Switching of Filamin Polypeptides during Myogenesis In Vitro

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ABSTRACT During chicken skeletal myogenesis in vitro, the actin-binding protein filamin is present at first in association with actin filament bundles both in myoblasts and in myotubes early after fusion. Later in mature myotubes it is found in association with myofibril Z disks. These two associations of filamin are separated by a period of several days, during which the protein is absent from the cytoplasm of differentiating myotubes (Gomer, R., and E. Lazarides, 1981, *Cell*, 23:524–532). To characterize the two classes of filamin polypeptides we have compared, by two-dimensional peptide mapping, ^{125}I-labeled filamin immunoprecipitated from myoblasts and fibroblasts to filamin immunoprecipitated from mature myotubes and adult skeletal myofibrils. Myoblast filamin is highly homologous to fibroblast and purified chicken gizzard filamins. Mature myotube and adult myofibril filamins are highly homologous but exhibit extensive peptide differences with respect to the other three classes of filamin. Comparison of peptide maps from immunoprecipitated ^{35}S-methionine-labeled filamins also shows that fibroblast and myoblast filamins are highly homologous but show substantial peptide differences with respect to mature myotube filamin. Filamins from both mature myotubes and skeletal myofibrils exhibit a slightly higher electrophoretic mobility than gizzard, fibroblast, and myoblast filamins. Short pulse-labeling studies show that mature myotube filamin is synthesized as a lower molecular weight variant and is not derived from a higher molecular weight precursor. These results suggest that myoblast and mature myotube filamins are distinct gene products and that during skeletal myogenesis in vitro one class of filamin polypeptides is replaced by a new class of filamin polypeptides, and that the latter is maintained into adulthood.

The development of myofibrils in skeletal muscle is an excellent system in which to study molecular morphogenesis in a eucaryotic system. The process has been studied by electron microscopy (11), immunofluorescence using antibodies to various myofibril proteins (13, 35), and biochemical identification of the contractile proteins present at different stages (8, 36). However, the molecular details of the assembly of the different myofibril substructures are at present poorly understood.

Filamin is a high molecular weight (250,000) actin-binding protein originally isolated from avian smooth muscle (39) and is closely related to an actin-binding protein isolated from mammalian macrophages (20). Immunofluorescence studies have shown that filamin is associated with actin filament bundles in the cytoplasm of cultured fibroblasts, and in vitro studies have shown that it interacts with actin filaments causing their gelation by cross-linking (3, 20, 33, 34, 40).

A protein with approximately the same molecular weight as filamin has been partially purified from adult skeletal muscle (1). This protein is antigenically related to gizzard filamin; by immunofluorescence it is localized at myofibril Z lines. We have previously studied the expression and distribution of filamin during myogenesis in vitro with respect to other known Z disk proteins such as α-actinin, desmin, and vimentin (15). By immunofluorescence, we observed that, in myoblasts and early after the onset of fusion, filamin is found in association with actin filament bundles. However, within 3 d after the onset of fusion, and before the development of α-actinin containing Z-line striations, filamin disappears from the cytoplasm. Several days after the development of α-actinin-containing Z-line striations, filamin is resynthesized and becomes associated with Z lines (15).

In this paper we have isolated myoblast and mature myotube filamins by immunoprecipitation and have compared them, by two-dimensional peptide mapping, to each other as well as to

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adult skeletal-muscle, adult smooth-muscle, and embryonic fibroblast filamins. We show that myoblast and mature myotube filamins differ from each other both in their peptide maps and in their electrophoretic mobilities. On the other hand, myoblast filamin and adult gizzard and fibroblast filamin are highly homologous, while mature myotube filamin and adult skeletal muscle filamin are highly homologous. These results suggest that myoblast and mature myotube filamins are distinct gene products, and that during myogenesis the synthesis of the myoblast filamin is switched off and replaced later on in myogenesis by a new class of filamin polypeptides.

MATERIALS AND METHODS

Cell Culture: Chicken embryonic fibroblasts and embryonic myogenic cells were grown as previously described (13), with the following modifications: To obtain myogenic cultures sufficiently pure for this study, we preplated primary cell cultures twice for ½ h on plastic tissue culture dishes (Falcon 3003; Falcon Labware, Oxnard, CA) and then plated onto collagen-coated petri dishes at a density of 1×10^7 cells/100-mm dish. After 18 h, secondary cultures were made by rinsing the cells three times with Ca- and Mg-free Earle's balanced salt solution, removing the mybolasts by the addition of trypsin for 3 min at 37°C, neutralizing the trypsin with growth medium, and washing the released cells by sedimentation twice in growth medium. Cells were again preplated twice for ½ h at 1×10^7 cells/100-mm dish and then plated onto collagen-coated dishes at a density of 3×10^6 cells/100-mm dish. Chick embryo fibroblasts were obtained by trypsinization of the first preplates after 3 d of incubation in complete medium and were grown in complete medium on noncollagenized petri dishes. Metabolic labeling of cell cultures with ^{35}S-methionine was carried out as previously described (13; see figure legends for details).

Sample Preparation: Skeletal myofibrils were prepared from adult chicken pectoral muscle by trimming a piece of muscle free of fat and connective tissue, and homogenizing it in a Lourdes blender at top speed for 30 s in ice-cold 20 mM Tris/HCl, 100 mM KCl, 5 mM EGTA, pH 7.5. Myofibrils were then purified by filtering the homogenate twice through two layers of cheesecloth, followed by centrifugation at 1,500 *g* for 5 min. The pellet was then immediately resuspended in 1% SDS, 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5, and boiled for 1 min at a concentration of ~10 mg wet weight of myofibrils/ml.

Adult chicken gizzard was trimmed free of fat and connective tissue and homogenized for 20 s at top speed in a Lourdes blender in ice-cold 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5, at a concentration of ~1 mg wet weight gizzard/ml, 20% SDS was added to a final concentration of 1% and the extract was then boiled for 1 min.

For immunoprecipitation, cell cultures were rinsed at room temperature with PBS, scraped into 1% SDS, 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5, and boiled for 1 min; typically, cells from one 100-mm culture plate were solubilized in 1 ml. Chicken gizzard filamin was purified as previously described by Wang (38), with the modification that all buffers contained an additional 1 mM EGTA.

