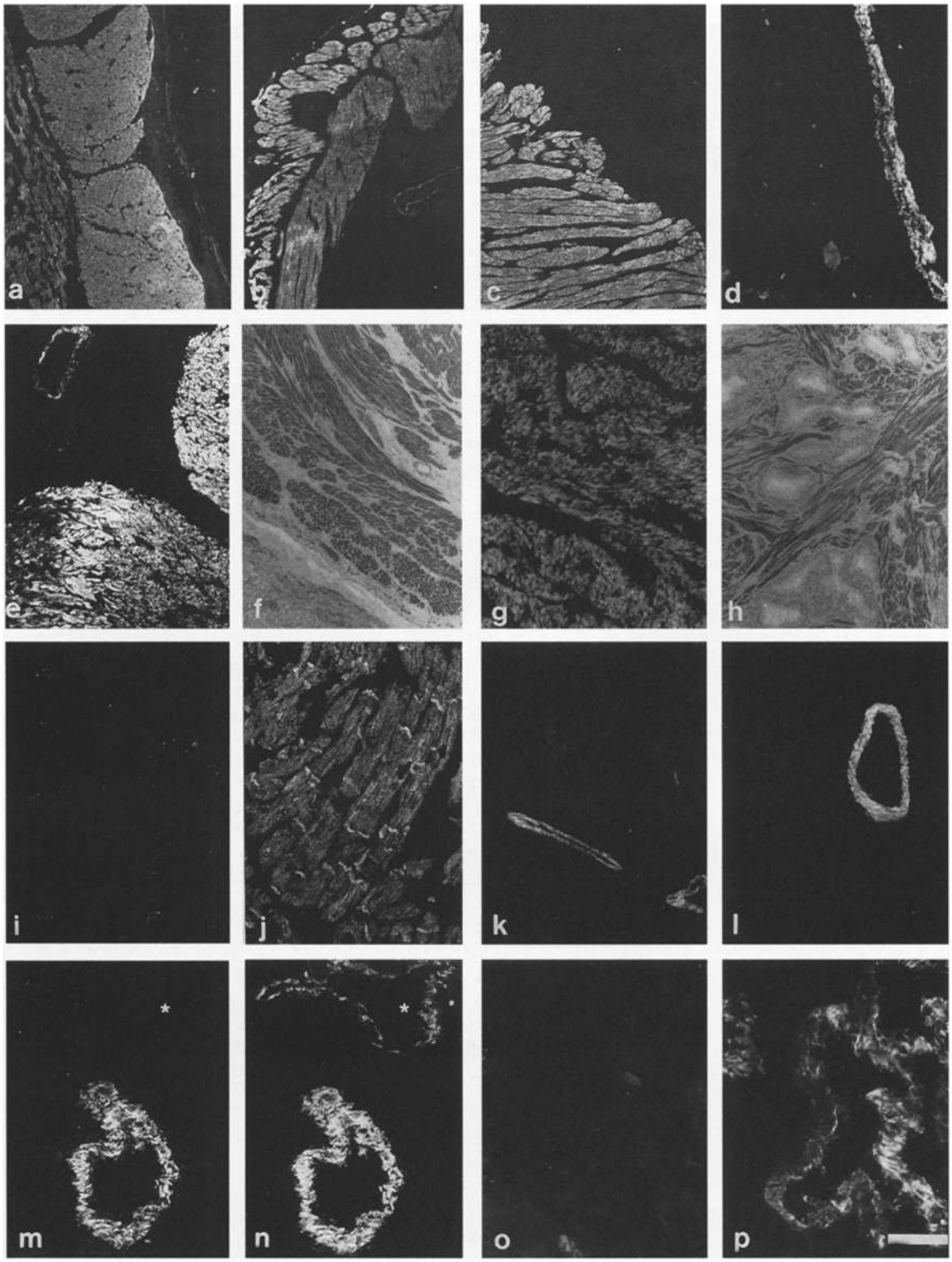

Figure 5. Immunohistochemical screening of different species and tissues for the presence of smoothelin. Intestinal smooth muscle tissue of human (*a*), goat (*b*), *Xenopus laevis* (*d*), and chicken oesophagus (*c*) were all positive for R4A. In human large intestine (*e*), stomach (*f*), uterus (*g*), 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 found. 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/a-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*).

