The Molecular Organization of Myosin in Stress Fibers of Cultured Cells

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Abstract. Antibodies to chicken gizzard myosin, subfragment 1, light chain 20, and light meromyosin were used to visualize myosin in stress fibers of cultured chicken cells. The antibody specificity was tested on purified gizzard proteins and total cell lysates using immunogold silver staining on protein blots. Immunofluorescence on cultured chicken fibroblasts and epithelial cells exhibited a similar staining pattern of antibodies to total myosin, subfragment 1, and light chain 20, whereas the antibodies to light meromyosin showed a substantially different reaction.

The electron microscopic distribution of these antibodies was investigated using the indirect and direct immunogold staining method on permeabilized and fixed cells. The indirect approach enabled us to describe the general distribution of myosin in stress fibers. Direct double immunogold labeling, however, provided more detailed information on the orientation of myosin molecules and their localization relative to α-actinin: α-actinin, identified with antibodies coupled to 10-nm gold, was concentrated in the dense bodies or electron-dense bands of stress fibers, whereas myosin was confined to the intervening electron-lucid regions. Depending on the antibodies used in combination with α-actinin, the intervening regions revealed a different staining pattern: antibodies to myosin (reactive with the head portion of nonmuscle myosin) and to light chain 20 (both coupled to 5-nm gold) labeled two opposite bands adjacent to α-actinin, and antibodies to light meromyosin (coupled to 5-nm gold) labeled a single central zone. Based on these results, we conclude that myosin in stress fibers is organized into bipolar filaments.

SINCE the beginning of tissue culture, cytoplasmic fibrils in the form of stress fibers or tension striae have been observed in various types of cultured cells (31, 35). They were believed to develop under tension and to be reversible when tension is altered or relaxed. Already at that time they were associated with "contractile substances" or "contractile protoplasm" that was thought to coagulate into fibrillae of various sizes (31). In addition to cells in culture, stress fibers have been described in various tissues in situ, including endothelial cells (1, 10, 17, 32, 36, 44, 47), retinal pigmented epithelium (22), and fish scale fibroblasts (6) (for a review see reference 7). In the electron microscope they are seen as parallel bundles of 5- to 7.5-nm microfilaments (4). Their major structural component has been identified as F-actin (2, 8, 24, 41). In addition, stress fibers or microfilament bundles contain proteins similar to smooth muscle filamin (43), α-actinin (15, 30), myosin (16, 45), tropomyosin (29, 46), myosin light chain kinase (11), caldesmon (34), and vinculin (18). In immunofluorescent preparations, some of these, such as α-actinin, myosin, myosin light chain kinase, and tropomyosin, show an alternating periodic arrangement similar to that seen in muscle sarcomeres. This regular arrangement is further reflected in electron microscope images of fixed and thin sectioned cells where regularly spaced electron-dense material resembling the dense bodies of smooth muscle cells can be observed in microfilament bundles (20, 42).

More recently, we as well as other investigators have been able to identify these dense bodies as α-actinin-containing structures (26, 37). Furthermore, we have shown that filamin is localized in both electron-dense and electron-lucid regions (26). Myosin also was thought to be present in dense bodies (23, 48). However, double label immunofluorescence and comparative light and electron microscopic studies have indicated that α-actinin and myosin are not co-localized (21, 48). Not only has it been unclear where myosin is located relative to α-actinin, but the molecular arrangement of myosin has remained largely unknown.

The use of colloidal gold as a marker for intracellular proteins (14) in combination with our permeabilization fixation procedure (26) has now enabled us to obtain a clear distribution pattern of myosin together with well-preserved microfilaments.

By using indirect immunogold staining with antibodies to chicken gizzard (ch.g.)[1] total myosin, myosin subfragment 1 (SF1), and light chain 20 (L_{20}), we have been able to localize myosin in the form of regular bands in microfilament bundles. This approach, however, could not provide the correct information on the precise localization of myosin relative to α-actinin, and double immunolabeling was needed to identify the two proteins. This was achieved by coupling the myosin antibodies to 5-nm colloidal gold and those to α-actinin to 10-nm gold and applying them simultaneously to permeabilized and fixed cytoskeletons. The results revealed an alternating pattern of α-actinin and two bands of myosin separated by an unlabeled region. This striking arrangement prompted further investigations of the molecular organization of myosin, now known to be located between the α-actinin bands. Antibodies to ch.g. L_{20} and light meromyosin (LMM) were coupled to 5-nm gold and used for double localization studies with α-actinin.

The results presented here indicate that myosin is present in the form of bipolar filaments. This bipolar organization suggests that myosin and microfilaments are in the necessary conformation to mediate a sliding filament interaction. To what extent, however, the complex architecture of stress fibers allows a sliding filament movement is not yet fully understood.

Preliminary results have been presented in abstract form (27, 28).

Materials and Methods

Cell Culture

Chicken embryonic heart fibroblasts and lung epithelial cells were isolated as described previously (26). Dissociated cells were plated onto glass coverslips for light microscopy and onto Permanox petri dishes (LUX, Lab-Tek Div., Miles Laboratories Inc., Naperville, IL) for electron microscopy (26). All cells were grown in Eagle's minimum essential medium with Earle's salts (Flow Laboratories, Irvine, Ayrshire, Scotland), supplemented with antibiotics and 10% fetal calf serum (Flow Laboratories). They were incubated in a humidified 5% CO_2/air atmosphere at 37 °C.

Preparation of Total Cell Lysates for SDS Electrophoresis and Electroblotting

Fibroblasts and epithelial cells were grown in ten 9-cm Petri dishes and allowed to reach confluence. A total cell lysate used for immunoblotting experiments was prepared as follows: the cells were washed twice in phosphate-buffered saline without Ca^{++} or Mg^{++} (Gibco Laboratories Inc., Grand Island, NY). They were collected with a razor blade and transferred to 5-ml ice-cold 10% trichloracetic acid, which was replaced by 5% trichloracetic acid after 30 min on ice. The precipitate was washed twice with cold (−20°C) acetone and the acetone was allowed to evaporate. The dried cell residue was then solubilized in 1 ml boiling SDS sample buffer for 3 min. This concentrated cell lysate was centrifuged (15,000 *g*, 5 min), aliquoted, and stored until use.

