Localization of smoothelin in avian smooth muscle and identification of a vascular-specific isoform

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Abstract Smoothelin is a smooth muscle-specific protein of minor abundance first identified via a monoclonal antibody obtained using an avian gizzard extract as antigen. Dual labelling of ultrathin sections with antibodies to smoothelin together with antibodies to other smooth muscle proteins showed that smoothelin was co-distributed with filamin and desmin in the cytoskeleton domain of the smooth muscle cell. From the finding that smoothelin, unlike desmin, was readily extracted by Triton X-100 as well as under conditions that solubilized myosin, β-actin and filamin, we conclude that smoothelin is most likely associated with the actin cytoskeleton. Western blot analysis of gizzard smooth muscle tissue revealed an immunoreactive protein band with an apparent molecular weight of 59 kDa that separated into 3–4 isolated variants, while avian vascular muscle showed a polypeptide band of 95 kDa. These results point to the presence of specific isoforms in visceral and vascular smooth muscles. The 59 kDa isoform was shown to be distinct from the 60 kDa filamin-binding protein, described by Maekawa and Sakai (FEBS Lett. 221, 68–72, 1987). As compared to other smooth muscle markers, such as calponin and SM22, smoothelin appeared very late during differentiation in the chick gizzard, on about the 18th embryonic day.

Key words: Smoothelin; Avian smooth muscle; Vascular-specific form

1. Introduction

Smooth muscle cells show a marked plasticity in their state of differentiation, both in vivo and in vitro (reviewed in [1]). This modulation of phenotype may play a leading role in lesions of the blood vessel wall implicating smooth muscle cells in processes leading to vascular disease [1]. However, progress in understanding the role of smooth muscle in such phenomena has been hampered in part by a lack of markers for different stages of smooth muscle cell differentiation.

Proteins characteristic of smooth muscle phenotype include smooth muscle myosin, metavinculin, heavy caldesmon and the smooth muscle actins (see review by [2]), as well as SM22 ([3]). These different protein constituents appear progressively during differentiation with the expression of myosin and caldesmon preceding that of calponin and SM22 ([1–3]).

More recently, van der Loop et al. [4] identified a protein, designated smoothelin, via a monoclonal antibody raised against a chicken gizzard extract, whose expression appeared to be restricted to highly differentiated smooth muscle cells. The protein, with an apparent molecular weight of 59 kDa, was found to be present in all vertebrate classes. The cDNA encoding for this protein has been cloned and sequenced and its putative primary structure has been deduced. To gain further insight into the possible function of smoothelin we undertook studies to determine its subcellular localization as well as its temporal appearance in gizzard smooth muscle cells. As we show, this protein is a minor component of the smooth muscle cell cytoskeleton that appears conspicuously late during differentiation. A high molecular weight variant of smoothelin, which is specifically expressed in avian vascular smooth muscle, is also described.

2. Materials and methods

2.1. Tissues and frozen sections

Freshly excised chicken gizzard from adult chicken was placed on ice and allowed to cool for at least 30 min. Thin (~1 mm) radial slices were cut from the main muscle layer using a razor blade, separated from the gizzard and transferred into cold cytoskeleton buffer (CB [5]). Narrow strips were then teased out of the slices and placed in a fixative containing 3% paraformaldehyde and 0.05% glutaraldehyde in CB for 5 min at room temperature. Alternatively, the slices were fixed directly in the same mixture and thin strips teased out after fixation. Subsequently the strips were washed several times in CB, followed by treatment with sodium borohydride (1.0 mg/ml) in the same buffer, for 3×10 min on ice to reduce free aldehyde groups, washed again in CB and transferred to the cryosectioning embedding mixture described by [6]: 20% polyvinyl pyrrolidone, 1.6 M sucrose in CB. After infiltration overnight, tissue pieces were mounted on cryopins, frozen in liquid nitrogen and sectioned on a Reichert cryomicrotome (FC 4E, Leica, Vienna), at an approximate thickness of 0.15 μm. Instead of using dry knives, sections were cut onto 40% DMSO [7] using a block temperature set at −60°C and a knife temperature set at −30°C. For glass knives, commercially available plastic boats (Leica, Vienna, Austria) were secured with superglue. Floating sections were transferred with a loop to a small, insulated reservoir (bottle cap) on the edge of the cryochamber containing a 1.2 M sucrose, 1% methylcellulose mixture [8] and then viewed under a dissecting microscope. Sections were retrieved with 4×4 mm coverslips coated with poly-L-lysine and stored floating in a petri dish containing 87% glycerol. Prior to labelling the sections were washed to remove glycerol by transferring them through several drops of CB.

