Localization of Filamin in Smooth Muscle

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Abstract. The distribution of contractile and cytoskeletal proteins in smooth muscle has been mapped by immunocytochemical methods, with special reference to the localization of the actin-binding protein, filamin. Immunolabeling of ultrathin sections of polyvinylalcohol-embedded smooth muscle distinguished two domains in the smooth muscle cell: (a) actomyosin domains, made up of continuous longitudinal arrays of actin and myosin filaments, and (b) longitudinal, fibrillar, intermediate filament domains, free of myosin but containing actin and α-actinin-rich dense bodies. Filamin was found to be localized specifically in the latter intermediate filament–actin domains, but

was excluded from the core of the dense bodies. Filamin was also localized close to the cell border at the inner surface of the plasmalemma-associated plaques. In isolated cells the surface filamin label showed a rib-like distribution similar to that displayed by vinculin.

It is speculated that the two domains distinguished in these studies may reflect the existence of two functionally distinct systems: an actomyosin system required for contraction and an intermediate filament–actin system, with associated gelation proteins, that is responsible, at least in part, for the slow relaxation and tone peculiar to smooth muscle.

WITH respect to its motile machinery, vertebrate smooth muscle occupies an interesting position between cross-striated muscle and motile, non-muscle cells. It expresses a contractile apparatus made up of actin and myosin filaments but whose component parts show several characteristics shared only by non-muscle tissues. Both smooth and non-muscle myosins differ from that of skeletal muscle in being Ca-regulated (see reviews in references 24, 29, and 38), and smooth muscle actin shows associated proteins that are found in non-muscle cells and absent or only minimally expressed in differentiated striated muscle. Smooth muscle actin-associated proteins include filamin (45) and the calmodulin binding protein caldesmon (7, 41). Smooth muscle also exhibits an intermediate filament system that is considerably more developed than in skeletal muscle (27, 37) and comparable in extent to that found in various non-muscle tissues (see review in reference 27).

Recent progress in smooth muscle biochemistry (see, e.g., review in reference 29) has tended to overshadow the fact that the structural organization of smooth muscle still remains poorly understood. The data relevant to this problem have been reviewed at length by Bagby (4), but despite the various conflicting points of view (4), a close analogy between smooth and striated muscle has generally been favored. Thus, from the common presence of α-actinin in both the dense bodies of smooth muscle (20, 33) and the striated muscle Z-line (30), the demonstration of actin filament insertion into dense bodies (6) and an apparent longitudinal relationship, deduced

from semithin serial sections, between dense bodies and small groups of myosin filaments (2), it has generally been assumed that some kind of sarcomeric organization of myofilaments exists in the smooth muscle cell (6). The evidence for a sarcomeric arrangement is not compelling, however, mainly due to the inherent difficulty in detecting long range, three-dimensional order by electron microscopy in the densely packed myofilament lattice. In other studies, evidence for sarcomeric units was not found (18, 34).

For non-muscle systems, particularly cultured cells, immunocytochemical methods have contributed significantly to studies of the structure and composition of the cytoskeleton (19, 47). In principle, the same approach should yield equally useful information about the organization of contractile and cytoskeletal elements in smooth muscle, and studies in this direction have already been initiated (3, 5, 18, 36). But here the small diameter (a few microns) and cylindrical form of the cells precludes, either in isolated cells or in conventional frozen sections, the clear resolution of subcellular detail. To circumvent this problem we have developed an embedding method (35) that readily yields ultrathin sections of muscle tissue suitable for both immunofluorescence microscopy and electron microscope immunocytochemistry with colloidal gold probes (15, 16). In this first report of the application of this method for electron microscopy we demonstrate the existence of two distinct domains in the smooth muscle cell: actomyosin domains and actin–intermediate filament domains. It is further shown that the actin-binding protein

filamin is specifically restricted to the actin–intermediate filament domains. This result is discussed in relation to the special contractile properties of smooth muscle.

Materials and Methods

Antigens

Contractile and cytoskeletal proteins from smooth muscle were purified to homogeneity according to procedures described previously: chicken gizzard actin (42); gizzard myosin 20,000-mol-wt light chain (40); hog stomach desmin (skeletin, 37); gizzard and hog stomach α-actinin, tropomyosin, and filamin (39). Gizzard actin, gizzard myosin light chain, and hog stomach filamin were gifts from Dr. A. Sobieszek.

Antibodies and Antibody Purification

Rabbits were immunized with native or denatured (using SDS–desmin only) proteins and the antisera fractionated and affinity purified as described (14). As necessary, antigens used for the affinity purification step were subjected to a final purification on an FPLC MONO Q HR 5/5 column (Pharmacia, Uppsala, Sweden).

Immunoblotting

Samples of whole muscle tissues were subjected to SDS PAGE on minislab gels according to Matsudaira and Burgess (31). The proteins were transferred electrophoretically (43) to nitrocellulose sheets (Schleicher & Schull, Dassel, FRG) and the blots processed for immuno-gold-silver staining as described (32). Protein patterns were visualized either by Coomassie Blue staining on a parallel SDS gel or by intermediate staining of the blots, after transfer, with Ponceau S (Sigma Chemical Co., St. Louis, MO).

Isolated Smooth Muscle Cells

Smooth muscle cells were isolated from guinea pig taenia coli muscle and prepared and processed for immunolabeling as described elsewhere (36). Cells were either extracted briefly at room temperature with 0.3% Triton X-100 in cytoskeleton buffer (see below) and then fixed in glutaraldehyde (1%, 10 min) or fixed directly in a glutaraldehyde–Triton mixture (0.125%/0.5%) for 20 min followed by fixation in 1% glutaraldehyde for 10 min.

Smooth Muscle Tissue Sections

Immunocytochemistry of tissue sections was carried out, for both light and electron microscopy, on ultrathin sections made from smooth muscle embedded in polyvinylalcohol (35).[1] Processing of muscle strips involved the following steps.

Dissection. Muscle strips were prepared from freshly dissected chicken gizzard (outer circumferential layer; Wagner, J., personal communication), chicken expansor secundariorum, or guinea pig taenia coli and transferred to a room temperature Ca++-, and Mg++-free balanced salt solution (avian Ringer's or mammalian Hanks', respectively) supplemented with 2 mM EGTA, 2 mM MgCl₂, 10 mM Pipes, pH 6.5–6.8. Single strips, 1–2-cm long, were tied straight onto small plastic plates (25 × 5 × 1 mm) and transferred directly to fixative. The dissection and tying procedure lasted typically 1–2 h.

Fixation. Fixation was carried out in aldehyde–detergent mixtures in a cytoskeleton buffer described previously (solution 1 in reference 34, composed of a Ca++-, Mg++-free Hanks' balanced salt soltuion with added 2 mM MgCl₂, 2 mM EGTA, 10 mM 2, [*N*-morpholino]ethane sulfonic acid pH 6.1) and at room temperature. A variety of aldehyde–detergent mixtures as well as a variety of fixation times were tested.[1] However, for the present purpose the most successful combination found for electron microscopy, as regards preservation of antigenicity and visualization of structure, was fixation for 1 h in a mixture of 3% paraformaldehyde (Polaron, London), 0.1% glutaraldehyde (Polaron, EM grade), and 0.2% Triton X-100 (Sigma Chemical Co.). Deviations from this scheme that resulted in preparations very adequate for light microscopy (depending on the antigen of interest) involved fixation in either paraformaldehyde–Triton (3%/0.2%; 1 h) or glutaraldehyde–Triton (e.g. 0.5%/0.5%; 40 min) mixtures.

