

α -Internexin, a 66-kD Intermediate Filament-binding Protein from Mammalian Central Nervous Tissues

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ABSTRACT In this paper we describe a 66-kD protein that co-purifies with intermediate filaments from rat optic nerve and spinal cord but can be separated further by ion-exchange chromatography. This protein is distinct from the 68-kD neurofilament subunit protein as judged by isoelectric focusing, immunoblotting, peptide mapping, and tests of polymerization competence. This protein is avidly recognized by the monoclonal anti-intermediate filament antigen antibody, previously demonstrated to recognize a common antigenic determinant in all five known classes of intermediate filaments. Also, when isolated this protein binds to various intermediate filament subunit proteins, which suggests an *in vivo* interaction with the intermediate filament cytoskeleton, and it appears to be axonally transported in the rat optic nerve. Because of this ability to bind to intermediate filaments *in situ* and *in vitro* we have named this protein α -internexin. A possible functional role for the protein in organizing filament assembly and distribution is discussed.

It is generally acknowledged today that the cytoskeleton of most eukaryotic cells is comprised of three filamentous systems. Two of these, microtubules and microfilaments, are composed of subunit proteins that are phylogenetically highly conserved. In contrast, the third constituent, the intermediate filament, is composed of at least five classes of polypeptides which vary in their biochemical constitution and immunologic specificity and are uniquely displayed in distinct tissue types. These five classes of intermediate filament are vimentin filaments, found primarily in cells of mesenchymal origin; desmin filaments, prevalent in all three types of muscle cells—skeletal, smooth, and cardiac; keratin filaments in cells of epithelial origin; glial filaments, expressed in fibrous astroglia, and Bergmann glia and neurofilaments, present in many, though not all, differentiated neurons of vertebrates and invertebrates (16).

Despite their biochemical heterogeneity, the five distinct intermediate filament types demonstrate similar ultrastructures (16) and contain a region with a similar amino acid sequence, which defines a common α -helical domain (7). All intermediate filament types are also highly insoluble in isotonic buffers in the physiological pH range, which has led to the supposition that they are present in the cytoplasm only in the polymeric state (16). In contrast to tubulin, intermediate filament proteins have been shown to associate with the cytoskeletal framework as nascent polypeptides (5). Organizing centers, similar to microtubule organizing centers, which

could assemble the elements of a cytoplasmic array of intermediate filament subunit proteins into polymers, have not been demonstrated in association with any type of intermediate filament network. However, the recent demonstrations that microinjection of antibodies to intermediate filament subunit proteins can effect the collapse of intermediate filament networks (4, 12) suggest that the physiology of the intermediate filaments might be more dynamic in nature than previously thought. Further evidence for the nonstatic nature of the intermediate filament network comes from the observation that treatment with colchicine and cytochalasin D causes conversion from a branching, even distribution of filaments to a series of starlike structures connected to the membrane at multiple attachment sites (13).

Potential candidates for effecting changes in the polymerization and/or distribution of intermediate filaments are the polypeptides found to associate with them *in situ* and through isolation (2, 10, 15, 22, 27, 33). Pruss et al. (26) have described a monoclonal antibody that recognizes all classes of intermediate filaments. This antibody also recognizes a 66-kD protein in a central nervous system intermediate filament fraction and proteins of similar molecular weight in many different cell types. Recently Geisler et al. (8) have shown that the epitope recognized by this monoclonal antibody is in the common α -helical domain of all intermediate filament proteins, which is presumably present also in this 66-kD protein. We have found a protein of similar molecular weight in

preparations of intermediate filaments from both bovine and rat spinal cord, optic nerve, and brain. This paper is concerned with the isolation and partial characterization of this apparent intermediate filament-binding protein from central nervous system tissue.

MATERIALS AND METHODS

Intermediate Filament Isolation: Rat spinal cord and optic nerve were used as starting material, and a fraction enriched in intermediate filaments was obtained by the axonal flotation technique as described by Liem et al. (20). Tissues were homogenized in solution A (10 mM phosphate buffer, pH 6.8, 1 mM EDTA, and 0.1 M NaCl), containing 0.85 M sucrose, and myelinated axons were floated by centrifugation in a Beckman SW 27 rotor (Beckman Instruments Inc., Palo Alto, CA) for 15 min at 10,000 rpm at 4°C. Myelinated axons were then extracted with 1.0% Triton X-100, diluted with an equal volume of 0.85 M sucrose in solution A, and centrifuged as before to pellet the insoluble filaments. The filaments were resuspended in 1.0% Triton X-100 in solution A and spun through a layer of 0.85 M sucrose in solution A by centrifugation in a Beckman SW 41 rotor for 1 h at 40,000 rpm at 4°C. This step was repeated to ensure the removal of any contaminating myelin. The final pellet, which was enriched in neurofilaments, glial filaments, vimentin, and the 66-kD protein in question was dissolved in a solubilization buffer (10 mM phosphate buffer, pH 7.4, containing 1% mercaptoethanol in 8 M urea). Urea was deionized as described previously (18) to remove contaminating cyanates. The crude filament suspension was cleared of insoluble debris by centrifugation at 20,000 rpm in a Sorvall SS-34 rotor (DuPont Instruments-Sorvall Biomedical Div., Wilmington, DE) for 30 min.