2.2. Immunocytochemistry and microscopy

The monoclonal antibody R4A to smoothelin has been described by van der Loop et al. [4]. Monospecific, polyclonal antibodies against desmin, myosin S1 and filamin were as used previously [9–11]. Antibody labelling of coverslips involved the following steps: (a) preincubation on a drop (10 μl) of 2% normal goat serum, 1% bovine serum albumin (BSA, Sigma, Vienna) in Tris-buffered saline (TBS) containing 155 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 20 mM Tris-base pH 7.6 for 10–30 min; this and the subsequent antibody incubations were carried out on parafilm stretched over a glass plate mounted on moist filter paper in a 15 cm diameter plastic petri dish; (b) transfer to 10 μl

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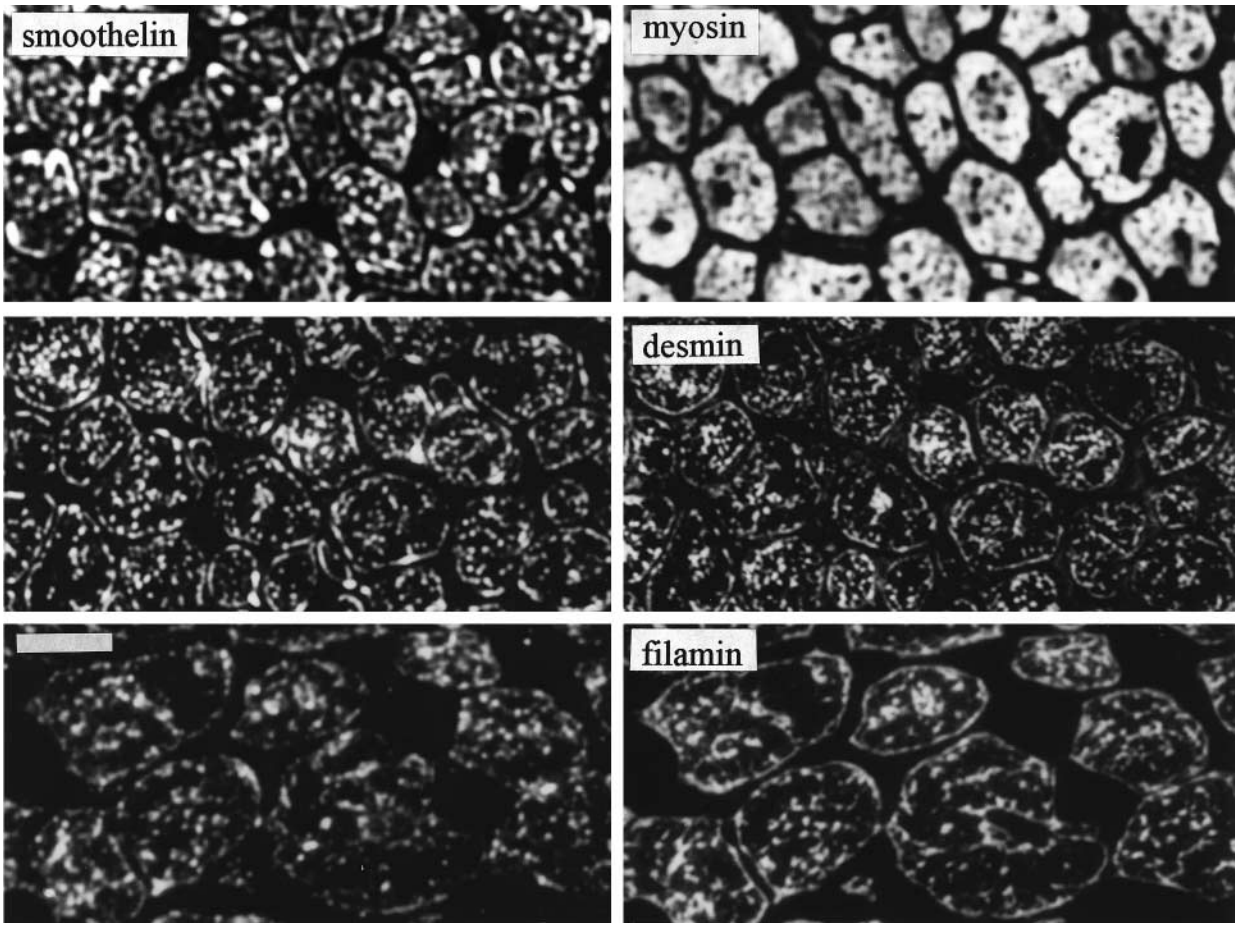

