β-Internexin, a Ubiquitous Intermediate Filament-associated Protein

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ABSTRACT In this article we show a Triton-insoluble, intermediate filament-associated protein of ~70 kD to be expressed ubiquitously in diverse mammalian cell types. This protein, assigned the name β-internexin, exhibits extreme homology in each of the various cell lines as demonstrated by identical limited peptide maps, similar mobilities on two-dimensional gels, and detection in Triton-soluble and -insoluble extracts. β-Internexin also shares some degree of homology with α-internexin, an intermediate filament-associated protein isolated and purified from rat spinal cord, which accounts for the immunologic cross-reactivity displayed by these polypeptides. Light microscopic immunolocalization of β-internexin with a monoclonal antibody (mAb-IN30) reveals it to be closely associated with the vimentin network in fibroblasts. The antigen is also observed to collapse with the vimentin reticulum during the formation of a juxtanuclear cap induced by colchicine treatment. Ultrastructural localization, using colloidal gold, substantiates the affinity of β-internexin for cytoplasmic filaments and, in addition, demonstrates its apparent exclusion from the intranuclear filament network. We examine also the resemblance of β-internexin to a microtubule-associated polypeptide and the constitutively synthesized mammalian heat shock protein (HSP 68/70).

Cytoskeletal-associated proteins are defined by their ability to co-isolate and interact in situ with one of the filamentous networks indigenous to most eukaryotic cell types. These polypeptides are thought to subserve a number of putative functions with regard to cytoskeletal organization and regulation. They may effect dynamic changes of cytoskeletal architecture in vivo by altering the polymerization state of cytoskeletal subunit components; engendering a redistribution of intracellular filaments; serving as potential cross-bridging agents between subcellular organelles (e.g., between neurofilaments and microtubules); or harboring specific catalytic activities (e.g., phosphokinase). Support for some of these hypothetical roles is derived from studies conducted in a number of laboratories. Numerous investigators have observed that high molecular weight microtubule-associated proteins (MAPs)[1] as well as lower molecular weight tau-proteins promote the in vitro assembly of tubulin subunits into polymeric microtubules (4, 26, 32). MAPs have also been implicated in mediating the interactions between microtubules and neurofilaments (1, 18). Immunoelectron microscopy studies have shown that the peripherally located 200-kD neurofilament-associated subunit appears to serve as an interfilamentous cross-linker in myelinated axons (11, 12, 19).

In this report we describe an intermediate filament (IF)-associated protein designated β-internexin which is present in many diverse eukaryotic cell types. We examine the relationship of this polypeptide to α-internexin, an IF-associated protein originally isolated from rat spinal cord and optic nerve, which was found to bind to several IF subunit proteins (22). The presence of β-internexin in all cell types investigated, and the homology of this protein isolated from the different cells were established by two-dimensional gel electrophoresis, peptide mapping, and antibody localization. Its solubility properties and ultrastructural localization render this protein a likely candidate for the class of IF-associated proteins that may play an integral role in the regulation of the IF-reticulum. In this paper we will also discuss the possible relatedness of β-internexin to a protein that co-isolates with microtubules and to the most commonly elicited, constitutively synthesized mammalian heat shock protein of ~70 kD (HSP 68/70).

[1] *Abbreviations used in this paper:* HSP, heat shock protein; IF, intermediate filament; MAP, microtubule-associated protein; SV8, *Staphylococcus aureus* V8.

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MATERIALS AND METHODS

Cell Culture and Cytoskeletal Extracts: Five culture lines were used to isolate IF-rich fractions and subsequently detect the IF-associated protein dubbed β-internexin: human glioma cells (U251-MG), Chinese hamster ovary fibroblasts (CHO), rat kangaroo kidney epithelial cells (PtK_2), human foreskin fibroblasts (FS-4), and neuroblastoma–glioma hybrids (NG108-15). All cultures were grown on 100-mm sterile Falcon tissue culture dishes (Falcon Labware, Oxnard, CA) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum except the neuroblastoma–glioma hybrid line, which was maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum containing 0.1 mM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine.

Cytoskeletal extracts from monolayer cultures were prepared by Dounce homogenization of freshly harvested cells in an ice-cold (4°C) buffer consisting of 10 mM phosphate (pH 6.8), 0.1 M NaCl, 1 mM EDTA containing 1% Triton X-100, 0.5 mg/ml DNase I (Worthington Biochemical Corp., Freehold, NJ), 10 mM $MgCl_2$, 2 mM phenylmethylsulfonyl fluoride, and 1 μM Aprotinin (Sigma Chemical Co., St. Louis, MO) followed by sedimentation in an Eppendorf centrifuge at 12,000 *g* for 10 min at 4°C. Triton-insoluble pellets were solubilized in SDS sample buffer (16), diluted in lysis buffer (21), and subjected to two-dimensional gel electrophoresis and fluorography as described below.

Labeling of Cell Cultures: Cultures grown in 100-mm Falcon dishes were labeled with [^{35}S]methionine by washing cells three times with phosphate-buffered saline (PBS) and preincubating the cultures with 3 ml methionine-free medium (Select-Amine Kit, Gibco Laboratories Inc., Grand Island, NY) for 1 h at 37°C. 100 μCi/ml of [^{35}S]methionine (>1,000 Ci/mmol, New England Nuclear, Boston, MA) was then added for another hour at 37°C. Cytoskeletal extracts were then prepared as described above.

Isolation of MAPs: MAPs were isolated from rat spinal cord by the method of Vallee (28). The spinal cords were homogenized in ice-cold reassembly buffer of 0.1 M 2-(*N*-morpholino)ethane sulfonic acid, 0.5 mM $MgCl_2$, and 1 mM EGTA, pH 6.8, with a Dounce homogenizer, and centrifuged at 40,000 rpm in a Beckman Ti 75 rotor (Beckman Instruments Inc., Palo Alto, CA) for 30 min in the cold. Microtubule proteins were obtained through one cycle of assembly and disassembly (25, 26). The resulting supernatant containing the disassembled microtubules was then removed, and taxol (from the National Cancer Institute) was added to a final concentration of 20 μM in the presence of 1 mM GTP. The solution was incubated for 30 min at 37°C and centrifuged at 40,000 rpm in a Beckman Ti 75 rotor at room temperature for 30 min. The MAPs were removed from the resulting pellet by washing the pellet with 0.3 M NaCl in the 2-(*N*-morpholino)ethane sulfonic acid buffer in the presence of 20 μM taxol, and then centrifuging it at 40,000 rpm for 30 min.

