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Coexistence of desmin and the fibroblastic intermediate filament subunit in muscle and nonmuscle cells: Identification and comparative peptide analysis

(cytoskeletons/two-dimensional gel electrophoresis)

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ABSTRACT **Extraction of chicken embryo fibroblasts (CEF) or baby hamster kidney (BHK) cells with 1% Triton X-100 and 0.6 M KCl leaves an insoluble cytoskeletal residue composed primarily of the 52,000 M_r subunit of intermediate filaments (F-IFP). In addition, CEF cytoskeletons exhibit a minor component with M_r of 50,000, identified as α-desmin, one of the two major isoelectric variants of the intermediate filament subunit from smooth muscle. BHK cytoskeletons contain the 50,000 M_r mammalian desmin variant. Cytoskeletons prepared from chicken embryonic myotubes contain F-IFP and both α- and β-desmin. These data suggest that two distinct 10-nm filament subunits coexist in a single cell. One-dimensional peptide analysis of F-IFP and desmin from avian and mammalian cells reveals significant interspecies homology, as well as homology between F-IFP and desmin from the same species. Peptide analyses of ^{32}P-labeled intermediate filament subunits suggest that there is considerable similarity in the phosphorylation sites of these proteins. These results indicate that F-IFP and desmin might be evolutionally related.**

Intermediate or 10-nm filaments have been observed in many cell types, including neural, glial, muscle, and fibroblastic cells of higher vertebrates (1–9). Although these filaments share several morphological and biochemical characteristics that suggest a common origin, recent studies suggest that the protein subunits of the filaments from different tissue sources are biochemically and immunologically distinct (6–8, 10–14). At least four distinct subclasses of 10-nm filaments can be identified: glial filaments, neurofilaments, muscle intermediate filaments, and 10-nm filaments in fibroblastic cells. The purified protein subunits of each of these filament types have distinct apparent molecular weights and are are immunologically distinguishable (2, 4–13). The filament subunit from fibroblastic cells, with a M_r of 52,000–55,000, has been studied in Triton X-100-extracted cytoskeletons of normal embryonic fibroblasts and has recently been isolated from filament caps formed in spreading or Colcemid-treated baby hamster kidney cells (1, 12). Intermediate filaments isolated from avian smooth muscle are composed of a protein of M_r 50,000–55,000 termed desmin (2, 5, 6, 9, 11), which has also been biochemically and immunologically identified in skeletal and cardiac muscle (6, 13, 15).

A fifth class of intermediate-sized filaments, characteristic of epithelial and epidermal cells, is composed of keratins. Although the keratin filaments (or tonofilaments) are similar in diameter to the intermediate filaments of other cell types, they are often found in wavy bundles within the epithelial cytoplasm (14, 16, 17) or are associated with desmosomes (16). These morphological characteristics and the role of keratin filaments in the terminal differentiation of epidermal cells distinguish them from other intermediate filament types (16, 17).

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The existence of these biochemically and immunologically distinct cell type specific subunits suggests that a cell might contain only one class of intermediate filament, determined by its tissue of origin. Recent reports, however, indicate that some cell types possess both fibroblastic 10-nm filaments and keratin tonofilaments (14). Thus, it seems possible that two distinct intermediate filament subunits coexist in a given cell type. Here we present biochemical and immunological evidence that some nonmuscle cell types possess both the fibroblastic (F-IFP) and muscle (desmin) intermediate filament subunits. Similarly, avian skeletal myotubes possess both F-IFP and desmin. These data support the conclusion that a given cell may possess more than one class of intermediate filaments. Additionally, we present evidence suggesting that F-IFP and desmin, though antigenically and biochemically distinct, exhibit significant peptide homologies.

MATERIALS AND METHODS

Cells. Cultures of nonmyogenic chicken embryo fibroblasts (CEF) were obtained by serial subculturing of fibroblastic cells derived from embryonic thigh during the preparation of myogenic cultures (see below). Three to four passages on noncollagenized petri plates were sufficient to eliminate all myogenic cells, as determined by the lack of α-actin in lysates analyzed by gel electrophoresis. Fibroblasts were maintained in Eagle's minimum essential medium (all culture media were from GIBCO) supplemented with 10% horse serum, 2% embryo extract, and antibiotics; cells were subcultured by brief trypsinization.