Fig. 1. Transverse ultrathin sections of chicken gizzard smooth muscle, double labelled for smoothelin (left panels) and myosin, desmin and filamin (right panels, as indicated). The smoothelin labelling pattern corresponds closely to that seen for desmin and filamin. For the lower row of photographs, sections were spread on TBS buffer, resulting in better separation of individual filaments. Bar, 5 μm.

of the primary antibody, also dissolved in 1% BSA in TBS and incubation for 45–60 min at room temperature; (c) brief rinsing by floating on TBS (by consecutive transfer onto the surface of three 10 μl drops) followed by flotation on drops of TBS for 3 times 15 min; (d) transfer to the secondary, conjugated antibody in 1% BSA in TBS on parafilm for 40 min to 1 h at room temperature; (e) rinsing and washing as described followed by mounting in Gelvatol (Vinol 203, Air Products Inc., Allentown, PA, USA) with 2.5 mg/ml *n*-propyl gallate [12] added as antibleach agent. Fluorescent labelling was performed using the biotin-streptavidin-FITC or Cy3-conjugated antibodies supplied by Amersham International (Amersham, UK) or Texas Red-conjugated secondary antibodies (Vector Laboratories Inc., Burlingame, CA). For dual labelling, the individual antibody concentrations were titrated to give quantitatively similar intensities of label for the separate staining patterns.

Semithin sections were evaluated using a Zeiss Axiovert 135TV inverted microscope equipped for epifluorescence microscopy, using a 100×/NA 1.3 Plan-Neofluar objective and up to 2.5× optivar intermediate magnification. Fluorescence images were acquired and stored as 16-bit sequences using a back illuminated, cooled CCD camera from Princeton Instruments driven by IPLabs software (both from Visitron Systems, Eichenau, Germany) on a MacIntosh Power PC7100/80.

2.3. Extraction of muscle strips

The muscle strips from freshly excised and ice-cooled chicken gizzard were cut into small pieces and homogenized sequentially in 40 ml of the following extraction buffers with intermediate centrifugation steps at 15000×*g* for 10 min: (1) 0.5% Triton X-100 in CB; (2) 60 mM KCl, 1 mM EDTA, 2 mM EGTA, 1mM dithiothreitol (DDT), 10 mM ATP, 40 mM imidazole, pH 7.1 (LAMES [13]); (3) LAMES plus 0.6 M NaCl; (4) LAMES; (5) 25 mM TRIS, 2 mM EDTA, 2 mM EGTA, 0.1 mM dithioerythritol, 2.5% sucrose, pH 9.0 (low salt, high pH). Homogenization was performed with an Ultra-Turrax blender in a 50 ml centrifuge tube and was repeated twice in fresh extraction buffer for each step. Samples of the supernatants after the first homogenization and pellets after the second were taken at each step and processed for SDS gel electrophoresis, as described previously [14].

2.4. Immunoblotting and electrophoresis

Freshly dissected pieces of either chicken aortic arch or jugular vein were frozen in liquid nitrogen, then shattered by hammering and dissolved in SDS-sample buffer [15]. Prior to gel electrophoresis, samples dissolved in sample buffer (0.05 M Tris-HCl, 0.03% glycerol, 0.01% β-mercaptoethanol, 10 mg/l bromophenol blue and 2.2 g/l SDS in 6 M urea) were boiled for 1 min to complete reaction with SDS.