Two-Dimensional Gel Electrophoresis: Two-dimensional separation of proteins was performed according to the method of O'Farrell (21). The pH gradient of the isoelectric focusing gel was established by 1.6% (vol/vol) pH 4–6 ampholines and 0.4 percent (vol/vol) pH 3.5–10 ampholines (LKB Instruments, Inc., Bromma, Sweden). The proteins were resolved in the second dimension on 7.5% SDS-polyacrylamide gels (16) which were either stained with Coomassie Blue or, in the case of ^{35}S-labeled proteins, subsequently processed for fluorography using Kodak X-omat film (3, 17). Radioactivity incorporated into specific proteins was determined by excision of individual labeled protein spots from the two-dimensional gels and quantitation in a Beckman scintillation counter.

Peptide Mapping: ^{35}S-radiolabeled proteins, extracted from each cell line investigated, were first resolved by two-dimensional electrophoresis. The protein spots whose isoelectric point and molecular weight approximated those of rat central nervous system α-internexin (22) were excised from the gel and loaded into the wells of a second 15% polyacrylamide gel. *Staphylococcus aureus* V8 (Sigma Chemical Co.) protease digestion was then carried out employing the method of Cleveland et al. (5). 0.2 μg protease was added to each well that contained protein, and electrophoresis was performed until the dye front approached the bottom of the stacking gel whereupon the current was turned off for 15 min to allow digestion to proceed. The limited peptide maps produced by the migration of the resultant fragments into the running gel were then visualized by fluorography.

Radioiodination of Proteins in Polyacrylamide Gel Slices: Polypeptides contained in polyacrylamide gel slices were iodinated to high specific activity by a variation of the procedure of Elder et al. (7). Protein spots were excised from Coomassie Blue–stained two-dimensional polyacrylamide slab gels (described above), placed in Eppendorf microcentrifuge tubes, and washed exhaustively with 10% methanol to remove SDS and other contaminants. The gel slices were then dried in a Speed-Vac concentrator (Savant Instruments, Inc., Hicksville, NY) at −70°C under vacuum. Radioiodination of proteins with Na^{125}I (17.4 Ci/mg; New England Nuclear, Boston, MA) was achieved by sequential addition of components used in the chloramine T method (10). After 20 μl phosphate buffer (0.5 M, pH 7.5) was pipetted into each tube, 20 μl Na^{125}I, resuspended in phosphate buffer (0.5 μCi/20 μl), was added. 20 μl chloramine T (1 mg/ml) was applied to each tube, and after the absorption of liquid was allowed to proceed for 30 min, 0.5 ml sodium metabisulfite (2 mg/ml) was added to terminate the reaction. 0.5 ml NaI (2 mg/ml) was placed into each sample tube, and the gel slices were then rinsed with several changes of 10% methanol. Radiolabeled proteins in these slices were used in peptide mapping studies by the method described above.

Antiserum Production: Polyclonal antisera directed against gel-purified α-internexin (22) derived from rat spinal cord were raised in rabbit and guinea pig by use of the following protocol: (*a*) A subcutaneous injection of 25–50 μg protein, emulsified in Freund's complete adjuvant, was injected subcutaneously followed by immediately by two intradermal injections into the hind paws. (*b*) 2 wk later, a second inoculation of protein, emulsified in Freund's incomplete adjuvant, was administered as before. (*c*) Step *b* was repeated for three more 2-wk intervals. (*d*) The animals were bled intermittently during these periods, and the immune sera were tested for specificity by immunoblotting and immunofluorescence assays described below.

Mouse monoclonal antibodies and ascites directed against α-internexin were produced as described by Wang et al. (30) employing the procedure of Kohler and Milstein (15) as modified by Dippold et al. (6). The schedule of immunization of female BALB/c mice was similar to that of the guinea pig. Approximately 50–100 μg of purified antigen, emulsified in complete Freund's adjuvant, was used for the initial immunization. In four subsequent inoculations, purified antigen was mixed with Freund's incomplete adjuvant and injected subcutaneously at 2-wk intervals. The last injection of antigen (without adjuvant) was administered intravenously 3 d before the fusion. Clonal supernatants were then screened for anti-α-internexin activity using the microspot assay described elsewhere (19).

Affinity Chromatography: Purification of the antisera by affinity chromatography was executed essentially as described in the pamphlet Affinity Chromatography, Principles and Methods (Pharmacia, Uppsala, Sweden). In brief, CNBr-activated Sepharose 4B (Sigma Chemical Co.) was swollen for 15 min in 1 mM HCl and then washed with coupling buffer (0.1 M $NaHCO_3$, pH 8.3, 0.5 M NaCl). The gel suspension was immediately transferred (for covalent coupling) to a solution of purified antigen (~1 mg/ml), dissolved in coupling buffer containing 0.1% SDS, and mixed in an end-over-end rotator overnight at 4°C. Active groups remaining on the gel were then blocked by incubation with 0.2 M glycine, pH 8.0, overnight at 4°C. Nonspecifically adsorbed protein was eluted from the gel suspension by 0.1 M acetate buffer, pH 4, containing 0.5 M NaCl and then rinsed extensively with coupling buffer. Antiserum, diluted in coupling buffer or PBS, was loaded onto the column with continued reflux (onto the suspension) for 16 h at 4°C, and the column was washed repeatedly with PBS. Specific immunoglobulins bound to the immobilized antigen on the gel were eluted with 0.1 M glycine adjusted to pH 2.5 with 0.2 M HCl, and fractions containing protein were immediately neutralized to pH 8.5 with solid Tris base.

Immunoblotting: Immunoblotting was performed by a modification of the method of Towbin et al. (27). Freshly prepared cytoskeletal proteins were separated on a 7.5% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose sheets (Schleicher & Schuell, Inc., Keene, NH). These nitrocellulose sheets were then incubated with primary antiserum and subjected to the avidin–biotin–peroxidase complex (Vectastain, Vector Laboratories, Inc., Burlingame, CA) method of Hsu et al. for visualization of bound antibody (13). Parallel nitrocellulose transblots, which were not used for immunostaining, were stained with amido black immediately after the electrophoretic transfer.

Fluorescence Microscopy: PtK_2, U251-MG, CHO, and FS-4 cells were grown on 12-mm glass coverslips and, where designated, pretreated with colchicine (5 μM) for 2 h at 37°C. Immunofluorescence was carried out according to the following protocol. Cells were washed three times for 10 min each in PBS at room temperature then fixed in ice-cold methanol for 20 min at −20°C. Cultures were incubated in primary antiserum at a 1:500 dilution in PBS for 1 h at room temperature then washed five times for 5 min each in PBS. Depending on the origin of the primary antiserum, cells were incubated with either fluorescein-conjugated goat anti-mouse IgG, fluorescein-conjugated goat anti-rabbit IgG, or fluorescein-conjugated goat anti-guinea pig (all from Miles Laboratories Inc., Elkhart, IN), at a dilution of 1:100 in PBS for 30 min at room temperature. Cells were again washed five times for 5 min each. Coverslips were then mounted onto a glass slide with a solution of 16% wt/vol polyvinyl alcohol, 33% vol/vol glycerol, and 0.02% sodium azide in PBS and viewed with Zeiss epifluorescence optics.

