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The 68,000-dalton neurofilament-associated polypeptide is a component of nonneuronal cells and of skeletal myofibrils

(intermediate filaments/immunofluorescence/two-dimensional isoelectric focusing/sodium dodecyl sulfate/polyacrylamide gel electrophoresis)

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ABSTRACT **Purified preparations of 10-nm neurofilaments from rat spinal cord and bovine or porcine brain contain a predominant 68,000-dalton polypeptide. This polypeptide is also a major component of the neurofilaments that copurify with brain tubulin isolated by cycles of polymerization and depolymerization. A protein that has the same isoelectric point and molecular weight as the neurofilament-associated polypeptide has also been identified as a cytoskeletal protein in a variety of avian and mammalian cell types, including baby hamster kidney (BHK-21) mouse 3T3, Novikoff rat hepatoma, chicken fibroblast, and chicken muscle cells. This protein is also a component of isolated chicken skeletal myofibrils. One-dimensional peptide maps of the 68,000-dalton proteins purified by two-dimensional isoelectric focusing/NaDodSO$_4$/polyacrylamide gel electrophoresis from myofibrils, cycled tubulin, purified neurofilaments, and various cultured cell types were identical. In immunofluorescence this protein was associated with cytoplasmic intermediate filaments and myofibril Z discs. These results indicate that the neurofilament-associated polypeptide is a conserved protein that is present in many different cell types in addition to neuronal cells. Because some of these cells contain the major components of two other intermediate filament classes, desmin and vimentin, a given cell type may contain the subunits of at least three distinct intermediate filament types.**

Intermediate or 10-nm filaments have been observed in many cell types of higher vertebrates. Although these filaments share several morphological and biochemical characteristics that suggest a common origin, the major protein components of the filaments from different tissue sources are biochemically and immunologically distinct. At least five subclasses of 10-nm filaments can be identified: muscle intermediate filaments, 10-nm filaments in cells of mesenchymal origin, neurofilaments, glial filaments, and keratin filaments in cells of epithelial origin (for a review see ref. 1). One of the most intriguing observations about intermediate filaments to date is that a large number of cell types possess two distinct major filament components. Most cell types contain vimentin, the 52,000-dalton intermediate filament component in cells of mesenchymal origin. In epithelial cells, vimentin and keratin make up distinct filament populations as demonstrated by immunofluorescence and electron microscopy (2, 3). In a number of other cell types, and in particular muscle cells, vimentin coexists with desmin, the 50,000-dalton muscle-specific intermediate filament component (4–7). In this case the cytoplasmic filamentous distributions of the two proteins are indistinguishable (4–6).

Purified mammalian neurofilaments are composed of three major polypeptides of 200,000, 160,000, and 68,000 daltons, known as the neurofilament triplet (8–13). These three proteins have been assumed to be the subunit constituents of neurofilaments because they copurify with the filaments and they are not dissociated from them under a variety of chemical treatments. Recently it has been demonstrated that the 68,000-dalton polypeptide is present in conventionally purified microtubules from brain that are contaminated with 10-nm neurofilaments (14–16).

In this paper we show that the 68,000-dalton polypeptide of purified mammalian neurofilaments is highly homologous, if not identical, to the protein that copurifies with microtubule protein isolated from brain by cycles of polymerization and depolymerization. In addition, the 68,000-dalton protein is enriched in the microtubule-free cytoskeletons prepared by high salt and detergent extraction of a variety of nonneuronal cell types. Because these cytoskeletons are highly enriched in intermediate filaments, the extraction results in conjunction with immunofluorescence indicate that the 68,000-dalton polypeptide is associated with cytoplasmic intermediate filaments also in a variety of nonneuronal cell types.

MATERIALS AND METHODS

Cells. Cultures of nonmyogenic chicken embryo fibroblasts (CEF), embryonic chicken myogenic cells, and baby hamster kidney (BHK) 21 cells were prepared and grown as described (4). Chicken skeletal myofibrils were prepared from fresh or glycerol-extracted adult chicken skeletal muscle as described (5).