Hydroxylapatite (HTP)¹ Chromatography: The clarified supernatant, containing 25–50 mg of protein, was applied to a hydroxylapatite (Bio-Rad HTP, Bio-Rad Laboratories, Richmond, CA) column, 1.5 × 10 cm, which had been previously equilibrated with a 10 mM phosphate buffer, pH 7.4, made up in freshly deionized 8 M urea. The column was washed with this buffer, at a flow rate of 25 ml/h, until no further protein was eluted. The column was then eluted with 0.1 M phosphate buffer, pH 7.0, in 8 M urea, followed by 0.3 M phosphate buffer, pH 7.0, in 8 M urea. The column fractions were assayed by absorbance at 280 nm, and the fractions containing the protein peaks were analyzed by SDS PAGE. The 0.3 M phosphate eluate, which contained the neurofilament triplet proteins (17), was concentrated by vacuum dialysis, dialyzed against assembly buffer as described below, and centrifuged in a Beckman Ti 70 rotor at 40,000 rpm for 1 h at 25°C to pellet the filaments. Neurofilaments were redissolved in solubilization buffer, as before, and separated into the individual triplet polypeptides (NF68, NF150, and NF200) as described by Liem and Hutchison (18). The fractions from 0.1 M phosphate elution, containing the peak amounts of the 66-kD protein, were also concentrated by vacuum dialysis, dialyzed against reassembly buffer, centrifuged as the glial and neurofilament fractions, and redissolved in solubilization buffer, pH 7.0. The solubilized 66-kD protein, plus minor contaminants, was then further separated by DEAE chromatography.

DEAE Chromatography: The partially purified 66-kD protein was placed on a DEAE-cellulose column (Whatman DE52, Whatman Chemical Separation Inc., Clifton, NJ), 0.75 × 5 cm, previously equilibrated with a 10 mM phosphate buffer, pH 7.0, in 8 M urea containing 0.1% mercaptoethanol. Protein was then eluted with a linear gradient of 10 mM–0.1 M phosphate, pH 7.0, in 8 M urea containing 0.1% mercaptoethanol. Fractions were analyzed as for HTP chromatography, and those containing the purified protein were combined and concentrated by vacuum dialysis.

Assembly Studies: Concentrated proteins (~1 mg/ml) were dialyzed against an assembly buffer which contained 10 mM phosphate, pH 6.8, 0.1 M KCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM ATP, and 1 mM phenylmethylsulfonyl fluoride (protease inhibitor) overnight at 4°C, then incubated for 1 h at room temperature. These conditions have previously been shown to be adequate to reassemble neurofilament triplet proteins, as well as the NF68 subunit (17, 18). Samples of the complete neurofilament triplet, the purified NF68 subunit, and the 66-kD protein were analyzed by negative-stain electron microscopy. The samples were applied to Formvar-coated grids which were glow discharged not more than 30 min before use. Grids were washed with two drops of distilled water, then with five or six drops of freshly filtered 1% aqueous uranyl acetate, and stained with a final drop of uranyl acetate for 30 s. The uranyl acetate was removed with a piece of filter paper, and the grids were allowed to dry. The grids were then viewed under a JEOL 100S electron microscope.

One- and Two-Dimensional PAGE: One-dimensional PAGE of proteins in the presence of SDS was performed by use of the discontinuous

¹ *Abbreviations used in this paper:* IFA, intermediate filament antigen antibody; HTP, hydroxylapatite; RSA, rat serum albumin.

buffer system of Laemmli (14) on a 7.5% acrylamide vertical slab gel. Before electrophoresis, protein samples were diluted with sample buffer consisting of 62.5 mM Tris pH 6.8, 1% SDS, 1% mercaptoethanol, and 10% glycerol, and boiled for 5 min. After electrophoresis was carried out at 40 mA/gel for ~2 h, the gels were fixed in 10% acetic acid/50% methanol/40% distilled water and stained with Coomassie Brilliant Blue R (Sigma Chemical Co., St. Louis, MO).