Baby hamster kidney 21 (BHK) cells were grown in monolayer culture in Glasgow minimal essential medium supplemented with 10% tryptose phosphate and 10% calf serum.

Primary cultures of embryonic chicken myogenic cells were prepared as described (18), with the indicated modifications. Ten-day embryonic thigh muscles were dissected free of skin and bone and were dissociated with trypsin at a final concentration of 0.05% for 30 min. Further dissociation was accomplished by repeated pipetting. The cell suspension was filtered through 5-ply sterile cheesecloth, and cells were washed twice by centrifugation (500 × *g* for 5 min) followed by resuspension in growth medium. Cells were then preplated twice for 10 min to remove adherent fibroblasts (used for CEF cultures above) and then plated on collagen-coated petri plates at densities of 0.8–1.5 × 10^6 cells per 100-mm plate.

Growth medium consisted of Eagle's minimal essential medium supplemented with 15% horse serum, nonessential amino

Abbreviations: F-IFP, fibroblastic intermediate filament subunit; CEF, chicken embryo fibroblasts; BHK cells, baby hamster kidney cells; P_i/NaCl, phosphate-buffered saline; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate; IEF, isoelectric focusing; $NaDodSO_4$, sodium dodecyl sulfate.

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acids, 5% chicken embryo extract, and antibiotics (100 mg of streptomycin and 100 μl of penicillin per ml). To prevent overgrowth of contaminating fibroblasts, we fed cultures on day 3 with growth medium containing 10 μM cytosine arabinoside (Calbiochem).

Plastic petri plates (Falcon) were collagen coated with a brief wash of soluble calf-skin collagen (Worthington) diluted to 0.5 mg/ml, followed by air-drying under a germicidal lamp.

Preparation of Triton/KCl Cytoskeletons for Isoelectric Focusing (IEF)–Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis. Triton/KCl cytoskeletons of CEF and BHK cells and myotube cultures were prepared by a modification of the procedure outlined by Starger *et al.* (12). Confluent cultures of CEF or BHK cells were primarily used, and no differences were observed in the cytoskeletal composition of spreading or Colcemid-treated cells. Cells were washed twice with phosphate-buffered saline [171 mM NaCl/3 mM KCl/6 mM NaK phosphate/2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate (EGTA), pH 7.4] (P_i/NaCl) and then scraped from the plates with a rubber policeman. The cell suspension was then pelleted at approximately 500 × *g* for 5 min. The cell pellet was lysed in 1 ml of lysis buffer (1% Triton X-100/0.6 M KCl/1 mg of *p*-tosyl-L-arginine methyl ester·HCl/0.1 mM phenylmethylsulfonyl fluoride/0.1 mM *O*-phenanthroline in P_i/NaCl) per 100-mm plate of cells. This lysate was then digested with 0.1 mg of DNase I per ml, in the presence of 10 mM Mg^{2+}, for 15 min. The cytoskeletal residue was then pelleted at 1000 × *g* for 5 min and reextracted twice with 0.5 ml of lysis buffer per plate, followed by several washes in P_i/NaCl supplemented with protease inhibitors. The cytoskeletons were then solubilized in 50–300 μl of isoelectric focusing buffer (see below).

Preparation of Crosslinked Cytoskeletons for Electrophoretic Analysis and Electron Microscopy. Crosslinked cytoskeletons were prepared by a modification of the procedure of Bell *et al.* (19). Monolayers of BHK cells were rinsed once with Dulbecco's phosphate-buffered saline (GIBCO) and then crosslinked by adding a freshly prepared solution of 4 mg of dimethyl-3,3′-dithiobispropionimidate·2HCl in Dulbecco's P_i/NaCl. Cells were incubated in dimethyldithiobispropionimidate for 10 min at 37°C, and the crosslinking reaction was quenched by replacing the dimethyldithiobispropionimidate with 50 mM NH_4Cl in P_i/NaCl for 5 min. Cells were then rinsed twice with P_i/NaCl and extracted with 0.5% Triton X-100 in P_i/NaCl for 5 min at room temperature. After two rinses with P_i/NaCl, the cytoskeletons were either fixed for electron microscopy (19) or solubilized in electrofocusing buffer.