Preparation of Triton X-100 KCl Cytoskeletons and Isoelectric Focusing (IEF)/NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Triton X-100 KCl cytoskeletons of CEF and BHK cells were prepared as described (4). Purified skeletal myofibrils were extracted with 0.6 M KI for 10 min at room temperature as described (5). Two-dimensional IEF/NaDodSO$_4$/polyacrylamide gel electrophoresis was performed also as described (17, 18). The rat spinal cord and brain extracts were precipitated with 8 vol of acetone at 0°C and the acetone was removed under reduced pressure prior to solubilization in the urea sample buffer. This treatment was found to reduce streaking in the first IEF dimension.

One-Dimensional Peptide Mapping by Limited Proteolysis. Peptide mapping by limited proteolysis followed the procedure outlined by Cleveland *et al.* (19) as described (4). Gel slices were placed in wells atop the mapping gel and overlayed with 10–20 ng of *Staphylococcus aureus* protease V8 (Miles). Electrophoresis was at 10 mA until the dye front reached the top of the resolving gel; electrophoresis was then continued at 35 mA until the dye front reached the bottom of the gel.

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Abbreviations: CEF, chicken embryo fibroblast(s); BHK, baby hamster kidney; IEF, isoelectric focusing; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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Purification of 10-nm Filaments from Brain. The method of Runge *et al.* (15) was followed for the purification of pig brain 10-nm filaments. All procedures were carried out at 4°C. Brain was homogenized in buffer consisting of 0.1 M 1,4-piperazinediethanesulfonic acid (Pipes), 2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM $MgSO_4$, and 10 mM 2-mercaptoethanol, pH 6.9. The homogenate was centrifuged first for 15 min at 7000 rpm in a Sorvall RC-2B centrifuge with an SS34 rotor. The supernatant was clarified again for 75 min at 29,000 rpm (approximately 100,000 × *g*) in a Beckman L5-75 ultracentrifuge with a type 42.1 fixed-angle rotor. The high-speed supernatant was applied to a column of Bio-Gel A-150m pre-equilibrated in the Pipes/EGTA/$MgSO_4$/2-mercaptoethanol buffer and eluted with the same buffer. The void volume was collected and centrifuged for 2 hr at 35,000 rpm as above. The high-speed pellets were either dissolved in the two-dimensional urea sample buffer or resuspended and precipitated by cold acetone (8:1). The precipitated material was collected at 15,000 rpm for 30 min in a Beckman SW50.1 rotor. The excess acetone was removed from the pellets under reduced pressure and the material was dissolved in urea sample buffer.

Purification of Rat Neurofilaments. Rat neurofilaments were isolated essentially according to the procedure of Schlaepfer (8, 10, 20). All experiments were carried out at 18–20°C. Rat spinal cords were excised and placed in isotonic saline after cardiac perfusion of etherized animals with saline to clear the vasculature of blood. The spinal cords were then minced into small pieces and subjected to hypotonic shock by immersing them into a low ionic strength buffer (1 mM sodium phosphate/2 mM EDTA/2 mM EGTA, pH 7.0). After 2 hr of slow agitation, the osmotically shocked and swollen tissue was homogenized. After the addition of 120 μl of 1 M NaCl per ml of extract, the homogenates were clarified at 12,000 rpm in a Beckman Ti50 fixed-angle rotor for 30 min. The supernatant was carefully decanted and spun again at 35,000 rpm for 2 hr. The high-speed pellets were washed once in the above buffer containing 0.1 M NaCl. The resultant pellets were either stored at −20°C or treated with acetone prior to two-dimensional electrophoresis as described above.

Purification of Brain Microtubule Protein. Bovine or porcine brain microtubule protein was purified by two cycles of polymerization-depolymerization according to the method of Shelanski *et al.* (21) and Borisy and Olmsted (22). The protein was a generous gift of W. Z. Cande (University of California, Berkeley).

Electron Microscopy. For electron microscopy, samples were applied to freshly carbon-coated grids by floating the grids upside-down on a drop of resuspended high-speed pellets from rat spinal cord or brain for 30 sec. The grids were then allowed to float on a drop of 0.5% aqueous solution of uranyl acetate for 3 min. After the excess liquid had been drawn off with a filter paper, the grids were air dried. The specimens were examined and photographed in a Phillips 201 electron microscope operated at 80 kV.

