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Cell Biology

Filamin, a new high-molecular-weight protein found in smooth muscle and non-muscle cells

(myosin-like proteins/filaments/immunofluorescence)

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ABSTRACT **A new high-molecular-weight protein, named filamin, was isolated from chicken gizzard. In chicken gizzard, filamin is present in an amount approximately 30–40% of that of myosin. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of highly purified filamin revealed a single polypeptide of about 250,000 daltons. Rabbit antibody directed against purified chicken gizzard filamin did not crossreact with myosin purified from the same source. By the use of microcomplement fixation and indirect immunofluorescent staining with antibodies to chicken gizzard filamin, an antigenically similar or identical protein was found to be widely distributed both in other organs of the chicken and in cultured cells of other species, but not in chicken skeletal muscle. In cultured cells, filamin was found largely to be arranged as a filamentous array very similar to that found for myosin. These data imply that filamin is a widely occurring and chemically conserved component of filaments in smooth muscle and non-muscle cells.**

A variety of contractile proteins, such as myosin, actin, and tropomyosin, have been isolated from many non-muscle cells (for review see ref. 1). Proposals have been made that these proteins play a role in shape change, motility, and other mechanochemical activities of cells. The molecular mechanisms for these activities are, however, largely undetermined.

We have previously reported that a human smooth muscle myosin-like component was found in several human non-muscle cells. This smooth muscle myosin-like component was found partly associated with the cytoplasmic surface of the plasma membrane of human WI38 cells (2). These observations have led us to investigate further the molecular and immunological properties of contractile proteins from smooth muscles.

During the isolation of myosin from chicken gizzard smooth muscle, we observed a major high-molecular-weight protein not previously identified. We have obtained a homogeneous preparation of this protein and a high titer monospecific rabbit antiserum against it. Further chemical and immunochemical studies showed that this protein is distinct from chicken gizzard myosin. However, by an indirect immunofluorescent staining technique, we have found that this protein forms part of the intracellular filamentous structure in a variety of cells from different species. The filamentous staining patterns are very similar to those of smooth muscle-like myosins of the same cell type. Because of its structural location and apparent ubiquity, we have named this new high-molecular-weight protein "filamin". This report is a preliminary survey of the properties of filamin; further details will be published elsewhere* †

MATERIALS AND METHODS

Purification of Chicken Gizzard Filamin and Myosin*. The detailed procedures will be published elsewhere. Briefly, both filamin and myosin were extracted by homogenizing freshly prepared chicken gizzard smooth muscle in a high salt buffer. Myosin precipitated and filamin remained soluble when the clarified extract was dialyzed against low salt buffer. Filamin was further purified by ammonium sulfate precipitation, gel filtration, and DEAE-cellulose ion exchange chromatography. Myosin from the low salt precipitation step was purified free of actin by gel filtration in the presence of KI and ATP (3), followed by DEAE-cellulose ion