Electrophoresis was carried out on minislab gels as described by Gimona et al. [14] and the gels blotted onto nylon membrane sheets (Immobilon-p transfer membrane, Millipore, Vienna) according to [16]. Antibody reactions were visualized using either the peroxidase 'Sigma Fast' system with a secondary peroxidase antibody from the chemiluminescence (ECL) kit from Amersham, or by the ECL method (Amersham, UK) according to the manufacturer's instructions. The monoclonal antibodies against calponin (CP-93), desmin (DU-10) and β-actin (AC-15) were from Sigma and the rabbit polyclonal antibodies against filamin, myosin light chain and vinculin were as described previously [11,17]. The polyclonal anti-60 kDa protein antibody was kindly provided by Dr. Shohei Maekawa (Kyoto Institute of Technology, Japan). In the case of muscle extracts, blots were reacted

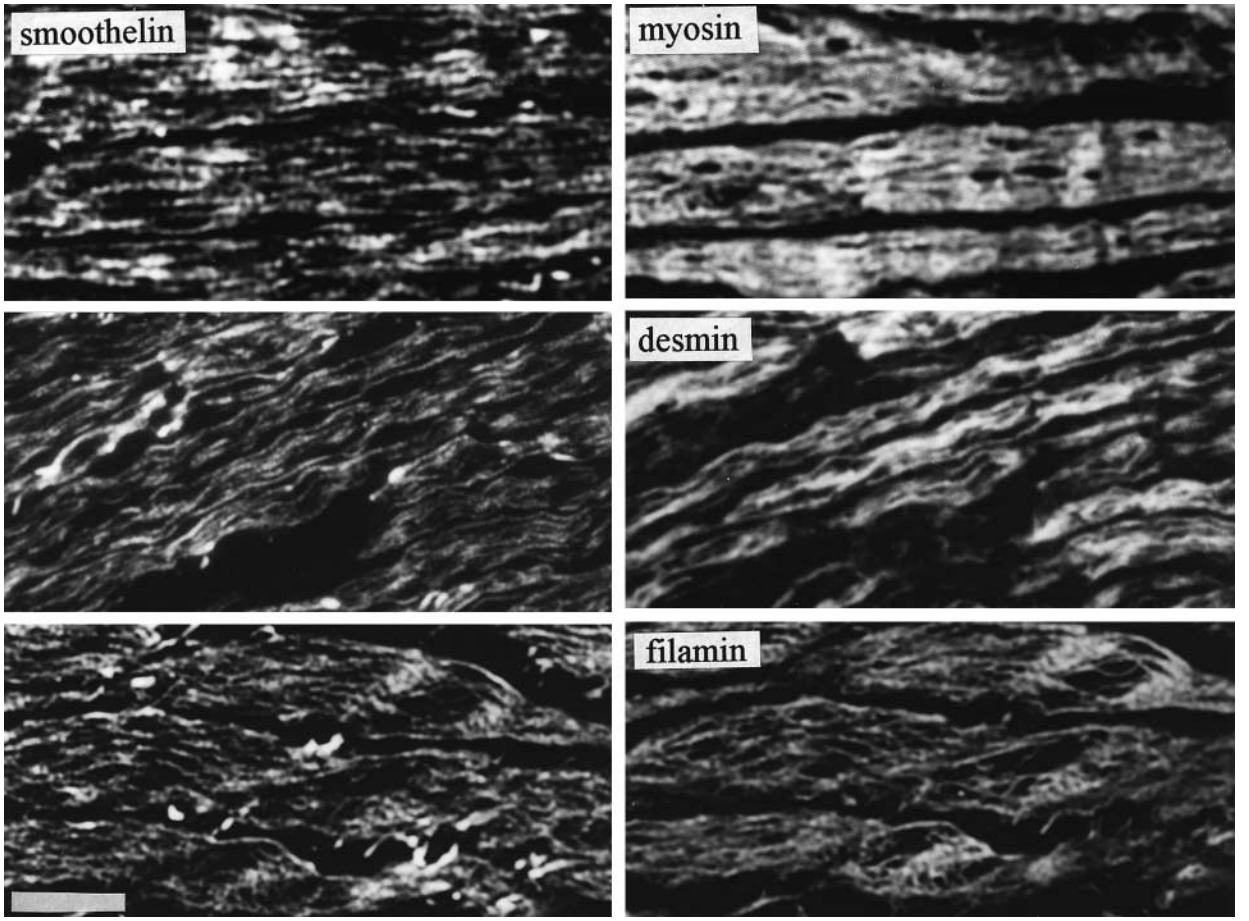

Fig. 2. Longitudinal ultrathin sections of chicken gizzard smooth muscle, double labelled for smoothelin (left panels) and myosin, desmin and filamin (right panels, as indicated). The fibrillar labelling pattern for smoothelin most closely resembles that for filamin. Bar, 5 μm.

simultaneously with two antibodies to facilitate comparison. The blots were scanned on a Mikrotek Scanmaker II SP and a composite blot created using Adobe Photoshop.