Indirect Immunofluorescence. Antibodies to the 68,000-dalton polypeptide were obtained against the antigen purified by preparative $NaDodSO_4$ gel electrophoresis from rat spinal cord. The material shown in Fig. 2*A* was electrophoresed on a preparative $NaDodSO_4$ slab gel. After staining and destaining the 68,000-dalton band was excised, neutralized with 0.1 M Tris·HCl at pH 8.7, homogenized in the same buffer, precipitated with $AlCl_3$, and emulsified for immunization as described (5). The immunization scheme was as described (5). Two-dimensional IEF of the antigen revealed the presence of no other detectable contaminants of the same molecular weight and different isoelectric point (not shown). The detailed characterization of this antiserum by two-dimensional immunoautoradiography will be presented elsewhere.

Indirect immunofluorescence on CEF was performed on cells fixed with formaldehyde in the presence of 0.6 M KCl and 1% Triton X-100 (4). Indirect immunofluorescence on myofibrils was as described (5).

RESULTS

Protein Composition of 10-nm Filaments Purified from Spinal Cord and Brain. The high-speed pellet of extracts from rat spinal cord contained numerous filaments with a diameter of approximately 10 nm as well as vesicles of various diameter (Fig. 1*A*). Analysis of this material by one-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis showed three prominent polypeptides of 200,000, 160,000, and 68,000 molecular weight in addition to a number of other polypeptides, consistent with the results of Schlaepfer and Freeman (8) (not shown). Two-dimensional IEF/$NaDodSO_4$/polyacrylamide gel electrophoresis revealed that the 68,000-dalton polypeptide is composed of a main isoelectric variant and a slightly more acidic minor variant (Fig. 2*A*). It is apparent from these gels that α and β tubulins are also present in the 10-nm filament preparations. The 68,000-dalton polypeptide is distinct from any rat serum albumin possibly present in these preparations, because the two proteins are clearly resolvable on these gels (Fig. 2*C*).

The high-speed supernatant from brain contained α and β tubulins as major components as well as a protein with the molecular weight and isoelectric point of the rat neurofilament

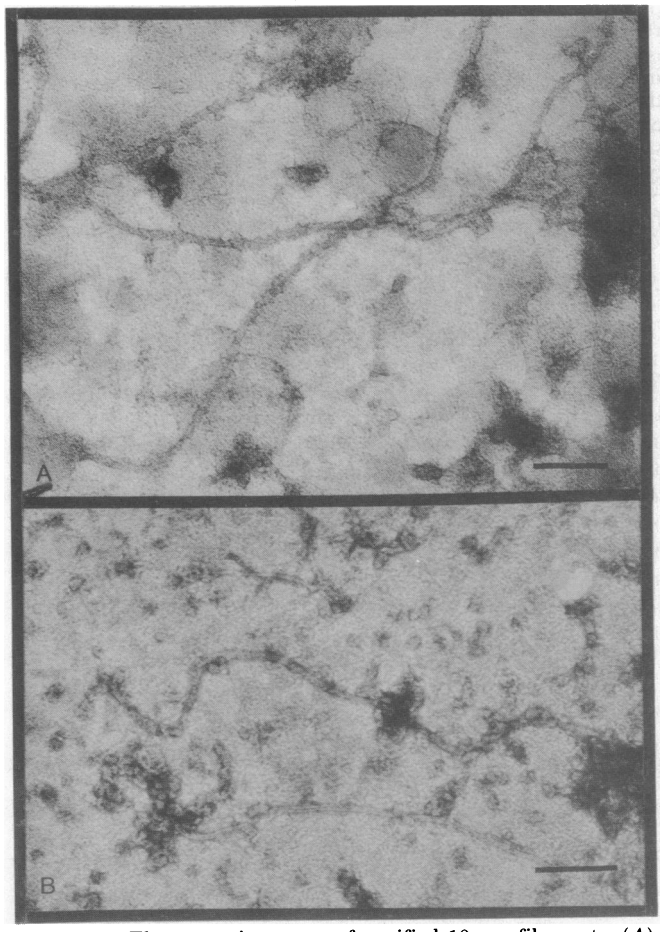

FIG. 1. Electron microscopy of purified 10-nm filaments. (*A*) High-speed pellet of rat spinal cord extracts. (*B*) High-speed pellet of pig brain extract purified by chromatography on Bio-Gel A-150m. Bars = 0.1 μm.