Two-dimensional gel electrophoresis was performed essentially according to the method of O'Farrell (24). The pH gradient was established by 1.6% (vol/vol) pH 4–6 ampholines and 0.4% (vol/vol) pH 3.5–10 ampholines (LKB Instruments, Inc., Bromma, Sweden). Protein samples were diluted with lysis buffer supplemented with 0.1% SDS, and ~10–20 µg was applied to the first dimension gel. A 7.5% SDS-polyacrylamide gel was used to resolve the second dimension.

Peptide Mapping: Peptide mapping of proteins using *Staphylococcus aureus* V8 protease (Sigma Chemical Co.) was carried out on excised gel bands by the procedure of Cleveland et al. (3). The bands to be digested were stained lightly with Coomassie Blue, rinsed with distilled water, excised, and equilibrated with a buffer consisting of 125 mM Tris, pH 6.8, 0.1% SDS, and 1 mM EDTA (equilibration buffer) for 0.5 h. The excised bands were then placed at the bottoms of the wells of a stacking gel. Equilibration buffer containing 10% (vol/vol) glycerol was placed in the wells surrounding the gel pieces, and 0.1 µg of enzyme in equilibration buffer containing 1% (vol/vol) glycerol was layered on top. Bromophenol blue (Sigma Chemical Co.) was added as tracking dye and proteolytic digestion was carried out by co-electrophoresing the proteins and enzyme at 10 mA/gel through the stacking gel. When the dye front reached the running gel (15% acrylamide, 10 × 13 cm), the current was turned off for 0.5 h, and enzymatic digestion was allowed to continue. Current was then turned back on and increased to 40 mA/gel for the remainder of the electrophoresis. Individual gel lanes, containing the proteolyzed fragments, were either fixed and stained with Coomassie Brilliant Blue R or immunoblotted with a polyclonal antibody against the NF68 subunit (25) as described below.

Immunoblotting: To determine if the 66-kD protein isolated in this work was either rat serum albumin (RSA) or the protein described by Pruss et al. (26), the purified proteins were reacted with the appropriate antibodies on immunoblots. For these experiments, the purified 66-kD protein was subjected to SDS PAGE, and transferred to nitrocellulose (Bio-Rad Laboratories) by a modification of the method of Towbin et al. (31) as described elsewhere (25). The blots were reacted either with anti-RSA (Antibodies Inc., Davis, CA) or with anti-intermediate filament antigen antibody (generously provided by Dr. Rebecca Pruss). The blots were then reacted with the avidin-biotin peroxidase complex (Vectastain kit, Vector Laboratories, Inc., Burlingame, CA) method of Hsu et al. (11), and visualized with diaminobenzidine (Sigma Chemical Co.).

Blot Overlay: Interaction of the purified 66-kD protein with various cytoskeletal proteins was done as follows. The neurofilament proteins and glial filament protein, isolated from rat spinal cord by the method of Liem (17); vimentin, isolated from bovine lens by the method of Geisler and Weber (6); and two cyclic microtubule proteins obtained from bovine brain (28), were tested by a blot overlay method. In addition, several noncytoskeletal proteins including RSA and casein (Sigma Chemical Co.) and α₂-macroglobulin (a gift from Dr. Fred Maxfield, New York University School of Medicine) were also assayed. The proteins were first subjected to SDS PAGE and transblotted as described above. The blots were then soaked overnight in overlay buffer (5% bovine serum albumin in a buffer consisting of 50 mM Tris, pH 7.4, 0.19 M NaCl, 5 mM EDTA, 2.5% Triton X-100, 0.1% SDS, 0.02% sodium azide, and 0.5 mM phenylmethylsulfonyl fluoride), and then incubated for 3 h with ¹²⁵I-labeled, purified 66-kD protein (2.5 × 10⁶ cpm), diluted in the same buffer, iodinated by the chloramine T procedure as described previously (19). The blot was then washed six times for 10 min, dried, and developed for autoradiography.

To characterize further the binding of this protein to the neurofilament protein, we performed a competition study. In this experiment, we incubated the blots in the presence of an excess (10,000-fold) amount of either cold NF68 or cold 66-kD protein, with the same amount of radioactive 66-kD protein as used in the experiment described above. The blots were then washed as described above, and the label bound to the separated proteins was visualized by autoradiography.

To quantitate the affinity of the 66-kD protein for blotted NF68 protein, a saturation analysis was performed. A tracer amount of ¹²⁵I-labeled 66-kD protein was diluted with purified unlabeled 66-kD protein (1 mg/ml) to a specific activity of 2.5 × 10⁶ cpm/mg protein and dialyzed against overlay buffer overnight. Volumes of 5, 25, 50, 75, 150, 300, and 600 µl of this labeled solution were diluted to 4 ml with overlay buffer yielding concentrations of 0.000125, 0.00625, 0.0125, 0.01875, 0.0375, 0.075, and 0.15 mg/ml, respectively. Using a molecular weight of 66,000, we calculated the corresponding molar concentrations. Individual nitrocellulose replicas of electroblotted neurofilament protein were incubated with these various solutions, and the area corresponding to the NF68 protein was cut out and counted for bound radioactivity in a Beckman 4000 gamma counter.