Cryostat sections of rat brain were also tested for immunofluorescent staining. Fresh brains were dissected from adult rats, cut into 2–3-mm-thick slices and immediately frozen in isopentane, which was first cooled in liquid nitrogen. The brain slices were transferred to dry ice and then sectioned in a cryostat at

−20°C. 8-μm tissue slices were placed on gelatin-coated slides and incubated with primary antiserum, diluted in PBS, overnight at 4°C. The sections were washed three times for 7 min each with PBS and reacted with FITC-conjugated secondary antibody, diluted in PBS, for 1 h at room temperature. Samples were rinsed in PBS as above, coverslipped with the polyvinyl alcohol solution, and viewed as above.

Immunoelectron Microscopy: Colloidal gold particles of ~5 nm diam were prepared following the method of Faulk and Taylor (8). Subsequent complexation of the gold particles to protein A (Sigma Chemical Co.) was performed according to the procedure outlined by Geuze et al (9).

Immunoelectron localization of the antigen in cultured cells was carried out by the following protocol. Subconfluent human foreskin fibroblasts (FS-4) were grown on 35-mm sterile Falcon dishes as described above. The cultures were washed extensively with PBS and then fixed with freshly prepared 4% paraformaldehyde for 30 min at room temperature. The fibroblasts were rinsed with PBS and extracted with 0.5% Triton X-100 in PBS for 10 min at room temperature then washed three times with PBS as above. Samples were then preincubated with 1% bovine serum albumin (Sigma Chemical Co.) in PBS (PBS-BSA) before immune sera were added. The primary antibody, diluted to 1:50 for AbVim (30) or 1:200 for mAb-IN30 ascites in PBS-BSA, was incubated with the cultures overnight at 4°C. Cultures were exhaustively washed in PBS-BSA, and secondary antibody (rabbit anti-mouse IgG, Antibodies Inc., Davis, CA), diluted 1:50 with PBS-BSA, was added for 1 h at room temperature. After they were rinsed repeatedly with PBS-BSA, cells were incubated with the protein A–colloidal gold complex at room temperature for 1–2 h with shaking, washed with PBS-BSA, and finally, rinsed with PBS alone.

The samples were processed for electron microscopy, first by fixation in 1% glutaraldehyde in PBS for 1 h at room temperature and then by thorough washing with PBS. Cells were postfixed with 1% osmium tetroxide in PBS, rinsed with PBS, dehydrated through graded ethanols, and embedded in Epon 812. Sections were cut on a diamond knife, stained with saturated aqueous uranyl acetate and Reynold's lead citrate, and viewed on a JEOL 100S electron microscope at 80 kV.

RESULTS

Two-Dimensional Gel Analysis

The ubiquity of internexin was demonstrated by its invariant appearance in all five cell lines examined. When [^{35}S]-methionine-labeled, Triton X-100-insoluble cytoskeletal and soluble protein fractions were subjected to two-dimensional electrophoresis followed by fluorography (Fig. 1, *a–e*), a polypeptide of ~66–70 kD with an isoelectric point of 5.8–5.9 was consistently observed. These biochemical properties, concomitant with the co-isolation of this protein with the IF-rich "cytoskeleton," suggested that it may be related to central nervous system α-internexin, an IF-associated protein recently described by our laboratory (22). To distinguish the protein in the cell extract from the central nervous system protein, we will refer to the ubiquitous cell protein as β-internexin. There also appears to be a Triton-soluble form of this protein in all cell types studied, the significance of which we elaborate upon in the Discussion.

Although β-internexin is a constituent of all cells investigated, it appears to be produced in varying amounts in the different culture lines tested. Since cell cultures were prelabeled with [^{35}S]methionine, the fluorograms depicted in Fig. 1 represent only the profiles of newly synthesized proteins and therefore do not reflect the absolute amount of endogenous protein. However, the amount of newly translated β-internexin present as compared with that of two major identifiable cytoskeletal elements, namely, actin and vimentin, was determined by excision of the individual gel spots corresponding to β-internexin, actin, and vimentin in each of the cell lines and measurement of radioactivity contained in each spot by scintillation counting. Ratios of newly synthesized actin to β-internexin and vimentin to β-internexin and actin to vimentin were then calculated for each cell line, and the results were compiled in Table I. CHO and neuroblastoma–glioma hybrid cells exhibited the lowest ratios of actin or vimentin to β-internexin, and the human glioma line (U251-MG) and FS-4 fibroblasts displayed the highest.

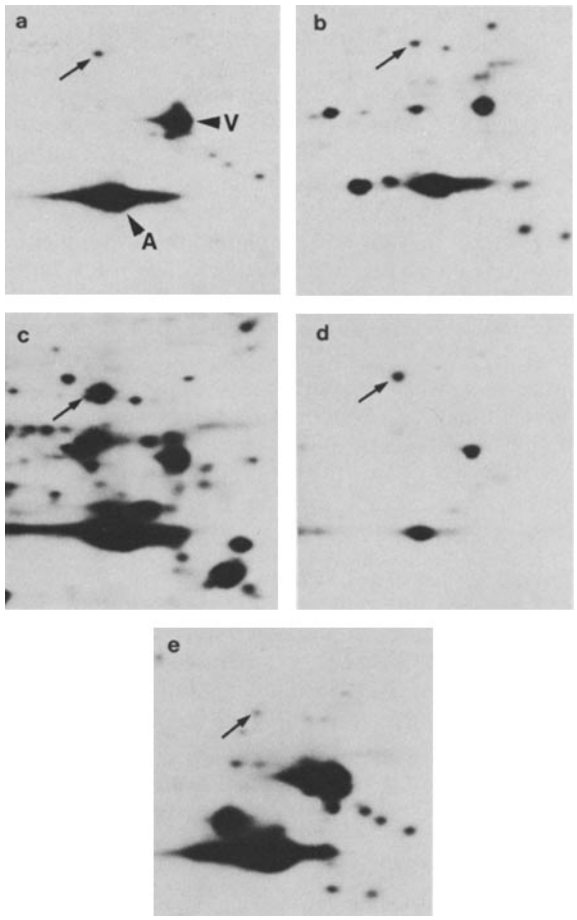

FIGURE 1 Two-dimensional gel electropherograms of [^{35}S]methionine-labeled proteins from IF-enriched "cytoskeletal" extracts (see Materials and Methods). (*a*) Human foreskin fibroblasts (FS-4). (*b*) Rat kangaroo kidney epithelial cells (PtK_2). (c) Neuroblastoma-glioma hybrids (NG108-15). (*d*) Chinese hamster ovary fibroblasts (CHO). (e) Human glioma cells (U251-MG). Isoelectric focusing is in the horizontal dimension, and SDS PAGE is in the vertical dimension with the basic pole to the left and the acidic pole to the right. Arrow indicates β-internexin with a pI of 5.8–5.9 in each cell type. *A*, actin. *V*, vimentin.