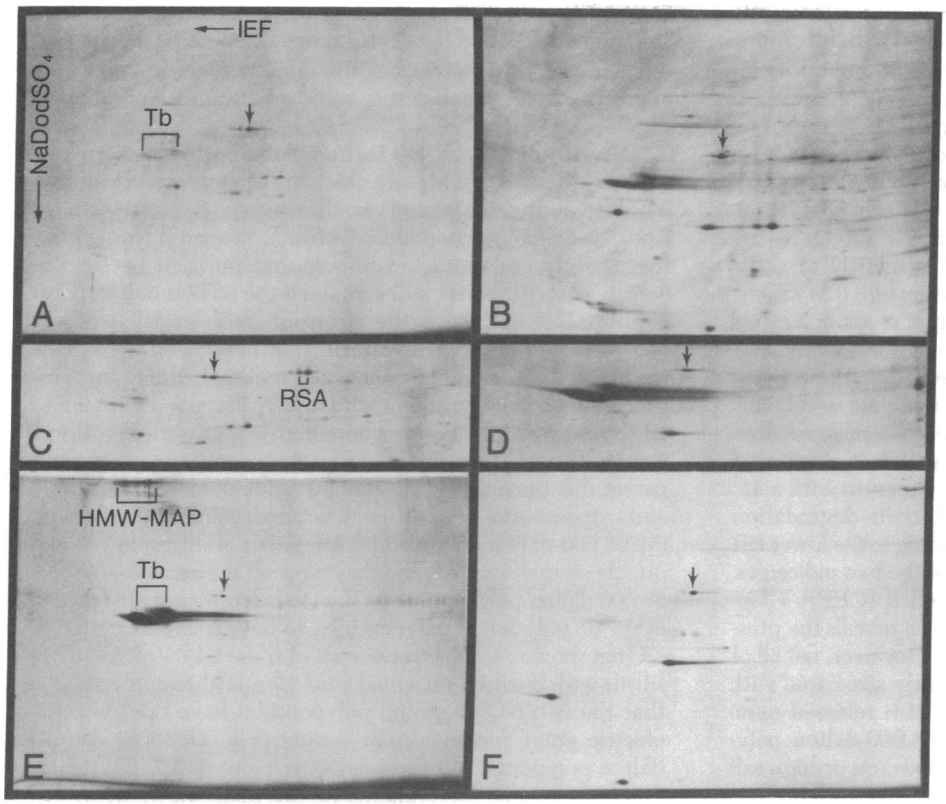

FIG. 2. Comparative two-dimensional gel electrophoresis of the 68,000-dalton polypeptide. In all electropherograms IEF was performed from right to left (acid on the left); pH range 4.5–7.0. The arrow points to the 68,000-dalton peptide. (*A*) Purified 10-nm filaments from rat spinal cord. Tb, α and β tubulins. (*B*) High-speed supernatant of a pig brain extract prior to gel filtration. (*C*) Coelectrophoresis of purified rat spinal cord 10-nm filaments and 1 μg of rat serum albumin (RSA). (*D*) High-speed pellet of the pig brain extract after gel filtration of the high-speed supernatant through a Bio-Gel A-150m column and high-speed centrifugation of the void volume material. (*E*) Microtubule protein purified by two cycles of polymerization–depolymerization from pig brain. The pellet after the second polymerization step was suspended directly in urea sample buffer. HMW-MAP, high molecular weight microtubule-associated proteins. (*F*) Coelectrophoresis of rat spinal cord 10-nm filaments and KI-extracted myofibrils (compare Figs. 2*A* and 3*D*).