For two dimensional gel electrophoresis total tissue samples were lysed in 9.8 M urea and soluble proteins separated on NEPHGE first dimension tube gels followed by second dimensions on 12.5% SDS PAGE mini slab gels as described earlier [15]).

2.5. Immunoprecipitation

Immunoprecipitations using chicken gizzard smooth muscle extracts were performed using protein A Sepharose beads as described [18] with the minor modifications indicated below. Briefly, 2 g of fresh chicken gizzard smooth muscle was homogenized in 25 ml buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2% (v/v) glycerol, 0.25% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA (IP buffer) and left 30 min on ice. Cellular residues were removed by centrifugation at $15\,000 \times g$. Prior to immunoprecipitation the supernatant was clarified by ultracentrifugation at $100\,000 \times g$ for 30 min and further cleared of non-specific reaction products by incubation for 60 min with protein A Sepharose (Pharmacia, Vienna) in IP buffer.

3. Results

3.1. Smoothelin is localized in the cytoskeleton of gizzard smooth muscle cells

In transverse ultrathin sections of gizzard muscle the smoothelin antibody produced a punctate pattern that co-distributed with that observed for desmin and filamin (Fig. 1). Both of these latter proteins are major components of the cytoskeleton domain of smooth muscle cells [19]. Smoothelin label contrasted with that for myosin, which is more generally distributed in the cell (Fig. 1), and defines the extent and localization of the contractile domain. Longitudinal sections revealed a fibrillar pattern for smoothelin that was again different from myosin (Fig. 2) and matched more closely that seen for filamin than for desmin (Fig. 2).

3.2. Smoothelin is co-extracted with actin-binding proteins

Although smoothelin immunostaining co-distributed with that for filamin, it also resembled the pattern obtained for desmin and a possible association of smoothelin with the intermediate filaments had thus to considered. It has formerly been shown that desmin is relatively insoluble and that the major part of desmin remains in the residue obtained after extraction of muscle cells with detergents and concentrated salt solutions [13]. By following the distribution of smoothelin in the pellet and supernatant fractions of a gizzard muscle homogenate extracted sequentially under different conditions, we could show that smoothelin does not co-fractionate with desmin. As can be seen in Fig. 3, smoothelin was readily extracted together with myosin, vinculin, filamin and β-actin under conditions that do not solubilize desmin. This is most evident in lane 1 of Fig. 3, corresponding to protein extracted in Triton X-100. In connection with the immunoprecipitation experiments described below we found, additionally, that smoothelin was readily extracted from fresh gizzard muscle

at physiological ionic strength, also in the absence of Triton X-100. After the sequential extractions described above a fraction of smoothelin was still retained in the pellet fractions, along with the residue of the other cytoskeletal and contractile proteins, presumably due to the incomplete homogenization of the tissue.

3.3. *Smoothelin is a minor protein that appears at a late stage of smooth muscle differentiation*

Immunoblots of two dimensional gels of chick gizzard identified smoothelin as a basic protein with a p*I* around 8.2 and a series of isoelectric variants (Fig. 4). In parallel gels stained with Coomassie blue the corresponding smoothelin spot was barely identified, being an order of magnitude smaller than those of calponin and SM22, that occur in the same pH range [15]. But the specificity and high affinity of the smoothelin antibody allowed us to follow the protein's expression during embryogenesis in the chick gizzard. As shown in Fig. 5B smoothelin was not detectable before embryonic day 18, whereas calponin appeared long before this, at around embryonic day 5 (Fig. 5A) [3]. In the last days of embryonic life smoothelin appeared as a doublet, the band of lower molecular weight (Fig. 5B), being absent, or reduced in the adult.