Antibody Production, Purification, and Characterization

Rabbit antibodies to ch.g. α-actinin were obtained and characterized as described in a previous study (26). Smooth muscle myosin and SF1 were made from ch.g. according to Sobieszek and Small (40) with the inclusion of an additional DEAE-ion-exchange column purification step for SF1. ch.g. myosin L_{20} was isolated according to Sobieszek and Barylko (39), and LMM of ch.g. myosin was purified by R. A. Cross as described elsewhere (9). Rabbits were immunized with these proteins, and the antibodies obtained were affinity purified as reported previously (12).

[1] *Abbreviations used in this paper:* ch.g., chicken gizzard; L_{17} and L_{20}, myosin light chain 17 and 20, respectively; LMM, light meromyosin.

The antibody specificity was tested with the immunogold silver staining method on protein blots (33). A typical blot unit consisted of the following lanes: (*A*) reference proteins at 0.5 μg per band; (*B*) chicken heart fibroblasts, 25 μl of total cell lysate; (*C*) lung epithelial cells, 20 μl of total cell lysate; (*D*) ch.g. myosin (0.25 μg); (*E*) ch.g. myosin rod (0.25 μg); (*F*) ch.g. SF1 (0.25 μg); (*G*) ch.g. LMM (0.25 μg); and (*H*) ch.g. L_{20} (0.25 μg). In brief, the proteins were separated on SDS polyacrylamide gels prepared as described by Blattler et al. (3) and electrotransferred in 25 mM Tris-glycine buffer without methanol (19) onto Zeta-probe (Bio-Rad Laboratories, Richmond, CA) using a Bio-Rad Trans-Blot cell, 200 V for 3 h, cooled to 4°C. The blot units were quenched with 10% bovine serum albumin (BSA) in 20 mM Tris-buffered saline, pH 8.2, at 37°C for 16 h (overnight) and further processed for immunogold silver staining with different antibodies (at concentrations of 0.5 μg/ml in Tris-buffered saline + 0.1% BSA (see legend to Fig. 1) as described earlier (33). The staining pattern of the blots was related to the total protein pattern in the blot unit by staining a duplicate blot with FerriDye (Janssen Life Sciences Products, Beerse, Belgium). This protein stain, the first to give satisfactory results on positively charged nylon membranes, involves an incubation of the blot unit with positively charged cacodylate-iron hydroxide particles, which selectively bind to the proteins, and a subsequent reaction (1 min) with fresh acid potassium ferrocyanide, which converts the colloid to Prussian blue. The details of the method will be described elsewhere (Moeremans, M., M. De Raeymaeker, G. Daneels, and J. De Mey, manuscript submitted for publication).

Immunocytochemistry

Cytoskeletons were prepared as reported elsewhere (26), using 0.1% Triton X-100 extraction (30 s for fibroblasts, 45 s for epithelial cells), 0.5% glutaraldehyde fixation (10 min), 0.5% Triton X-100 permeabilization (30 min), treatment with sodium borohydride (1 mg/ml, 20 min), and incubation in normal goat serum (diluted 1:20, 15 min). Indirect immunofluorescence and indirect immunogold staining were carried out exactly as described in our previous paper (26).

Direct Double Immunogold Staining. The antibodies to ch.g. α-actinin were coupled to 10-nm colloidal gold, and those to total ch.g. myosin, L_{20}, and LMM were coupled to 5-nm gold according to De Mey (13), as modified by Slot and Geuze (38).

Each of the 5-nm gold complexes was mixed with anti-α-actinin-10-nm gold, applied to permeabilized and fixed cytoskeletons (prepared as above), and incubated overnight. The best results were obtained with the following concentrations: anti-α-actinin-10-nm gold, optical density at 520 nm (OD_{520}) = 0.3 to 0.9; anti-myosin-5-nm gold, OD_{520} = 0.6 to 1.2; anti-L_{20} 5-nm gold, OD_{520} = 1.2 to 1.8; and anti-LMM-5-nm gold, OD_{520} = 1.2 to 1.8. Control cells for direct double immunogold staining were incubated with unlabeled primary antibodies in concentrations of 2 to 5 μg/ml for 2 h, then washed three times for 10 min in Tris-buffered saline + 0.1% BSA, pH 8.2, and further incubated with the corresponding antibody-gold complexes in concentrations as described above, overnight. Control cells and double immunogold stained preparations were washed three times for 10 min in Tris-buffered saline buffer + 0.1% BSA, pH 8.2, before postfixation.

Electron Microscopy. Indirect and direct immunogold preparations were processed for electron microscopy exactly as reported in our previous paper (26), involving postfixation with 2% glutaraldehyde/0.2% tannic acid (for 30 min), fixation with 0.5% osmium tetroxide (for 10 min on ice), impregnation with 1% phosphotungstic acid/0.5% uranylacetate (for 30 min), dehydration in ethanol, Epon embedding, and thin sectioning.

All experiments were carried out at room temperature, unless otherwise stated.

Results

Antibody Characterization

Fig. 1 illustrates the antibody reactions on immunoblots. The SDS-separated proteins (Fig. 1 *a*) were transferred to Zeta-probe membranes and visualized with FerriDye (Fig. 1 *b*). Fig. 1 *c* shows the reaction of antibodies to whole ch.g. myosin molecules. The antibody reacted with the 200-kD heavy chain of purified myosin, myosin in the reference protein mixture, and all subfragments except the light chains. Fibroblasts and epithelial cell extracts showed a reaction on bands that had the same relative mobility as the myosin heavy chain. Additional lower molecular weight bands were present in epithelial cell extracts. The antibody to SF1 of ch.g. myosin (Fig. 1 *d*)