68,000-dalton polypeptide (Fig. 2*B*). When this material was purified by gel filtration on Bio-Gel A-150m, the void volume contained numerous 10-nm filaments that could be pelleted by high-speed centrifugation, but no discernible 25-nm microtubules (Fig. 1*B*). Analysis of this material by two-dimensional gel electrophoresis revealed the presence of α and β tubulins as the major components and two other prominent polypeptides with molecular weights of 68,000 and 42,000 (Fig. 2*D*). Coelectrophoresis of these brain extracts with rat spinal cord purified 10-nm filaments showed that the 68,000-dalton polypeptides from the two sources have the same molecular weight and isoelectric point (not shown).

The pellets of microtubule protein purified from brain extracts by two cycles of polymerization–depolymerization contained α and β tubulins as their major component, a group of polypeptides with a very high molecular weight, corresponding to the high molecular weight microtubule-associated proteins, and another prominent polypeptide with a molecular weight of 68,000 (Fig. 2*E*). This is consistent with the results of Berkowitz *et al.* (14) and Runge *et al.* (15) [protein N4, figure

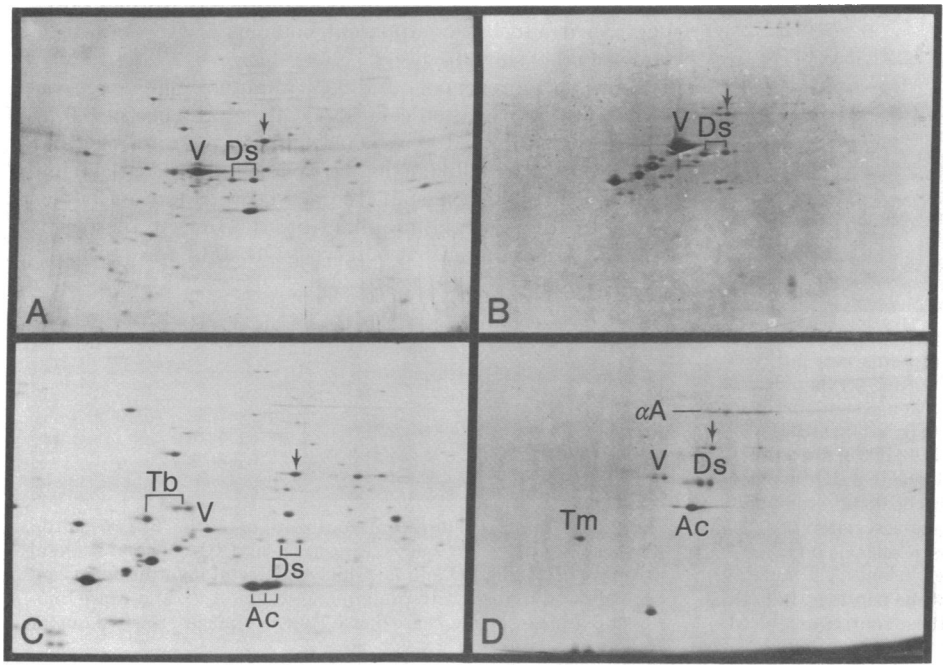

FIG. 3. Presence of the 68,000-dalton polypeptide in cytoskeletons and whole cell extracts of cells grown in tissue culture and in myofibrils. V, vimentin; Tm, tropomyosin; Ac, actin; Ds, α and β desmin; αA, α actinin. The arrow points to the 68,000-dalton polypeptide. (*A*) Cytoskeletal residues of BHK-21 cells. (*B*) Cytoskeletal residues of CEF. (*C*) Whole cell extracts of 7-day-old chicken embryonic skeletal myotubes. (*D*) Triton X-100/KI-extracted chicken pectoralis myofibrils.

2 of Berkowitz *et al.* (14)]. The microtubule-associated polypeptide is also composed of a major variant and a slightly more acidic variant. Coelectrophoresis of the microtubule protein with purified rat spinal cord 10-nm filaments (Fig. 2 *A* and *E*) showed that the two 68,000-dalton polypeptides have the same molecular weight and isoelectric point (not shown).