Some tissue or cell samples were treated with proteolysis inhibitors after solubilization in SDS. A solution of 25 mM phenylmethylsulfonyl fluoride (PMSF [Sigma Chemical Co., St. Louis, MO]), 0.5 M *p*-tosyl-L-arginine methyl ester (TAME [Sigma]) and 50 mM *o*-phenanthroline (Matheson, Coleman and Bell, Norwood, OH) in 100% ethanol was added to the samples at a concentration of 4 μl/ml of sample.

Antibody Preparation: Antifilamin antibodies used in this study were prepared in rabbits, using native chicken gizzard filamin as antigen. Blood serum was precipitated at 50% ammonium sulfate saturation at 0°C and the partially purified IgG was dialyzed against PBS in the presence of 10 mM sodium azide. The final protein concentration was ~20 mg/ml, assuming $E^{280}_{1\ mg/ml} = 1.4$. The IgG was further purified by dialysis against 10 mM $NaPO_4$, pH 7.5, for 2 d and then removing the insoluble material by centrifugation at 10,000 *g* for 15 min. 1 ml of the supernatant was then passed through a column containing 4 ml of Whatman DE-52 (Whatman Inc., Clifton, NJ) ion exchange resin equilibrated and run in the above buffer. The purified IgG was collected in the column flow-through peak; this was typically 4 ml containing 1 mg/ml protein. The IgG fraction was then dialyzed against PBS/10 mM NaN_3. Affinity-purified antiserum was prepared as described (15). Typical concentrations were 0.2 mg/ml protein.

Immunoprecipitation: Cells or myofibrils solubilized in 1% SDS were diluted with 4 vol of ice-cold 1.25% Nonidet P-40 (NP-40 [Particle Data Laboratories LTD, Elmhurst, IL]), 20 mM Tris/HCl, 130 mM NaCl, 5 mM EGTA, pH 7.5, to a final detergent concentration of 0.2% SDS and 1% NP-40. The solution was then clarified at 10,000 *g* for 10 min in a SS34 rotor. 2 ml of the above supernatant containing ~0.2 mg total protein (fibroblasts and myoblasts) or 2 mg total protein (myotubes and myofibrils) was added to either 20 μl of ammonium sulfate purified antifilamin antiserum, 150 μl of DEAE purified IgG, or 60 μl of affinity-purified IgG. After gentle rocking at 4°C for 2 h, 150 μl of a 10% (wt/vol) solution of fixed *Staphylococcus aureus* was added (23, 24). Before incubation, the bacteria were washed twice in 20 mM Tris/Cl, 130 mM NaCl, 5 mM EGTA, pH 7.5, resuspended to 10% (wt/vol) in the same buffer containing 1% SDS, and then placed in a boiling water bath for 1 min. After pelleting in an Eppendorf centrifuge (Brinkman Instruments, Westbury, NY) for 4 min, the bacteria were washed an additional three times in precipitation buffer (20 mM Tris/HCl, 130 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% NP-40).

The bacteria were rocked with the immunoprecipitation solutions for 2 h at 4°C and the pelleted in a Sorval J6 centrifuge using a JS5.2 rotor (DuPont Instruments, Newtown, CT) for 15 min at 2,000 *g*. The pellets were resuspended at 4°C in precipitation buffer and washed by pelleting three times in an Eppendorf centrifuge for 4 min. Immunoprecipitates to be run on SDS polyacrylamide gels were resuspended in 50 μl of sample buffer (1% SDS, 0.5% β-mercaptoethanol, 20 mM Tris/HCl, pH 6.8, 0.2 mM EDTA, 10% glycerol, 0.005% bromphenol blue), boiled for 2 min, and cleared of bacteria by centrifuging for 5 min in an Eppendorf centrifuge. Immunoprecipitates to be iodinated (see below) were washed additionally twice in 100 mM sodium phosphate, pH 7.5, after having been washed in precipitation buffer and then resuspended in 100 μl of 0.5 sodium phosphate, pH 7.5, containing 1% SDS, boiled for 15 s, and cleared of bacteria by spinning twice for 5 min in an Eppendorf centrifuge.

Iodination: Iodination of proteins in polyacrylamide gel slices was performed by the method of Elder et al. (10). For the peptide maps shown in this study, proteins were iodinated in solution following the method of Greenwood et al. (19). 0.3 mCi of carrier-free ^{125}I (New England Nuclear O33H [Boston, MA]) in 10 μl of 0.5 M $NaPO_4$, pH 7.5, was added to the 100 μl of immunoprecipitate in 0.5 M $NaPO_4$, pH 7.5, 1% SDS, followed by 20 μl of 1 mg/ml chloramine T (Sigma Chemical Co.). The reaction proceeded for 2 min at room temperature and was stopped by the addition of 10 μl of 10 mg/ml sodium metabisulfite. 30 μl of 5× SDS sample buffer was added, and the mixture was boiled for 15 s and then loaded on a SDS polyacrylamide gel and electrophoresed. After removal of the dye front, the gel was stained and destained normally, and the filamin bands were then cut out.

The advantages of this method over the method of Elder et al. (10) are that the inclusion of the excess immunoglobulin in the iodination mixture appears to act as a carrier, so that differences in the quantity of precipitated filamin have a negligible effect on the total protein concentration. The Elder et al. (10) method has the added disadvantage of generating iodinated variants of contaminants present in the polyacrylamide gel slice which then give a reproducible background pattern in all of the peptide maps. Finally, performing the iodination on the protein in solution in the presence of 1% SDS should help to eliminate differences in labeling due to differences in protein tertiary structure or the presence of the polyacrylamide matrix.

Peptide Mapping: Polyacrylamide gel slices containing either ^{125}I- or ^{35}S-labeled proteins were washed for 48 h in two changes of 10% methanol and then for 6 h in 100% methanol at room temperature; they were then dried under vacuum. Following the method of Elder et al. (10), digestion of labeled proteins in gel slices was carried out by adding 400 μl of 0.05 mg/ml protease in 200 mM ammonium bicarbonate to each dried gel slice for 12 h at 37°C. 600 μl of freshly prepared protease solution was then added and the digestion allowed to proceed for an additional 12 h, after which time the eluted peptides were lyophilized. Proteases used were thermolysin (Sigma P1512), alpha-chymotrypsin (Worthington Type CDS; Worthington Biochemical Co., Freehold, NJ) and trypsin-TPCK (Millipore Worthington TRTPCK; Worthington Biochemical Co., Bedford, MA). Two-dimensional peptide mapping was performed on cellulose 20-×-20-cm Chromagram sheets (Eastman 13255 [American Scientific Products, McGraw Park, IL]). The electrophoresis buffer for the first dimension was 11.4:10:379 acetic acid:formic acid:water, and the chromatography buffer for the second dimension was 5.5:3.3:1:3 butanol:pyridine:acetic acid:water. Autoradiography was done on Kodak AR-5 film (Eastman Kodak, Rochester, NY) using, for ^{125}I, DuPont Cronex Lightening-Plus intensifying screens (E. I. DuPont de Nemours and Co., Wilmington, DE). ^{35}S peptide maps were sprayed with Enhance (New England Nuclear, Boston, MA) and the x-ray film was preflashed (26).