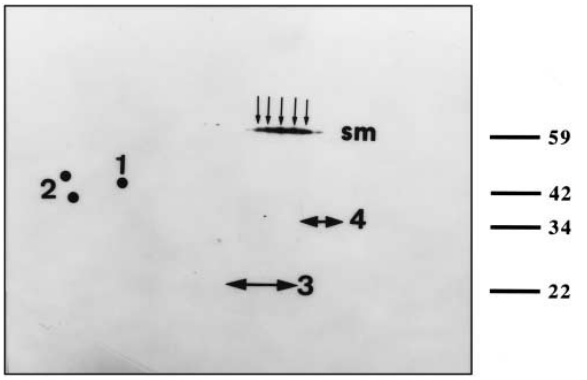

Fig. 4. Immunoblot of a two dimensional NEPHGE gel of total chicken gizzard smooth muscle extract probed with the R4A anti-smoothelin antibody. Smoothelin (sm) appears as multiple isoelectric variants (vertical arrows) of similar molecular weight. The estimated p*I* values range from 8.9 to 7.9. The positions of other smooth muscle marker proteins were determined by Ponceau S stain of the nitrocellulose following transfer and are indicated: 1, actin; 2, tropomyosin; 3, SM22; 4, calponin. Molecular weights in kDa.

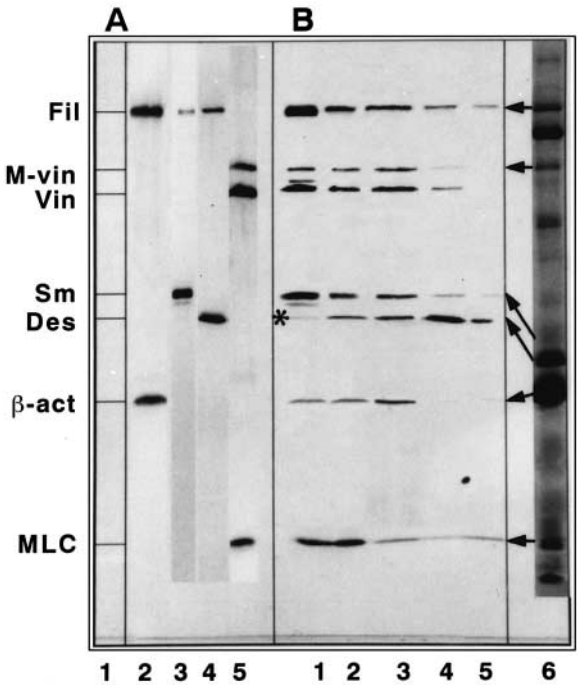

Fig. 3. Immunoblots of whole chicken gizzard homogenates (A) and of the supernatants obtained under different extraction conditions (B). Lanes 2–5 in A show immunoblots of the same chicken gizzard sample (see Coomassie blue stained gel, panel B, lane 6) labelled with the different pairs of antibodies to the antigens indicated on the left. B shows a composite immunoblot of the different supernatant fractions obtained during the different extraction steps as described in Section 2. The original immunoblots of these supernatants after double antibody labelling (as in lane A) were scanned in Adobe Photoshop and edited into single lanes, for clarity. Hence the positions of proteins in the Coomassie lane 6 do not match. Lanes 1–5 correspond to the supernatants obtained after sequential extractions of the same sample using the conditions 1–5 given in Section 2. These were briefly: lane 1, Triton X-100 in CB; lane 2, ATP in low salt; lane 3, ATP in high salt; lane 4, ATP in low salt; lane 5, low salt, high pH. Note that smoothelin was not co-extracted with desmin but co-fractionated with the actin-associated proteins. Abbreviations: Fil, filamin; M-vin, metavinculin; vin, vinculin; SM, smoothelin; Des, desmin; β-act, β-actin; MLC, myosin light chain.

3.4. *A high molecular weight isoform of smoothelin is expressed in vascular smooth muscle*

Avian vascular smooth muscle of both veins and arteries exhibited a single polypeptide with an apparent molecular weight of 95 kDa reactive in immunoblots with the smoothelin antibody (Fig. 5C). The same samples were equally positive for calponin (Fig. 5D). On the presumption that the high molecular weight band could be a dimer of the 59 kDa smoothelin protein, various strategies were employed to maintain the samples in a reduced state prior to electrophoresis. However, none of these, including the addition of high concentrations of dithioerythritol or β-mercaptoethanol, had any effect on the intensity of the 95 kDa band. We conclude therefore that the occurrence of this band may be indicative of a high molecular weight variant of smoothelin. Immunofluorescence labelling by the smoothelin antibody in chicken blood vessels was restricted to the layers of smooth muscle in the vessel wall (not shown).