Embedding. After two washes in cytoskeleton buffer (2× 15 min) to remove the fixative, muscle strips were treated with 0.5 mg/ml sodium borohydride, freshly dissolved in ice-cold cytoskeleton buffer, 3× for 15 min. This treatment

was necessary to reduce free aldehyde groups (48) that would otherwise interact chemically with the embedding medium (1). The strips were then washed again in the same buffer and transferred to 3-ml plastic tubes containing an embedding mixture of 20% wt/vol polyvinylalcohol (10,000 mol wt; Janssen Chimica, Belgium) dissolved in cytoskeleton buffer diluted five times in water (i.e., 20% salts) supplemented with trehalose (Sigma Chemical Co.) at a concentration of 5–10% (wt/vol). Typically muscle strips were then left in closed tubes for 24–48 h at 40°C to allow penetration of the embedding medium. Thereafter the strips were transferred face up in fresh medium in 3.5-cm-diam petri dishes that were left open in the 40°C oven to allow drying to take place. The embedding medium was replenished two or more times at ~48 h intervals so that the final embedding layer on the strip was thick enough (>1 mm) to allow further manipulation. The plastic plate carrying the muscle was cut or sawn out of the dish (after about 1 wk of drying at 40°C) and the embedding layer allowed to dry further until it could be easily released with the muscle from the plate.

Sectioning. The muscle strips were mounted into home made holders for flat embedded specimens, compatible with the microtome (model UM 3; Reichert, Vienna, Austria), and trimmed with razor blades in the same manner as for epoxy resin–embedded material. To achieve the necessary hardness at this stage it was normally necessary to subject the blocks, before trimming, to a final drying step involving incubation at 60°C for 4–12 h. Sectioning was carried out as for epoxy sections but using glycerol (87%, Merck, Darmstadt, FRG) as the flotation medium (35).[1] Ultrathin sections (silver-gold interference color) were picked up, by contact from above, on either 4-mm-square glass coverslips (previously coated with polylysine using a 1 mg/ml aqueous solution) or on pioloform-coated silver 150–200-mesh electron microscope grids (Teepe-Brandsma, Holland). For electron microscopy the support films needed to be relatively thick to give the necessary stability in the microscope. These were cast on glass slides from a 1.2% solution of pioloform in dichloroethylene to give a silver–silver gray interference color when floated onto a water surface. Coverslips or grids carrying sections were floated onto glycerol in small petri dishes and stored at 4°C before use.

Immunocytochemistry

The basic buffer used for immunolabeling for light and electron microscopy was a Tris-buffered saline containing 155 mM NaCl, 2 mM MgCl₂, 2 mM EGTA, 20 mM Tris-base pH 7.6. We shall refer to this solution as gold buffer (GB).[2] Before immunolabeling, the coverslips or grids (stored by flotation on glycerol, see above) were washed free of glycerol by flotation on GB (two changes) in over-filled 5-ml beakers and incubated further for at least 12 h (at room temperature) on GB in a petri dish to effect extraction of the embedding medium.

Coverslips. Antibody labeling of coverslips involved the following steps: (*a*) Preincubation on a drop (5–10 μl) of 2% normal goat serum (Nordic, Holland), 1% bovine serum albumin (BSA, Sigma Chemical Co.) in GB for 10–30 min; this and the subsequent antibody incubations were carried out on a layer of parafilm stretched over a glass plate mounted on a moist filter paper in a 15-cm-diam plastic petri dish. (*b*) Transfer to 10 μl of the primary antibody, also dissolved in 2% normal goat serum, 1% BSA in GB. Incubation time was 30–60 min at room temperature. (*c*) Brief rinsing by flotation on GB (by consecutive transfer on the surface of three full 5-ml beakers) followed by flotation on GB in multiwell dishes (24 wells; NUNC, Roskilde, Denmark) filled to the brim and mounted on a rotating, tilting table. Washing was done two times for 15 min (one buffer change). With the dishes filled level, without a meniscus, the coverslips rotated slowly around the multiwells during the washing steps. (*d*) Transfer to the second antibody (rhodomine-conjugated goat anti-rabbit IgG, a gift from Dr. B. Geiger) in 1% BSA in GB on parafilm for 40 min to 1 h at room temperature. (*e*) Rinsing and washing as after the first antibody, followed by mounting in Gelvatol 20–30 (Monsanto Co., St. Louis, MO). The diluted first and second antibodies were centrifuged at top speed for 15 min in a Beckman Airfuge just before use.

Grids. Sections on electron microscope grids were labeled in a manner similar to that used for coverslips but with the following modifications. Unless otherwise stated the second antibody was a gold-conjugated goat anti-rabbit IgG coupled with 5-nm or 10-nm colloidal gold according to the procedure of De Mey and Moeremans (15). To minimize non-specific labeling extra washes of 15 min each, in 0.1% BSA/GB, were made directly before (one wash) and directly after (two washes) the second antibody step. (The diluted gold-conjugated antibody [in 1% BSA/GB] was subjected to 4-min centrifugation in an Eppendorf-type centrifuge before use to remove any possible aggregates.) After the final two washes in GB alone, the grids were processed for negative staining.

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[1] Small, J. V. Manuscript in preparation.

[2] *Abbreviation used in this paper:* GB, gold buffer.
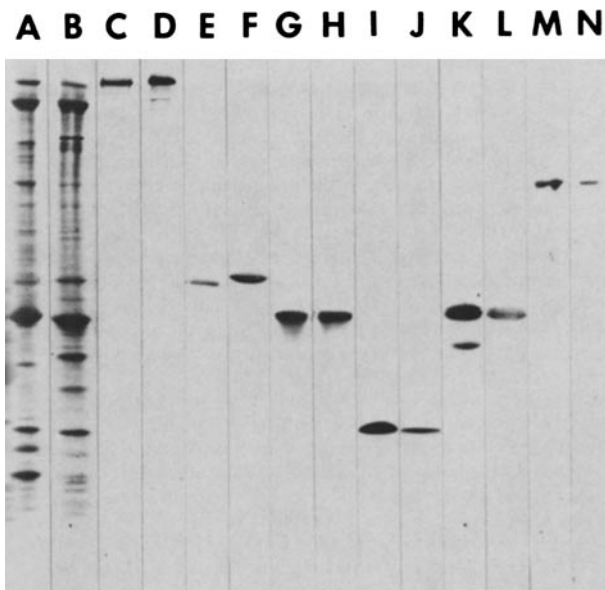
A B C D E F G H I J K L M N

Figure 1. Immunoblot analysis of antibody specificity. The same pair of samples, shown after Coomassie Blue staining in *A* and *B*, was applied for the corresponding immunoblots shown in *C* and *D*, *E* and *F*, and so on. The two samples were chicken gizzard whole muscle (*A*) and guinea pig taenia coli whole muscle (*B*). The affinity purified antibodies applied to the respective lanes were: (*C*) anti-chicken gizzard filamin; (*D*) anti–hog stomach filamin; (*E* and *F*) anti–hog stomach desmin; (*G* and *H*) anti–chicken gizzard actin; (*I* and *J*) anti–chicken gizzard myosin L_{20} light chain; (*K* and *L*) anti-chicken gizzard tropomyosin; (*M* and *N*) anti–hog stomach α-actinin. Parallel lanes run with purified antigens (not shown) showed immunoreactivity on bands in identical positions to those in the whole muscle samples. The slab gels contained a gradient of 12–20% acrylamide.

Negative Staining

Contrasting for electron microscopy was achieved by negative staining in aqueous 2% sodium silicotungstate, as follows. Each grid, held in a pair of forceps clamped together with a large paper clip, was first rinsed with a few drops of bacitracin (40 μg/ml in water with added 0.1% amyl alcohol; 22) and then with a few drops of the negative stain solution, leaving one drop on the grid. 3–4 μl of a solution of 0.015% octadecanol in hexane was then injected into the final stain drop to form a surface skin (21). The stain drop was then drained slowly away by touching a torn filter paper edge close to the tip of the forceps. When dry, the grids were ready for inspection in the electron microscope.

Light and Electron Microscopy

Light microscopy was carried out using a Zeiss photomicroscope III (Carl Zeiss, Oberkochen, FRG) equipped with epifluorescence optics and a side-mounted 50-W mercury lamp. Electron microscopy was performed on a Zeiss 10 A electron microscope operating at 80 kV and with a 50-μm objective aperture.