Quantitation of Radioactivity: 10 μl of a cell extract containing ^{35}S-labeled proteins was added to 1 ml of 4 mg/ml L-methionine (Sigma Chemical Co.), 0.1 mg/ml BSA (Sigma Chemical Co.) in 1 N sodium hydroxide. After a 10-min incubation at 37°C, 2 ml of ice-cold 30% trichloroacetic acid was added and the mixture allowed to stand on ice for 2 h with occasional vortexing. The precipitated protein was collected by filtration through GF/C filters (Whatman, Inc.), washed with 3% trichloroacetic acid, and dried. All samples were counted in Aquasol-2 (New England Nuclear) using an LS-233 scintillation counter (Beckman Instruments Inc., Fullerton, CA). Counting efficiency for ^{35}S was 100% with the full width isoset, using ^{35}S-methionine from New England Nuclear as a standard.

Quantitation of Protein: Purified chicken gizzard filamin was quantitated using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

Ovalbumin (Sigma A5503) weighed on a calibrated Mettler H 34 (Mettler Instruments Corp., Highstown, NJ) scale was used to construct a curve of O.D.595 vs. weight of protein. To determine the amount of protein in 1% SDS extracts, we performed the assay using 1 ml of the diluted Bio-Rad dye reagent mixed with 5 μl of sample in 1% SDS. Ovalbumin was weighed as described above, boiled in 1% SDS, and then used to construct a standard curve.

Gels stained with Coomassie Blue were scanned with a Joyce-Loebl MK III C densitometer (National Instrument Laboratories Inc., Rockville, MD), using a 0-1 O.D. wedge. Several scans were made across each filamin band, the traces being recorded on paper. The area under each peak was cut out, weighed, and an average taken. Scans of known quantities of filamin (as determined above) were used to construct a standard curve for each gel.

PAGE: SDS PAGE was based on a modification of the method of Laemmli (25) as described by Hubbard and Lazarides (22). Gels contained 10% acrylamide and 0.13% *N,N'*-methylene bisacrylamide.

RESULTS

Immunoprecipitations

Filamin for peptide maps was obtained by immunoprecipitation with antifilamin antibody and fixed *S. aureus* following the method developed by Kessler (23, 24). The method was altered to effect a complete solubilization of the protein to ensure an accurate representation of the filamin in a given tissue or culture; this solubilization consisted of boiling the sample in 1% SDS for 1 min. After dilution of the sample to 0.2% SDS and addition of NP-40 to 1% final concentration (see Materials and Methods), antibody was added, followed by the fixed *S. aureus* bacteria. The immunoprecipitates were then electrophoresed on SDS polyacrylamide gels.

To ensure accurate representation of the filamin present in a given tissue or cell type, we used an excess of antibody over antigen and an excess of fixed *S. aureus* over antibody to precipitate all of the filamin present in a given extract. The saturation point of fixed *S. aureus* over antibody was determined by precipitating serial dilutions of antibody; a twofold excess of the *S. aureus* was then used for all subsequent precipitations. Similarly, the saturation point of antibody for filamin was determined by precipitating serial dilutions of filamin with a fixed amount of antibody. These precipitates were run on SDS polyacrylamide gels along with known quantities of purified chicken gizzard filamin, and a Joyce-Loebl densitometer was used to scan the gels (see Materials and Methods). In this manner we determined that the amount of purified chicken gizzard filamin that could be precipitated by 20 μl of ammonium sulfate-purified antibody was at least 13 μg (data not shown). Fig. 1 *a*, lane *4*, shows an immunoprecipitation starting from 50 μg of purified gizzard filamin, thereby showing the saturation level of the antibody. Bands below filamin are degradation products of filamin. For quantities of filamin below the saturation point, the gel scans show that we obtained at least 95% recovery of filamin in the immunoprecipitations (data not shown).

Gel scans of filamin immunoprecipitated from known quantities of myoblast, fibroblast, adult gizzard, or adult skeletal muscle protein in 1% SDS extracts gave the following approximate quantities expressed as g filamin/g total protein: myoblast, 2×10^{-3}; fibroblast, 1×10^{-2}; gizzard, 3×10^{-2}; skeletal myofibrils, 1×10^{-3}. As shown in Fig. 1, purified chicken gizzard, fibroblast, and myoblast filamins all have the same electrophoretic mobility (Fig. 1 *a*, lanes *3*, *4*, *5*, *8*, and *9*). Filamin immunoprecipitated from 7–14-d-old cultured chick myotubes has the same electrophoretic mobility as filamin immunoprecipitated from adult chicken pectoral muscle myofibrils (Fig. 1 *a*, lanes *6* and *7*). This apparent molecular weight is lower than that of gizzard, myoblast, or fibroblast