Axonal Transport Studies: For the axonal transport studies, adult rats were anesthetized with methofane, and 0.5 mCi of [35 S]methionine was injected intraocularly. We then identified the retinal ganglion cell proteins by letting them be transported into the optic nerve for 30 d. After this time, the optic axons were removed and dissolved in lysis buffer, and the samples were run on two-dimensional isoelectric focusing-SDS PAGE to analyze the protein composition as described above. The gels were then soaked with salicylate, dried, and exposed to x-ray film to visualize the radioactively labeled proteins.

RESULTS

Isolation of the 66-kD Protein

The electrophoretic profile of crude intermediate filament proteins isolated from adult rat optic nerve and spinal cord is shown in Fig. 1, lanes *a* and *b*. In both preparations, the neurofilament triplet polypeptides along with the glial filament protein are decidedly enriched in the adult filament preparation. The 66-kD protein is clearly present, as is vimentin. The relative amounts of the proteins are different in the optic nerve and spinal cord. In the optic nerve, there is almost as much 66-kD protein as NF68, whereas in the spinal cord, the latter clearly predominates.

The HTP column successfully separated the 66-kD protein from most of the glial filament protein and the neurofilament triplet proteins (Fig. 1, lanes *c-e*). Glial filament protein eluted in the void volume (Fig. 1, lane *c*), the 66-kD protein with 0.1 M phosphate buffer (Fig. 1, lane *d*), and the neurofilament triplet at 0.3 M phosphate (Fig. 1, lane *e*). A small amount of contaminant, which probably represents glial filament protein, also elutes with the 66-kD protein. The 0.1 M phosphate peak was concentrated and further purified by DEAE chromatography, which successfully removed the contaminating protein (Fig. 1, lane *f*). This protein clearly migrates faster than the NF68 protein (Fig. 1, lane *g*).

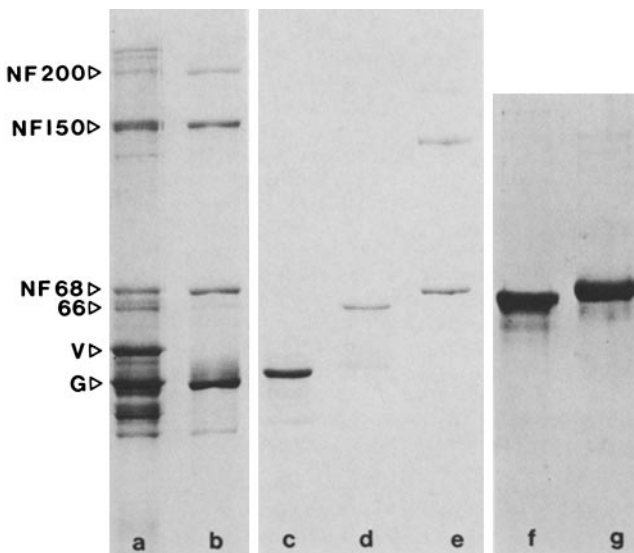


FIGURE 1 A gel electropherogram of crude filament proteins and the various fractions from the columns shows the purification of α -interneixin. Lane *a*, cytoskeletal extract from adult rat optic nerve. Lane *b*, cytoskeletal extract from adult rat spinal cord. Lane *c*, the fractions from the HTP column that elute with 10 mM phosphate buffer, containing the glial filament protein. Lane *d*, the fractions eluted with 0.1 M phosphate, pH 7.0, in 8 M urea, which contain α -interneixin. Lane *e*, the fractions obtained from the 0.3 M phosphate buffer elution, which contain the neurofilament triplet proteins. Lane *f*, purified α -interneixin after DEAE chromatography. Lane *g*, purified NF68 protein. The neurofilament triplet proteins (NF200, NF150, and NF68) are indicated as are α -interneixin (66), vimentin (V), and glial filament protein (G).

Immunoblotting Analysis of the 66-kD Protein

Immunoblotting of the partially purified 66-kD protein (obtained by HTP chromatography) with an antiserum prepared against RSA, showed that the 66-kD protein is not immunologically related to the serum protein of equivalent molecular weight (Fig. 2, lane *a*). On the other hand, immunoblotting with the anti-IFA antibody revealed binding to the 66-kD protein, as well as to what is most probably some contaminating glial filament protein (Fig. 2, lane *b*).