Results

Antibody Characterization

The monospecific polyclonal antibodies used in these studies were obtained using either hog stomach or chicken gizzard antigens (see Materials and Methods). With the exception of the filamin antibodies all cross-reacted with both avian and mammalian proteins (Fig. 1).

Domains of Contractile and Cytoskeletal Proteins

Immunolabeling of ultrathin cross-sections revealed characteristic and distinctive patterns for the different antigens that were recognizable even in the light microscope (Fig. 2). Fig. 2 shows two sets of sections: longitudinal sections from chicken gizzard and transverse sections from guinea pig taenia coli. The corresponding longitudinal and transverse sections of these avian and mammalian muscles were equivalent with respect to the details observed. The latter transverse series (Fig. 2, *g–l*) shows labeling of a set of pseudo, ~0.1-μm serial sections (collected from a set of around 40 consecutive ultra-thin sections) to allow comparison of the staining patterns within the same cells.

For actin, as well as for tropomyosin (not shown), the cytoplasm was labeled more or less homogeneously in both transverse and longitudinal sections. In marked contrast, the patterns for myosin, desmin, and filamin were distinctly punctate in cross-sections (Fig. 2). For myosin, the pattern showed slight variations according to the muscle origin and preparation protocol and ranged from finely punctate to more patchy in appearance. In longitudinal sections filamin and desmin occurred in longitudinally oriented fibrils that were continuous over several microns, the absolute lengths depending on the exactness of orientation of the sectioning plane. Myosin antibodies also revealed longitudinal fibrillar units (Fig. 2*b*), but these commonly fused together and thus appeared less distinct than the fibrils stained by filamin or desmin antibodies. The longitudinal fibrillar distribution of filamin was also clearly discernable in isolated smooth muscle cells from the guinea pig taenia coli stained with the antibody against hog stomach filamin (Fig. 3). An additional feature recognized in whole cells was the labeling of longitudinal surface ribs (Fig. 3*c* and *e*) of appearance similar to that seen with antibodies to vinculin (36). This surface distribution was consistent with the punctate labeling observed at the periphery of cells in cross-sections (Fig. 2*j*).

From the collected data it was apparent that filamin, an established actin-binding protein, was not bound to all actin filaments, like tropomyosin, but was localized in a subset of thin filaments within the cell. By labeling the same sections with pairs of antibodies, some indication of the relative distributions of the different proteins could be established at the light microscope level (Fig. 2, *e, f, k,* and *l*). For such dual labeling the individual antibody concentrations were titrated to give qualitatively similar intensities of label for the separate staining patterns. As shown in Fig. 2 the result of combining the filamin and desmin antibodies (Fig. 2, *e* and *k*) contrasted with the pattern obtained with the filamin/myosin antibody combination (Fig. 2, *f* and *l*). The fibrillar pattern obtained with filamin plus desmin suggested a co-localization of these two proteins, whereas the homogeneous pattern with filamin plus myosin suggested that the latter two proteins occupy complementary positions within the cell. As shown in the following sections this conclusion was confirmed by electron microscopy.

Localization of Filamin in the Intermediate Filament Domains

The different factors affecting filament visibility in thin sections of polyvinylalcohol-embedded muscle will be dealt with in some detail elsewhere.[1] Suffice it to say here that with the appropriate fixation and grade of polyvinylalcohol (see Materials and Methods), the four filamentous elements in the smooth muscle cells, microtubules, myosin, actin, and intermediate filaments, could be readily distinguished in negatively
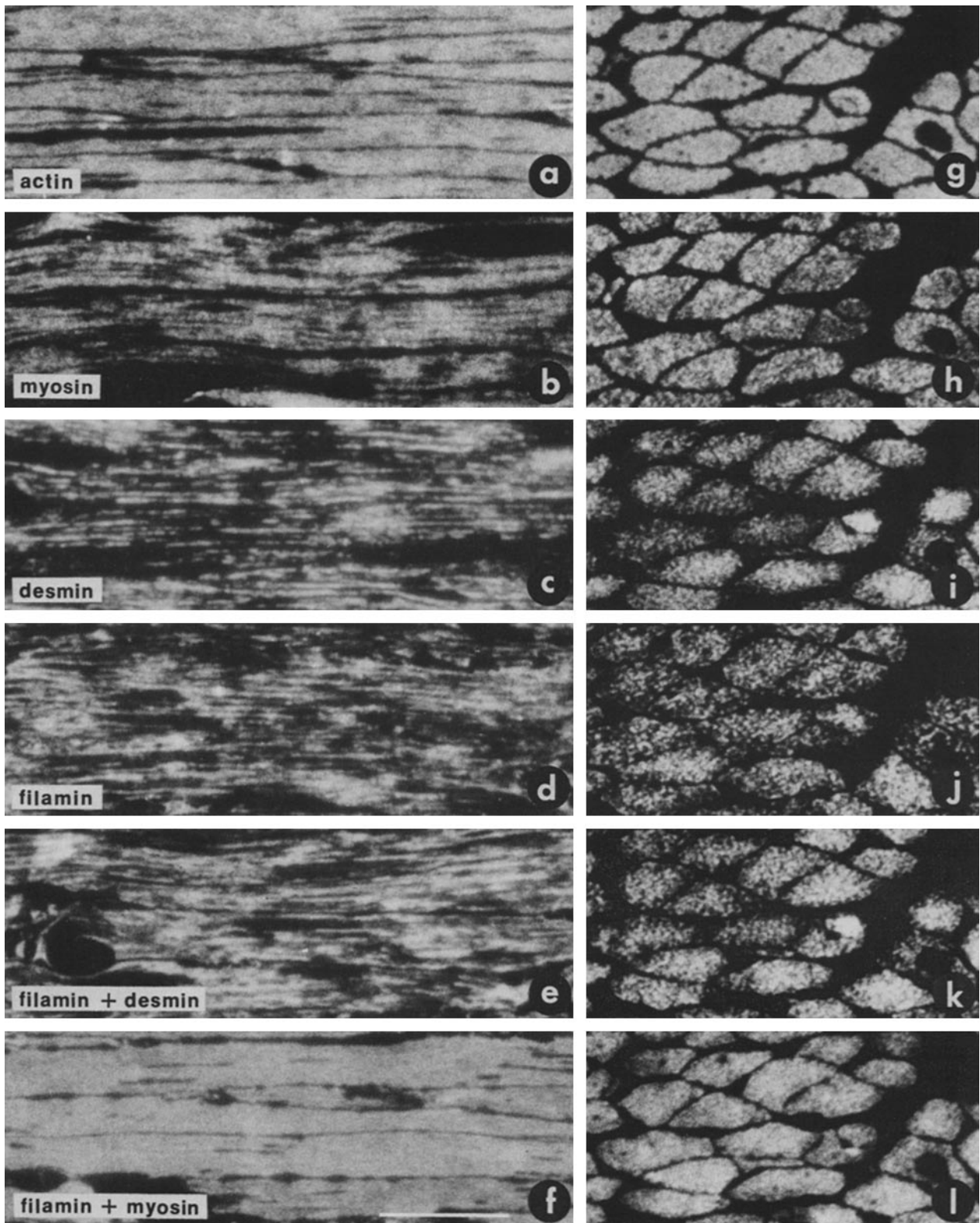
Figure 2. Ultrathin sections of polyvinylalcohol-embedded smooth muscle stained with the antibodies and antibody combinations indicated. The longitudinal sections (*a–f*) are from chicken gizzard, and the transverse sections (*g–l*) from guinea pig taenia coli. Note the homogeneous label obtained for actin (*a* and *g*) and the fibrillar (*b–d*) or punctate (*h–j*) label obtained with myosin, desmin, and filamin. The combination staining for filamin with either desmin (*e* and *k*) or myosin (*f* and *l*) indicates a co-localization of filamin and desmin (see text for details). Bar (for all), 10 μm.