Preparation of Cytoskeletons for Immunofluorescence Microscopy. Cells for immunofluorescence microscopy were grown on 18-mm circular glass coverslips. These cells are rinsed once in P_i/NaCl and then extracted for 1.5–2 min in a lysis buffer containing 1% Triton X-100, 0.6 M KI, and 0.37% formaldehyde. A small amount (≈1 mM) of sodium thiosulfate was also added to the lysis buffer to prevent oxidation of iodide to iodine. Cells were then briefly rinsed in P_i/NaCl and fixed in P_i/NaCl containing 3.7% formaldehyde for an additional 7 min. All of the above steps were at 37°C. The subsequent processing for immunofluorescence, including the preparation and characterization of desmin antibody, has been published (6, 20).

Two-Dimensional IEF–$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Two-dimensional electrophoresis followed a modification of the O'Farrell procedure (21) as described by Hubbard and Lazarides (11). IEF sample buffer contained 8 M urea, 1% Nonidet P-40, 0.5% 2-mercaptoethanol, and protease inhibitors (phenylmethylsulfonyl fluoride and phenanthroline).

One-Dimensional Peptide Mapping by Limited Proteolysis. Peptide mapping by limited proteolysis followed the procedure outlined by Cleveland *et al.* (22). Proteins to be digested were isolated by two-dimensional IEF–$NaDodSO_4$ gel electrophoresis; gels were stained briefly with Coomassie brilliant blue, and the spots of interest were cut out with razor blades. These protein bands were then equilibrated in 100 mM Tris·HCl, pH 6.8/2 mM EGTA and frozen until use. Gel slices were placed in wells atop the mapping gel and overlayed with differing amounts (see figure legends) of *Staphylococcus aureus* protease V8 (Miles). Electrophoresis was at 20 mA until the dye front reached the top of the resolving gel, at which point the power was turned off for 25 min; electrophoresis was then continued at 35 mA until the dye front reached the bottom of the gel.

The gel system used for peptide mapping consists of a 3.5-cm stacking gel of 5% acrylamide/0.13% bisacrylamide as described (11) and a 15% resolving gel (23), modified by the inclusion of 8 M urea. Gels were stained overnight, destained, and photographed as described (11).

RESULTS

Protein Composition of Triton/KCl Cytoskeletons of CEF. Extraction of CEF with 1% Triton X-100 leaves an insoluble cytoskeletal residue, composed primarily of actin filaments and 10-nm filaments (ref. 1; see below). Inclusion of 0.6 M KCl in the extraction buffer facilitates the extraction of actin, leaving predominately 10-nm filaments. Comparison of two-dimensional IEF–$NaDodSO_4$ gel electrophoreograms of this cytoskeleton with an 8 M urea whole cell extract shows enrichment of a small number of protein species in the residual cytoskeleton (Fig. 1). The major polypeptide species possesses a M_r of 52,000, and has been tentatively identified as the F-IFP, as described by Brown *et al.* (1) in similar CEF cultures and by Starger *et al.* (12) in BHK cells. Two peptides with M_r of 50,000 are also apparent in the cytoskeletal sample. The more basic of these, slightly more basic than actin, has been identified as α-desmin by coelectrophoresis of CEF cytoskeletons with desmin isolated from avian smooth muscle (ref. 11, data not shown). The more acidic 50,000 M_r protein yields a one-dimensional peptide profile similar to α-desmin (data not shown) and thus represents a new desmin variant. Previous reports indicate that desmin from smooth muscle and skeletal muscle myofibrils consists of two isoelectric variants, α and β (5, 6); no β-desmin is identifiable in cytoskeletons from CEF. Small quantities of β,γ-actin also remain in the cytoskeletal residue, though the majority of actin is solubilized during the extraction. Several degradation products of F-IFP are apparent, forming a diagonal line extending to the lower left (acidic, lower molecular weight) of F-IFP. The presence of these degradation products is not affected by inclusion of *p*-tosyl-L-arginine methyl ester, phenylmethylsulfonyl fluoride, or phenanthroline, but is eliminated by heating samples to 100°C in 1% $NaDodSO_4$ and 0.5% 2-mercaptoethanol for 3 min prior to IEF (data not shown).

Immunofluorescence of CEF with rabbit antisera raised against chicken gizzard desmin reveals little antibody-specific fluorescence. However, if the CEF are pretreated with 5 μM Colcemid for 16 hr prior to fixation and antibody staining, the induced 10-nm filament cap is weakly positive for desmin-specific antibody. This fluorescence is more easily visualized in cells that have been extracted with Triton X-100 and KI prior to fixation (see Fig. 2), which is analogous to the preparation of cytoskeletons for electrophoretic analysis. Absorption of the antiserum with purified desmin (11) blocks the specific fluorescence (not shown). No crossreaction of desmin antisera with F-IFP is detectable in immunolabeling of IEF–$NaDodSO_4$ gels (not shown), in spite of the homology exhibited by these proteins (see below).