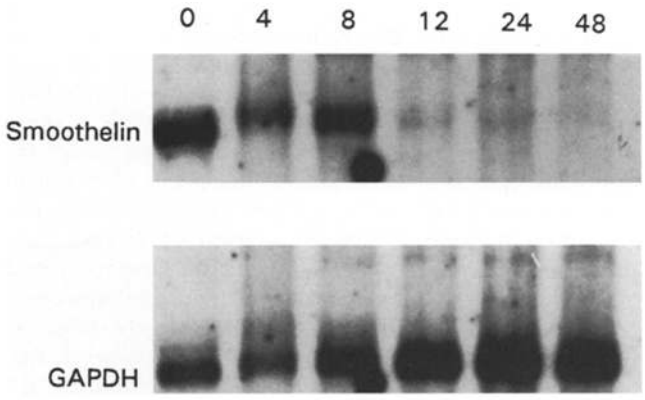

Figure 6. Northern blot of RNAs extracted from human myoma explants kept in a culture medium for 0, 4, 8, 12, 24, and 48 h. Hybridization with smoothelin (*upper panel*) and GAPDH (*lower panel*) cDNAs shows that smoothelin mRNA disappears between 8 and 12 h after removal of the tissue from its natural environment. Hybridization with GAPDH cDNA demonstrates that this is not due to RNA degradation.

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, h-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-

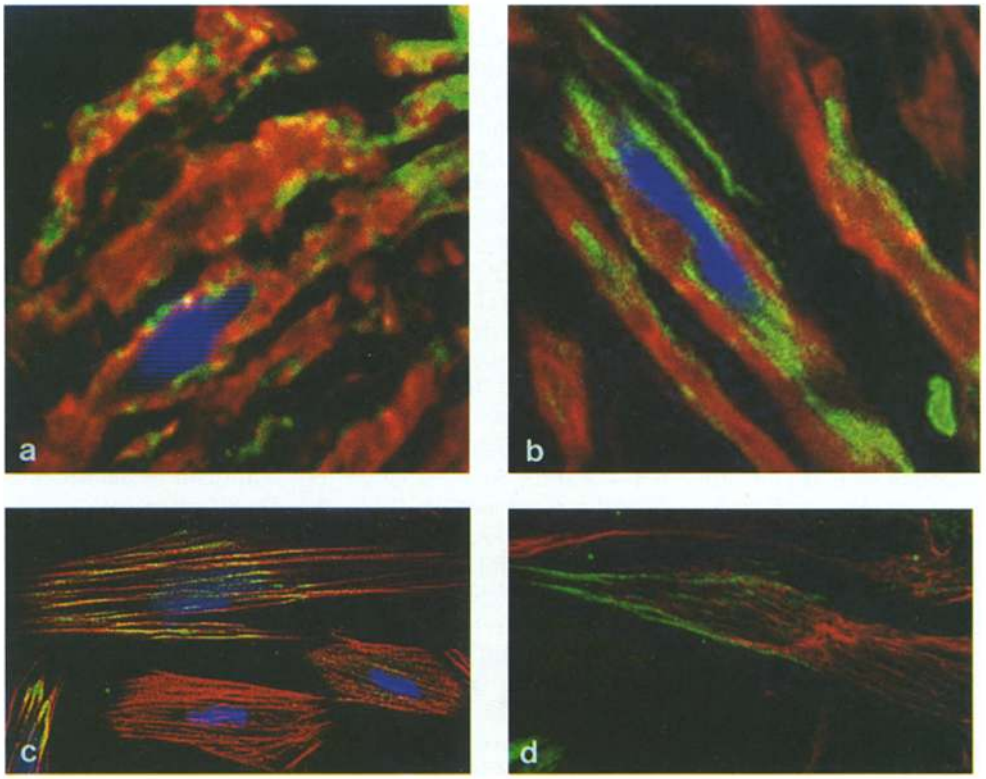

Figure 7. Confocal laser scanning microscopy of double-stained sections of human artery (*a* and *b*) and primary embryonic chicken gizzard cells (*c* and *d*). Cells and tissues were immunostained for smoothelin (*red*) and α-smooth muscle actin (*a* and *c*) or desmin (*b* and *d*) (*green*). In *a–c*, the position of the nucleus is indicated by blue staining (DAPI).