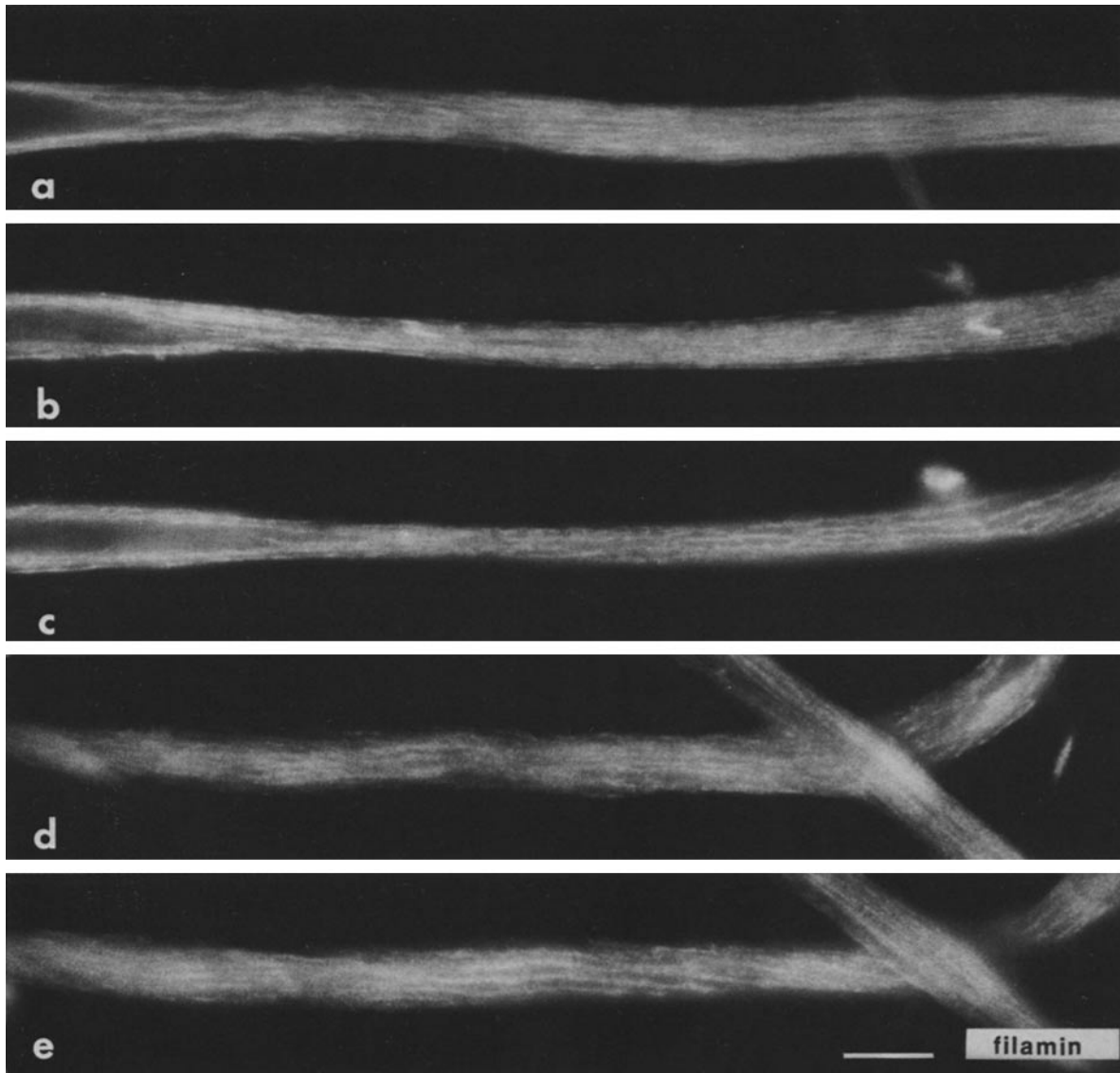Small et al. *Localization of Filamin in Smooth Muscle*

Figure 3. Isolated smooth muscle cells from the guinea pig taenia coli stained for the antibody against hog stomach filamin. Note fibrillar pattern in the body of the cells (*a*, *b*, and *d*) and a rib-like label on the cell surface (*c* and *e*). Fixations (see Materials and Methods): (*a*) glutaraldehyde–Triton mixture followed by glutaraldehyde; (*b–e*) Triton extraction followed by glutaraldehyde. Bar (for *a–e*), 10 μm.

stained cross-sections (Figs. 4 and 6). In favorable instances diffuse patches, commonly surrounded by intermediate filaments and clearly corresponding to the dense bodies seen in positively stained material, could also be recognized (Fig. 4).

The micrographs in Fig. 4 show cross-sections of chicken gizzard muscle labeled with the antibody to chicken gizzard filamin, as visualized by the immunogold staining procedure (15, 16). Using in this case 10-nm gold particles, about the right compromise could be achieved between the density of label (number of particles) and visibility of the gold probe in the filament lattice. As is evident from Fig. 4, filamin is clearly localized together with the intermediate filaments and is excluded from the domains containing actin and myosin filaments. In addition, filamin label occurred at the cell edge along the inner face of a membrane-associated layer (Fig. 4), taken as equivalent to the dense plaques seen after positive staining (4). Particularly at this location, filamin label occurred without a clear co-distribution of recognizable intermediate filaments. This disparity was confirmed by the results

obtained with the antibody to desmin (see also below). As illustrated also in Fig. 4, filamin label was excluded from the cytoplasmic dense bodies.

The continuous, longitudinal distribution of filamin, suggested by the immunofluorescence data, was substantiated by immunoelectron microscopy of longitudinal sections (Fig. 5, *a* and *b*). Longitudinal arrays of filamin label were found in the main body of the cytoplasm as well as beneath the plasma membrane (Fig. 5*a*). No evidence for a punctate type of distribution, as observed in the stress fiber bundles of cultured cells (26), was obtained.