Peptide Mapping

Limited protease digests of the 66-kD protein and the NF68 protein produced dissimilar peptide maps (Fig. 3*a*). This dissimilarity was further accentuated by immunoblotting these maps with a polyclonal antibody to the NF68 subunit (25), which did not recognize any of the peptide fragments of the 66-kD protein (Fig. 3*b*), indicating that this protein is not a degradation product of the neurofilament 68-kD protein.

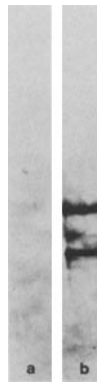


FIGURE 2 Immunoblotting characterization of the α -interneixin. Partially purified α -interneixin was reacted with (lane *a*) anti-RSA and (lane *b*) anti-IFA. Anti-rat serum albumin does not recognize the protein, whereas the anti-IFA antibody does. Anti-IFA also recognizes a band that corresponds to the glial filament protein.

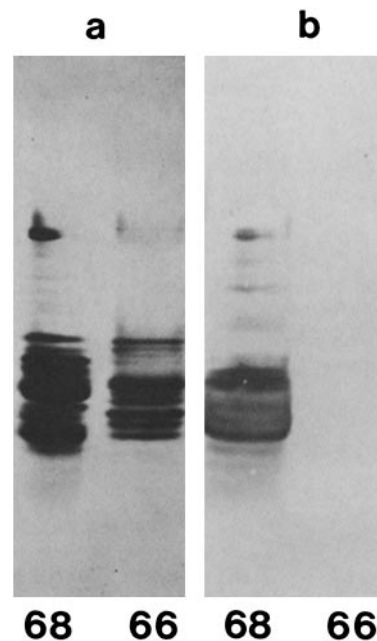


FIGURE 3 One-dimensional peptide mapping of the α -interneixin and the NF68 subunit. The proteins were separated by SDS PAGE and cut out of the gel. Digestion was with the *S. aureus* protease as described in the text. (a) Coomassie Blue-stained peptide maps and (b) peptide maps, after transfer to nitrocellulose and reaction with anti-NF68.

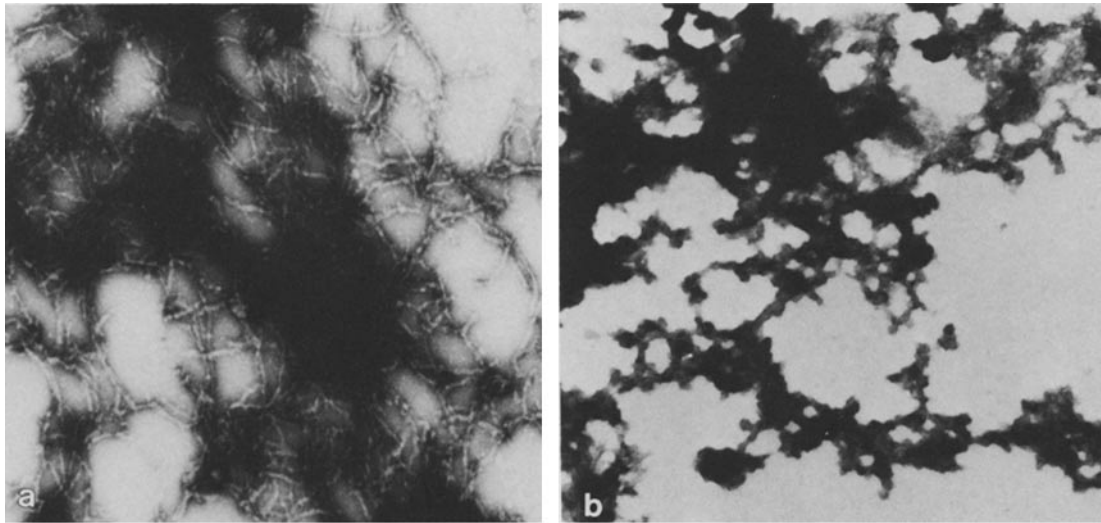


FIGURE 4 Electron micrographs of negatively stained purified NF68 and 66-kD proteins under conditions of neurofilament assembly. (a) NF68 forms 10-nm filaments upon the removal of urea. (b) Purified α -internexin forms only aggregates and no apparent filaments. $\times 50,000$.

Assembly Studies

Unlike the purified NF68 subunit, the purified 66-kD protein could not form 10-nm filaments under the assembly conditions used here (Fig. 4). Instead, large aggregates of proteinaceous material were observed upon removal of urea by dialysis against assembly buffer.

Two-Dimensional Gel Electrophoresis

The isoelectric point of the 66-kD protein was 5.8–5.9, with some additional minor, more acidic isovariants (Fig. 5). The pI of this protein is therefore also different from the pI of the NF68 protein, which on our gel system is 5.3–5.4 (25).