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 (Berbers 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-Jeuken 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 concen-

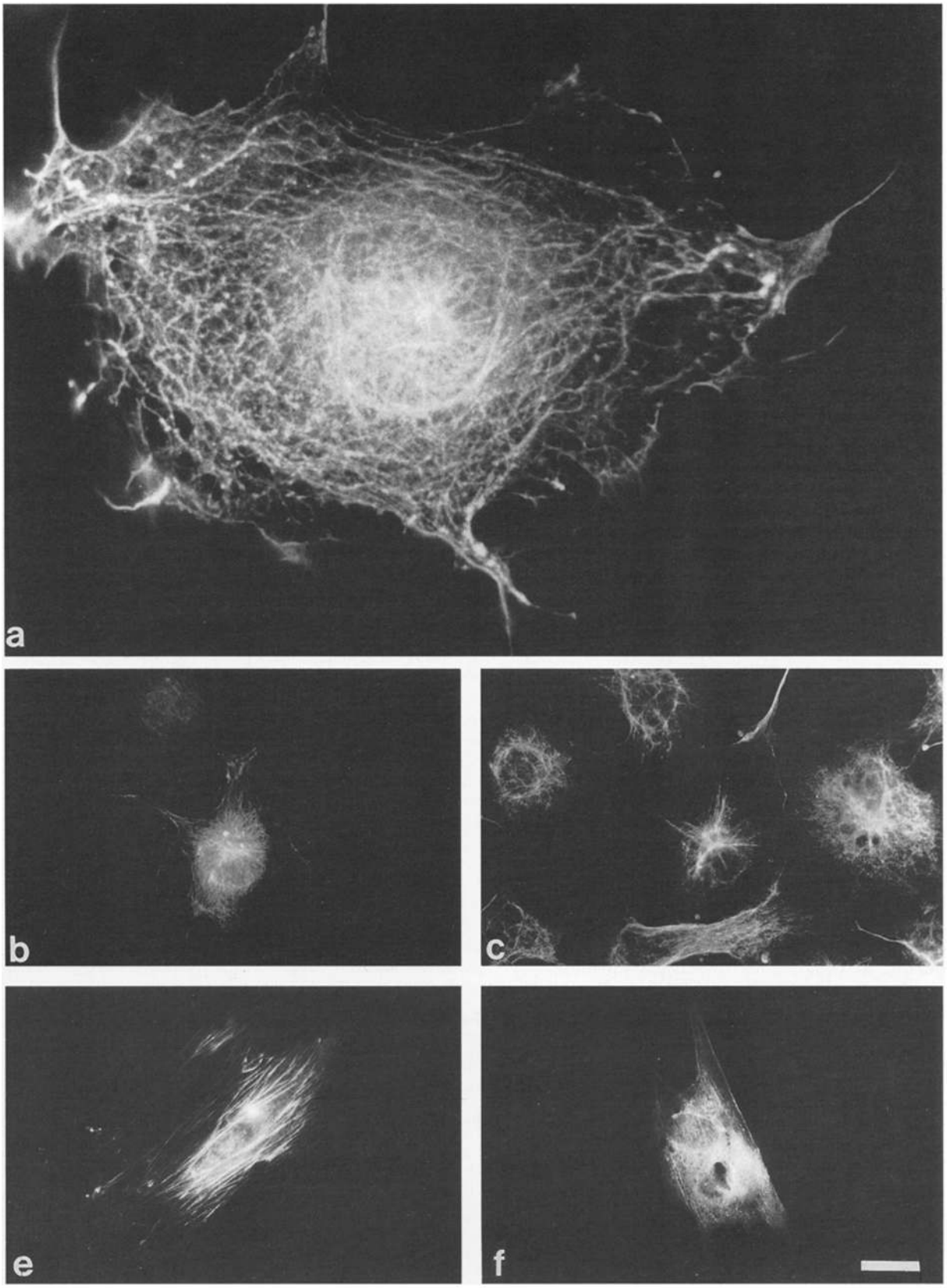

Figure 8. Immunofluorescence micrographs of COS7 cells transfected with the full-size human smoothelin cDNA. Transfected cells clearly show filamentous structures reactive with anti-smoothelin R4A (*a*). Double immunofluorescence staining of COS7 cells with R4A (*b*) and anti-vimentin (pVim) (*c*). Transfection 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.