In the longitudinal sections, elongated ovoid bodies, similar in size and shape to typical dense bodies, could be recognized (Fig. 5, *b* and *c*). Their identity with dense bodies was demonstrated by their positive reaction with antibodies to α-actinin (Fig. 5*c*). As shown in Fig. 5*b* and consistent with the data from cross-sections, the filamin label was co-linear with, and abutted, the dense bodies but was excluded from the dense body material.
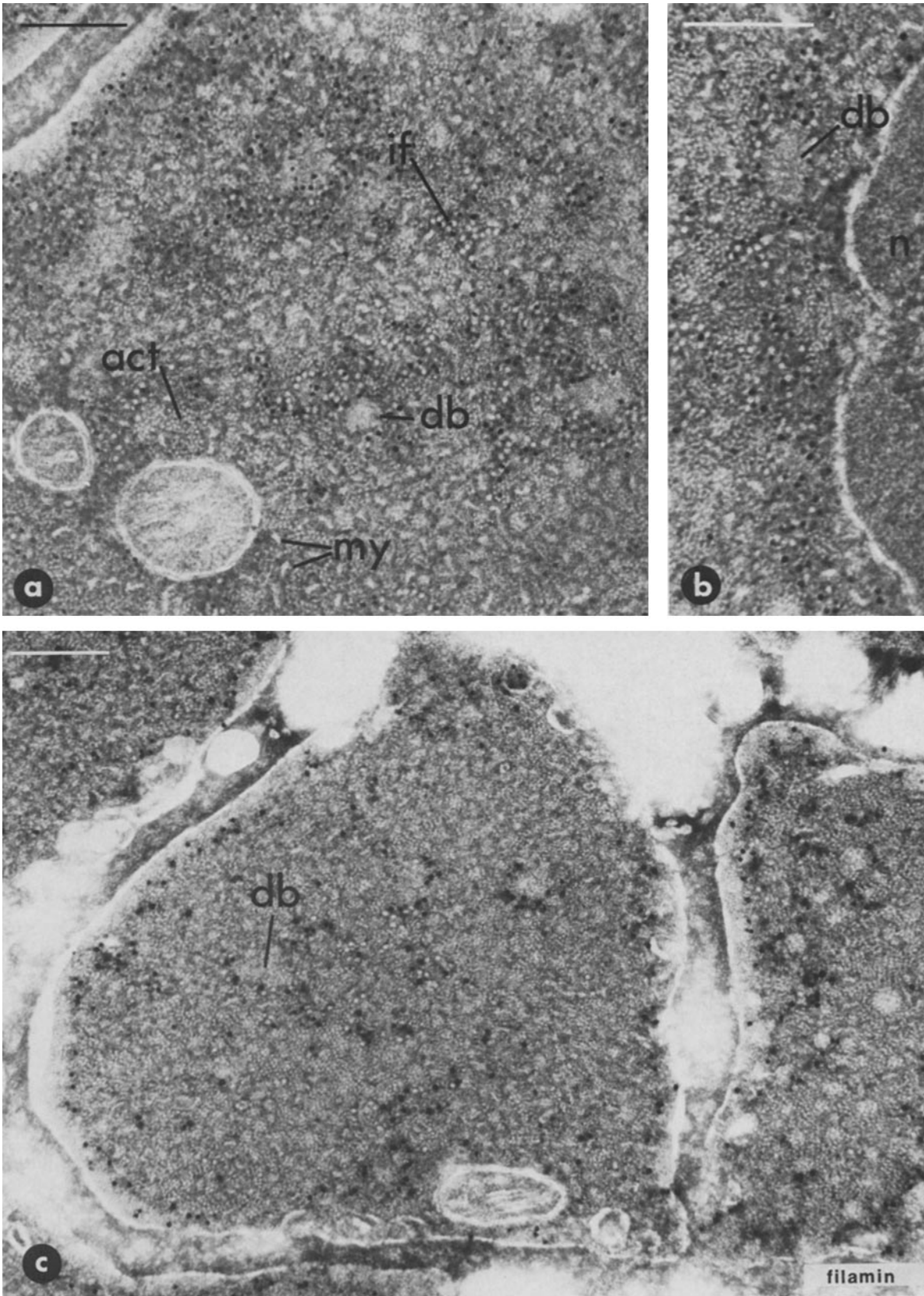
Figure 4. Transverse sections of smooth muscle cells from chicken gizzard labeled with the antibody to chicken gizzard filamin. *my*, myosin filaments; *act*, actin filaments; *if*, intermediate filaments; *db*, dense bodies; *n*, nucleus. Note localization of antibody label (10-nm gold particles) over the intermediate filament groups and along the inner surface of the plasmalemma plaques. Bars, 0.2 μm.
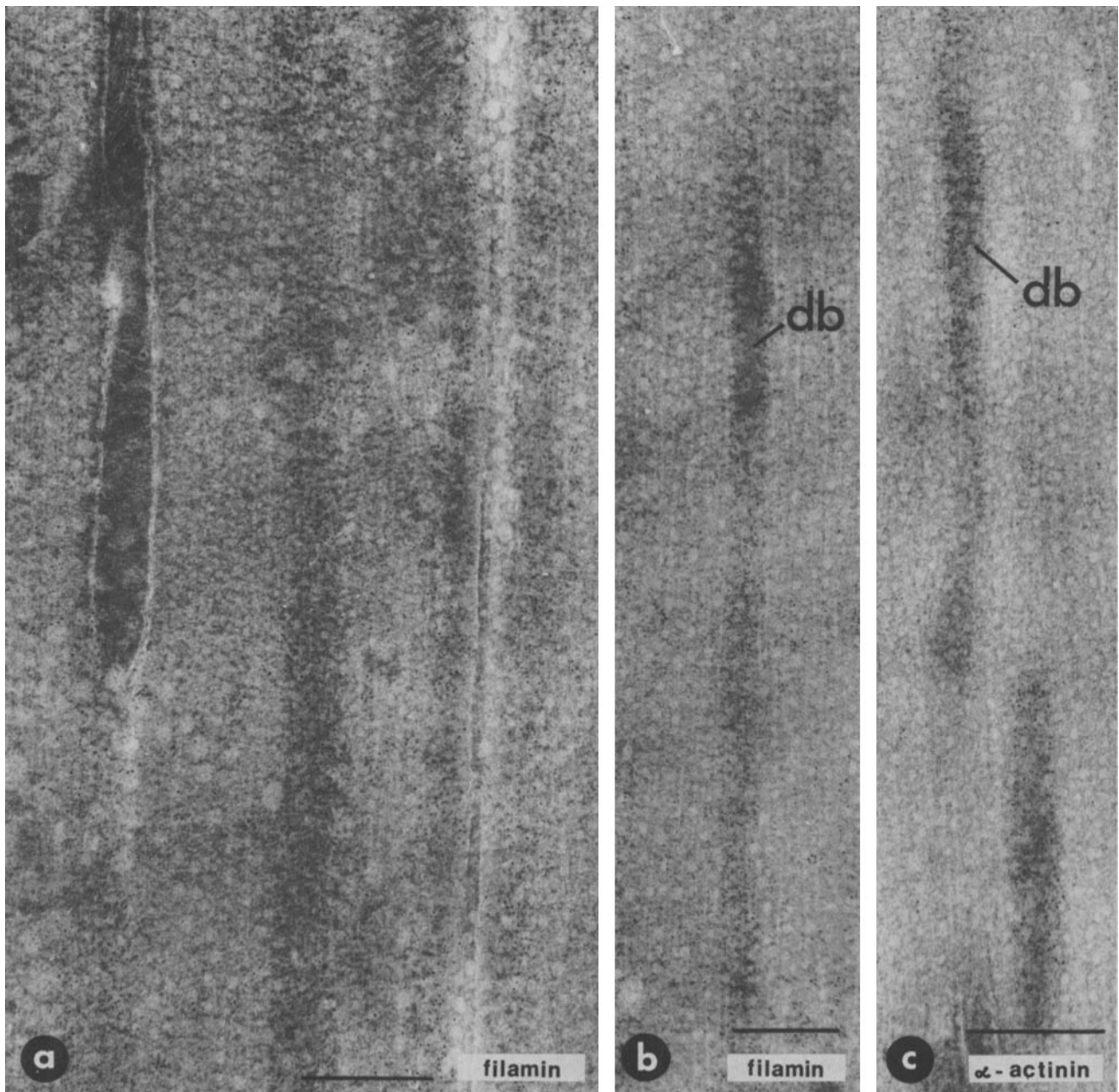
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Figure 5. Longitudinal sections of smooth muscle cells from chicken gizzard labeled for filamin (*a* and *b*) and α-actinin (*c*). 5-nm gold. Filamin shows a continuous longitudinal distribution (*a* and *b*) and is continuous with but excluded from the dense bodies (*db* in *b*) identified by their reaction with antibodies to α-actinin (*c*). Note also in *a* the labeling close to the plasma membrane in the region on the right where two cells are apposed. Bars, 0.4 μm.

Distribution of Other Contractile and Cytoskeletal Proteins

The labeling patterns obtained in the electron microscope with the antibodies to desmin and myosin demonstrated the restricted localization of these two proteins in their respective filaments (Fig. 6, *a* and *b*). Specifically, it could be shown that myosin is excluded from the intermediate filament domains (Fig. 6*a*). Also, and as indicated above, intermediate filaments were not distributed in the same continuous layer at the cell surface as observed for filamin. Instead, they occurred less frequently at this location and when they did commonly as small groups rather than in extended layers

parallel to the membrane plaques (not shown). In longitudinal sections, the desmin antibody revealed continuous longitudinal bundles (Fig. 7*a*) of similar dimension to those observed with anti-filamin. However, and consistent with the result from transverse sections, desmin label in longitudinal sections (not shown) was less commonly associated with the cell membrane than was the case for filamin (Fig. 5*a*).