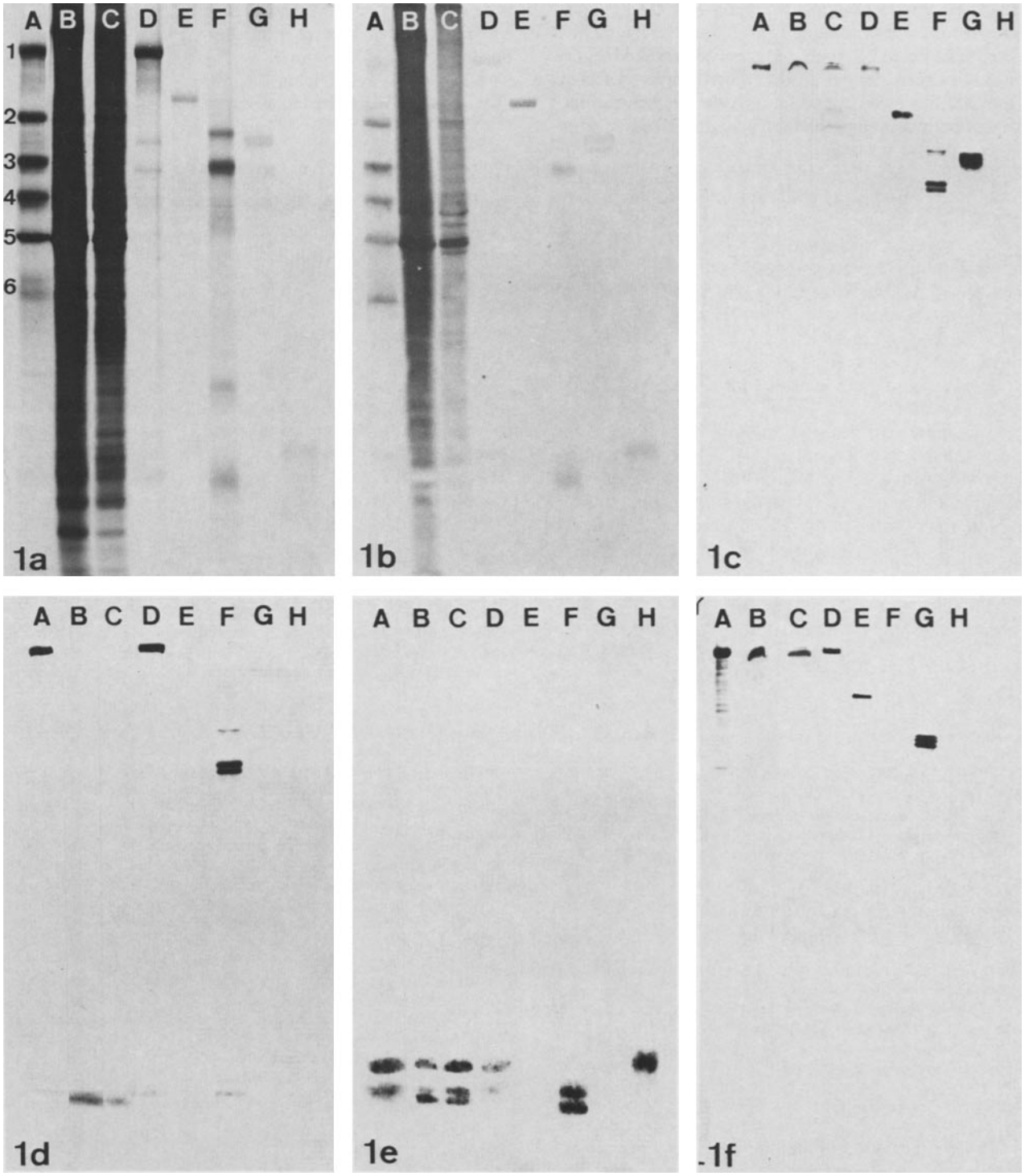

Figure 1. Characterization of antibodies to ch.g. total myosin, myosin SF1, L_{20}, and LMM. (*a*) Electrophoretic pattern of polypeptides in SDS polyacrylamide gels (12%) according to Blattler (3). Lane *A*, purified reference proteins: (*1*) ch.g. myosin heavy chain; (*2*) ch.g. α-actinin; (*3*) BSA; (*4*) rat brain tubulin; (*5*) ch.g. actin; and (*6*) pig stomach tropomyosin. Lane *B*, chicken heart fibroblasts. Lane *C*, chicken lung epithelial cells. Lane *D*, ch.g. myosin heavy chain. Lane *E*, ch.g. myosin rods. Lane *F*, ch.g. myosin SF1. Lane *G*, ch.g. LMM. Lane *H*, ch.g. L_{20}. (*b–f*) Immunoblots of electroeluted proteins on Zeta-Probe: (*b*) vizualization of the total protein pattern by FerriDye staining enhanced to Prussian blue. (*c–f*) Immunogold silver staining of protein blots reacted with antibodies to (*c*) total ch.g. myosin, (*d*) ch.g. myosin SF1, (*e*) ch.g. L_{20}, and (*f*) ch.g. LMM.

reacted with the myosin heavy chain in both myosin and the reference proteins containing myosin, SF1, and low molecular weight bands corresponding to the 17-kD light chain. In fibroblast and epithelial cell extracts it reacted with bands that had the same relative mobility as the light chain 17 (L_{17}) bands. Myosin rods and LMM fragments did not show any reaction. Fig. 1 *e* demonstrates the reactions of antibodies to ch.g. L_{20} with purified L_{20} and with both light chains (ch.g.

L_{20} and L_{17}) of purified myosin, myosin in the reference proteins, and the corresponding bands in whole cell extracts (fibroblasts and epithelial cells). Myosin rods and LMM fragments did not react. Antibodies to LMM (Fig. 1*f*) reacted with the 200-kD heavy chain of myosin in the reference proteins, pure myosin, and the corresponding bands in fibroblast and epithelial cell extracts. Bands slightly lower than the myosin heavy chain in the protein mixture probably represent breakdown products of myosin. The antibody did not react with SF1 or the light chains.

Light Microscopy. Immunofluorescence images of chicken heart fibroblasts (Fig. 2, *a–d*) and lung epithelial cells (Fig. 2,