Blot Overlay

When different cytoskeletal proteins were overlaid with ^{125}I -labeled 66-kD protein, the NF68 subunit, vimentin, and the glial filament protein all bound the label (Fig. 6). Tubulin and the 150-kD neurofilament subunit bound the label relatively weakly, and the 200-kD neurofilament subunit did not bind. Similarly, several noncytoskeletal proteins, i.e., casein, α_2 -macroglobulin, and RSA did not bind any radioactivity (data not shown). To see if the labeled protein, which bound to the intermediate filament protein, was indeed the 66-kD protein, the bands were eluted from the blot and rerun on a gel. The autoradiogram of this gel showed that only the 66-kD protein had bound to the intermediate filament protein bands.

To show the specificity of the binding of the 66-kD protein to the neurofilament protein under these conditions, the experiment described above was repeated in the presence of a 10,000-fold excess of either NF68 or 66-kD protein. Under these conditions, the reaction can clearly be competed by either of the two cold proteins.

The concentration curve in Fig. 7 shows the number of counts of ^{125}I -labeled 66-kD protein, which was bound to the area representing NF68 as a function of ^{125}I -labeled 66-kD protein. The binding can be seen to approach saturation at the upper limit of concentrations assayed. As no more than 4% of the labeled 66-kD protein applied actually bound at any one point, zone A behavior was assumed throughout the

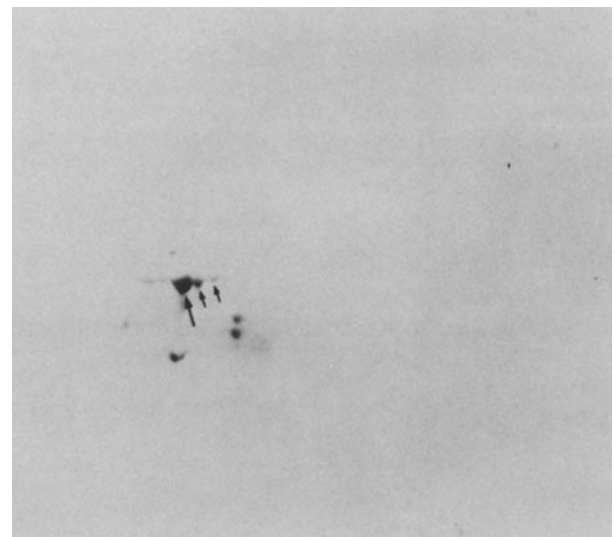


FIGURE 5 Two-dimensional gel electropherogram of the partially purified 66-kD protein. The pH gradient was from 6.5 to 5.5 (left to right). The large arrow indicates the α -internexin (pI ~ 5.8), and the small arrows indicate minor, more acidic isovariants.

curve (9). Hence, the concentrations of free 66-kD protein at the completion of binding was taken to be equal to that at the beginning. Extrapolation to the point at which half maximal binding was obtained yielded a rough estimate of $K_D \approx 4.0 \times 10^{-7}$ M. Note that this is only an approximate K_D , which is included primarily to show the relatively strong binding of this protein to NF68.

Axonal Transport

To determine if the 66-kD protein is an axonally transported protein, we subjected optic nerve, labeled by injection of ^{35}S methionine intraocularly, to two-dimensional isoelectric focusing-SDS PAGE, 30 d after the injection. The resulting autoradiogram and a Coomassie Blue-stained gel of optic nerve filaments are shown in Fig. 8. A protein with the identical isoelectric point and molecular weight is transported

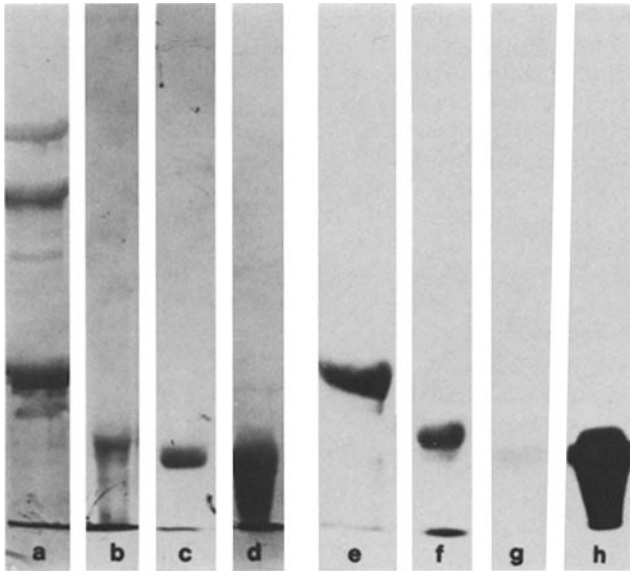


FIGURE 6 Gel overlay of separated filament proteins with ^{125}I -labeled purified α -internexin. Purified neurofilament triplet proteins (lanes a and e), vimentin (lanes b and f), tubulin (lanes c and g), and glial filament proteins (lanes d and h) were subjected to SDS PAGE, transblotted to nitrocellulose, and either stained with amido black (lanes a–d) or reacted with ^{125}I -labeled α -internexin and subjected to autoradiography to visualize the label (lanes e–h). All of the major intermediate filament subunits were found to react with the labeled 66-kD protein.