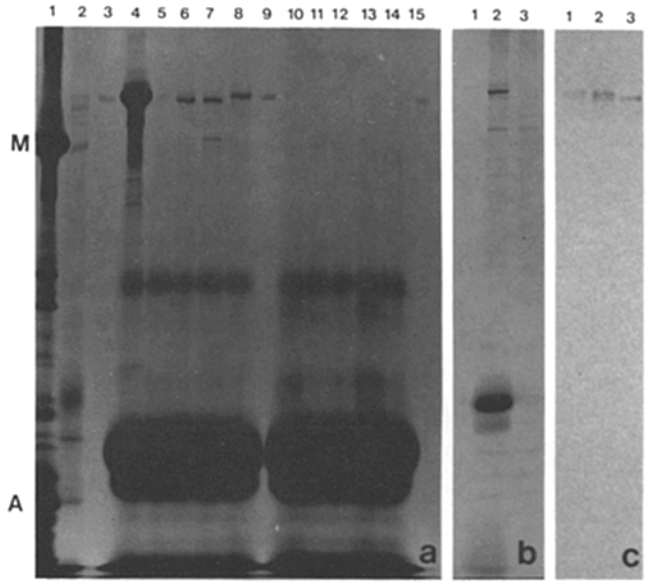

FIGURE 1 Filamin was immunoprecipitated and then electrophoresed on 10% SDS polyacrylamide gels (*a*, *b*, and *c*). *a* and *b* are Coomassie-Blue-stained gels; *c* is an autoradiogram. (*a*) Molecular weight markers are purified chicken skeletal muscle myofibrils (lane *1*) showing myosin heavy chain (*M*) (200,000) and actin (*A*) (43,000). 10-d cultured chick myotubes (lane *2*) and purified chicken gizzard filamin (lanes *3*, *9*, and *15*: 250,000). Lanes *4–8* show immunoprecipitations using rabbit antifilamin antisera; lanes *10–14* show parallel precipitations of the same antigens with preimmune sera. The heavy bands above actin in the immunoprecipitates are immunoglobulin heavy chains. In *a*, filamin is precipitated from purified chicken gizzard filamin, starting with 50 μg of filamin (lane *4*, immune serum; lane *10*, preimmune serum), cultured myoblasts (lane *5*, immune serum; lane *11*, preimmune serum), 10-d cultured myotubes (lane *6*, immune serum; lane *12*, preimmune serum), purified skeletal myofibrils (lane *7*, immune serum; lane *13*, preimmune serum), and cultured chick fibroblasts (lane *8*, immune serum; lane *14*, preimmune serum). (*b*) Filamin immunoprecipitated from skeletal myofibrils using antibody affinity purified with purified chicken gizzard filamin. Lane *1* is a purified gizzard filamin marker, lane *2* is the immunoprecipitate from the myofibrils, and lane *3* is a control precipitate using PBS instead of affinity-purified antiserum. The affinity-purified antiserum precipitates both the lower molecular weight myofibril filamin and a small amount of contaminating fibroblast filamin. (*c*) Immunoprecipitated filamin from myoblasts (lane *1*) and pure myotubes (lane *3*) labeled with ^{35}S-methionine at 40 μCi/ml for 5 min; lane *2* is a mixture of lanes *1* and *3*. Both myoblasts and myotubes were from a single 100-mm culture dish originally plated at 3×10^6 cells/plate.

filamin by approximately 5,000. A protein with an apparent molecular weight slightly lower than that of purified chicken gizzard filamin occasionally appears in immunoprecipitates of cultured myoblasts, using both immune (Fig. 1 *a*, lane *5*) and preimmune (Fig. 1 *a*, lane *11*) sera. Although there is less of this protein in the preimmune than in the immune immunoprecipitate, in other experiments the opposite is seen. Thus, we believe that this is a nonspecific contaminant rather than a filamin-associated protein or filamin degradation product. A similar protein is also occasionally seen in immunoprecipitates from fibroblasts (Fig. 1 *a*, lanes *8* and *14*). A different protein with an electrophoretic mobility near that of myosin heavy chain is also nonselectively immunoprecipitated from myofibrils and occasionally from myoblasts and myotubes (Fig. 1 *a*, lanes *7* and *13*). This protein appears in immunoprecipitates

from myofibrils, using immune or preimmune antibodies to other myofibril proteins such as α-actinin and synemin, and is also precipitated by fixed *S. aureus* alone (Fig. 1 *b*, lane *3*).

To demonstrate that the protein immunoprecipitated from skeletal muscle is immunologically related to purified chicken gizzard filamin, we affinity-purified the anti-chicken gizzard filamin antibody using immobilized chicken gizzard filamin. This was then used to immunoprecipitate filamin from an SDS solubilized myofibril preparation as shown in Fig. 1 *b*. This antibody precipitates the lower molecular weight myofibril filamin, showing that the same antibodies that bind to purified gizzard filamin will bind to the lower molecular weight myofibril filamin molecules.

Synthesis of Skeletal Muscle Filamin

Using cultures of skeletal myoblasts and myotubes essentially free of fibroblasts (99% fibroblast free), we performed an experiment to determine whether myoblasts synthesize only the high molecular weight variant of filamin whereas mature myotubes synthesize only the lower molecular weight variant. Fig. 1 *c* shows an autoradiograph of filamins immunoprecipitated from cell cultures metabolically labeled with ^{35}S-methionine and electrophoresed on a 10% polyacrylamide SDS gel. Cells were labeled for 5 min and then immediately solubilized by boiling in SDS. Lane *1* of Fig. 1 *c* shows the filamin from pulse-labeled myoblasts, lane *3* shows the filamin from myotubes, and lane *2* is a mixture of lanes *1* and *3*. These results have also been obtained when the cell samples were treated with the protease inhibitors TAME, PMSF, and *o*-phenanthroline immediately after solubilization, indicating that the lower molecular weight of myotube filamin is not due to proteolysis during the immunoprecipitation.

^{125}I Peptide Maps

Fig. 2 shows peptide maps of immunoprecipitated filamins iodinated in solution. Precipitations, iodinations, and digestions were performed in parallel for all samples. Maps generated by three proteases, trypsin, chymotrypsin, and thermolysin, all show that fibroblast, skeletal myoblast, and mature skeletal myotube filamins are related to chicken gizzard filamin as purified by the method of Wang (38). Cultured chick embryo fibroblast filamin appears essentially identical to purified chicken gizzard filamin when the pairs Fig. 2 *a* and *b*, Fig. 2 *e* and *f*, and Fig. 2 *i* and *j* are compared. Cultured chick skeletal myoblast filamin (Fig. 2 *c*, *g*, and *k*) is closely related, but not identical, to purified gizzard and fibroblast filamin. With each protease a small number of differences can be seen between myoblast and gizzard or fibroblast filamin. For instance there is a spot (*) in the tryptic map of myoblast filamin (Fig. 2 *c*) that is not present in the tryptic maps of gizzard or fibroblast filamins (Figs. 2 *a* and *b*). Finally, the protein precipitated from 10-d-old cultured chicken skeletal muscle myotubes by anti-chicken gizzard filamin antibodies shows a large number of peptide differences with respect to gizzard, fibroblast, and myoblast filamins. This can be seen by comparing Fig. 2 *a*, *b*, and *c* with *d*; Fig. 2 *e*, *f*, and *g* with *h*; and Fig. 2 *i*, *j*, and *k* with *l*.