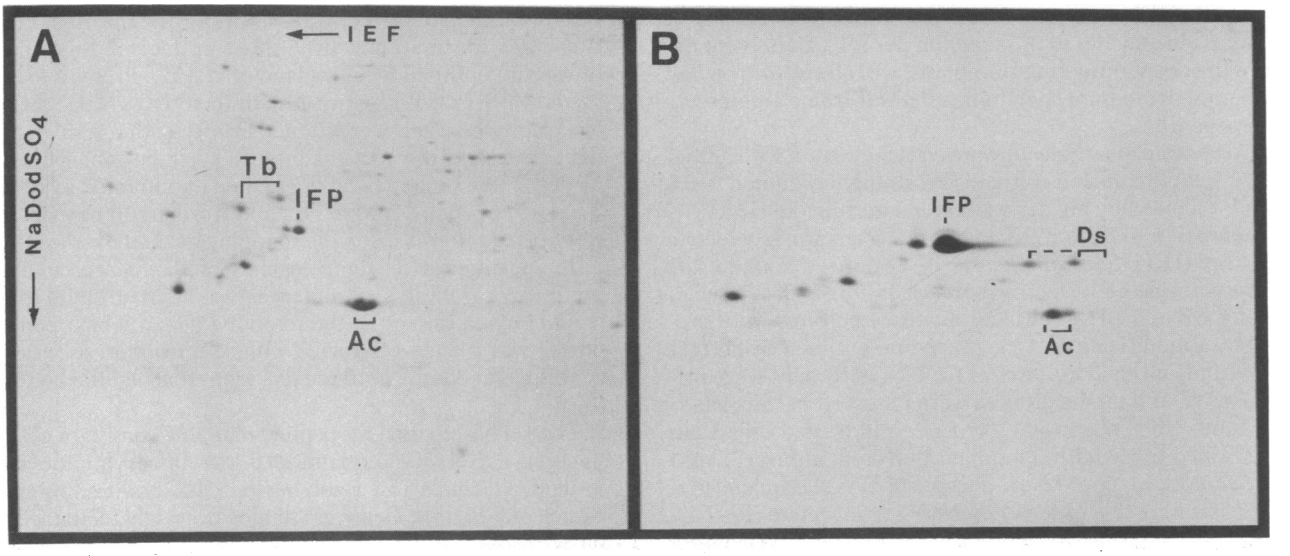

FIG. 1. Comparison of whole cell extracts with Triton/KCl cytoskeletons of CEF. IEF was from right (basic) to left (acidic) in all two-dimensional gels presented. (*A*) The whole cell extract contains tubulin (Tb), fibroblastic intermediate filament protein (IFP), and β,γ-actin (Ac) as well as many unidentified proteins. No desmin can be identified in the whole cell extract. (*B*) The insoluble residue of Triton/KCl extraction is enriched in IFP, actin, and two desmin variants (Ds). The more basic variant (middle bracket) has been identified as α-desmin (see text and Fig. 4). The acid variant (dotted bracket) has not been identified in adult muscle. No β-desmin (right bracket) is detectable in CEF. The proteins referred to in the text as the "diagonal" proteins are seen to the acidic side of IFP and with lower molecular weights.

Cytoskeletons from BHK Cells. Analysis of BHK cytoskeletons prepared by using the reversible crosslinker dimethyl-3,3′-dithiobispropionimidate prior to extraction with Triton X-100 yields results similar to those described above for CEF (Fig. 3*A*). F-IFP and β,γ-actin are clearly identifiable, as well as two proteins with M_r of 50,000. The more basic of these two proteins has been identified as the mammalian desmin species by coelectrophoresis with mammalian and avian muscle desmin. The single mammalian desmin species differs slightly from avian α-desmin in both molecular weight and isoelectric point (13). The more acidic protein of 50,000 M_r in BHK cytoskeletons corresponds to the new acidic variant of desmin described above for CEF. It is interesting to note the lack of the "diagonal" proteins in crosslinked cytoskeletons. Electron microscopy of crosslinked cytoskeletons reveals numerous 10-nm filaments within the cytoplasm (Fig. 3*B*).