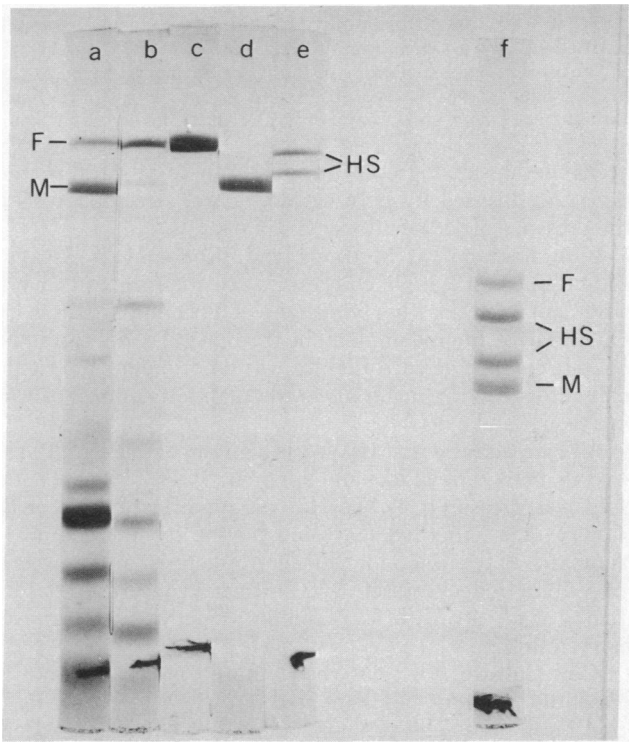

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of: (a) whole chicken gizzard homogenate (~50 μg of protein); (b) high salt extract of chicken gizzard (~50 μg); (c) purified chicken gizzard filamin (~10 μg); (d) purified chicken gizzard myosin (~10 μg); (e) purified human erythrocyte spectrin (~5 μg); and (f) a mixture (3 μg of each) of purified chicken gizzard filamin (F), chicken gizzard myosin (M), and human erythrocyte spectrin (HS). In (f), the duration of electrophoresis was about three times longer than that necessary for the tracking dye to reach the bottom of the gel; the ink mark near the bottom of this gel represents only the direction of electrophoresis, but *not* the position of tracking dye, as is the case in (a) to (e).

* Wang, Ash, and Singer, manuscript in preparation.
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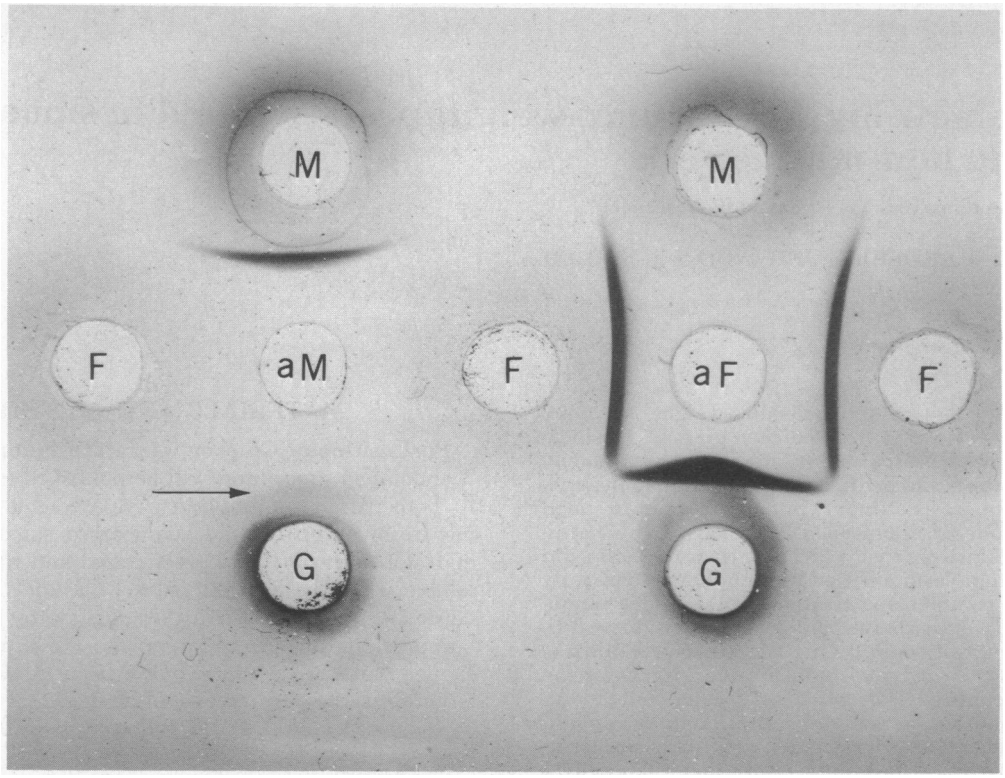

FIG. 2. Double diffusion test of rabbit antibody against chicken gizzard filamin and rabbit antibody against chicken gizzard myosin. F, purified chicken gizzard filamin (3 mg/ml); M, purified chicken gizzard myosin (2 mg/ml); G, high salt extract of chicken gizzard (15 mg/ml); aF, antiserum against filamin; aM, antiserum against myosin. The gel medium was 0.75% agarose in 0.6 M KCl, 25 mM phosphate buffer at pH 7.5. The gel was washed, dried, and stained with Amido black. Note that both sera are monospecific when tested against the crude gizzard extract (arrow shows band of weak reaction of antibody against myosin) and show no crossreactions.

exchange chromatography (4). The purity of various fractions was assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Immunochemical Methods and Biochemical Assays*. Rabbit antisera against chicken gizzard filamin and myosin were obtained by the lymph node injection method (5). Quantitative microcomplement fixation (6) with antibodies against chicken gizzard filamin was used to detect antigenically crossreacting materials in the high salt extracts of various tissues and cells.