To further examine the similarities and differences between filamins from early and late stage myogenic skeletal muscle cells, we made peptide maps of mixtures of cultured myoblast and myotube filamins as shown in Fig. 3 to more accurately determine which peptides from the myoblast and myotube maps co-migrate. An analysis of the tryptic peptides (Fig. 3 *a* and *c*) shows 10 spots that match, 13 spots unique to the myoblast filamin, and 14 spots unique to the myotube filamin. Similarly, in the chymotryptic maps, 19 s spot match, 13 are unique to myoblasts, and 16 are unique to myotubes (Fig. 3 *e*, and *g*). With both proteases the matching and unique peptides were fairly evenly distributed over the maps, indicating no segregation of any of the three classes of peptides according to charge, size, or hydrophobicity. For unknown reasons, some matching spots differ in intensity between maps (arrows, Fig. 3 *a* and *c*). Because of the large percentage of nonmatching spots in both the tryptic and chymotrypic peptide maps, myoblast and myotube filamins appear to be quite different polypeptides despite their antigenic similarity.

A similar analysis of the purified chicken gizzard filamin peptides in Fig. 2 shows that all but one of 24 tryptic peptides exactly match the 24 fibroblast filamin peptides, and all of 27 gizzard filamin chymotryptic peptides match the fibroblast filamin chymotryptic peptides. A comparison of purified gizzard filamin with myoblast filamin shows that 16 tryptic and 23 chymotryptic peptides match. At least eight gizzard and fibroblast filamin tryptic peptides do not correspond to any myotube filamin spots. Similarly, four gizzard filamin chymotryptic peptides do not co-migrate with any myotube filamin peptide. Of the seven tryptic peptides present in myoblast but not in purified gizzard filamin, five are peptides common to myoblast and myotube filamins and two are unique to myoblasts. Similarly, nine chymotryptic peptides are present in myoblast but not gizzard or fibroblast filamins. Six of these peptides are common with myotube filamin chymotryptic peptides and three are not.

Peptide maps of filamins iodinated by the method of Elder et al. (10) rather than the method described above gave results identical to those described here for the similarities and differences of the various filamins (data not shown). Similar results were also obtained when comparing peptide maps of filamins digested under different conditions such as varying the concentration of protease from 50 to 1,000 μg/ml, or the concentration of the digestion buffer from 50 to 200 mM ammonium bicarbonate.

Finally, a comparison of iodinated cultured skeletal myotube and adult myofibril filamins (Fig. 4) shows very few differences between the two filamins when they are digested with three different proteases. This indicates that the filamin present in the late stage of in vitro myogenesis is indistinguishable from the corresponding filamin present in in vivo myogenesis.

^{35}S Peptide Maps

The close similarity of fibroblast and skeletal myoblast filamins and their difference with respect to myotube filamin, shown by peptide maps of filamins labeled with ^{125}I in vitro, are also seen in peptide maps of filamins metabolically labeled with ^{35}S-methionine (Fig. 5). Because the digested proteins were metabolically labeled, differences observed in these maps cannot be artifacts of an in vitro labeling system. An analysis of chymotrypic peptides of myoblast and fibroblast filamins shows that, out of 40 peptides, only one is different between the two, while a comparison of myoblast and myotube filamins shows 30 matching peptides, with an additional 10 peptides unique to myoblasts and 11 unique to myotubes. No peptides from myotube filamin co-migrate with the two peptides that distinguish fibroblast from myoblast filamin. The close similarity of fibroblast and myoblast ^{35}S peptide maps shows that the difference between myoblast and myotube ^{35}S filamin maps