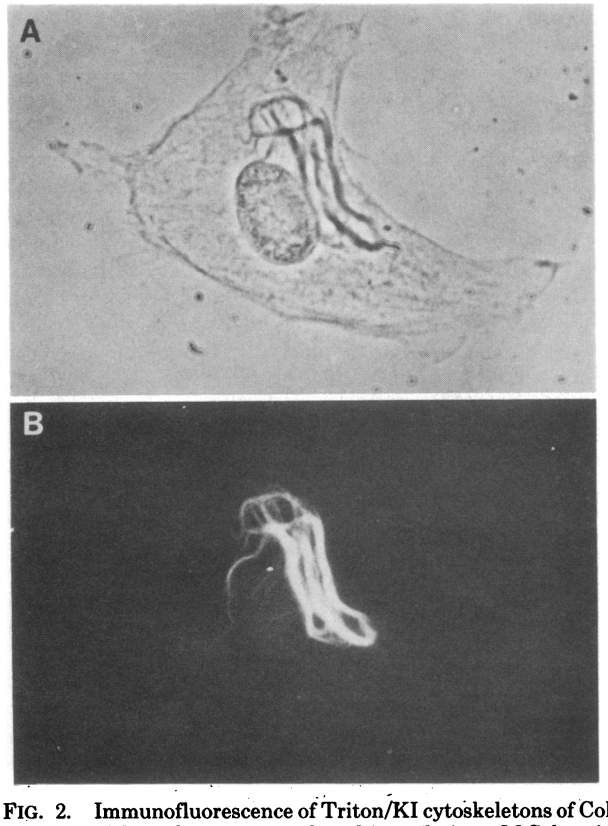

FIG. 2. Immunofluorescence of Triton/KI cytoskeletons of Colcemid-treated CEF. Cells were incubated for 16 hr in 5 μM Colcemid, and cytoskeletons were examined by phase (*A*) and immunofluorescence (*B*) microscopy by using antibodies raised against smooth muscle desmin. (×800.) The Colcemid-induced aggregation of intermediate filaments into a perinuclear filament cap is apparent. Desmin-specific fluorescence is localized to this structure.

Cytoskeletons from Skeletal Muscle Myotubes. Triton cytoskeletons prepared from cultures of skeletal muscle myotubes low in contaminating fibroblasts were compared to 8 M urea extracts of whole myotubes (Fig. 4) and to CEF cytoskeletons. The protein constituents of the myotube cytoskeleton are quite similar to those obtained from fibroblasts (compare Figs. 1 and 4). The two major features that distinguish myotube cytoskeletons are the presence of both α- and β-desmin, and the predominance of the muscle specific α-actin variant. IFP, the diagonal proteins, and the new acidic desmin variant are all identifiable in quantities that cannot be accounted for by a slight (<5%) contamination with mononucleate fibroblastic cells.

Comparative Peptide Analysis of Desmin and IFP. One-dimensional peptide analysis of cytoskeletal proteins was performed with protease V8 from *S. aureus*. Due to the similarity in the electrophoretic mobilities of IFP and desmin, it was found necessary to isolate these proteins from two-dimensional IEF–$NaDodSO_4$/polyacrylamide gels. Extensive homology is evident between avian (CEF) F-IFP and mammalian (BHK) F-IFP, with 11 of 14 generated peptides exhibiting closely similar molecular weights (Fig. 5, lanes A–D). Similarly, avian and mammalian desmin exhibit several similar peptides (Fig. 5, lanes E–H), implying some interspecies homology between these proteins, though less than the homologies exhibited by the F-IFPs. Homologous peptides are also apparent when desmin and IFP from the same cells (either CEF, myotubes, or BHK) are compared (Fig. 5, lanes C–F). Neither IFP nor desmin showed homology with actin or tropomyosins. Examination of the phosphopeptides generated from ^{32}P-labeled F-IFP and