3.5. *Smoothelin is distinct from the 60 kDa filamin-binding protein*

Maekawa and Sakai [20] described a 60 kDa protein from smooth muscle that bound to filamin. In view of smoothelin's apparent molecular weight of 60 kDa and its co-localization with filamin it was relevant to establish whether or not smoothelin and the 60 kDa protein were identical. The results of two sets of experiments indicated that this was not the case. We first took advantage of the absence of the 59 kDa molecular weight smoothelin in arterial smooth muscle. As shown in Fig. 6 the anti-60 kDa antibody labelled only a 60 kDa product in these vascular samples, in which the smoothelin antibody labelled only the 95 kDa band (Fig. 6). In a second experiment, smooth muscle extracts were subjected to immunoprecipitation with either the smoothelin or the 60 kDa antibody. Each antibody specifically precipitated only its respective antigen (Fig. 7). Thus, smoothelin is not the 60 kDa filamin-binding protein.

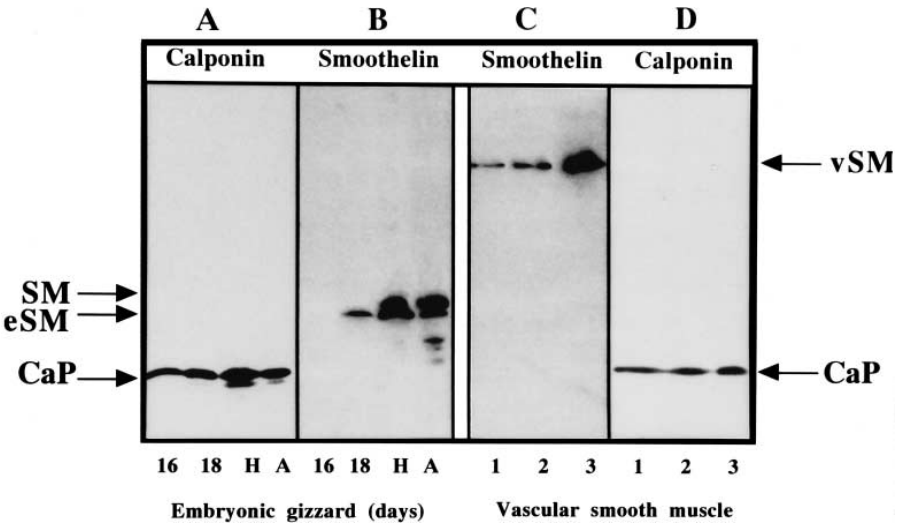

Fig. 5. A,B: Immunoblots of gizzard samples taken from chick embryos at embryonic days 16 and 18, at hatching (H) and from an adult chicken (A), made using the calponin and smoothelin antibodies. Note the late appearance of smoothelin (SM) at around 18 days, as compared to calponin (CaP) and the occurrence of a lower molecular weight, embryonic form (e SM). C,D: Vascular smooth muscle samples from adult chickens blotted with the smoothelin and calponin antibodies and showing the high molecular weight variant of smoothelin (vSM). Samples were: 1, jugular vein; 2, carotid artery; 3, ascending aorta.

4. Discussion

Studies on the localization of different smooth muscle proteins have revealed a partitioning of contractile and structural proteins into complementary domains in the smooth muscle cell, i.e. the contractile domains and cytoskeletal domains [19]. Desmin containing intermediate filaments constitute a major component of the cytoskeleton domain. The contractile domain, responsible for the generation of mechanical forces in the muscle cells, mainly consists of actin and myosin. Although the presence of two domains within the smooth muscle cell is distinct, the filament systems that make them up appear to be integrated into a functional unit through mechanical coupling via the α-actinin containing dense bodies [19].

Information about the binding partners of smoothelin is currently lacking. However, the noted homology of part of its primary sequence to the consensus actin binding domain of proteins of the spectrin family suggests that it could be actin associated [4]. We have earlier demonstrated that non-muscle type actin is specifically localized in the cytoskeleton domain of smooth muscle cells, along with filamin and desmin [5]. Since smoothelin colocalizes with filamin in chicken gizzard cells we presume it is associated, like filamin, with the non-muscle actin component of the cytoskeleton. This conclusion would be in line with the present extraction data, and with the observed co-localization of smoothelin with stress fibers in primary chicken culture cells as well as in cardiomyocytes and certain smooth muscle cell lines [4]. Binding studies with purified smoothelin will, however, be necessary to support these conclusions. As we show, smoothelin is not related to the 60 kDa filamin-binding protein described by Maekawa and Sakai [20]. The lack of identity of the two proteins is also supported by the observed expression of the 60 kDa protein in heart and skeletal muscle [20], from which smoothelin is absent.