TABLE I. *Ratios of Cytoskeletal Proteins*

Cell type	Actin/β-internexin	Vimentin/β-internexin	Actin/vimentin
FS-4	23.9	12.2	1.95
PtK_2	26.5	8.4	3.18
NG108-15	9.3	1.5	6.23
CHO	5.5	2.2	2.50
U251-MG	68.6	65.7	1.04

Ratios of newly synthesized cytoskeletal proteins were calculated by excision of ^{35}S-labeled protein spots from two-dimensional isoelectric focusing–SDS polyacrylamide gels and quantitation of radioactivity in a scintillation counter.

To compare the ubiquitous β-internexin with the IF-associated protein recently described in our laboratory from central nervous system tissue as well as MAPs of similar molecular weight and isoelectric point, we performed a number of two-dimensional gel analyses of mixtures of these proteins. The results are shown in Fig. 2. Fig. 2*a* shows the pattern

obtained from the optic nerve IFs, which consist of NF68, vimentin (≃58 kD), glial filament protein (≃50 kD), and actin (≃45 kD), as well as a spot, which we have identified as α-internexin (designated *1* in Fig. 2). When MAPs are added to this mixture, a prominent spot of ~70 kD and a slightly more basic isoelectric point is observed (Fig. 2 *b*, *2*). Careful examination of Fig. 2 *a* also reveals a faint spot in this area. The two proteins, although similar in molecular weight and isoelectric point, clearly do not co-migrate on two-dimensional gels. On the other hand, when a preparation of the MAPs is mixed with a ^{35}S-labeled CHO cell extract and the same gel is first stained with Coomassie Blue and then processed for autoradiography, the results in Fig. 2, *c* and *d* are obtained. The spot obtained from the MAP preparation co-migrates exactly with the CHO β-internexin spot (designated *2* in Fig. 2, *c* and *d*). α-internexin appears to be absent from the CHO cell extract and the MAP preparation.

Peptide Mapping

Homology between the distinct β-internexins derived from each of the disparate cell types was established by peptide mapping techniques. Radiolabeled spots indicated by the arrows in Fig. 1, *a–e* were excised from duplicate two-dimensional gels and subjected to limited SV8 protease digestion according to the method of Cleveland et al. (5). The pattern of proteolytic fragments subsequently generated was identical for each of the tissue-specific β-internexin homologues, revealing a high degree of conservation of this polypeptide across various mammalian species (Fig. 3 *a*).

Since rat central nervous system α-internexin could not be labeled with [^{35}S]methionine to high enough specific activity for detection in the analysis described above, we employed a different experimental approach to circumvent this problem. Extracts from unlabeled rat optic nerve, which contain large quantities of α-internexin (22), IF-enriched cytoskeletal fractions from CHO fibroblasts, and MAPs were resolved on two-dimensional gels, and the Coomassie Blue–stained spots corresponding to α- or β-internexin were excised. The IF-associated proteins contained in these gel slices were then radioiodinated to high specific activity with chloramine T in a modified version of the procedure described by Elder et al. (7) and partially digested with SV8 protease as before. The peptide maps of CHO-derived β-internexin and rat brain MAP were indistinguishable from one another (Fig. 3 *b*, lanes *2* and *3*), whereas the proteolytic cleavage pattern of rat optic nerve α-internexin revealed a more limited homology than did the other two (Fig. 3 *b*, lane *1*). Thus, β-internexin from cultured cells and rat brain MAP probably are indeed highly homologous if not identical, whereas rat optic nerve α-internexin and CHO β-internexin appear to be distinct polypeptides whose limited number of common cleavage fragments (arrowheads in Fig. 3 *b*) could account for the immunologic cross-reactivity described below.

Immunoblotting and Indirect Immunofluorescence

Both polyclonal (guinea pig and rabbit) and monoclonal antisera raised against purified antigen (α-internexin) from rat spinal cord (22) were used for immunological characterization of α- and β-internexin. Specificity of the various antisera was demonstrated by immunoblotting shown in Fig. 4. The monoclonal antibody mAb-IN30 exhibits binding to α-internexin (Fig. 4, lane *c*, band at 66 kD) in the optic nerve extract, and to a second polypeptide of ~60 kD, which is believed to represent a degradation product of α-internexin. This monoclonal antibody also recognizes a protein in the 66–70-kD range in CHO extracts, which we have identified as β-internexin (Fig. 4, lane *d*).

A guinea pig–derived polyclonal antibody, AbIN-GP2, recognized a major band at 66–70 kD when blotted against a crude IF preparation from rat optic nerve (Fig. 4, lane *e*). After affinity purification against α-internexin, AbIN-GP2 reacts strongly against only one polypeptide in the CHO cell extract in this molecular weight range (Fig. 4 *f*). The rabbit polyclonal antibody, AbIN-R1, when tested in this assay, produced intense staining of β-internexin (Fig. 4, lane *g*);