Presence of the 68,000-Dalton Polypeptide in Triton/KCl Cytoskeletons of Cells Grown in Tissue Culture. Extraction of BHK-21 cells and CEF with 1.0% Triton X-100 leaves an insoluble cytoskeletal residue, composed primarily of actin filaments and 10-nm filaments (23). Inclusion of 0.6 M KCl in the extraction buffer facilitates the extraction of actin, leaving predominantly 10-nm filaments. Electron microscopy and two-dimensional gel electrophoresis shows that these cytoskeletal residues contain no detectable microtubules or tubulins (4, 24). The major polypeptide species enriched in these residues have been identified by two-dimensional IEF/NaDodSO$_4$/polyacrylamide gel electrophoresis (4) as vimentin with a M_r of 52,000, desmin with a M_r of 50,000, their degradation products, which form a diagonal line extending to the lower left (acidic, lower molecular weight) of each of the two molecules, and residual actin [Fig. 3 *A* (BHK-21) and *B* (CEF)]. Close examination of these electropherograms also reveals the presence of a polypeptide with a M_r of 68,000. However, not all of the 68,000-dalton polypeptide may be tightly associated with these cytoskeletons, because a fraction of it is released upon prolonged extraction (not shown). The 68,000-dalton polypeptide has been identified in whole cell extracts or high salt detergent cytoskeletons of a number of cell types grown in tissue culture, including chicken embryonic skeletal myotubes (Fig. 3*C*), mouse 3T3, and Novikoff rat hepatoma cells (not shown). In each case this protein has the same isoelectric point and molecular weight as the rat spinal cord 10-nm filament-associated polypeptide.

Presence of the 68,000-Dalton Polypeptide in Chicken Skeletal Myofibrils. Mature chicken skeletal myofibrils give a pattern of protein spots on two-dimensional gels that includes a 68,000-dalton polypeptide. Myofibrils, prepared from either fresh or glycerol-extracted muscle and purified by sucrose density centrifugation, still contained the 68,000-dalton polypeptide. Fig. 3*D* shows the electrophoretic profile of glycerol-extracted chicken myofibrils that have been detergent-washed to remove membranous organelles, banded on a discontinuous sucrose gradient, and briefly extracted with 0.6 M KI to remove actomyosin, whose high quantity in myofibrils would otherwise obscure minor protein components. It is apparent that these myofibrils contain residual myosin, α-actinin, and tropomyosin, in addition to actin, desmin, vimentin, and the 68,000-dalton polypeptide. A number of different types of muscle were examined, and they were all shown to contain the 68,000-dalton polypeptide. As in cytoskeletons, a small fraction of the 68,000-dalton polypeptide can be extracted with 0.6 M KI (not shown). Coelectrophoresis of these KI-extracted myofibrils with purified rat spinal cord 10-nm filaments indicates that the two 68,000-dalton polypeptides have the same isoelectric point and molecular weight (Fig. 2*F*). The 68,000-dalton polypeptide has an isoelectric point of 5.7 (25), which agrees well with that estimated for this molecule by Berkowitz *et al.* (protein N4 in figure 2 and table 2 of ref. 14).

Comparative Peptide Analysis of the 68,000-Dalton Polypeptide. One-dimensional peptide analysis of the 68,000-dalton polypeptide was performed with protease V8 from *S. aureus*. In order to maximize the purity of the 68,000-dalton polypeptide, it was isolated from two-dimensional IEF/NaDodSO$_4$/polyacrylamide gels. As shown in Fig. 4, the peptide maps of this protein isolated from rat spinal cord 10-nm filament preparations (Fig. 4*C*), BHK-21 cells (Fig. 4*D*), CEF (Fig. 4*E*), brain 10-nm filament preparations (Fig. 4*F*), skeletal myofibrils (Fig. 4*G*), and cycled brain microtubule protein (Fig. 4*H*) are highly homologous. Comparison of the peptide map of this protein with the maps of rat serum albumin (Fig. 4*A*) and chicken serum albumin (Fig. 4*B*) indicates that the 68,000-dalton polypeptide is a completely distinct polypeptide; it also has no apparent homology with desmin or vimentin (not shown).