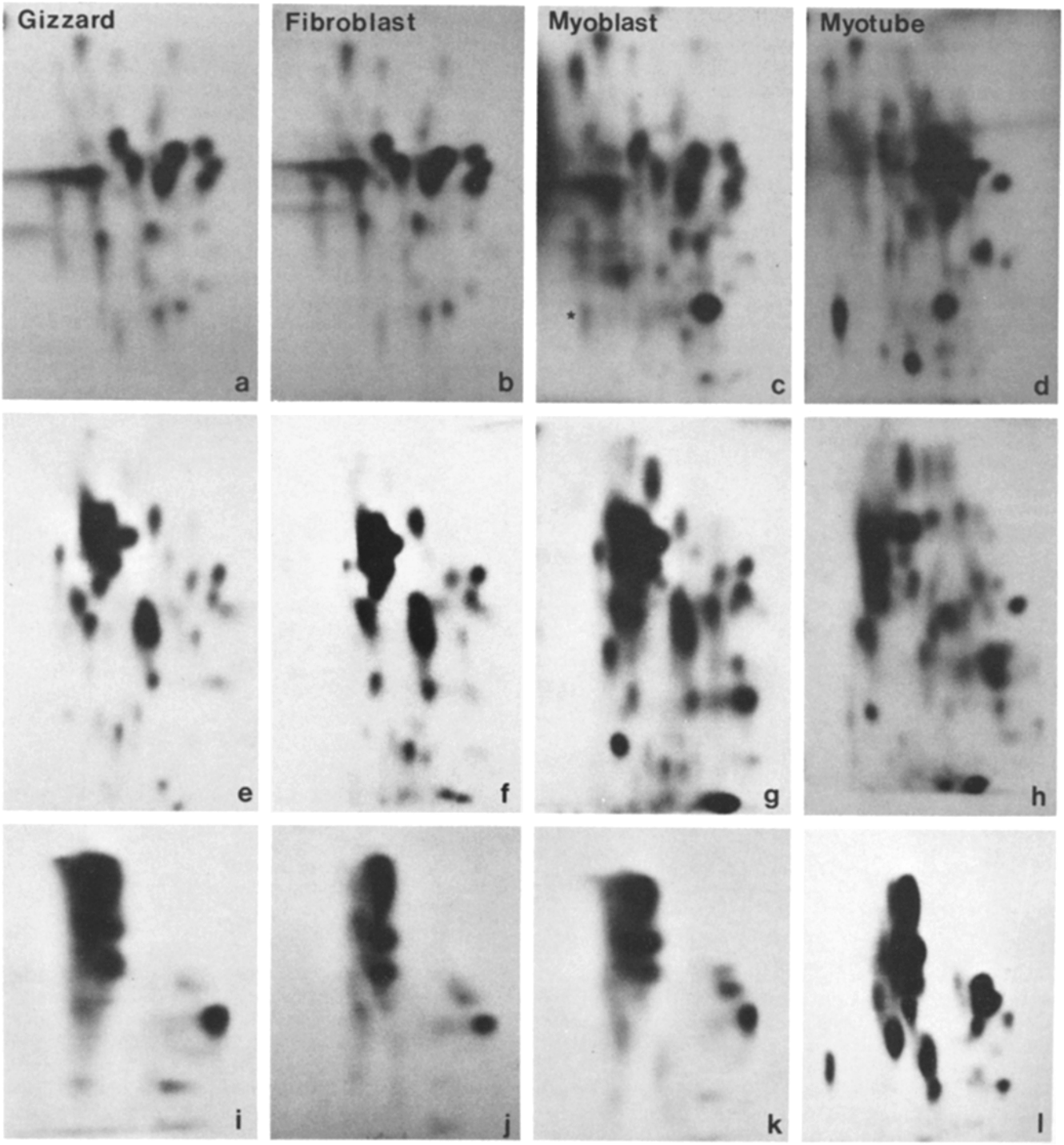

FIGURE 2 Two-dimensional peptide maps of iodinated gizzard filamin (*a*, *e*, and *i*), chick embryo fibroblast filamin (*b*, *f*, and *j*), chick skeletal myoblast filamin (*c*, *g*, and *k*), and 10-d-old cultured chick myotube filamin (*d*, *h*, and *l*). Digests were performed with Trypsin-TPCK (*a–d*), α-chymotrypsin (*e–h*), and thermolysin (*i–l*). Peptide maps were of typically one-fifth of the immunoprecipitated filamin (0.2 μg) labeled at ~0.2 mol I/mol protein. Electrophoresis was from left to right and ascending chromatography from bottom to top. Maps contained ~10^6 cpm ^{125}I labeled peptides and were exposed at −70°C with intensifying screens typically for 48 h. Star in *c* shows a peptide present in myoblast and absent from gizzard or fibroblast filamin.

cannot be due to random variations caused by the mapping procedure.

DISCUSSION

Patterns of protein synthesis change markedly during skeletal myogenesis. At the time when myoblasts fuse to form myotubes, several proteins begin to be synthesized, such as the α variant of actin (42), desmin (13), the acetylcholine receptor (29), the sodium channel (12) and creatine kinase and aldolase isozymes (9, 36). Other proteins exhibit changes from one variant to another well after functional myofibrils have developed. These proteins include some myosin light chains (5, 14, 43), myosin heavy chains (21, 32, 44), tropomyosins (30) and

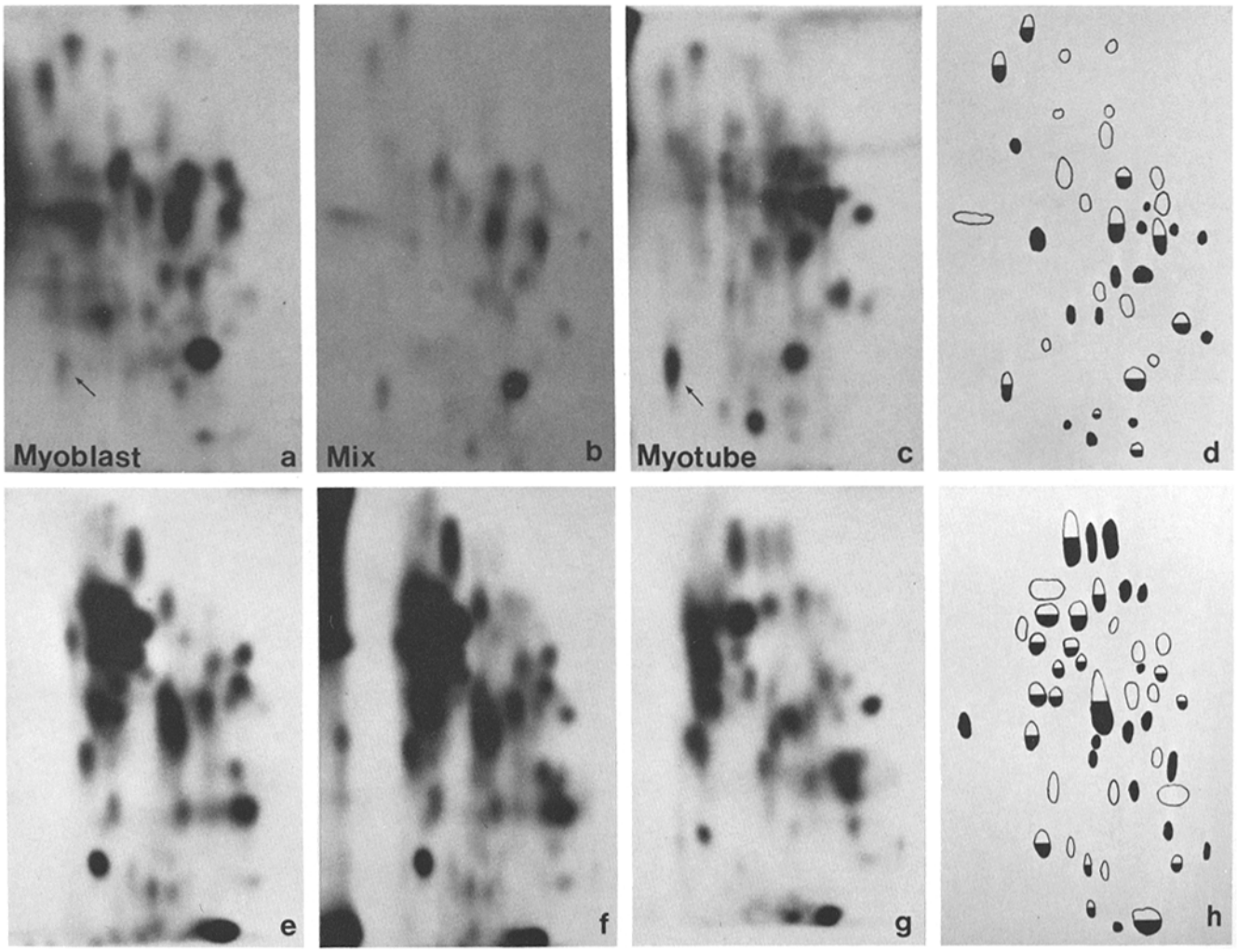

FIGURE 3 Peptide maps comparing iodinated chick skeletal myoblast filamin (*a* and *e*) with 10-d-old myotube filamin (*c* and *g*) by means of a coelectrophoresis map showing a combination of the two filamins (*b* and *f*). Proteases are trypsin-TPCK in the top row and α-chymotrypsin in the bottom row. Tracings (*d* and *h*) show spots unique to myoblasts (○), unique to myotubes (●), or common to both (◒). Arrows (*a* and *c*) show an example of matching spots that differ in intensity.

troponin T (28, 35). Finally, a small number of proteins exhibit changes of variants between myoblasts and late embryonic myotubes, for example, myosin light chains exhibit a loss of three minor variants between day 10 and day 18 in chick embryos (14).