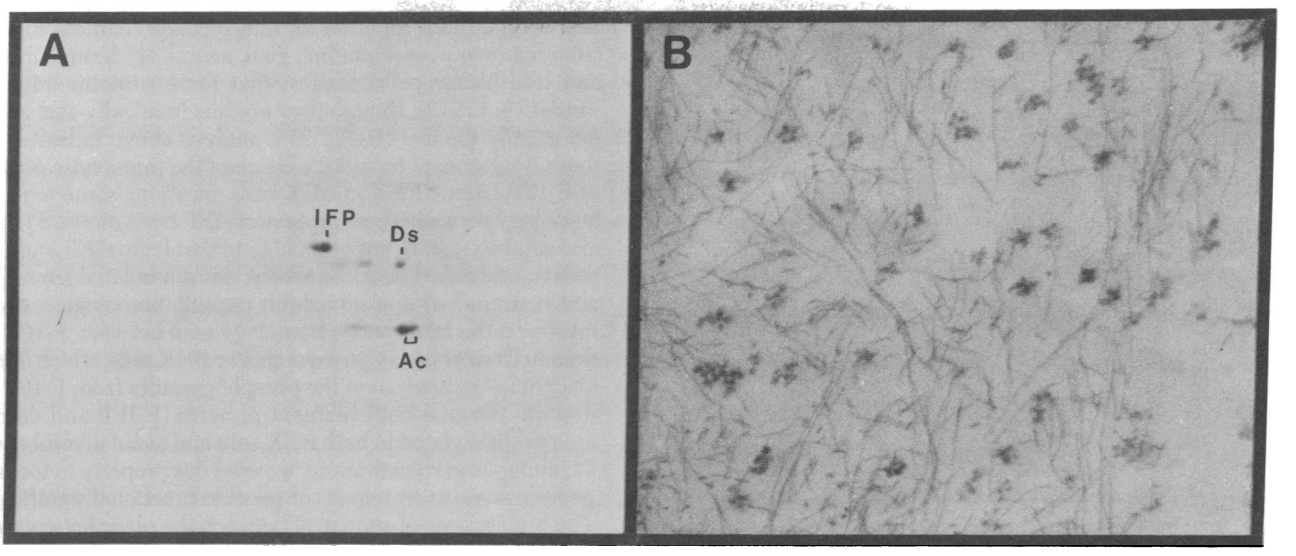

FIG. 3. Analysis of dimethyldithiobispropionimidate-crosslinked cytoskeletons of BHK cells. (*A*) Cytoskeletal residue of BHK cells crosslinked prior to Triton extraction contains predominantly IFP, β,γ-actin (Ac), and desmin (Ds). Note the lack of diagonal proteins in the crosslinked cytoskeleton (see text). (*B*) Thin sections of the cytoskeleton reveal numerous intermediate filaments with diameters of 7–8 nm. (×62,000.)

desmin (24) further supports the conclusion that these proteins are homologous. The autoradiogram of the peptide mapping gel (Fig. 6) shows that the patterns of phosphopeptides of these two proteins from both myotubes and BHK cells are nearly identical; phosphopeptides from α,β-tropomyosin (24) are not homologous to either IFP or desmin.

DISCUSSION

Coexistence of Desmin and F-IFP in CEF, BHK, and Muscle Cells. We have demonstrated that nonmuscle cells of both avian (CEF) and mammalian (BHK) species contain desmin and that avian skeletal muscle myotubes contain the F-IFP. These results are interesting in several regards. First, the presence of identifiable desmin in CEF and BHK cells indicates that desmin is not a muscle-specific protein, having a wider distribution than originally believed (5, 6, 11, 13). A protein tentatively identified as desmin has also been observed in cytoskeletons of mouse 3T3 cells (unpublished observations). Second, the observation that these cell types contain both desmin and F-IFP indicates that two proteins capable of forming morphologically similar filaments may coexist in the same cell. Analogous results have been obtained by Franke *et al.* (14), who have reported the coexistence of keratin filaments and fibroblastic (termed vimentin) filaments in PTK_2 and HeLa cells. Closer examination of the results published by Brown *et al.* (1) also reveal desmin in CEF cytoskeletons. Analysis of BHK 10-nm filament caps by Starger *et al.* (12) revealed the presence of two major components of M_r 55,000 and 54,000 which exhibited some peptide homologies. Data presented above suggest that these components represent F-IFP and desmin, respectively, which migrate with M_r of 52,000 and 50,000 in our gel systems.