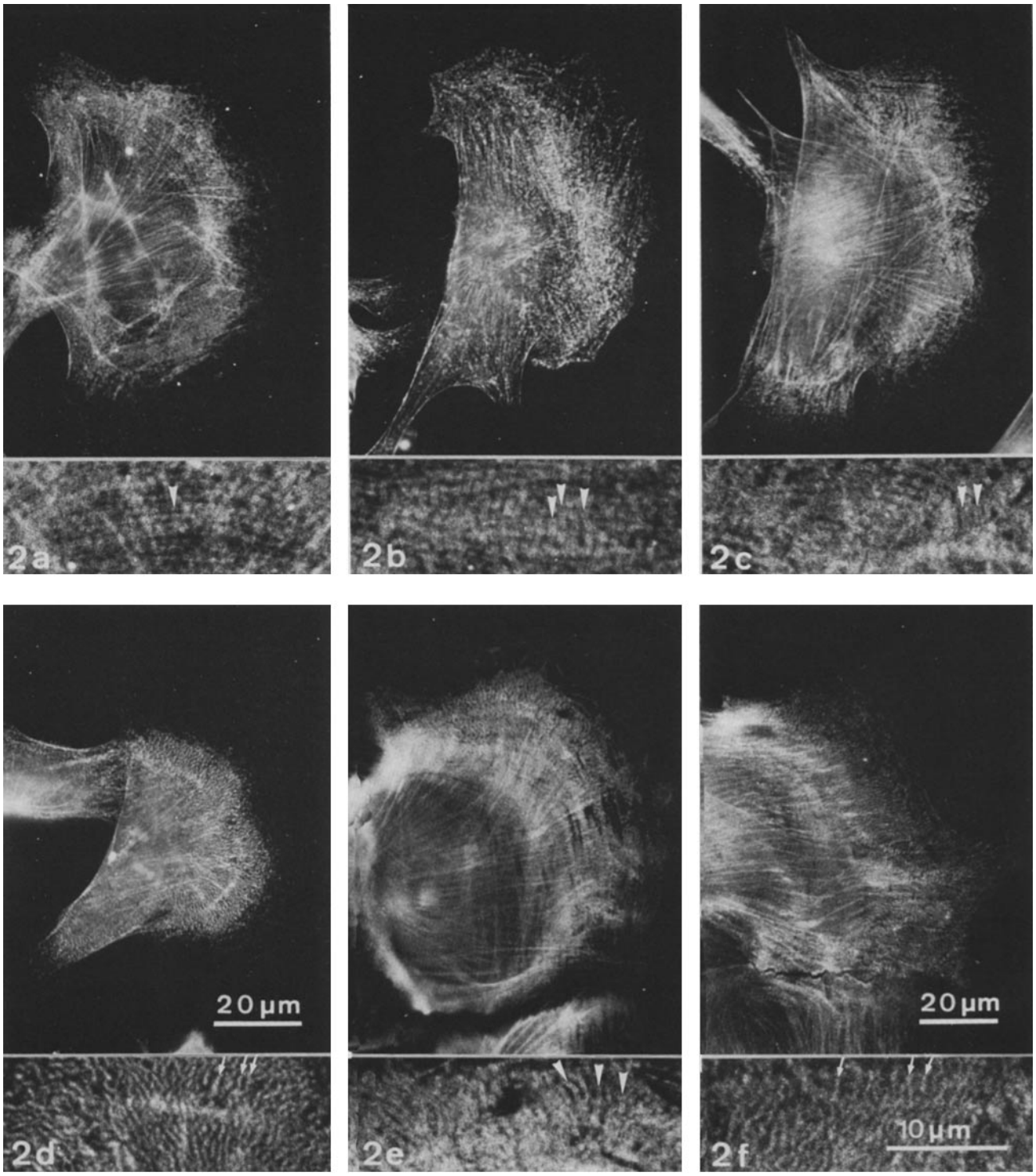

Figure 2. Immunofluorescence images of 0.1% Triton X-100 extracted chick heart fibroblasts (*a–d*) and lung epithelial cells (*e* and *f*) stained with antibodies to (*a*) ch.g. total myosin, (*b*) myosin SF1, (*c*) L_{20}, (*d*) LMM, (*e*) L_{20}, and (*f*) LMM. *a–c* and *e* exhibit a similar staining pattern of long fluorescent bands interrupted by shorter unstained regions. *d* and *f*, in contrast, show a more uniform banding pattern of dark and fluorescent regions. Insets show the different staining patterns in more detail: long fluorescent regions (*a–c* and *e*) often running in doublet bands (arrowheads), on the one hand, and single fluorescent bands (*d* and *f*, arrows), on the other.

e and *f*) show the reaction of antibodies to smooth muscle myosin and its subfragments with cultured cells. Cells stained with antibodies to total myosin (Fig. 2*a*), myosin SF1 (Fig. 2*b*), and L_{20} (Fig. 2, *c* and *e*) exhibit a similar pattern of relatively long fluorescent bands interrupted by shorter unstained periods. Antibodies to LMM, on the other hand, display a more uniform banding pattern of dark and fluorescent regions (Fig. 2, *d* and *f*). This characteristic difference is seen with particular clarity at higher magnifications (Fig. 2, insets). In addition, we have noted that some of the long fluorescent regions seen in the insets to Fig. 2, *a–c* and *e* appear in doublets, whereas the bands in Fig. 2, *d* and *f* do not show such subdivisions.

The fixation permeabilization procedure used here involved 0.1% Triton X-100 extraction and 0.5% glutaraldehyde fixation. As reported in previous research (26), the protein distribution in stress fibers is not significantly altered by this procedure. The organization of myosin is particularly well preserved under these conditions.

Electron Microscopy

Controls. The method specificity of the indirect immunogold procedure has been demonstrated previously, using 1% normal goat serum as negative and anti-tubulin staining as positive control (26). As described in our earlier report, the background staining of indirect immunogold preparations reflecting the unspecific binding of goat anti-rabbit antibodies coupled to gold is negligible (26). Staining of anti-α-actinin-10-nm gold and anti-myosin-5-nm gold was largely reduced by blocking the antigenic sites with unlabeled primary antibodies to α-actinin and myosin (Fig. 3.). The remaining gold labeling may partly be due to specific staining (because a certain amount of primary antibodies may have dissociated from the antigen and allowed the antibody-gold complex to bind to that site) and partly reflect the unspecific binding of the anti-α-actinin and -myosin gold complexes.

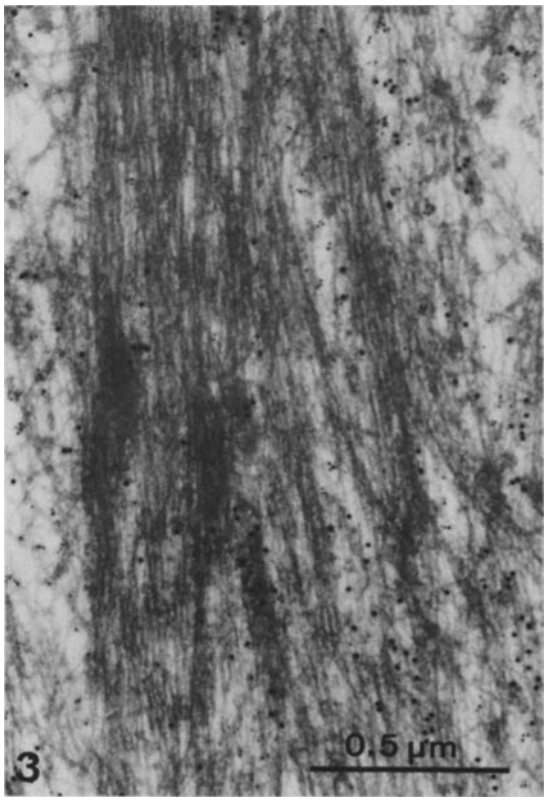

Figure 3. Control for double immunogold staining: fibroblast incubated with primary antibodies to smooth muscle α-actinin and myosin and then with anti-α-actinin-10-nm gold and anti-myosin-5-nm gold. Note the morphological details of stress fibers preserved under these conditions.