Having found an actin-binding protein in the intermediate filament domains, it was important to establish whether actin and tropomyosin also occurred in these zones; from morphological data alone the presence of additional filaments thinner than the intermediate filaments could not be definitively
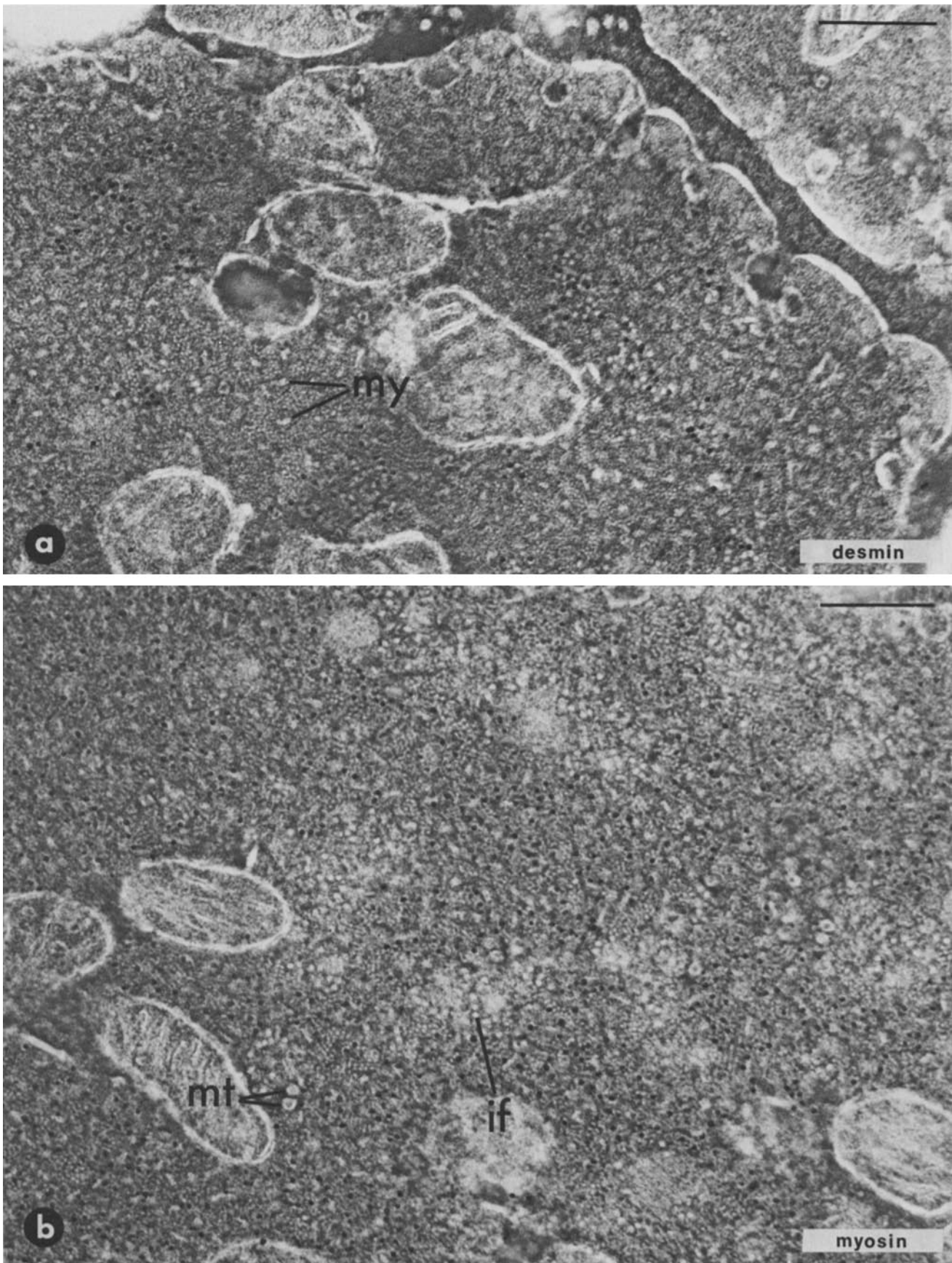
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Figure 6. Transverse sections of chicken gizzard smooth muscle cells labeled with antibodies to desmin (*a*) and myosin (*b*). 10-nm gold. The desmin antibody is localized over the intermediate filament groups (*if*) and the myosin antibody exclusively over the myosin filaments (*my*). Microtubules (*mt*) are also evident. Bars, 0.2 μm.

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demonstrated. In cross-sections the intensity of gold label obtained with the polyclonal antibodies against actin and tropomyosin was, for an as yet unknown reason, insufficient to settle this question. However, with longitudinal sections the density of label with both antibodies was sufficient to indicate that both actin and tropomyosin are distributed more or less uniformly across the cell (Fig. 7, *b* and *c*). There was no obvious exclusion of either protein from longitudinal channels similar to those populated with filamin and desmin.

We concluded, then, that thin actin filaments, bearing tropomyosin, exist in the intermediate filament domains. Consistent with the earlier results of Geiger and co-workers (20), the tropomyosin antibody did not label the dense body structures (Fig. 7*c*). For actin, the result regarding dense bodies was less clear (Fig. 7*b*), but from parallel experiments using an actin monoclonal antibody (28), it was apparent that actin was indeed a dense body component (not shown). However, more experiments are needed to substantiate this conclusion.
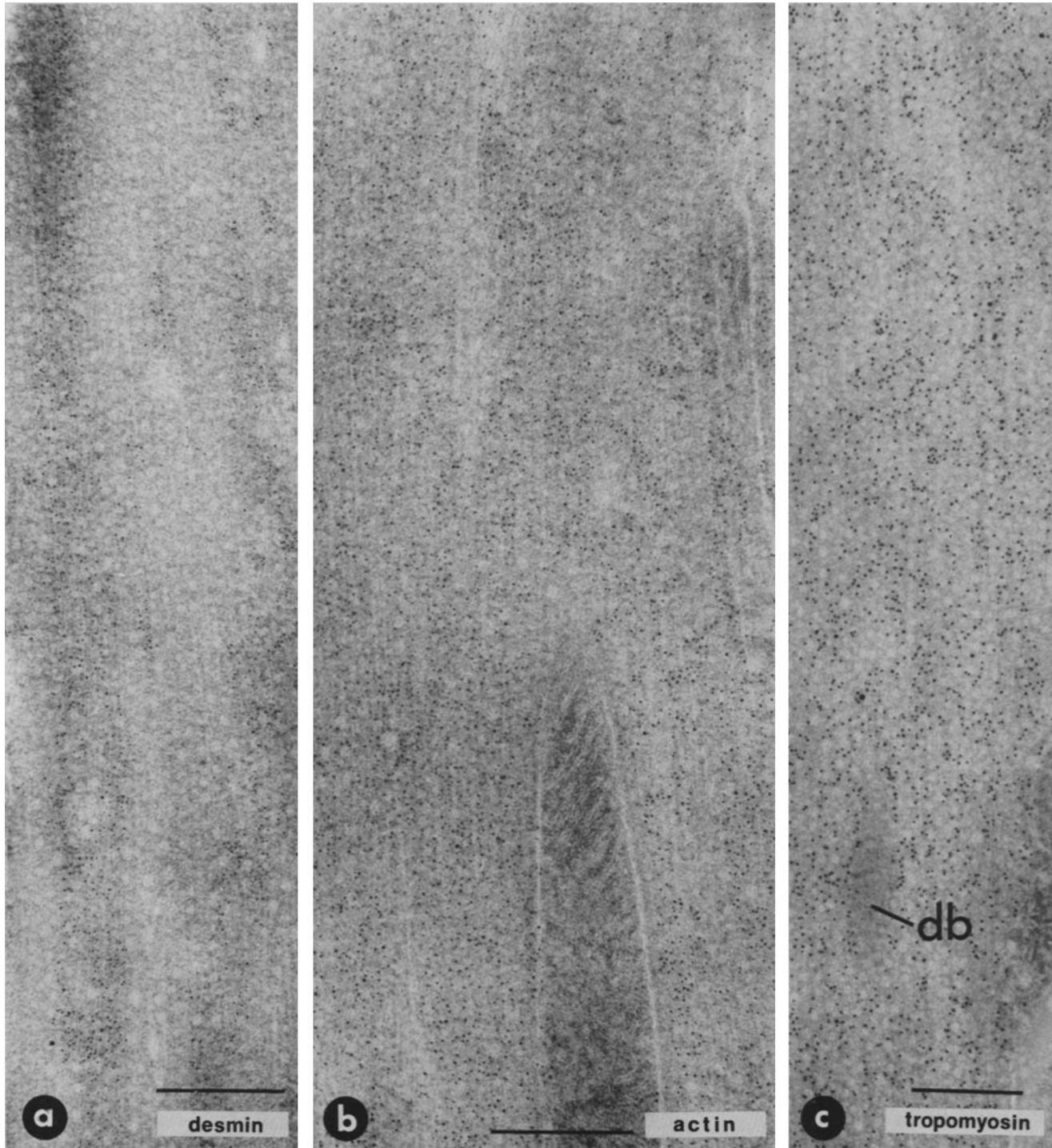