The relationship between the desmin variants described to date remains to be established. Desmin from avian smooth and skeletal muscle myofibrils consists of two isoelectric variants, α and β (5, 13). In this paper, we identify a third, more acidic, variant of desmin present in CEF and skeletal myotubes. Recently we have reported that α-desmin and the new acidic variant are phosphorylated both in *in vitro* differentiating myotubes and in embryonic muscle *in vivo* (24). β-Desmin is not phosphorylated in myotubes and is not observed in CEF. It is possible that β-desmin represents a nonphosphorylated

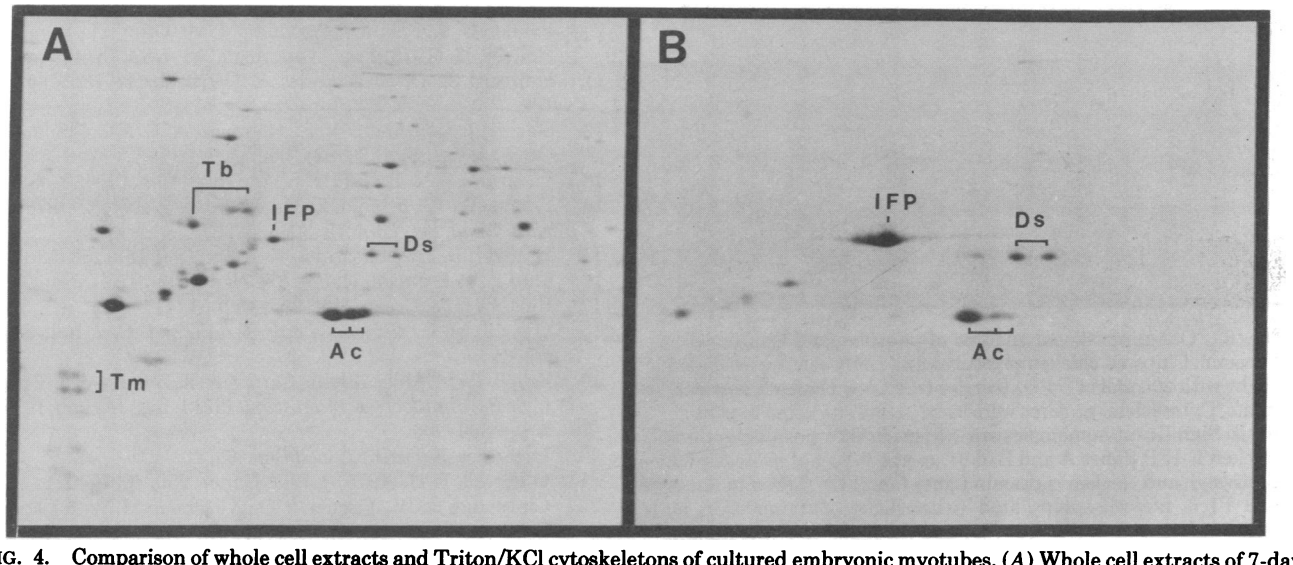

FIG. 4. Comparison of whole cell extracts and Triton/KCl cytoskeletons of cultured embryonic myotubes. (*A*) Whole cell extracts of 7-day myotubes contain tubulin (Tb), IFP, α- and β-desmin (Ds), α,β,γ-actins, α,β-tropomyosins, and many unidentified protein species. (*B*) Cytoskeletal residue consists of IFP, α,β-desmin (Ds), and actin (Ac). Note that α-actin is the major actin species of the muscle cytoskeleton.

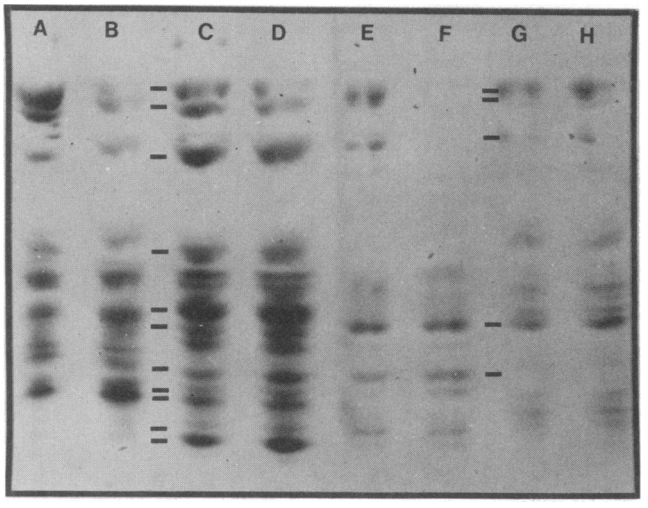

FIG. 5. One-dimensional peptide analysis of cytoskeletal proteins with *S. aureus* protease V8. Lanes A and B, hamster IFP with 0.02 μg and 0.1 μg of protease, respectively; lanes C and D, chicken IFP; lanes E and F, chicken desmin (myotube); lanes G and H, hamster desmin. Significant homology is evident between IFP from hamster and chicken (compare lanes A and B with C and D). Desmins from the two species also show several homologous peptides (compare lanes E and F with G and H). IFP and desmin from chicken show less homology, although several similar peptides are apparent.