trations 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, β-spectrin, and α-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 sequences 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. 2 *B*). 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 extend) 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 α-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.

The authors are grateful to Luc Nieland and Jack Leunissen (CAOS/CAMM Centre, Nijmegen) for assistance given with the data bank screening, to Christine Voorter and Monique Vallinga for in situ hybridization, and to Nicole Caberg and Paul Stassen for experimental contribution. We like to thank Dr. Jan-Willem Arends and Mr. Sjaak Joosten (Department of Pathology, Maastricht) for providing human and Dr. H. van Straaten (Department of Anatomy, Maastricht) for the chicken tissues. We are indebted to Dr. Guilio Gabbiani (Department of Pathology, University of Geneva) for providing the long-term cultured smooth muscle cells and human tissue samples.

This work was financially supported by the Dutch Heart Foundation (FvdL). Monoclonal antibody against β-tubulin (E7) was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD) and the Department of Biology, University of Iowa (Iowa City, IA) under contract NO1-HD-2-3144 from the NICHD.

Received for publication 11 October 1995 and in revised form 14 April 1996.

References

Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.

Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* 107:393–314.

Ausma, J., G. Schaart, F. Thoné, D. Shivalkar, W. Flameng, C. Depré, J.-L. Vanover-schelde, F. Ramaekers, and M. Borgers. 1995. Chronic ischemic viable myocardium in man: aspects of dedifferentiation. *Cardiovasc. Pathol.* 4: 29–37.

Aviv, A., and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose. *Proc. Natl. Acad. Sci. USA.* 69:1408–1412.

Babai, F., J. Musevi-Aghdam, W. Schurch, A. Royal, and G. Gabbiani. 1990. Coexpression of α-sarcomeric, α-smooth muscle actin and desmin during myogenesis in rat and mouse embryos. *Differentiation.* 44:132–142.

Baron, M.D., M.D. Davison, P. Jones, and P.R. Critchley. 1987. The sequence of α-actinin reveals homologies to spectrin and calmodulin. *J. Biol. Chem.* 262:17623–17629.

Beggs, A.H., T.J. Byers, J.H.M. Knoll, F.M. Boyce, G.A.P. Bruns, and L.M. Kunkel. 1992. Cloning and characterization of two human skeletal muscle α-actinin genes located on chromosomes 1 and 11. *J. Biol. Chem.* 267:9281–9288.

Berbers, G.A.M., W.A. Hoekman, H. Bloemendal, W.W. de Jong, T. Kleinschmidt, and G. Braunitzer. 1983. Proline- and alanine-rich N-terminal extension of the basic bovine β-crystallin B_1 chains. *FEBS Lett.* 161:225–229.

Borrione, A.C., A.M.C. Zanellato, L. Giurato, G. Scannapieco, P. Pauletto, and P. Sartore. 1990. Non-muscle and smooth muscle myosin isoforms in bovine endothelial cells. *Exp. Cell Res.* 190:1–10.

Campbell, G.R., and J.H. Chamley-Campbell. 1981. Smooth muscle phenotypic modulation: role in atherogenesis. *Med. Hypothesis.* 7:729–735.

Campbell, J.H., O. Kocher, O. Skalli, G. Gabbiani, and G.R. Campbell. 1989. Cytodifferentiation and expression of α-smooth muscle actin mRNA and protein during primary culture of aortic smooth muscle cells: correlation with cell density and proliferative state. *Atherosclerosis.* 9:633–643.