Figure 7. Longitudinal sections of chicken gizzard smooth muscle stained for (*a*) desmin (5-nm gold); (*b*) actin (5-nm gold); and (*c*) tropomyosin (10-nm gold). Desmin is organized in longitudinal arrays, as is filamin (see also text). Actin and tropomyosin are distributed more or less uniformly, tropomyosin being excluded from the dense bodies (*db*). Bars, 0.4 μm.

Discussion

Filamin belongs to a class of rod-shaped Ca^{++}-insensitive actin gelling proteins that include α-actinin, spectrin, and spectrin-related proteins (11, 19). Under physiological conditions filamin exists as an elongated (8, 44) 500,000-mol-wt dimeric molecule (45) formed from the antiparallel end-to-end attachment of its monomer chains (13, 44). The molecule is highly flexible due to a predominance of β-structured and unstructured domains (25) and possesses, in its dimeric form, actin-binding sites at each end (23, 44) that are responsible for its potent actin gelling activity. Apart from being present in smooth muscle, filamin has been identified in various other cells and tissues (39), but in smooth muscle it is particularly plentiful, occurring in amounts estimated as 30–40% of the myosin content (46).

The demonstrated absence of filamin from the actomyosin system in smooth muscle and its localization within the intermediate filament zones is not only of interest in its own right but has brought to light the existence of two spatially separate domains that were not formerly appreciated; an actomyosin domain and an intermediate filament–actin domain. Indeed, this initial analysis of the composition of these two domains provides strong evidence against the type of sarcomeric model for the contractile apparatus proposed by others (see the introduction). Since myosin filaments are excluded from the intermediate filament–actin domains, a model that incorporates thick myosin filaments interdigitating between actin filaments that emanate from the cytoplasmic dense bodies is rendered very unlikely. More detailed evidence concerning the organization of the actomyosin system will be the subject of a later report. We may already conclude, however, that filamin plays neither a direct role in the organization of the contractile elements, for example in the spacing or polar arrangement of the thin filaments in the myofilament lattice, nor in the regulation of the actin–myosin interaction (12). More recently, we have shown that combined staining with α-actinin and filamin antibodies gives the same fibrillar staining pattern as with filamin antibodies alone, further confirming the presence of α-actinin (and hence the dense bodies) in the filamin-containing domains.

As was argued by one referee of this paper, the actin filaments within the intermediate filament domains and that contain filamin may simply arise by intrusion from the periphery of the actomyosin domains. A more likely alternative conclusion is that the actin filaments that emanate from the dense bodies (6) contain filamin and that these splay into the periphery of the actomyosin domains. While we cannot rigidly exclude these possibilities, the consistent, high concentration of labeling by filamin antibodies across the width of even the largest intermediate filament arrays suggests the existence of a mainly separate subset of actin filaments that presumably possesses a non-contractile function. Intuitively, these filaments, being co-distributed with the intermediate filaments and apparently originating from the dense bodies contained within the intermediate filament arrays, serve some kind of cytoskeletal role. It is conceivable for example that the intermediate filament–dense body system may be fixed, transitorily, at any length through the cross-linking by filamin of interdigitating arrays of thin filaments originating from the dense bodies (6). In this way filamin could serve a role in stress maintenance and thus act as an important modulator of the mechanical response. Alternatively, or additionally, filamin may act as a coupler between the contractile and cytoskeletal and membrane domains. The membrane sites are here envisaged as comprising a layer of actin filaments, or oligomers, that is bound to the membrane by membrane-actin-linkers and that is functionally separated from the actomyosin system. Filamin, as a long range cross-linker, could couple the myofilaments and membrane sites through side binding on actin filaments located in the two domains. A possible, direct interaction of filamin with intermediate filaments also remains to be tested.

In studies of the mechanical properties of smooth muscle, considerable attention has been paid to the role of myosin phosphorylation and its modulation during the contractile cycle (see, e.g., reference 10). Further, to explain the characteristic ability of smooth muscle to maintain stress with low expenditure of energy, two possible modes of cross-bridge cycling have been invoked, a latched state and a cycling state (17). It has further been suggested, from studies on skinned muscles, that these two states respond to different levels of free Ca^{++} concentration (9). But these considerations have been made without regard to the possible involvement of actin-associated proteins that exist in significant amounts in smooth muscle. Among these, filamin represents a serious candidate for the performance of modulating functions leading to actomyosin-independent stress maintenance. It is proposed that actin–actin interactions mediated by such proteins constitute a much more likely basis for the tonic response than a dual activity of myosin.

Note added in proof: More recent studies have shown that caldesmon is specifically localized in the actomyosin domains (Fürst, D. O., R. A. Cross, J. De Mey, and J. V. Small, manuscript in preparation).

References

1. Anonymous. 1976. Gelvatol Polyvinyl Alcohol Resin: Properties and Uses. Tech. Bull. No. 6082 G, Monsanto Polymers and Petrochemicals Co., St. Louis, MO. 1–21.

2. Ashton, F. T., A. V. Somlyo, and A. P. Somlyo. 1975. The contractile apparatus of vascular smooth muscle: intermediate high voltage stereo electron microscopy. *J. Mol. Biol.* 98:17–29.

3. Bagby, R. M. 1980. Double immunofluorescent staining of isolated smooth muscle cells. *Histochemistry.* 69:113–130.

4. Bagby, R. M. 1983. Organization of contractile/cytoskeletal elements. *In* Biochemistry of Smooth Muscle. Volume 1. N. L. Stephens, editor. CRC Press Inc., Boca Raton, FL. 1–84.

5. Bagby, R. M., and F. A. Pepe. 1978. Striated myofibrils in anti-myosin stained, isolated chicken gizzard smooth muscle cells. *Histochemistry.* 58:219–235.

6. Bond, M., and A. V. Somlyo. 1982. Dense bodies and actin polarity in vertebrate smooth muscle. *J. Cell Biol.* 95:403–413.

7. Bretscher, A. 1984. Smooth muscle caldesmon: rapid purification and F-actin cross-linking properties. *J. Biol. Chem.* 259:12873–12880.

8. Castellani, L., G. Offer, A. Elliot, and E. J. O'Brien. 1981. Structure of filamin and F-actin-heavy merofilamin complex. *J. Muscle Res. Cell Motil.* 2:193–202.

9. Chatterjee, M., and R. A. Murphy. 1983. Calcium-dependent stress maintenance without myosin phosphorylation in skinned smooth muscle. *Science (Wash. DC)*. 22:464–466.

10. Cooke, R., and J. T. Stull. 1981. Myosin phosphorylation: a biochemical mechanism for regulating contractility. *In* Cell and Muscle Motility. Volume 1. Robert M. Dowben and Jerry W. Shay, editors. Plenum Publishing Corp., New York. 99–133.