ATPase activity was assayed using radioactive [γ-^{32}P]ATP by the method of Clarke and Spudich (3). Protein concentration was measured by the Hartree modification (7) of the Lowry *et al.* method (8) using bovine serum albumin as standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Fairbanks *et al.* (9), with 0.2% sodium dodecyl sulfate and 4% acrylamide.

Cell Culture. All cells were cultured in Eagle's Minimal Essential Medium supplemented with 10% fetal calf serum and antibiotics. The primary cultures of chick gizzard cells and dorsal root ganglian cells were obtained from 9-day embryos. The Balb 3T3 cells (strain BN 31A) were a gift of Dr. James Robb.

Indirect Immunofluorescent Staining†. Cells were cultured for at least 1 day after plating onto cover slips. The medium was removed and the cells were fixed with 2% formaldehyde in phosphate buffered saline and prepared for antibody staining†. The cells were reacted with rabbit antibody against filamin or antibody against myosin and then with fluorescein isothiocyanate conjugated goat antibody against rabbit IgG. The stained cells were observed with dark field ultraviolet optics on a Zeiss Photomicroscope with a BG 38 exciter filter and barrier filters 44 and 50.

RESULTS

Molecular properties of chicken gizzard filamin

When whole chicken gizzard homogenate was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, filamin appeared as a band with slower mobility than the heavy chain of myosin (200,000 daltons) (Fig. 1a). By staining intensity this band was present in an amount approximately 30–40% of that of myosin heavy chain. High salt extraction, which is generally used to purify myosin, simultaneously extracted both myosin and filamin (Fig. 1b). Further purification gave a final protein fraction at least 95% pure, with a molecular weight of about 250,000 (Fig. 1c). Unlike myosin, filamin apparently does not contain light chains in the 20,000 dalton region of sodium dodecyl sulfate-polyacrylamide gels. Filamin migrated slightly more slowly than the large chain of human spectrin (Fig. 1d and e). Prolonged coelectrophoresis of filamin, human spectrin, and chicken gizzard myosin clearly showed the distinctive mobilities of each of these components (Fig. 1f). Purified chicken gizzard filamin is soluble at both high (0.6 M KCl) and low (0.05 M KCl) ionic strength. The addition of up to 10 mM Ca^{++} or Mg^{++} did not cause any visible precipitation. In solution filamin is highly viscous at both high and low ionic strength even at low protein concentration (0.1 mg/ml). Although this is indicative of a highly extended structure, we have not been able to detect any filaments in a negatively stained preparation of filamin examined with the electron microscope.