Immunofluorescence. Indirect immunofluorescence on CEF was performed on cells fixed in the presence of 0.6 M KCl/1.0% Triton X-100, which solubilizes microtubules and the majority of actin filaments but leaves intermediate filaments insoluble (4). The rat spinal cord 68,000-dalton antibodies reacted with a cytoplasmic filamentous system in these cells (Fig. 5*A*) resembling that observed with antibodies to inter-

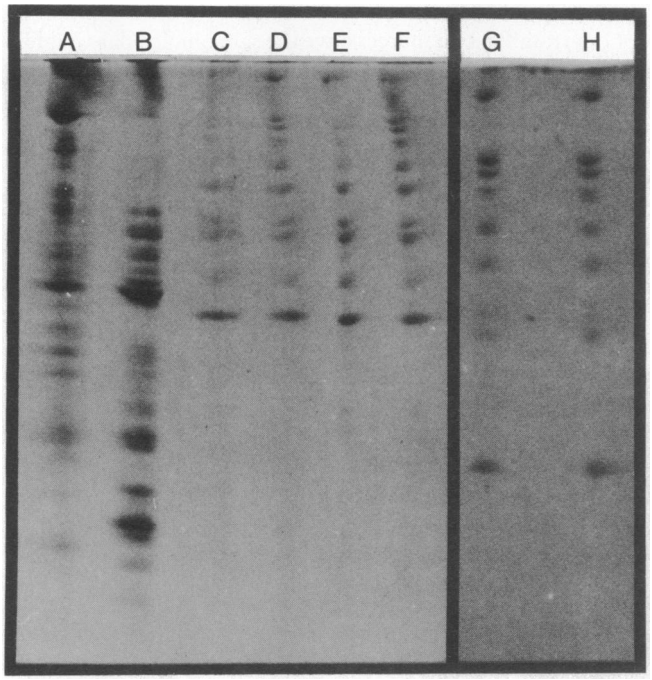

FIG. 4. One-dimensional peptide analysis of the 68,000-dalton polypeptide with *S. aureus* protease V8. The proteins were purified in each case by two-dimensional IEF/NaDodSO$_4$/polyacrylamide gel electrophoresis. A, Rat serum albumin; B, chicken serum albumin; C, rat spinal cord 68,000-dalton polypeptide (Fig. 2*A*); D, BHK-21 68,000-dalton polypeptide (Fig. 3*A*); E, CEF 68,000-dalton polypeptide (Fig. 3*B*); F, pig brain high-speed pellet 68,000-dalton polypeptide (Fig. 2*D*); G, chicken skeletal myofibril 68,000-dalton polypeptide (Fig. 3*D*); H, cycled brain microtubule-associated 68,000-dalton polypeptide (Fig. 2*E*). The peptide maps in lanes G and H were prepared at a different time than those depicted in lanes A–F and the gel was run longer to increase the resolution of the peptides. In both cases the 68,000-dalton polypeptide from all these sources exhibited indistinguishable peptides.

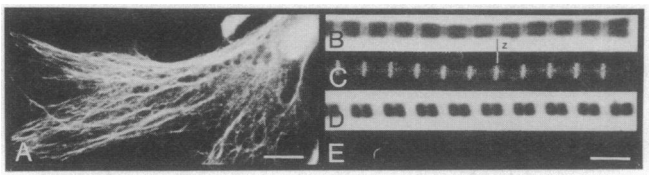

FIG. 5. Immunofluorescent localization of the 68,000-dalton polypeptide. (*A*) CEF; bar = 10 μm. (*B* and *C*) Chicken skeletal myofibril. Z, Z line. (*D* and *E*) Chicken skeletal myofibril that had been allowed to react with preimmune serum. *A*, *C*, and *E* are fluorescence images; *B* and *D* are phase-contrast images. Bar = 3 μm for *B–E*.

mediate filaments (2, 26). In isolated myofibrils, immunofluorescence indicated that the 68,000-dalton polypeptide is concentrated in the Z-line region of the myofibril (Fig. 5 *B* and *C*). The antigen also remains associated with the Z lines after solubilization of the actin and myosin filaments with 0.6 M KI (not shown). Preimmune sera were uniformly negative both with myofibrils (Fig. 5 *D* and *E*) and with fibroblasts (not shown).