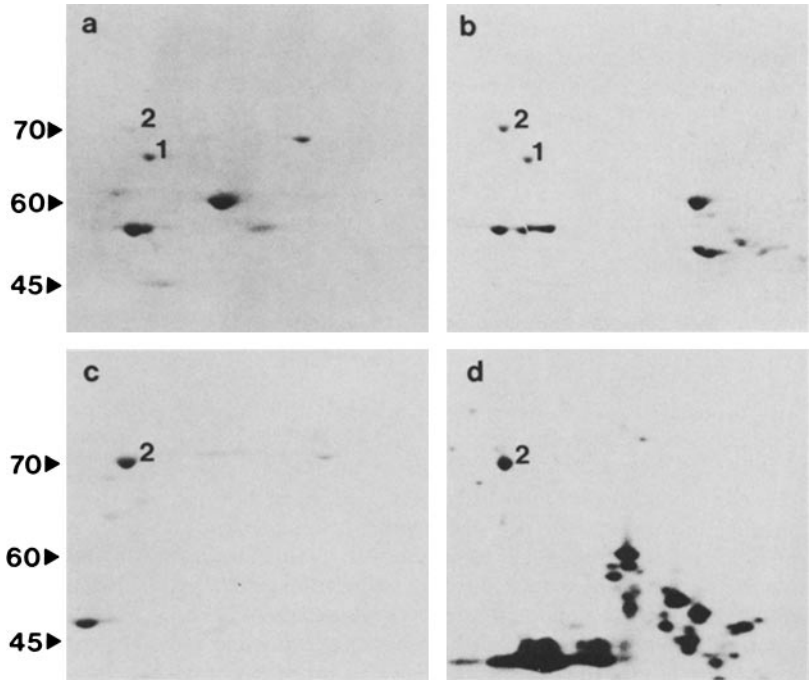

FIGURE 2 Two-dimensional gel electropherograms show the differences in mobility of α- and β-internexin. (a) Optic nerve cytoskeletal extract. (*b*) A mixture of optic nerve cytoskeletal extract and a MAP preparation. (c) A MAP preparation. (*d*) An autoradiogram of a CHO cytoskeletal extract. There are clearly two proteins present with different mobilities, designated *1* and *2*. In the optic nerve preparations, α-internexin (*1*) is the primary protein, whereas in the MAP preparation and CHO cell extracts, β-internexin (*2*) is the primary protein. The extracts in *c* and *d* were run together to show exact co-migration of spot *2* in the radioactively labeled CHO preparation (*d*) and Coomassie Blue–stained microtubule-associated protein preparation (*c*). Molecular weight markers are based on the optic nerve preparation. The gels are shown with the basic end on the left and the acidic end on the right.

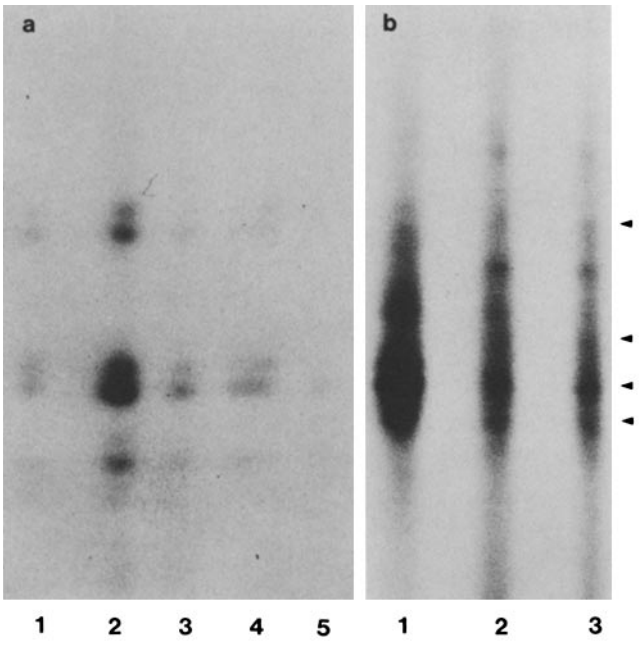

FIGURE 3 (*a*) SV8 limited protease digest of ^{35}S-labeled β-internexin (arrow in Fig. 1) excised from duplicate gels depicted in Fig. 1. Lane *1*, PtK_2. Lane *2*, CHO. Lane *3*, NG108-15. Lane *4*, FS-4. Lane *5*, U251-MG. (*b*) SV8 limited protease digest of ^{125}I-labeled proteins. Lane *1*, rat optic nerve α-internexin. Lane *2*, CHO β-internexin. Lane *3*, rat brain–MAP that co-isolates with microtubules through two cycles of assembly-disassembly. Arrows indicite fragments common to all three polypeptides.

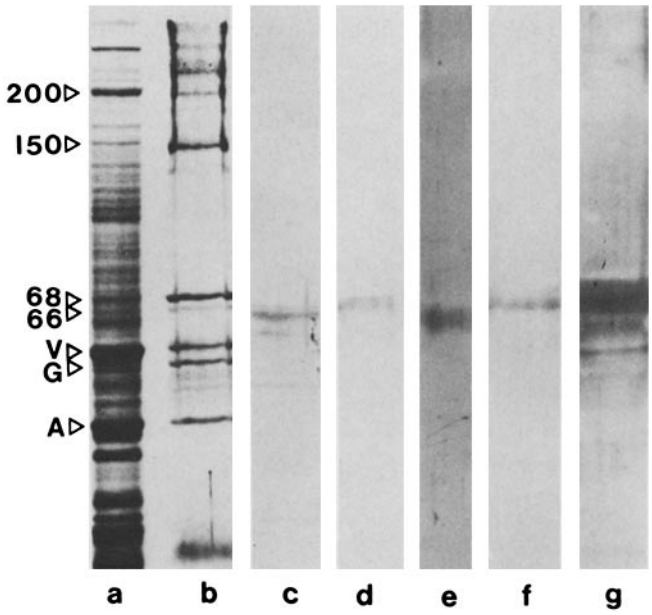

FIGURE 4 Immunoblot analysis of various polyclonal and monoclonal anti-α-internexin antisera. Lane *a*, Coomassie Blue–stained CHO "cytoskeletal" extract. Lane *b*, Coomassie Blue–stained optic nerve cytoskeleton. Lane *c*, mAb-IN30 (monoclonal Ab) reacted against proteins in lane *b*. Lane *d*, mAb-IN30 reacted against proteins in lane *a*. Lane *e*, AbIN-GP2 (polyclonal guinea pig Ab) after reaction with proteins in lane *b*. Lane *f*, affinity-purified AbIN-PG2 reacted against proteins in lane *a*. *Lane g*, AbIN-R1 (rabbit polyclonal Ab) blotted against proteins in lane *b*. Molecular weight markers are based on the optic nerve preparation. *V*, vimentin; G, glial filament protein; *A*, actin.

however, weak cross-reactivity of this immune serum with a number of other polypeptides (possibly) including vimentin, even after affinity purification, precluded the use of this antibody in localization studies. These studies indicate that all of the antibodies we obtained against α-internexin also cross-react with β-internexin, indicating that the two proteins are related. These cross-reactivities confirm the peptide mapping data.

Indirect immunofluorescent localization of the antigen in FS-4 cells with mAb-IN30 yielded an IF-like fibrillar pattern (Fig. 5*c*) virtually indistinguishable from that of vimentin (Fig. 5*a*). Pretreatment of the cultured cells with colchicine induced the formation of a juxtanuclear "cap" engendered by the collapse of the IF-network (Fig. 5, *b* and *d*).

Incubation of mAb-IN30 with sections of rat optic nerve produced strong staining of both neuronal and glial processes (Fig. 6*a*), whereas fluorescence micrographs of other central nervous system regions revealed a diffuse, almost granular, cytoplasmic staining of neuronal perikarya and some occasional glial cells (Fig. 6, *b–d*). Cross-reactivity of the antibody with α- and β-internexin may be responsible for the punctate, diffuse staining pattern observed in some brain areas, since mAb-IN30 may be recognizing an epitope on the cytosolic (soluble) form of β-internexin (probably MAP), thus eliciting a cytoplasmic fluorescence.