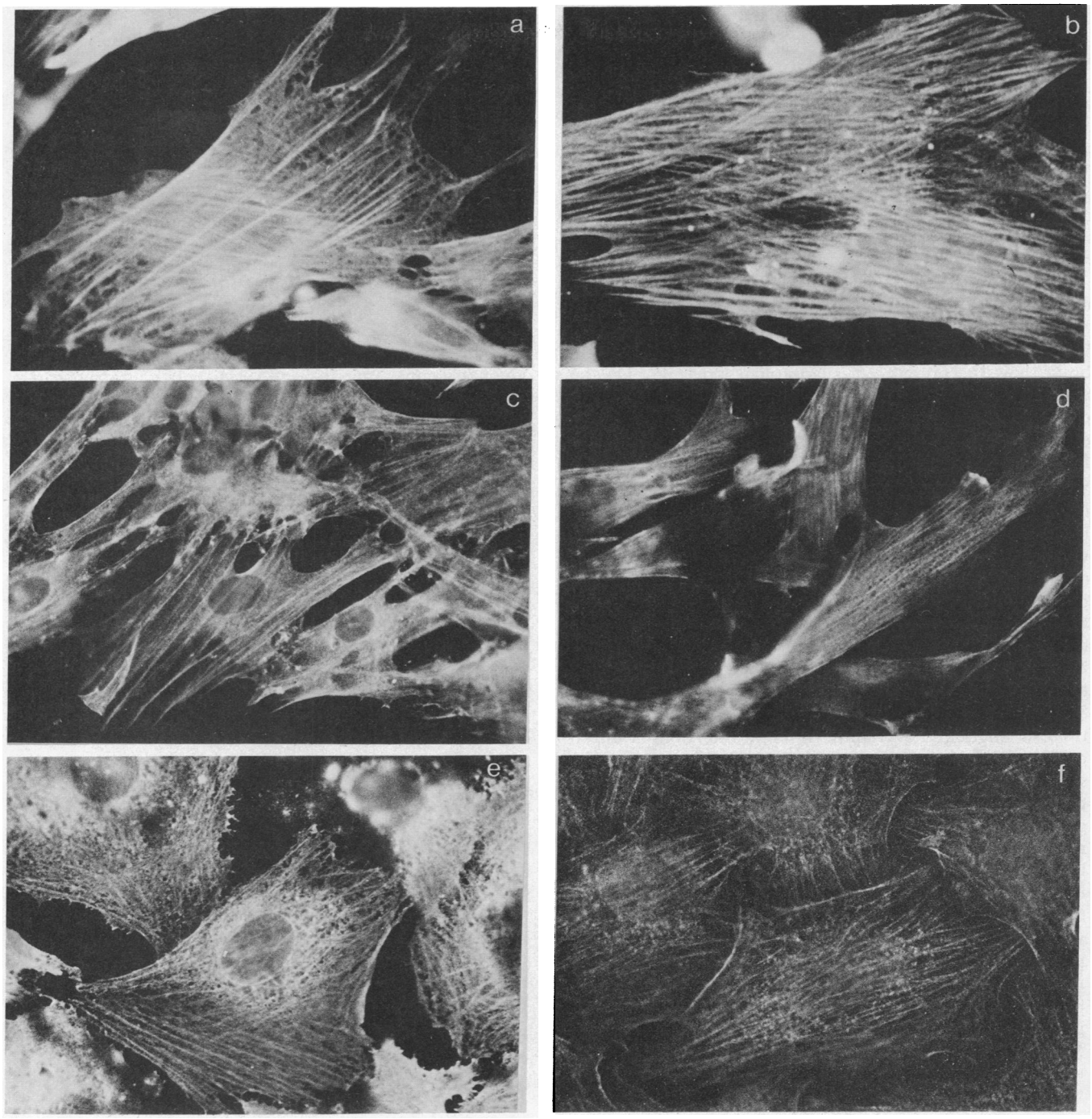

FIG. 3. Indirect immunofluorescent observation of cells stained with anti-filamin and anti-myosin. Chick embryo gizzard cells stained with (a) antiserum against filamin diluted 1:5 and (b) antiserum against gizzard myosin diluted 1:5. Chick embryo dorsal root ganglion cells stained with (c) anti-filamin IgG, 1 mg/ml, and (d) antiserum against gizzard myosin diluted 1:5. Mouse 3T3 cells stained with (e) anti-filamin IgG, 5 mg/ml, and (f) anti-uterine myosin IgG (2), 3 mg/ml. Magnification for all figures is 600×.

Purified chicken gizzard filamin exhibited no detectable high salt, K^+, and EDTA-activated ATPase activity nor actin-activated Mg^{++}-ATPase activity (specific activity $< 2 \times 10^{-3}$ μmol of P_i/mg per min at 37°). Preliminary amino-acid analysis of filamin showed that it contains no amino sugars or hydroxyproline, and that the composition is different from the published analysis of chicken gizzard myosin (10).

The above physical and chemical properties demonstrate that filamin is distinct from other well characterized high-molecular-weight proteins such as myosin and procollagen, and is most likely not a glycoprotein.

Immunological properties of filamin*

In order to identify any relatedness between chicken gizzard filamin and myosin, we have prepared rabbit antisera directed against highly purified chicken gizzard filamin and myosin. The monospecificity of these antisera was demonstrated by the Ouchterlony double diffusion tests, as shown in Fig. 2. Antisera directed against filamin gave a strong sin-

gle line of identity against purified filamin and the high salt extract of gizzard muscle. Similarly, antisera directed against gizzard myosin gave a single precipitin line with purified gizzard myosin and a weak line with the same high salt extract of gizzard. However, there was no detectable crossreaction between either anti-myosin and filamin or between anti-filamin and myosin. Thus gizzard filamin and myosin are distinctly different antigenically. Using the more sensitive immunochemical method of quantitative microcomplement fixation, we have also been unable to observe significant crossreaction between anti-filamin and gizzard myosin.