DISCUSSION

The 68,000-Dalton Polypeptide as a Component of 10-nm Neurofilaments. It has been well documented in the literature that mammalian 10-nm neurofilaments contain a 68,000-dalton polypeptide as their major constituent (8, 12, 15, 16). This polypeptide remains associated with the filaments under a variety of chemical treatments (8, 12, 15) and thus appears to be an integral component of the filaments. However, it is not clear whether this polypeptide serves as a subunit for these filaments, because it has not yet been purified to homogeneity and shown to assemble into intermediate filaments *in vitro*. On the basis of its purification with 10-nm filaments from brain, Berkowitz *et al.* (14) and Runge *et al.* (15) have concluded that the 68,000-dalton polypeptide is not a component of microtubules but rather of the 10-nm filaments that copurify with them during the purification of the microtubule protein by cycles of polymerization–depolymerization. Our results confirm the conclusions of these authors and show clearly that the 68,000-dalton polypeptides isolated from preparations of 10-nm filaments from rat spinal cord or pig brain, as well as from microtubule protein purified by cycles of polymerization–depolymerization, are highly homologous if not identical proteins. The presence of tubulin as the major component of isolated brain 10-nm filaments remains unexplained but is in accordance with the observations of Runge *et al.* (15).

Presence of the 68,000-Dalton Polypeptide in Nonneuronal Cells and in Skeletal Myofibrils. The most interesting conclusion to be reached from the results presented here is that the 68,000-dalton neurofilament-associated polypeptide is a major component of many different nonneuronal cell types of both avian and mammalian origin. In particular, this protein is present in cytoskeletal preparations, which are composed predominantly of intermediate (10-nm) filaments and do not contain any detectable microtubules or tubulin. Thus it seems likely that the 68,000-dalton polypeptide is also associated with intermediate filaments in nonneuronal cells. Indeed, immunofluorescence microscopy with antibodies raised against this protein confirms this contention.

A 68,000-dalton polypeptide designated tubulin assembly protein (TAP) has been purified from microtubule protein isolated from brain by cycles of polymerization–depolymerization as a protein that stimulates microtubule assembly *in vitro* (27). Antibodies to this protein were shown by immunofluorescence to decorate cytoplasmic filamentous structures that resemble microtubules. It was therefore concluded that this polypeptide was associated with cytoplasmic microtubules even though the fluorescent images would also be consistent with intermediate filament staining (27). From our results it appears that at least a portion of this polypeptide is associated with intermediate filaments in a number of cell types grown in tissue culture as judged by immunofluorescence and its presence in the microtubule-free, intermediate-filament-rich, cytoskeletons prepared from these cells.

The comparative peptide map analysis of the 68,000-dalton polypeptide from a variety of avian and mammalian cell sources indicates that it is a conserved protein and a major cytoplasmic constituent. We have previously reported that the cell types studied here, namely BHK-21 cells, CEF, and embryonic chicken skeletal myotubes contain and synthesize simultaneously two distinct major intermediate-filament components, desmin and vimentin (4, 6). The presence of the 68,000-dalton polypeptide in these cells indicates that a third major intermediate-filament component can also coexist in the cytoplasm of these cells. This observation raises interesting and intriguing questions about the functional specificity and regulation of assembly of intermediate filaments in higher eukaryotic cells.

We have previously demonstrated that desmin and vimentin associate with the peripheries of myofibril Z discs and that they remain associated with the Z disc after extraction of actomyosin with 0.6 M KI. Furthermore, the two proteins are deposited initially as cytoplasmic filaments early during muscle differentiation and myofibril assembly, and later during myogenesis they transit onto the Z disc (5, 6). From the results presented here, the 68,000-dalton polypeptide is also a component of the myofibril Z disc and remains associated with this structure after short extraction of the actomyosin with 0.6 M KI. The function of this polypeptide during myogenesis and myofibril assembly or as a component of cytoplasmic intermediate filaments remains to be elucidated.

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