Indirect Immunogold Staining. By using antibodies to smooth muscle myosin, SF1, and L_{20} we could localize myosin in the form of regular bands interrupted by longer and shorter unlabeled regions. The localization of SF1, shown in Fig. 4*a* illustrates the type of reaction obtained with these antibodies. Some of the stained regions are arranged into doublet bands.

The permeabilization fixation procedure is identical to that used for light microscopy and has been shown to provide the optimal conditions for the preservation of ultrastructural details and the penetration of antibodies and gold probes into densely packed microfilament bundles (26).

Fig. 4*a* illustrates that the organization of myosin and the general appearance of microfilament bundles is well preserved under these conditions. Due to the accumulation of primary and secondary antibodies, however, and the following postfixation and contrasting procedures, the labeled regions were greatly enhanced in contrast. The dense bodies of stress fibers seen in glutaraldehyde-fixed cells and controls for indirect immunogold staining could no longer be discerned from the dark anti-myosin bands. Consequently, the sites of α-actinin could not be identified by morphologic criteria. Simultaneous localization of α-actinin and myosin with primary antibodies followed by immunogold labeling resulted in an homogeneous distribution of gold particles occasionally interrupted by unlabeled regions (Fig. 4*b*). Although dense bodies could be detected in these preparations, this approach did not provide enough evidence for a possible antiperiodicity of myosin and α-actinin.

Direct Double Immunogold Staining. The essential information on the distribution of myosin relative to α-actinin was obtained after double immunolabeling: simultaneous localization of α-actinin and myosin resulted in a periodic staining pattern of α-actinin (10-nm gold) flanked by two bands of myosin (5-nm gold) and unlabeled zones separating the confronting myosin regions (Fig. 5). This pattern was observed in all microfilament bundles of the cell body, including very fine rays of parallel actin filaments. Double immunolabeling with anti-L_{20}-5-nm gold and anti-α-actinin-10-nm gold revealed the same distribution pattern in fibroblasts and epithelial cells. A significantly different reaction was obtained by using anti-LMM-5-nm gold in combination with anti-α-actinin. These antibodies labeled the middle bands, which were unstained in the previous experiments (see Figs. 6, and 7, *c* and *f*). The two bands adjacent to α-actinin were largely unlabeled by the LMM antibodies. From these experiments and the results of indirect immunogold staining we conclude that the antibodies raised against total myosin and reactive with the whole molecule of smooth muscle myosin detect only the head regions of fibroblast and epithelial cell myosin. Myosin rods are located in the middle of stress fiber units and can only be identified with antibodies specific to smooth muscle LMM. This could indicate a basic difference between the rods of nonmuscle cell and smooth muscle myosin.