We have shown that filamin exhibits a novel pattern of expression and possibly a different mode of regulation of its expression during myogenesis (15). This protein is present in skeletal myoblasts and early fused myotubes in association with actin filament bundles as shown by immunofluorescence. However, within 2 d after the onset of fusion and before the transition of α-actinin from a punctate distribution along the actin filament bundles to striated Z lines, filamin disappears from the cell. Several days after the appearance of α-actinin-containing Z line striations, filamin reappears at the Z lines and maintains this distribution into adulthood. These studies, in conjunction with previous studies (1, 39), have also established that filamins from gizzard, fibroblast, myoblast, mature myotube, and adult skeletal muscle are antigenically related.

In the present study we have isolated filamins from cultured chicken skeletal myoblasts and myotubes by immunoprecipitation and compared them to each other and to filamins immunoprecipitated from adult gizzard, adult skeletal muscle, and fibroblasts. Tryptic, chymotryptic, and thermolytic peptide maps have shown that myoblast and mature myotube filamins labeled with ^{125}I in vitro exhibit substantial peptide differences (Figs. 2 and 3), implying that they are distinct polypeptides, which may be either distinct gene products or arise as a result of differential RNA processing of the same gene product. Myoblast and mature myotube filamins also exhibit differences in their chymotryptic peptide maps when metabolically labeled with ^{35}S-methionine before immunoprecipitation (Fig. 5). These latter results show that the presence of peptide differences between these two forms of filamin is not due to a peculiar charge modification of any proteins during the iodination procedure. Comparison of ^{125}I-labeled peptides from myoblast and mature myotube filamins with those of adult gizzard, adult skeletal muscle and cultured fibroblast filamins shows that myoblast filamin and gizzard and fibroblast proteins are highly homologous while mature myotube filamin and adult skeletal muscle protein are highly homologous. However, even in the case of gizzard, fibroblast and myoblast filamins, a close examination of their peptide maps reveals the presence of some peptide differences. At present, we do not know

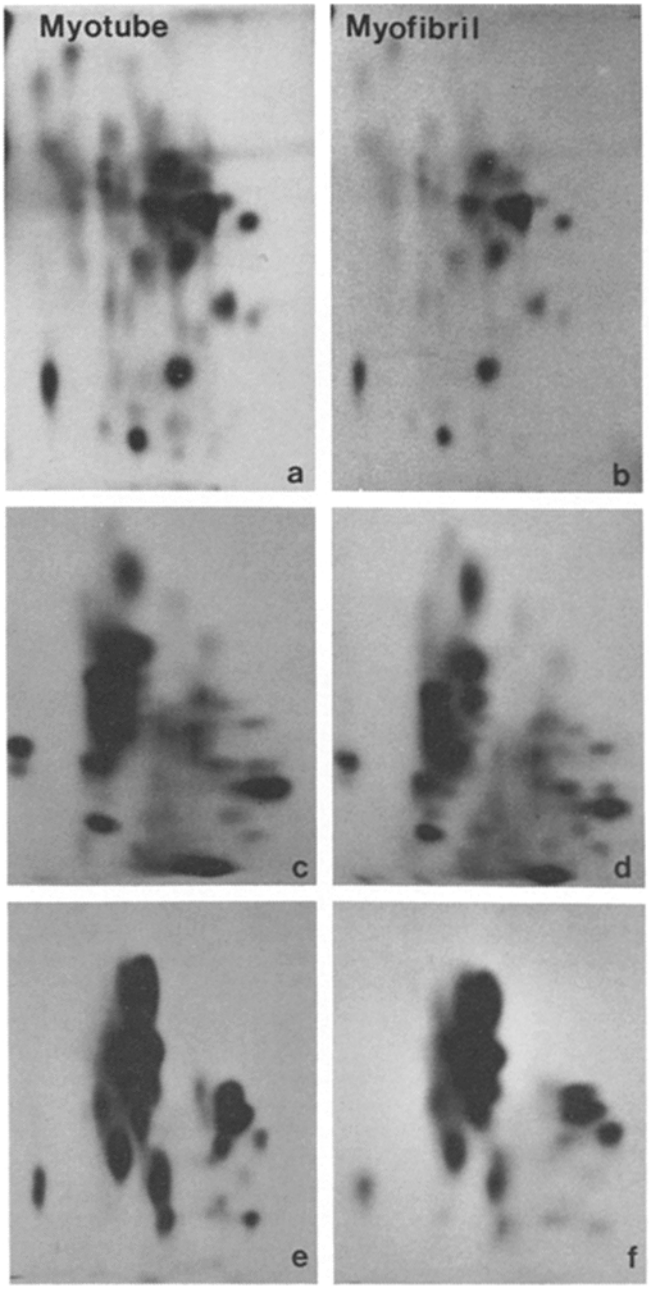

FIGURE 4 A comparison of 10-d-old cultured chick skeletal myotube filamin (*a*, *c*, and *e*) and filamin from purified adult skeletal myofibrils (*b*, *d*, and *f*); both were labeled with ^{125}I. Proteases used were trypsin-TPCK (*a* and *b*), α-chymotrypsin (*c* and *d*), and thermolysin (*e* and *f*). Quantities of ^{125}I-filamin used for peptide maps and exposure times were as in Fig. 2.

whether these differences represent heterologous amino acid sequences or posttranslational modifications of identical sequences. These small peptide differences provide convenient markers in the peptide maps which enabled us to be confident that myoblast, myotube, and myofibril filamins were not contaminated to any substantial extent by fibroblast filamin. This was especially important in the case of mature myotubes and adult myofibrils where filamin represents a much lower percentage of total protein compared to that of gizzard, fibroblasts, or myoblasts. However, the extensive peptide differences between myotube or myofibril filamins and fibroblast filamin and the absence from their peptide map of peptides characteristic of fibroblast filamin render this possibility unlikely. The results presented here have been consistently obtained with several different preparations of immunoprecipitated filamins from all five sources. In addition, closely similar peptide maps have been obtained with the antigens either iodinated directly in the gel slices according to the procedure of Elder et al. (10) or iodinated after immunoprecipitation, dissociation of the immune complexes by SDS, and subsequent purification of the ^{125}I-labeled filamin by SDS gel electrophoresis. Thus, we are confident that the peptide homologies and differences seen between the five forms of the filamins indicate that myoblast filamin and gizzard and fibroblast filamins are homologous, that mature myotube filamin and adult skeletal myofibril filamin are highly homologous, but that mature myotubule filamin is very different from myoblast filamin. These results, in conjunction with the immunofluorescence results obtained earlier (15), suggest that the synthesis of the myoblastic form of filamin ceases with a concomitant removal of the molecule from the cytoplasm, presumably by protein turnover, and that later in myogenesis a different filamin polypeptide is synthesized and becomes localized at the Z disk. The homology of mature myotube and adult myofibril filamins suggests that this form of filamin does not change substantially after its association with the Z disk.