The presence of the 95 kDa molecular weight form of smoothelin in vascular muscle is intriguing and potentially significant. We have not yet localized this isoform by high resolution immunocytochemistry in vascular muscle but presume that it is also associated with the cytoskeleton. Since a reduction in apparent molecular weight could not be achieved using reducing agents, the 95 kDa smoothelin does not appear to arise from a homo- or heterodimer species. The possibility that vascular and visceral smoothelin may be the product of different genes has been refuted since fluorescent in situ hybridization experiments revealed only one signal on chromosome 22 (van Eys, personal communication), suggesting that the differences in molecular weight arise either from extensive post-translational modifications, from alternative splicing or from a dual promoter system. Current studies are aimed at establishing the origin of the vascular isoform of smoothelin.

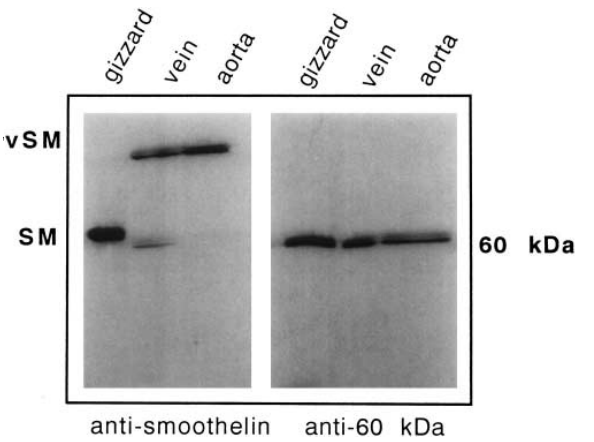

Fig. 6. A: Immunoblot of total homogenates from chicken gizzard and vascular smooth muscle samples (femoral vein, ascending aorta) with the smoothelin antibody showing the 95 kDa molecular weight isoform of smoothelin (vSM). B: The same samples as in A blotted with the antibody to the 60 kDa filamin-binding protein. In this case gizzard and vascular samples show only one band at 60 kDa.

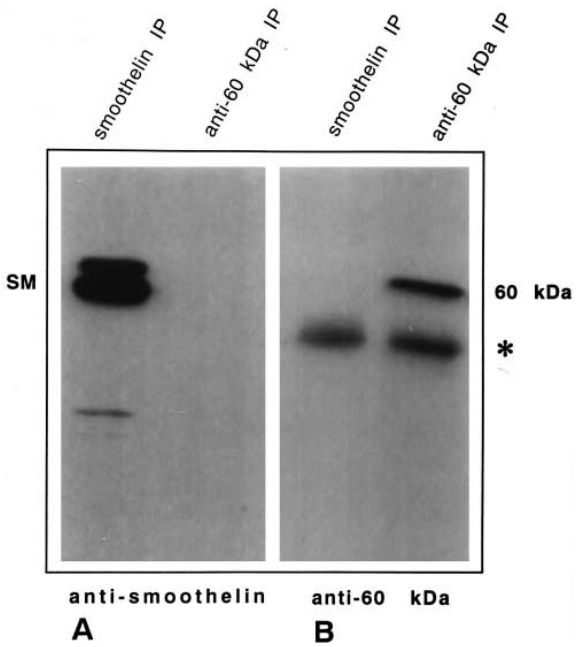

Fig. 7. Western blots of immunoprecipitates (IP) from the same chicken gizzard smooth muscle extract probed with either the monoclonal anti-smoothelin antibody R4A (A) or the polyclonal anti-60 kDa filamin-binding protein (B). The blots demonstrate that smoothelin and the 60 kDa protein are unrelated polypeptides of similar molecular weight. The lower reactive band in B (asterisk) represents a non-specific cross-reaction of the polyclonal anti-60 kDa antiserum. SM and 60 kDa indicate the positions of smoothelin and the 60 kDa filamin-binding protein.

In conclusion, smoothelin is here identified as a minor component of the smooth muscle cell cytoskeleton that is most likely associated with actin. More data on the biochemistry of smoothelin are required to establish its in vitro properties and its binding partners, as a necessary further step towards elucidating its role in the smooth muscle cell.

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