11. Craig, S. W., and T. D. Pollard. 1982. Actin binding proteins. *Trends Biochem. Sci.* 7:88–92.

12. Davies, P., P. Bechtel, and I. Pastan. 1977. Filamin inhibits actin activation of heavy meromyosin ATPase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 77:228–232.

13. Davies, P. J. A., D. Wallach, M. C. Willingham, I. Pastan, M. Yamaguchi, and R. M. Robson. 1978. Filamin-actin interaction: dissociation of binding from gelatin by Ca^{2+}-activated proteolysis. *J. Biol. Chem.* 253:4036–4042.

14. De Mey, J. 1983. Raising and testing antibodies for immunocytochemistry. *In* Immunocytochemistry: Practical Applications in Pathology and Biology. J. M. Polak and S. von Noorden, editors. John Wright & Sons Ltd., Bristol, UK. 43–52.

15. De Mey, J., and M. Moeremans. 1986. The preparation of colloidal gold probes and their use as markers in electron microscopy. *In* Advanced Techniques in Biological Electron Microscopy. Volume 3. G. K. Koehler, editor. Springer-Verlag, Heidelberg. In press.

16. De Mey, J., M. Moeremans, G. Guens, R. Nuydens, and M. De Brabander. 1981. High resolution light and electron microscopic localization of tubulin with the IGS (Immuno Gold Staining) method. *Cell Biol. Int. Rep.* 5:889–899.

17. Dillon, P. F., M. O. Aksoy, S. P. Driska, and R. A. Murphy. 1981. Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. *Science (Wash. DC).* 211:495–497.

18. Fay, F. S., K. Fujiwara, D. D. Rees, and K. E. Fogarty. 1983. Distribution of α-actinin in single isolated smooth muscle cells. *J. Cell Biol.* 96:783–795.

19. Geiger, B. 1983. Membrane-cytoskeleton interaction. *Biochim. Biophys. Acta.* 737:305–341.

20. Geiger, B., A. H. Dutton, K. T. Tokuyasu, and S. J. Singer. 1981. Immunoelectron microscope studies of membrane-microfilament interactions: distribution of α-actinin, tropomyosin and vinculin in intestinal epithelial brush border and chicken gizzard smooth muscle cells. *J. Cell Biol.* 91:614–628.

21. Gordon, C. N. 1972. The use of octadecanol monolayers as wetting agents in the negative staining technique. *J. Ultrastruct. Res.* 39:173–185.

22. Gregory, D. W., and B. J. S. Pirie. 1973. Wetting agents for biological electron microscopy. I. General considerations and negative staining. *J. Microsc.* 99:261–265.

23. Hartwig, J. H., and T. P. Stossel. 1981. Structure of macrophage actin binding protein molecules in solution and interacting with actin filaments. *J. Mol. Biol.* 145:563–581.

24. Kendrick-Jones, J., and J. M. Scholey. 1981. Myosin-linked regulatory systems. *J. Muscle Res. Cell Motil.* 2:347–372.

25. Koteliansky, V. E., M. A. Glukhova, V. P. Shirinsky, V. N. Smirnov, T. L. Bushueva, V. V. Filiminov, and S. Yu. Venyaminov. 1982. A structural study of filamin, a high molecular weight actin-binding protein from chicken gizzard. *Eur. J. Biochem.* 121:553–559.

26. Langanger, G., J. De Mey, M. Moeremans, G. Daneels, M. De Brabander, and J. V. Small. 1984. Ultrastructural localization of α-actinin and filamin in cultured cells with the immunogold staining (IGS) method. *J. Cell Biol.* 99:1324–1334.

27. Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. *Nature (Lond.).* 283:249–256.

28. Lin, J.J.-C. 1981. Monoclonal antibodies against myofibrillar compo-

nents of rat skeletal muscle decorate the intermediate filaments of cultured cells. *Proc. Natl. Acad. Sci. USA.* 78:2335–2339.

29. Marston, S. B. 1982. The regulation of smooth muscle contractile proteins. *Prog. Biophys. Mol. Biol.* 41:1–41.

30. Masaki, T., M. Endo, and S. Ebashi. 1967. Localization of 6S component of α-actinin and Z-band. *J. Biochem. (Tokyo).* 62:630–632.

31. Matsudaira, P. T., and D. R. Burgess. 1978. SDS microslab linear gradient polyacrylamide gel electrophoresis. *Anal. Biochem.* 87:386–396.

32. Moeremans, M., G. Daneels, A. Van Dijck, G. Langanger, and J. De Mey. 1984. Sensitive visualization of antigen-antibody reactions in dot and blot immune overlay assays with immunogold and immunogold/silver staining. *J. Immunol. Methods.* 74:353–360.

33. Schollmeyer, J. E., L. T. Furcht, D. E. Goll, R. M. Robson, and M. H. Stromer. 1976. Localization of contractile proteins in smooth muscle cells and in normal and transformed fibroblasts. *In* Cell Motility. Volume A. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 361–388.

34. Small, J. V. 1977. Studies on isolated smooth muscle cells: the contractile apparatus. *J. Cell Sci.* 24:327–349.

35. Small, J. V. 1984. Polyvinylalcohol, a water soluble resin suitable for electron microscope immunocytochemistry. *Proc. Eur. Congr. Electron Microsc., 8th, Budapest.* 3:1799–1800.

36. Small, J. V. 1985. Geometry of actin-membrane attachments in the smooth muscle cell: the localizations of vinculin and α-actinin. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:45–49.

37. Small, J. V., and A. Sobieszek. 1977. Studies on the function and composition of the 10-nm (100-A) filaments of vertebrate smooth muscle. *J. Cell Sci.* 23:243–268.

38. Small, J. V., and A. Sobieszek. 1980. The contractile apparatus of smooth muscle. *Int. Rev. Cytol.* 64:241–306.

39. Small, J. V., and A. Sobieszek. 1983. Contractile and structural proteins of smooth muscle. *In* Biochemistry of Smooth Muscle. Volume 1. N. L. Stephens, editor. CRC Press Inc., Boca Raton, FL. 85–140.

40. Sobieszek, A., and B. Barylko. 1985. Enzymes regulating myosin phosphorylation in vertebrate smooth muscle. *In* Smooth Muscle Contraction. N. L. Stephens, editor. Marcel Dekker Inc., New York. 283–316.

41. Sobue, K., Y. Muramoto, M. Fujita, and S. Kakiuchi. 1981. Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. *Proc. Natl. Acad. Sci. USA.* 78:5652–5655.

42. Strzelecka-Golaszewska, H., E. Prochniewicz, E. Nowak, S. Zmorzynski, and W. Drabikowski. 1980. Chicken gizzard actin: polymerization and stability. *Eur. J. Biochem.* 104:41–52.

43. Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

44. Tyler, J. M., J. M. Anderson, and D. Branton. 1980. Structural comparison of several actin-binding macromolecules. *J. Cell Biol.* 85:489–495.

45. Wang, K. 1977. Filamin, a new high-molecular-weight protein found in smooth muscle and non-muscle cells. Purification and properties of chicken gizzard filamin. *Biochemistry.* 16:1857–1865.

46. Wang, K., J. F. Ash, and S. J. Singer. 1975. Filamin, a new high-molecular weight protein found in smooth muscle and non-muscle cells. *Proc. Natl. Acad. Sci. USA.* 72:4483–4486.

47. Weber, K., and M. Osborn. 1981. Microtubule and intermediate filament networks in cells viewed by immunofluorescence microscopy. *In* Cytoskeletal Elements and Plasma Membrane Organization. G. Poste and G. L. Nicolson, editors. 1–53. Elsevier/North Holland Biomedical Press, Amsterdam.

48. Weber, K., P. C. Rathke, and M. Osborn. 1978. Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells by electron microscopy and by immunofluorescence microscopy. *Proc. Natl. Acad. Sci. USA.* 75:1820–1824.