Immunoelectron Microscopy

Monoclonal antibody mAb-IN30 was employed for the ultrastructural localization of β-internexin in FS-4 cells by immunogold microscopy (Fig. 7). At the electron microscopic level, the antibody unequivocally decorated intermediate filaments; however, the distribution of gold particles along the filament does not display any apparent periodicity. Since the integrity of microtubules was not preserved under these conditions, it is not possible at this time to ascribe a microtubule-IF cross-linking function to this molecule, although the data are consonant with the classification of this protein as IF associated. The data also do not exclude the possibility that the antigen is present in some other area of the cell, which was sensitive to the detergent extraction.

DISCUSSION

We recently described an IF-binding protein, isolated and purified from rat spinal cord, which was assigned the name α-internexin (22). In the present report, we demonstrate by a number of criteria that homologues of β-internexin, a related but not identical protein, exist in many if not all cell types. Two-dimensional isoelectric focusing–SDS PAGE analysis of five independent cell lines revealed that a protein whose molecular weight and isoelectric point approximate those of rat central nervous system α-internexin, is always detected in the Triton-insoluble, IF-enriched fraction of all cell lines studied. In addition, peptide mapping experiments and immunological cross-reactivity provide confirmatory evidence that these polypeptides are indeed homologous in all mammalian cell types analyzed, which indicates that β-internexin is a highly conserved protein of potential evolutionary significance (discussed below).

We have examined the relationship between α- and β-internexin in greater detail by comparing their mobilities on two-dimensional gels, peptide maps, and immunologic cross-

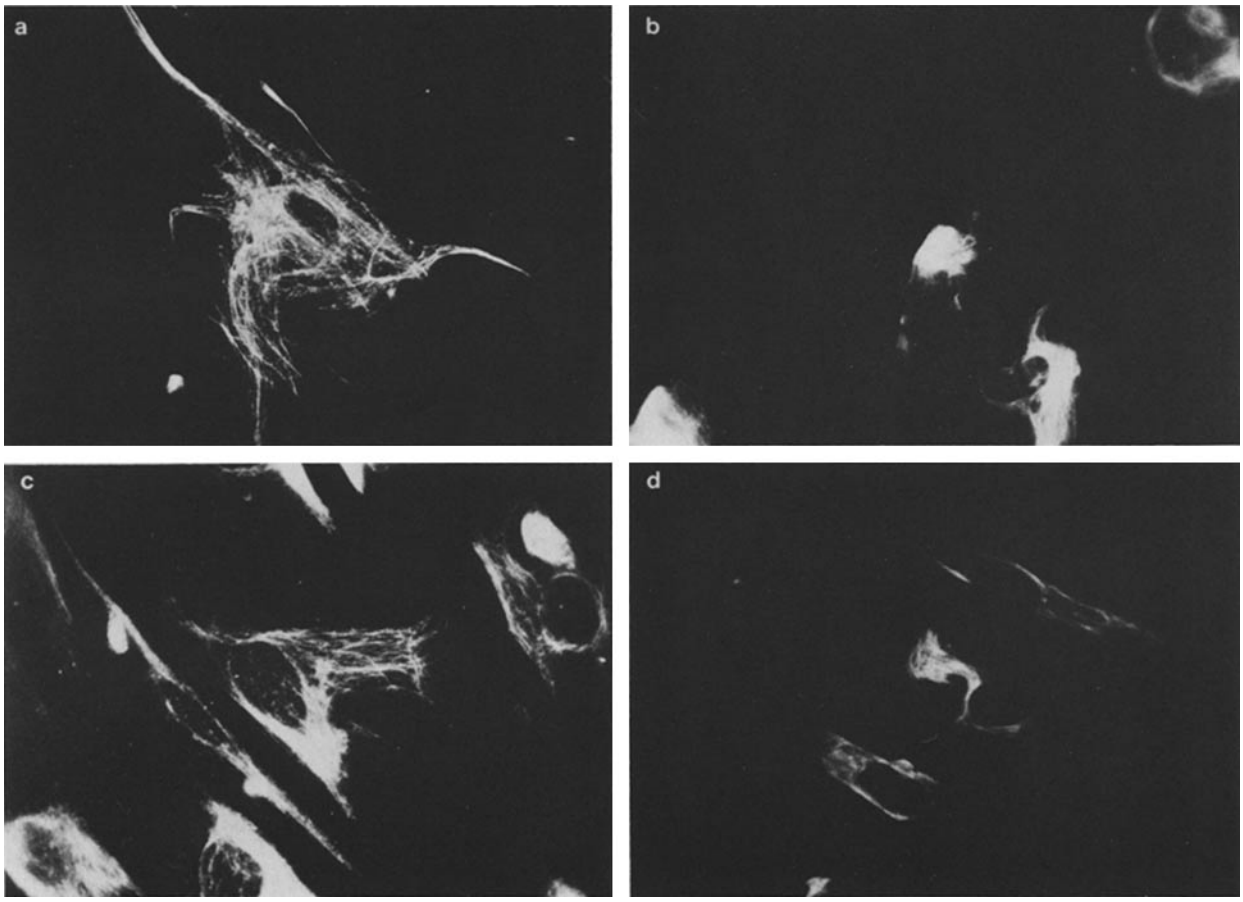

FIGURE 5 Indirect immunofluorescence of FS-4 cells with anti-vimentin antibodies and mAb-IN30. (*a*) Anti-vimentin. (*b*) Anti-vimentin after pretreatment with colchicine (5 μM) for 2 h. (*c*) mAb-IN30. (*d*) mAb-IN30 after pretreatment with colchicine as in *b*. Note the co-distribution of the antigen (β-internexin) with the vimentin IF-network before and after colchicine treatment. × 1,500.

reactivity. The two-dimensional gel analysis indicates that although the mobilities of the two proteins are similar, they are not identical. β-Internexin is slightly more basic and has a slightly higher molecular weight than does α-internexin. Peptide maps reveal differences between the two proteins as well, but immunological cross-reactivity was demonstrated using a variety of antibodies.

On the other hand, β-internexin turns out to be identical to a protein found in preparations of MAPs from spinal cord. Although the molecular weight of the MAP in the 50–70-kD range could classify it as a tau-protein, tau-proteins are considerably more basic and more highly phosphorylated than β-internexin. In addition, monoclonal and polyclonal antibodies to tau-proteins fail to recognize β-internexin on immunoblots (unpublished results). Thus, although β-internexin can be found in MAP preparations, it is not yet possible to conclude that it is a MAP protein, since it is not clear how well the protein can cycle with tubulin or if it is competent to initiate microtubule polymerization. These studies will require purification of this protein in much larger quantities under nondenaturing conditions.