Higher magnifications of stress fiber units show the orga-

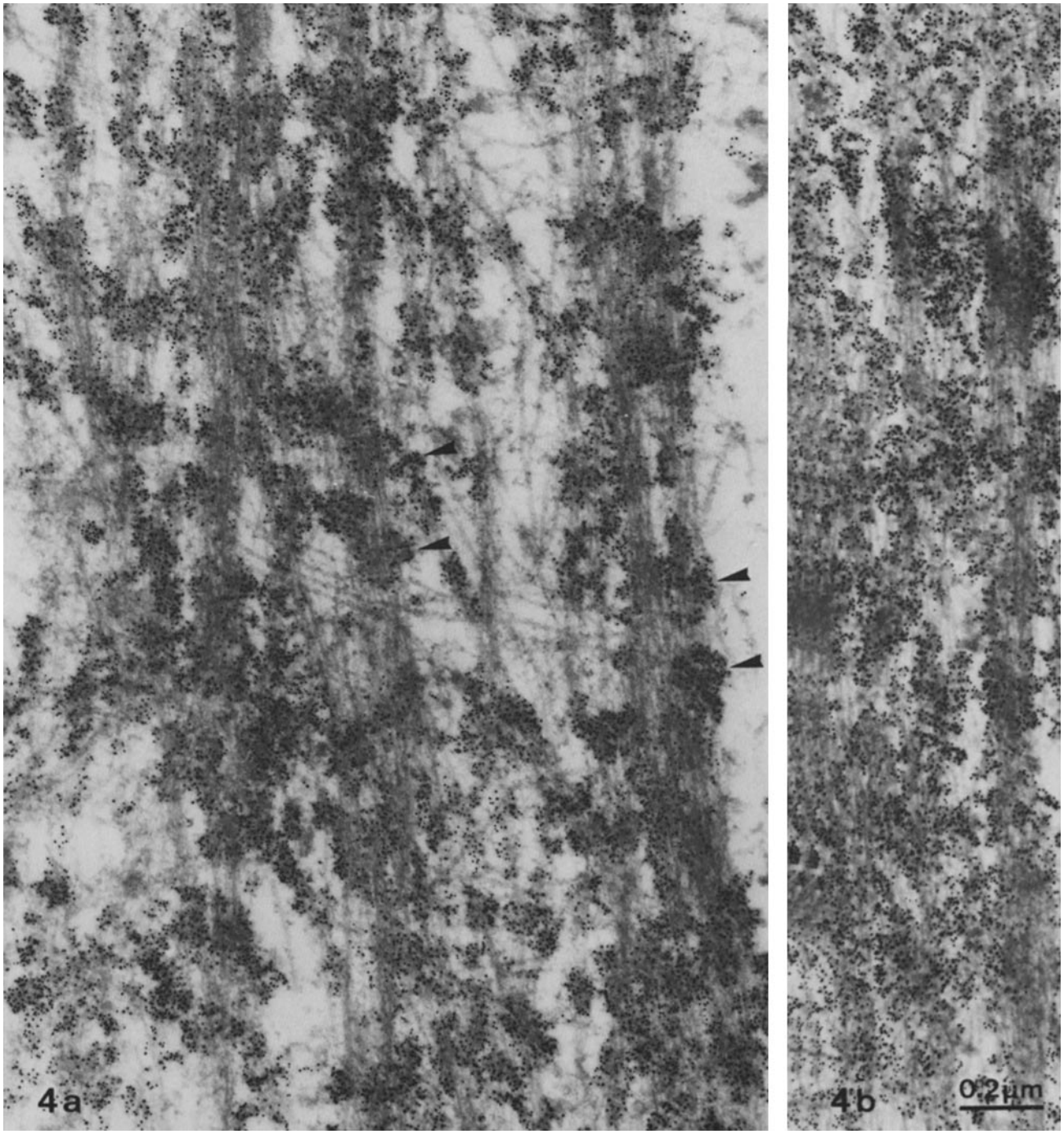

Figure 4. Thin section of microfilament bundles (stress fibers) labeled with (*a*) antibodies to myosin SF1 and goat anti-rabbit antibodies coupled to 5-nm gold (GAR-G5), and (*b*) antibodies to SF1, α-actinin, and GAR G5. Arrowheads in *a* indicate doublet bands.

nization of myosin in fibroblasts (Fig. 7, *a–c*) and epithelial cells (Fig. 7, *d–f*) in more detail: dense bodies are identified with indirect α-actinin staining in Fig. 7, *a* and *d* and direct 10-nm gold labeling in Fig. 7, *b*, *c*, *e*, and *f*. Morphological details of the intervening regions such as two electron-dense bands containing cross-bridges and a more electron-translucent central region are seen in Fig. 7, *a* and *d*. Regions corresponding to the two dense bands of cross-bridges are labeled with L_{20} antibodies (5-nm gold, Fig. 7, *b* and *e*) and the central zone with anti-LMM-5-nm gold (Fig. 7, *c* and *f*). The schematic drawing (Fig. 8) summarizes our results on the organization of actin, myosin, α-actinin, and filamin in stress fibers (see also reference 26): Actin filaments with opposite polarity emerge from α-actinin- and filamin-containing dense bodies. Filamin and myosin are localized in the intervening electron-lucid regions. Myosin is organized into bipolar filaments with a maximal length of 0.4 μm. Since Triton X-100 is used before fixation, the filaments could be slightly affected by the detergent. Therefore the exact diameter cannot be given.

The organization of myosin has mainly been described in fibroblasts. The thicker epithelial cells were less easily pene-

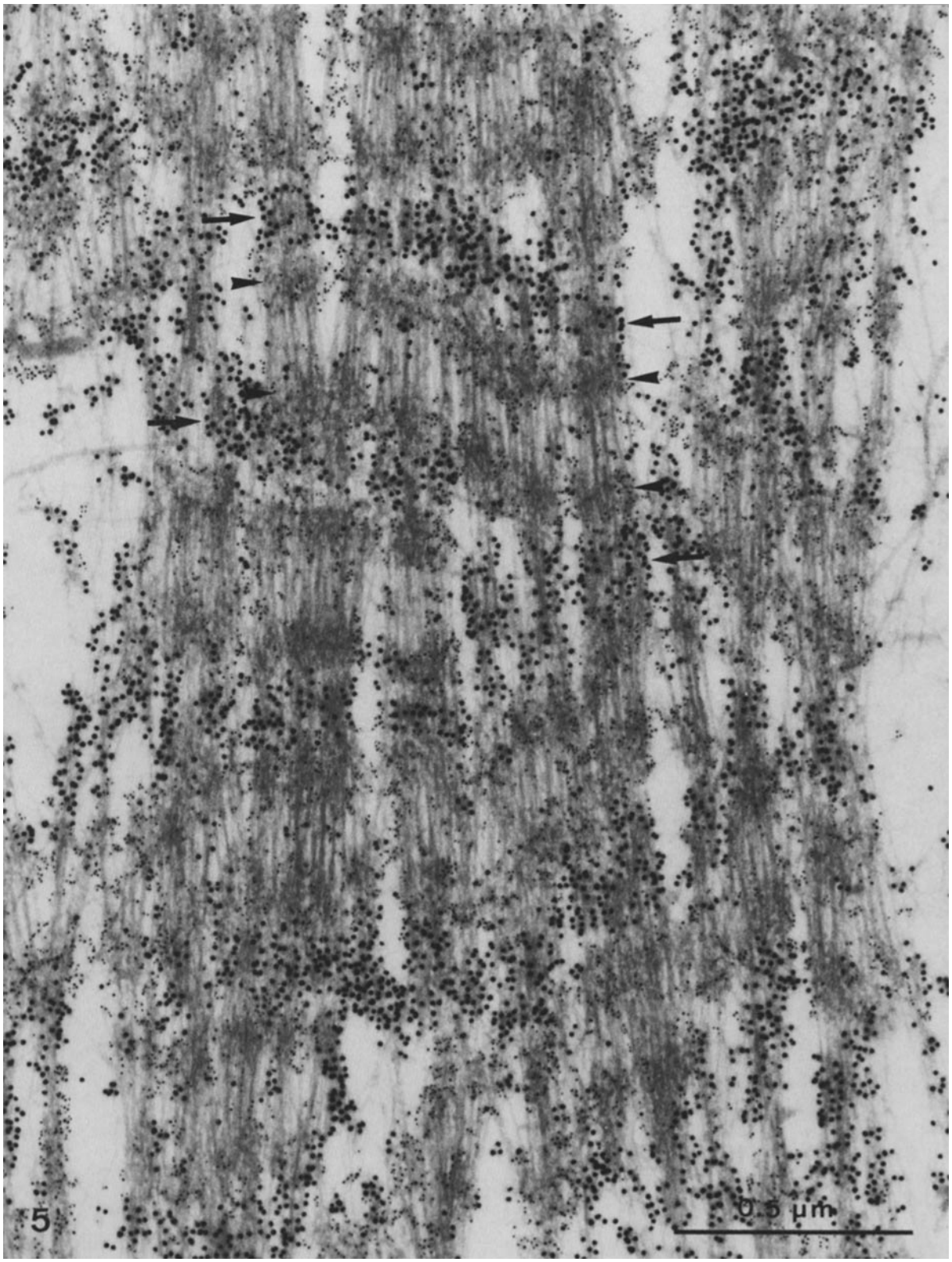

Figure 5. Double immunogold staining of α-actinin and myosin in a chick heart fibroblast: myosin, labeled with 5-nm gold (arrowheads) is confined to the regions between α-actinin (10-nm gold, arrows). Note the presence of doublet bands similar to those observed in SF1 and L_{20} stained cells and the bare zones in between.

trable for antibody-gold probes, and it was very difficult to obtain large areas in which two antibodies showed an optimal label density. The antibody distribution patterns observed in immunofluorescence preparations and in the small well-labeled areas, however, indicate that stress fibers of epithelial cells are organized like those in fibroblasts. The unit length however, may vary among different types of cells (see also reference 37).