precursor to the more acidic variants, with the addition of phosphate responsible for the observed differences in isoelectric point. A similar situation may exist for F-IFP, where multiple phosphorylated variants and a single more basic variant that is not phosphorylated has been described (24). A new acidic variant of mammalian desmin is apparent in BHK cells, which also possess the mammalian species of desmin previously described in rat and guinea pig myofibrils (13). As in avian cells, the acidic desmin variant is phosphorylated, while the basic mammalian desmin species is not phosphorylated, suggesting that it is analogous to avian β-desmin (unpublished data).

Peptide Homologies between Desmin and F-IFP. Amino acid compositions reported for intermediate filament subunits from many sources (including glial, neural, epidermal, muscle, and fibroblastic cells) suggest that these proteins might be similar (8, 11, 12), though they are biochemically and immunologically distinct (6–12). The analysis above indicates that there is significant homology between the proteolytic peptides of F-IFP from CEF and BHK cells, implying some sequence homology between these proteins. F-IFP from mouse 3T3 cells also exhibits significant homology to that from CEF and BHK cells (unpublished data). Similarly, desmin isolated from avian and mammalian sources exhibit peptide homologies. Also of interest is the intraspecies homology seen between F-IFP and desmin from either avian myotubes or BHK cells, which is more evident in the analysis of the phosphopeptides from F-IFP and desmin. Because both filament proteins (F-IFP and desmin) are phosphorylated in both BHK cells and avian myotubes (ref. 24; unpublished observation), we used this property to focus our peptide analysis on regions of possible functional significance. The high degree of similarity between the phosphopeptides of F-IFP and desmins from both BHK cells and avian myotubes strengthens the conclusion that desmin and F-IFP have some homologous sequences and, in particular, suggests that these homologies span the sites of phosphorylation. Though these data are not yet conclusive, they suggest that the subunits of 10-nm filaments form a family of evolutionally related proteins.

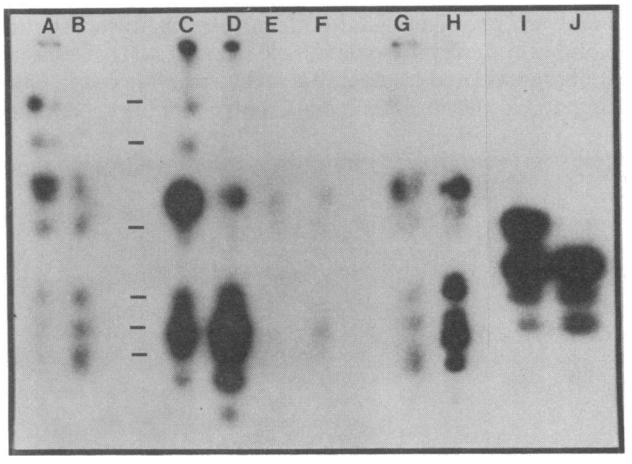

FIG. 6. One-dimensional analysis of phosphopeptides from IFP and desmin. Cultured chicken myotubes and BHK cells were labeled for 20 hr with 200 μCi of $^{32}PO_4$ (carrier free, New England Nuclear) per plate. Cytoskeletal proteins were isolated and subjected to peptide analysis. Significant homologies are evident in the phosphopeptides of chicken F-IFP (lanes A and B, 0.01 μg and 0.1 μg of protease V8, respectively), and chicken α-desmin (lanes C and D); β-desmin (lanes E and F) is not phosphorylated (with slight contamination by α-desmin). Hamster F-IFP (lane G) and the hamster acidic desmin variant (lane H, see text) also show striking homology to each other and to the analogous chicken proteins. The pattern of phosphopeptides from chicken myotube tropomyosin (lanes I and J) is distinct.

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