The relative amounts of cellular β-internexin with respect to two major cytoskeletal subunit polypeptides, actin and vimentin, varies from one cell line to another. CHO fibroblasts and neuroblastoma–glioma hybrids contain the highest relative quantities of β-internexin, whereas human glioma cells and human fibroblasts exhibit the lowest. The possible significance of this finding is discussed below.

The fluorescent staining pattern obtained in FS-4 and PtK_2 cells after reaction with mAb-IN30, a monoclonal antibody originally raised against α-internexin, but which reacts with β-internexin, was like that of vimentin, although somewhat more punctate at higher dilutions, which suggests that this protein may bedeck IFs in vivo. Even though vimentin and keratin (IFs) are both expressed in PtK_2 cells, the antibody always co-localized with the vimentin network (unpublished results). More specifically, this co-localization became evident when pretreatment with colchicine, which characteristically effects the collapse of the IF-network, produced intense staining of the juxtanuclear cap. Since the antibody reacts only with β-internexin in a cell extract, it is reasonable to assume that the antigen recognized by the immunolocalization studies is β-internexin.

The staining pattern derived from cryostat sections of rat optic nerve was characterized by strong fluorescence of neuronal and glial processes, whereas only neuronal somata and a paucity of glial cells were recognized by the antibody in other central nervous system areas. Diffuse, cytoplasmic staining observed in some central nervous system areas may be attributed to the recognition by the antibody of an epitope on the soluble form of β-internexin.

Immunogold staining of β-internexin in FS-4 cells unambiguously localizes this antigen to 10-nm IFs composed of vimentin. Since the antibody decorates vimentin in a random,

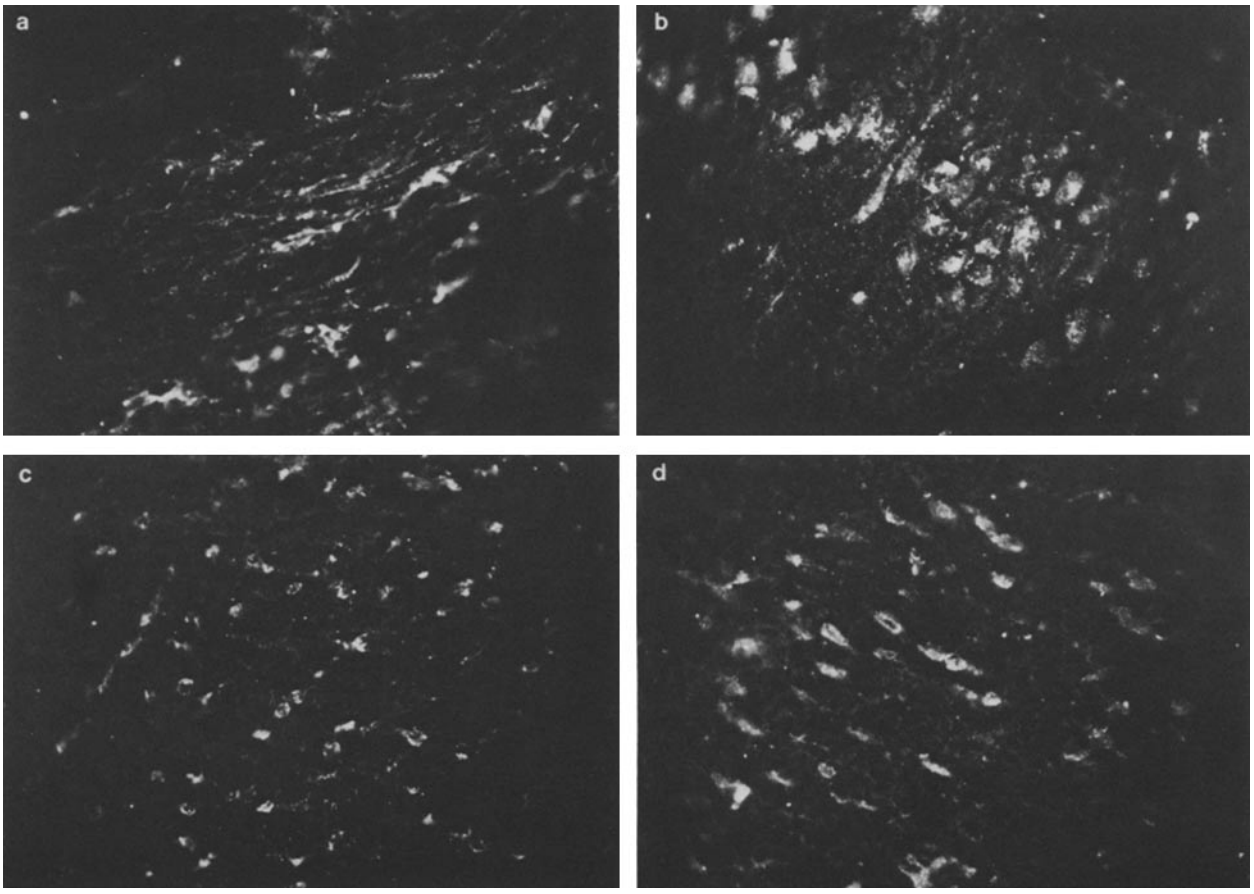

FIGURE 6 Indirect immunofluorescence of cryostat sections from various rat brain regions with mAb-IN30. (*a*) Optic nerve. (*b*) Forebrain. (*c*) Midbrain. (*d*) Hindbrain. The more punctate pattern produced in certain areas may represent recognition by the antibody of the soluble form of β-internexin. × 750.

nonperiodic manner, it generates a pattern indistinguishable from that produced by an anti-vimentin antiserum. In light of the high in vitro binding activity of α-internexin to disparate IF-subunit polypeptides capable of self-assembly into filaments, i.e., vimentin, glial filament, and NF68 (22), this is not a surprising result for a related protein. Similar binding studies with β-internexin have not been done, since these studies require purification of the protein to homogeneity.

In addition to a cytoskeletal-associated pool of β-internexin, which partitions into the Triton-insoluble subcellular compartment, there appears to be a cytosolic pool, since a polypeptide with identical biochemical properties in two-dimensional isoelectric focusing–SDS PAGE is also recovered in the Triton-soluble cell fraction (manuscript in preparation). This situation closely resembles that of actin and tubulin, the subunit proteins of microfilaments and microtubules, respectively, which also exist subcellularly in two distinct pools, i.e., unpolymerized, soluble polypeptide subunits or oligomers and assembled, polymeric filaments.