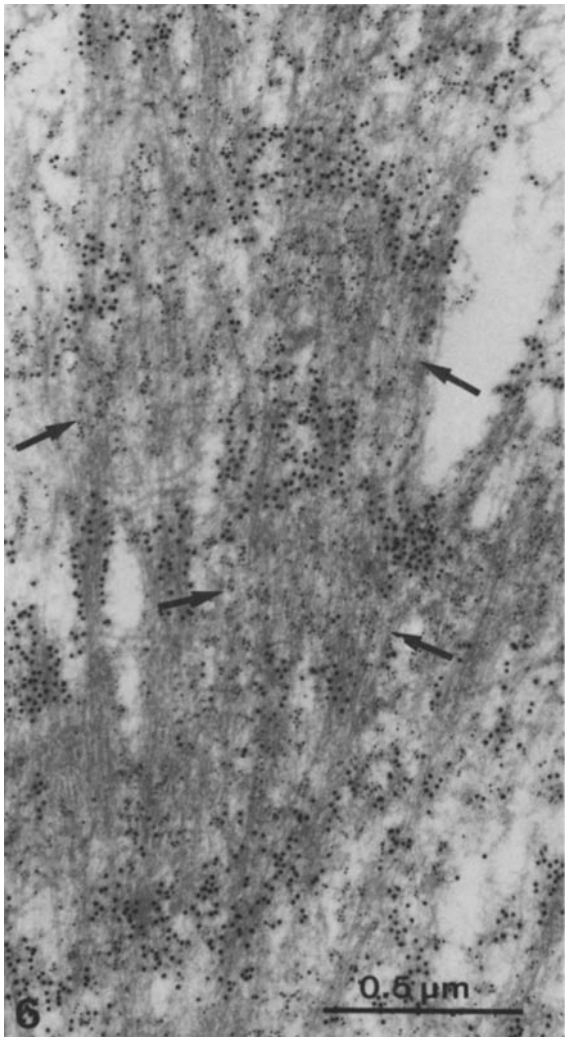

Figure 6. Double immunogold staining of α-actinin and myosin rods in a chick heart fibroblast: antibodies to LMM coupled to 5-nm gold decorate the middle bands (arrows) between the α-actinin regions identified by 10-nm gold.

Discussion

Antibody Characterization

The results document the specificity of all antibodies in both purified proteins and whole cell extracts. Anti-myosin, however, shows a cross-reaction in epithelial cells (Fig. 1 *c*, lane *C*) presumably corresponding to a reaction with keratin filaments (or associated proteins) seen in immunofluorescence and immunogold-stained cells (results not shown). Fibroblast intermediate filaments did not cross-react with anti-myosin. The antibody was not used for the localization of myosin in epithelial cells. Antibodies to SF1 strongly react with the myosin heavy chain of ch.g. myosin (in both purified myosin and reference proteins). Fibroblasts and epithelial cells, however, show a reaction only with the lower molecular weight light chain (L_{17}). The absence of immunoreactive 200-kD bands in both cell types suggests a significant difference between cellular and smooth muscle myosin. Our cells do not react with antibodies to striated muscle myosin (results not shown). Hence, we conclude that the cells used in this study are true nonmuscle cells.

Immunogold Staining

Whereas the light microscopic distribution of actin and associated proteins has been excellently documented by fluorescent techniques, the electron microscopic investigations have been greatly hampered by technical problems. Using the indirect immunogold staining method we have been able to overcome some of the difficulties. The technique has been adapted to the localization of actin and associated proteins (α-actinin, filamin) in either thin microfilament networks or bundles in cultured cells (26).

However, the conclusions obtained by the indirect method are always restricted to the one protein studied. An investigation of the distribution of two proteins with antibodies of the same species requires the use of antibodies directly coupled to different sizes of gold. Cells stained with these antibody-gold complexes revealed a somewhat higher background than usual. This may be caused by the rather high concentrations of gold probes that had to be used to obtain a significant label density. The use of homologous high affinity antibodies (especially anti-myosins) will probably overcome that problem. However, the double labeling approach using antibodies that cross-react relatively weakly with nonmuscle cell myosin has allowed us to gain more insight into stress fiber organization.

Microfilament Bundles: Architecture and Composition

Electron microscope images of microfilament bundles or stress fibers establish the existence of a substructure of alternating electron-dense and electron-lucid regions (20, 42). This arrangement is believed to reflect the periodic distribution of actin-associated proteins such as α-actinin, myosin, tropomyosin, and others.

Although double immunofluorescence and simultaneous localization studies of α-actinin and myosin (21, 48) have made it quite clear that these two proteins are not co-localized and arranged alternately along stress fibers, it has been more difficult to determine precisely their electron microscopic localization and correlate it with the typical fine structure of microfilaments. Both α-actinin (26, 37) and myosin (23, 48) have been found in electron-dense bands. This apparent contradiction can in part be explained by an artifact due to antibody staining and contrasting procedures: When α-actinin present in electron-dense material usually visible in glutaraldehyde-fixed preparations (20, 26, 42) is detected by gold-labeled antibodies and subsequently submitted to contrasting procedures, the general morphology of microfilament bundles is not altered but enhanced in its typical appearance (26). However, when myosin is localized by antibodies coupled to colloidal gold (see Fig. 4 *a*) or ferritin (23) or by protein A-gold complexes (25), additional proteins are accumulated and subsequently contrasted, resulting in structures often as dense or denser than the α-actinin containing dense bodies.