The occurrence of filamin has been examined by the quantitative microcomplement fixation technique using antibodies against filamin and the high salt extracts of various tissues of chicken. Of the tissues tested, kidney, liver, and brain show the presence of filamin-like molecules. On the other hand, under the same conditions filamin-like molecules were not detectable in chicken breast and leg muscle extracts.

Localization of filamin in cultured cells†

We next attempted to localize filamin incultured cells by the indirect immunofluorescent technique. In all cells examined, antibodies against filamin revealed a filamentous network similar to that observed with anti-myosin (11). If the cells were fixed, but not rendered permeable to antibody, no detectable staining resulted. This suggests that most, if not all, of the filamin is intracellular. Several examples of anti-filamin and anti-myosin staining of cultured cells are shown in Fig. 3. Staining of secondary cultures of embryonic chick gizzard cells with anti-filamin (Fig. 3a) or anti-myosin (Fig. 3b) both show long fiber bundles that traverse nearly the entire cell length and overlap in different directions at different levels of focus. These cultures should contain both smooth muscle cells and fibroblasts, although we did not distinguish between these two cell types. The dorsal root ganglion cells, presumably mostly glial cells derived from the neural crest, when stained with anti-filamin (Fig. 3c) exhibited both sparse long fibers and a uniform background staining; the latter could be due to either a finer or nonfilamentous distribution of filamin. A similar distribution is seen when cultures are stained with anti-myosin (Fig. 3d) with, perhaps, larger numbers of fibers present. When nearly confluent cultures of 3T3 cells are stained with anti-filamin (Fig. 3e) or anti-myosin (Fig. 3f) a characteristic pattern of groups of parallel fibers was observed with both antibodies. While the detailed staining patterns observed with both anti-filamin and anti-myosin require more detailed study, the general picture is clear.

DISCUSSION

We have shown that there exists a major hitherto undetected high-molecular-weight protein in extracts of chicken gizzard muscle which is chemically and immunologically distinct from chicken gizzard myosin. With antibodies to this protein, we have made two other important observations. First, using microcomplement fixation and indirect immunofluorescent staining, we found an antigenically similar or identical protein to be widely distributed both in other organs of the chicken and in tissue culture cells of other species. Second, in cultured cells this protein was generally found in a filamentous array closely similar to that of myosin (11). This information implies that the protein is a widespread and chemically conserved component of filaments in smooth muscle and in non-muscle animal cells, and we have therefore named the protein filamin.

Although we have no direct evidence as yet to support any functional role for filamin in smooth muscle or non-muscle cells, its ubiquity and organization imply that filamin might play a role in the regulation of mechanochemical activities of these cells by interacting with other contractile proteins or with cell membranes. If this is the case, absence of filamin in extracts of chicken skeletal muscle suggests that it is not essential for the basic sliding filament mechanism (12). It is to be emphasized that the name filamin does not necessarily imply that filamin is able to self-assemble into filaments; in fact, we have no evidence for this *in vitro*. The staining patterns observed would also be obtained if individual molecules of filamin were uniformly associated with another filamentous cellular structure such as an actin- or myosin-containing fiber. The low resolution of light microscopy does not allow an easy assessment of the possible relationship among filamin-, myosin-, and actin-containing structures in cells.

We initially explored the extent of the relationship between filamin and myosin because they are both high-molecular-weight components of gizzard muscle. Our findings, however, reveal that filamin is more similar to human spectrin, a high-molecular-weight protein from erythrocytes (13, 14), than to myosin. Aside from their similar but not identical molecular weights, they have similar solubilities at high and low ionic strength, they lack myosin-like ATPase activities, and they do not readily form filaments *in vitro*.

While this manuscript was in preparation, Hartwig and Stossel (15) reported the isolation of a high-molecular-weight actin-binding protein from rabbit alveolar macrophages. Its physical properties were shown to be similar to erythrocyte spectrin. The possible relationship, if any, between filamin and this actin-binding protein remains to be determined.

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