The dynamic equilibrium established between the soluble component and the cytoskeletal-associated pool in vivo may prove to be critically significant in the physiological regulation of cytoskeletal organization. Localized shifts in equilibrium of this system may favor either the binding of cytosolic β-internexin to IFs or, conversely, its dissociation from the filament, thereby influencing the disposition of the various cytoskeletal elements and, subsequently, their interactions with other subcellular systems. In this regard, the varying quantities of cytoskeletal-associated β-internexin found in different cell types may reflect an equilibrium condition peculiar to that particular cell lineage which, in turn, may correspond to a functional state of the cell population (e.g., degree of cell density, contact growth inhibition, change in cellular morphology). Ben-Ze'ev (2) has recently reported marked alterations in the expression of cytoskeletal proteins during various stages of cell growth and morphological differentiation. In concordance with his findings, we have also observed variations in the profile of newly synthesized cytoskeletal and heat shock proteins (HSPs) when subconfluent and confluent cultures were compared (manuscript in preparation).

Another interesting property of β-internexin is its striking resemblance to one of a class of commonly elicited mammalian stress or heat shock proteins which are believed to confer protection to the cell upon environmental insult (23, 24, 29, 33). There is accumulating evidence that suggests a correspondence between β-internexin and the constitutively synthesized mammalian HSP of 68–72 kD. In this regard, we noted that a 68-kD cytoskeleton-associated protein isolated from HeLa cells (31) had the same isoelectric point as an HSP (70–72 kD), inducible in NIH/3T3 cells, and a similar tryptic peptide map (14). Welch et al. (34) have also recently proposed that the constitutively produced HSP 68/70 (designated in their report as HSP73) may belong to the family of MAPs, a result consistent with our findings. Experiments conducted in our laboratory demonstrate the association of mammalian

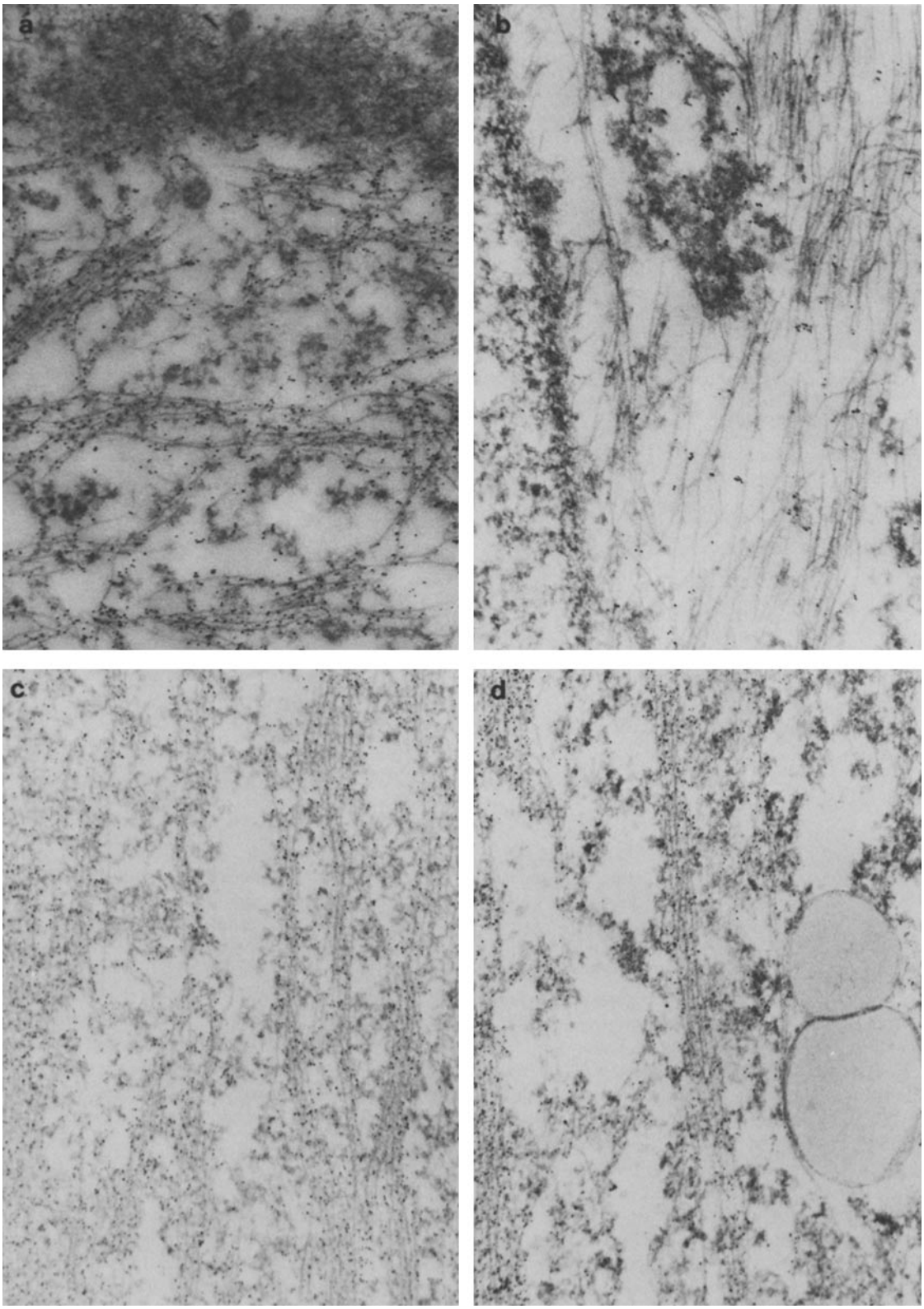

FIGURE 7 Ultrastructural localization of β-internexin (with mAb-IN30) in FS-4 cells using immunogold microscopy. (a) Anti-vimentin. (*b*) Preimmune mouse serum. (*c* and *d*) mAb-IN30. mAb-IN30. mAb-IN30 decorates 10-nm filaments in a nonperiodic fashion virtually identical to that of vimentin. × 50,000.

HSPs in this molecular weight range and of similar biochemical properties with the Triton-insoluble cytoskeleton, as well as detection of their Triton-soluble counterparts (manuscript in preparation). Studies are in progress to ascertain the relatedness between these polypeptides and the physiological roles they subserve in cytoskeletal regulation.

In summary, β-internexin is an IF-associated protein that is conserved across diverse mammalian species. It co-isolates

with Triton-insoluble cytoskeletons and is also found in a Triton-soluble cellular form. Preliminary data relate this protein to MAPs, as well as to the most commonly elicited constitutively synthesized mammalian HSPs of 68–72 kD. Incontrovertible evidence linking these observations awaits further investigation.

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