On the other hand, we have shown here that the intervening electron-lucid regions are subdivided into two zones of slightly increased electron-density that contain cross-bridges and a central, more electron-translucent, band. The use of our extraction procedure followed by tannic acid fixation and heavy metal contrasting allowed the visualization of structures formerly not seen in glutaraldehyde-fixed cells. Immunogold labeling with antibodies specific to either head or tail regions of myosin molecules has shown that the electron-dense bands adjacent to α-actinin correspond to myosin heads and the central more electron-translucent bands to myosin tails. The

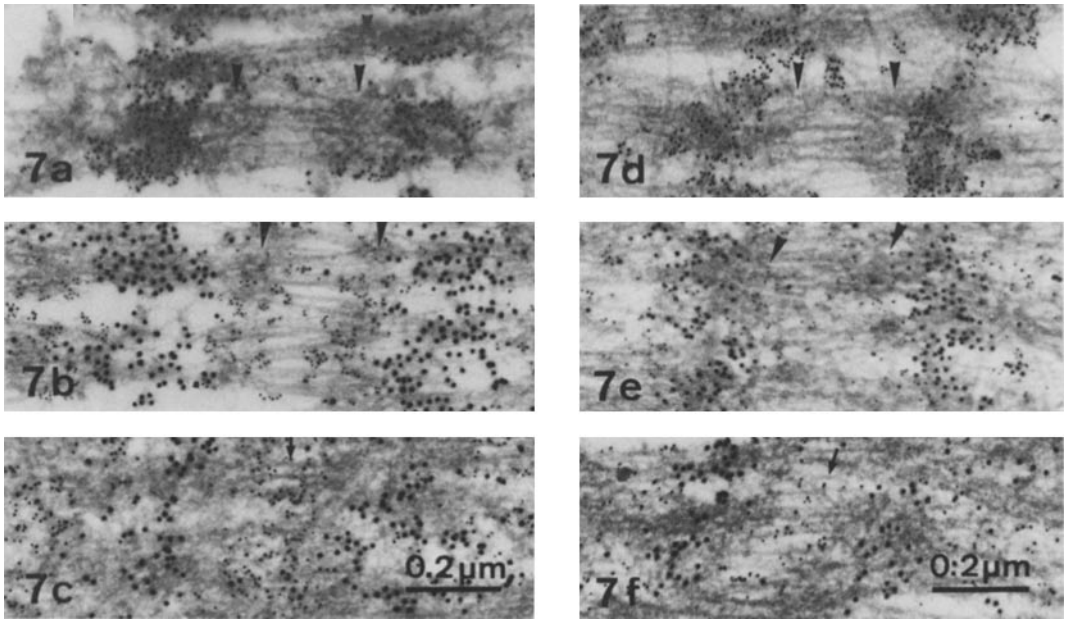

Figure 7. Analysis of stress fiber units in fibroblasts (*a–c*) and epithelial cells (*d–f*). Dense bodies are identified with anti-α-actinin and GAR-G5 in *a* and *d* and anti-α-actinin coupled to 10-nm gold in *b*, *c*, *e*, and *f*. (*a* and *d*) Fine structural details of the electron-lucid bands: two electron-dense regions containing cross-bridges (arrowheads) are adjacent to a bare, more electron-translucent zone in the middle. (*b* and *e*) Decoration of the two bands of cross-bridges by antibodies to L_{20} coupled to 5-nm gold (arrowheads). (*c* and *f*) Identification of the bare zones by antibodies to LMM bound to 5-nm gold (arrows).

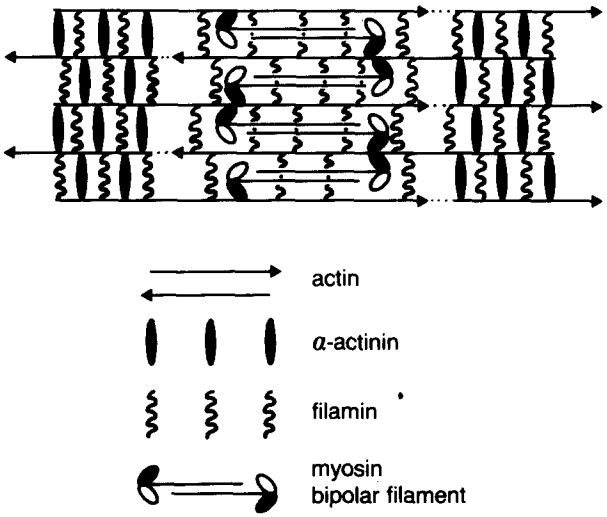

Figure 8. Schematic diagram illustrating our results on the organization of actin, α-actinin, filamin, and myosin in stress fibers. Maximal length of myosin filaments, 0.4 μm. Diameter not significantly wider than that of F-actin.

double-label experiments have established that α-actinin and myosin are not co-localized: α-actinin is concentrated in the electron-dense bands or dense bodies, and myosin is confined to the intervening (electron-lucid) regions. In summary, the questions regarding the localization of myosin in electron-dense bands can be answered as follows: A part of the myosin molecule, more specifically the myosin heads, is localized in bands of slightly increased electron density but not in the dense bodies that contain α-actinin. Due to their pronounced electron-opaque nature the latter are seen more clearly and much more frequently in fixed and thin-sectioned cells.

Based on the double label experiments with antibodies specific to either the head or tail regions of myosin molecules (anti-myosin, anti-L_{20}, and anti-LMM respectively), we conclude that myosin in stress fibers is present in the form of bipolar filaments. They could be dimers or organized into oligomers with a maximal length of 0.4 μm and a diameter not significantly wider than that of F-actin (~7 to 8 nm). Similar filaments 13 to 15 nm in diameter have been found in *N*-ethylmaleimide-treated negatively stained human glioma cells (25).

The ability of stress fibers to undergo either isotonic or isometric contractions has been a matter of discussion (5, 7). At present, it is not clear if myosin mainly serves as a cross-linker or mediates a sliding actomyosin movement. If stress fibers indeed undergo a contraction in vivo, the changes in unit length might be too small to be seen under the light microscope. Thus, new experimental conditions (e.g., the use of compounds favoring cell relaxation or contraction) and improved preparation techniques for immunocytochemistry are needed to document by electron microscopy these possible changes.

We are indebted to Dr. R. A. Cross (Department of Physics, Institute of Molecular Biology, Austrian Academy of Sciences, Salzburg) for his generous gift of purified myosin rod and LMM fragments; Lambert Leijssen, Guy Jacobs, and Hans Henderickx for their excellent photographic assistance, and C. Verellen for typing the manuscript.

These studies were supported by grants from the Belgian I.W.O.N.L. (Institut ter bevordering voor het Wetenschappelijke Onderzoek in Nijverheid en Landbouw) fund Brussels, the Austrian Research Council, and the Muscular Dystrophy Association, Inc.

Received for publication 18 June 1985, and in revised form 27 September 1985.

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