Chu, D.T.W., and M.W. Klymkowsky. 1987. Experimental analysis of cytoskeletal function in early *Xenopus laevis* embryos. *Development.* 8:140–142.

Church, G.M., and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA.* 81:1991–1995.

Dhermy, D. 1991. The spectrin super-family. *Biol. Cell.* 71:249–254.

Duband, J.-L., M. Gimona, S. Scatena, and J.V. Small. 1993. Calponin and SM22 as differentiation markers of smooth muscle markers: spatiotemporal distribution during avian embryonic development. *Differentiation.* 55:1–11.

Feinberg, A.P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6–12.

Frid, M.G., B.V. Shekhonin, V.E. Koteliansky, and M.A. Glukhova. 1992. Phenotypic changes of human smooth muscle cells during development: late expression of heavy caldesmon and calponin. *Dev. Biol.* 153:185–193.

Gabbiani, G., E. Schmid, S. Winter, C. Chaponnier, C. de Chastonay, J. Vandekerckhove, K. Weber, and W.W. Franke. 1981. Vascular smooth muscle cells differ from other smooth muscle cells: predominance of vimentin filaments and a specific a-type actin. *Proc. Natl. Acad. Sci. USA.* 78:298–302.

Glukhova, M.A., A.E. Kabakov, A.M. Belkin, M.G. Frid, O.I. Ornatsky, N.I. Zhidkova, and V.E. Koteliansky. 1986. Meta-vinculin distribution in adult human tissues and cultured cells. *FEBS Lett.* 207:139–141.

Gluzman, Y. 1981. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell.* 23:175–182.

Gunning, P., M. Gordon, R. Wade, R. Gahlmann, C.-S. Lin, and E. Hardeman. 1990. Differential control of tropomyosin mRNA levels during myogenesis suggests the existence of an isoform competition-autoregulation compensation control mechanism. *Dev. Biol.* 138:443–453.

Haeberle, J.R., D.R. Hathaway, and C.L. Smith. 1992. Caldesmon content of mammalian smooth muscles. *J. Muscle Res. Cell Motil.* 13:81–89.

Karinch, A.M., W.E. Zimmer, and S.R. Goodman. 1990. The identification and sequence of the actin-binding domain of human red blood cell β-spectrin. *J. Biol. Chem.* 265:11833–11840.

Kimes, B.W., and B.L. Brandt. 1976. Properties of a clonal muscle cell line from rat heart. *Exp. Cell Res.* 98:367–381.

Koening, M., A.P. Monaco, and L.M. Kunkel. 1988. The complete sequence of dystrophin predicts a rod shaped cytoskeletal protein. *Cell.* 53:219–228.

Köhler, G., and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature (Lond.).* 256:495–497.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the heads of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lees-Miller, J.P., D.H. Heeley, and L.B. Smillie. 1987. An abundant and novel protein of 22 kDa (SM22) is widely distributed in smooth muscle. *Biochem. J.* 244:705–709.

McHugh, K.M., K. Crawford, and J.L. Lessard. 1991. A comprehensive analysis of the developmental and tissue-specific expression of the isoactin multigene family in the rat. *Dev. Biol.* 148:442–458.

Nagai, R., M. Kuro-O, P. Babij, and M. Periassamy. 1989. Identification of two types of smooth muscle myosin heavy chain isoforms by cDNA cloning and immunoblot analysis. *J. Biol. Chem.* 264:9734–9737.

Nanaev, A.K., V.P. Shirinsky, and K.G. Birukov. 1991. Immunofluorescent study of heterogeneity in smooth muscle cells of human fetal vessels using antibodies to myosin, desmin, and vimentin. *Cell Tissue Res.* 266:535–540.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of protein. *J. Biol. Chem.* 230:4007–4021.

Owens, G.K. 1995. Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* 75:487–517.

Pless, D.D., and W.J. Lennarz. 1977. Enzymatic conversion of proteins to glycoproteins. *Proc. Natl. Acad. Sci. USA.* 74:134–138.

Quax-Jeuken, Y.E.F.M., W.J. Quax, and H. Bloemendal. 1983. Primary and secondary structure of hamster vimentin predicted from the nucleotide sequence. *Proc. Natl. Acad. Sci. USA.* 80:3548–3552.