The Z line form of filamin also exhibits a slightly greater electrophoretic mobility in SDS polyacrylamide gels compared to its myoblast counterpart. A calcium-activated protease purified from skeletal muscle has been shown to cleave filamin into proteins of 240,000 and 9,500 molecular weight (7). Other studies have shown that platelet filamin is cleaved by an endogenous calcium-activated protease (41) to an unknown set of cleavage products. Immunoprecipitation of filamin from extracts containing a variety of protease inhibitors prepared from myoblasts or mature myotubes metabolically labeled with ^{35}S-methionine for a short period of time showed that myotubes exclusively synthesize the lower molecular weight filamin (Fig. 1 *c*). This observation argues that the myotube form of filamin is synthesized as a polypeptide slightly shorter than myoblast filamin and that this electrophoretic difference among them is not due to a higher susceptibility of the myotube molecule to proteolysis.

The differences in peptide maps between adult skeletal muscle filamin and adult smooth muscle (gizzard) filamin correlate with the differences observed between these two muscle types for other structural and contractile proteins such as the intermediate filament protein desmin (31), actin (37), myosin (4), and α-actinin (2). Such differences may be due to the existence of distinct genetic programs for the assembly of smooth and skeletal muscle.

It has been shown previously that purified chicken gizzard filamin inhibits the actin-activated myosin ATPase (6) and blocks the binding of tropomyosin to actin (27, 45). The similarity between myoblast and gizzard filamins supports our previous hypothesis that filamin is removed from early myogenic cells because it blocks the interaction between tropomyosin or myosin and actin which is necessary for sarcomere assembly. The synthesis of a new form of filamin later in myogenesis and its association with Z lines indicate that there is a functional difference between the myoblast and the Z line forms of this protein. As we have demonstrated previously, skeletal muscle filamin associates with the periphery of the myofibril Z disk (15) which also contains actin and the intermediate filament proteins desmin, vimentin and synemin (16–18). The differences between Z line and myoblast filamins

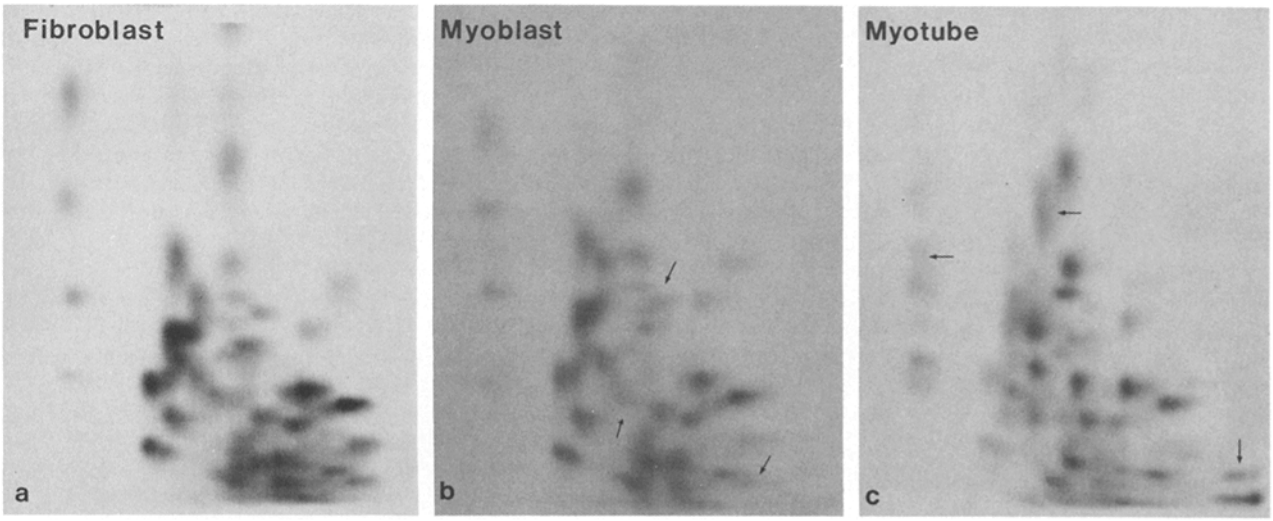

FIGURE 5 Chymotryptic digests of ^{35}S-methionine-labeled filamins isolated from chick embryo fibroblasts (*a*), chick skeletal myoblasts (*b*), and 10-d-old cultured chick skeletal myotubes (*c*). Two 100-mm plates containing ~5 × 10^6 cells of each type were labeled for 18 h in methionine-free minimum essential medium with 0.2 mCi ^{35}S-methionine (1,000 Ci/mM) in 5 ml medium in each plate. Approximately 3 × 10^8 cpm of total TCA-precipitable counts were recovered from each pair of plates. 3 μg of each filamin was immunoprecipitated, using 300 μl of 10% (wt/vol) *S. aureus* and 40 μl of ammonium sulfate-purified antifilamin antiserum (see Materials and Methods), and electrophoresed on a 10% SDS polyacrylamide gel which was stained and destained normally. Filamin bands were cut out and washed for 48 h in 10% methanol, dried, and digested with α-chymotrypsin as described in Materials and Methods. Maps contain ~2 × 10^4 cpm ^{35}S and were exposed for 15–30 d. Electrophoresis was from left to right and chromatography from bottom to top. Arrows in *b* indicate three of the peptides present in fibroblast and myoblast filamins and absent from myotube filamins. Similarly, arrows in *c* indicate peptides unique to myotube filamin.

might restrict the former to binding only the actin present at the periphery of the Z disk, or to mediate the binding of desmin and vimentin to the Z disk.

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