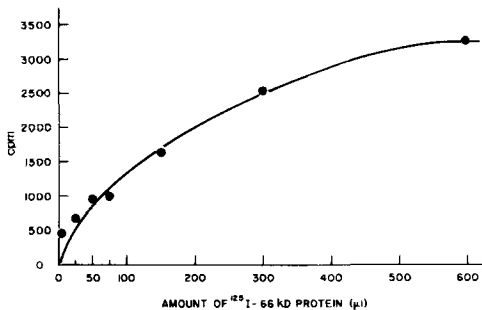


FIGURE 7 A binding curve of ^{125}I -labeled α -internexin with NF68 shows that binding approaches saturation and that a $K_d \approx 4 \times 10^{-7}$ can be estimated for the binding (see text).

at roughly the same rate as tubulin and the NF68 subunit. Several other proteins in the 50–70-kD range are also clearly visualized at 30 d after transport, which are at this point not identified.

DISCUSSION

The 66-kD protein described here can easily be resolved from the known intermediate filament subunit proteins by ion-exchange chromatography. Immunoblotting analysis of the isolated 66-kD protein demonstrated that it is not RSA but is recognized by the anti-IFA antibody described by Pruss et al. (26), which also reacts with each of the five classes of intermediate filaments.

Limited protease digestion of the 66-kD protein and the NF68 subunit, followed by immunoblotting the peptide maps with a polyclonal antiserum directed against the NF68 subunit, reveals that the 66-kD protein is not a proteolytic cleavage product of the neurofilament protein. This was also

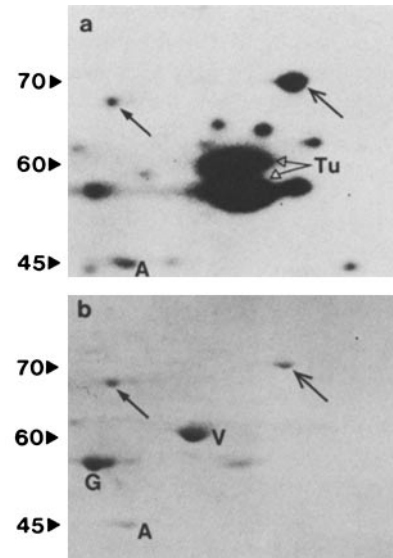


FIGURE 8 Axonal transport study. Rats were injected intraocularly and after 30 d the transported proteins were analyzed by two-dimensional isoelectric focusing–SDS PAGE. (a) Coomassie Blue-stained preparation of optic nerve filaments and (b) autoradiogram of the labeled transported proteins. Notice that the position of α -internexin (\blacktriangledown) relative to NF68 (\blacktriangledown) is identical in both gels. Molecular weight markers in thousands are based on the crude cytoskeletal preparation from optic nerve including the neurofilament proteins, vimentin, and actin. Tu, tubulin; G, glial filament protein; V, vimentin, A, actin. The pH gradient is from pH 6.5 to 5.0 (left to right).

found to be the case when the 66-kD protein was compared with the 150- and 200-kD neurofilament subunits (21).

Tests of polymerization competence showed that the purified 66-kD protein, unlike the purified NF68 subunit, cannot polymerize into 10-nm filaments under the assembly conditions used here. Upon removal of urea by dialysis against neurofilament assembly buffer, the solution containing the 66-kD protein became extremely opalescent, and when examined by negative stain electron microscopy it was found to consist of proteinaceous aggregates. More than 95% of the dialyzed 66-kD protein was pelletable under the same conditions employed to sediment the reassembled neurofilaments, attesting to the extreme insolubility of this protein in physiological buffers.

Although this 66-kD protein is isolated from a Triton-insoluble intermediate filament preparation, it cannot assemble into 10-nm filaments under conditions that promote the assembly of glial filaments and neurofilaments from their purified subunits (17). It appears therefore that the 66-kD protein does not constitute an independent filamentous system, even though it is recognized by the anti-IFA antibody and therefore presumably contains the common α -helical rod segment present in all intermediate filaments and necessary for polymerization (8). Instead, it may represent an intermediate filament-binding protein, which associates with filaments in vivo. In this regard, it may behave similarly to the 200-kD neurofilament subunit, which also contains the common α -helical rod segment of all intermediate filaments (8), but cannot form 10-nm filaments by itself upon isolation (6, 18). In addition, the keratins can form only co-polymers and not homopolymers, which indicates that the presence of the highly conserved α -helical rod segment may be a necessary,

but not sufficient, condition for formation of intermediate filaments. Of course, an alternative explanation may be that upon removal of urea, the 66-kD protein becomes irreversibly denatured, and/or that the most efficient conditions for polymerization have not been met by our assay.