Ramaekers, F.C.S., J.J.G. Puts, O. Moesker, A. Kant, P.H.K. Jap, C. Herman, and G.P. Vooijs. 1983. Antibodies to intermediate filament proteins in the immunohistochemical identification of human tumours: an overview. *Histochem. J.* 15:691–713.

Ramaekers, F.C.S., O. Moesker, A. Huijsmans., G. Schaart, G. Westerhof, Sj.Sc. Wagenaar, C.J. Herman, and G.P. Vooijs. 1985. Intermediate filament proteins in the study of tumor heterogeneity: an in-depth study of tumors of the urinary and respiratory tracts. *Ann. NY Acad. Sci.* 455:614–634.

Rost, B., and C. Sander. 1993. Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* 232:584–599.

Rost, B., and C. Sander. 1994. Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins.* 19:55–77.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanger, F., A.R. Coulson, B.G. Barrell, A.J.H. Smith, and B.A. Roe. 1980. Cloning a single stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* 143:161–178.

Schaart, G., F.R. Pieper, H.J.H. Kuijpers, H. Bloemendal, and F.C.S. Ramaekers. 1991. Baby hamster kidney (BHK-21/C13) cells can express striated muscle type proteins. *Differentiation.* 46:105–115.

Skalli, O., P. Ropraz, A. Trzeciak, G. Benzonana, G. Gillesen, and G. Gabbiani. 1986. A monoclonal antibody against α-smooth muscle actin: a new probe for smooth muscle differentiation. *J. Cell Biol.* 103:2787–2796.

Takahashi, K., K. Hiwada, and T. Kokubu. 1988. Vascular smooth muscle calponin: a novel T-like protein. *Hypertension.* 11:620–626.

Takeuchi, K., K. Takahashi, M. Abe, W. Nishida, K. Hiwada, T. Nabeya, and K. Maruyama. 1991. Co-localization of immunoreactive forms of calponin with actin cytoskeleton in platelets, fibroblasts and smooth muscle. *J. Biochem.* 109:311–316.

Tinsley, J.M., D.J. Blake, A. Roche, U. Fairbrother, J. Riss, B.C. Byth, A.E. Knight, J. Kendrick-Jones, G.K. Suthers, D.R. Love, et al. 1992. Primary structure of dystrophin-related protein. *Nature (Lond.).*360:591–593.

Turner, C.E., and K. Burridge. 1989. Detection of metavinculin in human platelets using a modified talin overlay assay. *Eur. J. Cell Biol.* 49:202–206.

Vaughan, K.T., F.E. Weber, S. Einheber, and D.A. Fischman. 1993. Molecular cloning of chicken myosin-binding protein (MyBP) H (86-kDa protein) reveals extensive homology with MyBP-C (C-protein) with conserved immunoglobulin C2 and fibronectin type III motifs. *J. Biol. Chem.* 268:3670-3676.

Van der Loop, F.T.L. 1996. Cell Biological Aspects of Muscle Differentiation. Ph D. Thesis. University of Limburg, Maastricht, The Netherlands. pp. 101.

Voorter, C., S. Joos, P.-P. Bringuier, M. Vallinga, P. Poddighe, J. Schalken, S. Du Manoir, S.F. Ramaekers, P. Lichter, and A. Hopman. 1995. Detection of chromosomal imbalances in transitional cell carcinoma of the bladder by comparative genomic hybridization. *Am. J. Pathol.* 146:1341–1354.

Winder, S.J., J. Himmings, S.K. Maciver, S.J. Bolton, J.M. Tinsley, K.E. Davies, D.R. Crithley, and J. Kendrick-Jones. 1995. Utropin actin binding domain: analysis of actin binding and cellular targeting. *J. Cell Sci.* 108:63–71.

Winkelman, J.C., J.-G. Chang, W.T. Tse, A.L. Scarpa, V.T. Marchesi, and B.G. Forget. 1990. Full-length sequence of the cDNA for human erythroid β-spectrin. *J. Biol. Chem.* 265:11827–11832.