The affinity of the 66-kD protein for various intermediate filament subunit proteins is clearly demonstrated in our ligand blotting assays. The binding of the labeled 66-kD protein to various intermediate filament subunits appears to be specific and approaches saturation. In addition, binding is inhibited by co-incubation of labeled 66-kD protein with unlabeled, purified neurofilament protein or 66-kD protein. On the other hand, the 66-kD protein does not bind to several noncytoskeletal proteins, and iodinated protein A does not bind to the blotted cytoskeletal proteins. It is also apparent that this affinity is reserved for subunit proteins that are competent to assemble into intermediate filaments in vitro: the NF68 subunit, the 50-kD glial filament subunit protein, and the 57-kD vimentin filament subunit protein. The 66-kD protein, therefore, is not requisite for the formation of any of these filaments but, instead, may function in an associative manner. It is interesting, however, that the 66-kD protein has little affinity for tubulin.

A physiological role for the 66-kD protein has yet to be determined. The evidence provided by the gel overlay experiments suggests that the NF68 subunit, as well as vimentin and glial filament protein, bind the isolated 66-kD protein. The biological consequence of the binding of intermediate filament subunits to the 66-kD protein might be to organize them into a cytoskeletal framework. In this regard, it is interesting that Soifer et al. (29) observed the disappearance of a 65-kD polypeptide, with an isoelectric point similar to that determined for the 66-kD protein, coincident with the disappearance of the three neurofilament triplet proteins, from both optic and sciatic nerves undergoing Wallerian degeneration. Further studies on the appearance of this protein during development and various disease states may give us further clues as to its importance in both Wallerian degeneration and cytoskeletal dissolution, as well as in growth and cytoskeletal organization.

The results of the axonal transport studies presented here show that a protein of electrophoretic mobility on two-dimensional isoelectric focusing-SDS PAGE identical to that of the 66-kD protein isolated from spinal cord is labeled in the optic nerve and still present 30 d after intravitreal injection. That, together with the fact that an antibody to the 66-kD protein also recognizes axonal processes in the optic nerve (21, 23), leads us to conclude that this protein is axonally transported, possibly with neurofilaments and microtubules. Moreover, Strocchi et al. (30) have described the slow axonal transport of a 61-kD protein whose solubility characteristics and isoelectric point closely resemble those of the protein described here. The extreme insolubility of the 66-kD protein and its demonstrated lack of affinity for tubulin in our binding assay, however, render it unlikely to be related to the 65-kD microtubule-associated protein, recently described by Tytell et al. (32) to be transported in the slow components of axonal transport along with neurofilaments and microtubules. As can be seen from the two-dimensional autoradiogram, a number of other proteins are also transported in the rat optic nerve in the 50–70-kD range, some of which may in fact be microtubule-associated proteins (1, 32).

The association of the 66-kD protein with intermediate

filament proteins through isolation in situ and in vitro have led us to propose the name α -internexin for this protein. Even though binding to vimentin and glial filament protein was observed, the data in this paper do not show that this protein is both neuronal and glial. The data also do not address the question of whether or not this protein is present in a wide variety of cell types as described by Pruss et al. (26). In the accompanying paper we describe a related protein, which is present in a wide variety of cell types (23) but has different a mobility on two-dimensional gels and a different peptide map from α -internexin, described in this paper. The co-purification of α -internexin with intermediate filaments from rat central nervous system tissue, its ability to bind to a number of intermediate filaments, and the fact that it contains the epitope recognized by an anti-IFA (26) do, however, support a theoretical construct in which α -internexin is ascribed an intermediate filament-organizing function.

We are especially grateful to Mr. Eugene Napolitano for his critical evaluation and advice of the work and the manuscript, and Ms. Susan Babunovic for her assistance in preparing this manuscript. We also thank Dr. Michael Shelanski for his helpful suggestions and criticisms throughout this work.

This work was supported by a grant EY03849 from the National Institutes of Health (NIH). R. K. H. Liem is the recipient of research career development award (NS00487) from the NIH and the Irma T. Hirsch Charitable Foundation. J. S. Pachter was an NIH predoctoral trainee for 1981–1982.

Received for publication 12 December 1984, and in revised form 6 March 1985.

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