Biochemistry of the filaments of brain
(neurofilaments/glial fibrillary acidic protein/tubulin)

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ABSTRACT Intermediate filaments—cytoplasmic filaments with a diameter of 8-9 nm—have been described in a large variety of cell types. In this study, the subunit protein of the neurofilament and the presumptive subunit of the astroglial filament are compared by immunological and biochemical methods and are found to be very similar. Strong cross-reactions are also found between these proteins in a variety of mammalian species. These results suggest that the intermediate filaments may, like microtubules and microfilaments, represent a highly conserved and widely distributed fibrous protein system.

Four major classes of intracellular filaments are seen on electron microscopic examination of the normal mammalian central nervous system: microtubules, microfilaments, neurofilaments, and astrocytic filaments (1-3). Tubulin, the major protein subunit of the microtubule, is a dimer with a sedimentation velocity of 6 S and a molecular weight of 110,000 (4-6). The monomers are nonidentical (7-9) and have apparent molecular weights of 53,000 (α-tubulin) and 55,000 (β-tubulin) on dodecylsulfate-urea polyacrylamide gel electrophoresis. The nervous system is especially rich in tubulin, and tubulin may exist in the brain in membrane-associated as well as soluble and microtubular forms (10, 11). However, tubulin is in no way unique to the nervous system and is found in most, if not all, eukaryotic cells. Microfilaments are generally described as being 5-6 nm in diameter and, in general, are thought to be composed of actin. This is based both on heavy meromyosin binding (12, 13) and on biochemical studies (14, 15). As in the case of tubulin, actin is present in the nervous system (16, 17) but is in no way specific for it.

Neurofilaments have been isolated and characterized from the giant axon of the squid (18) and from the axons of the sea worm, myxica (19). Both these proteins differ markedly in molecular weight from the filaments isolated from mammalian brain. These latter filaments, with a diameter of 9 nm, have a subunit weight of 51,000 and are soluble only in detergents or denaturing agents, such as urea or guanidine-HCl (20-22). Glial filaments are found in fibrous astroglial cells and have a diameter of 8-9 nm (1). The one report of the isolation of presumptive glial filaments from mammalian brain (23) reported their subunit molecular weight as 57,000 and found a peptide fingerprint that closely resembled tubulin. On the other hand, a soluble protein with a molecular weight of 54,000 has been found in regions of the brain rich in fibrous astrocytes and has been reported to be limited to these cells by immunofluorescent techniques (for review see ref. 24). Early studies on this glial fibrillary acidic protein (GFA) reported molecular weights as low as 40,000, but it now appears that these lower molecular weight forms are all the result of proteolytic degradation of the native 54,000 form (25).

In order to avoid confusion with the well-established terminology in which the term microfilament is reserved for the 5- to 6-nm filaments, we have adopted the term "intermediate filament" for the 8- to 10-nm filaments found in neurons, glia, and other cell types.

Although tubulin, actin, GFA, and the neurofilament protein differ markedly with respect to morphology and their solubility properties, their similarity in subunit molecular weight and the ability of tubulin to form a variety of different polymeric structures (26) suggests that there might be a close relationship between two or more of these proteins.

In this study, we use both peptide fingerprinting and immunological techniques to examine the relationships between these proteins in neural tissue.

MATERIALS AND METHODS

Isolation of Neurofilaments, GFA, and Tubulin. Neurofilaments were isolated and purified from a calf brain using a modification of the method originally described by Shelanski et al. (20). All procedures were carried out at 4°. Brains were obtained at a local slaughterhouse and were processed within 2 hr. Routinely, 40 g of brain white matter was carefully dissected and finely minced with scissors. The minced tissues were homogenized in 600 ml of 0.85 M sucrose, 0.03 M KCl in 0.02 M phosphate buffer pH 6.5 (solution A) using a Dounce homogenizer. Three passes were made with the A pestle, followed by three with the B pestle. The homogenate was centrifuged in Beckman SW-27 rotors at 10,000 rpm for 15 min. The pads of myelinated axons, which had floated to the top of the tubes, were collected and rehomogenized in 600 ml of solution A. The flotation was repeated several times until the pellet was free of red or pink color. The pads of myelinated axons from the final flotation were then resuspended and agitated overnight in a solution containing 0.01 M phosphate buffer and 0.01 M mercaptoethanol, pH 6.5 (solution B) to remove the myelin. After rehomogenization, the suspension was mixed with an equal volume of 1.70 M sucrose, 0.01 M mercaptoethanol, 0.01 M Tris pH 8.6 (solution C) and centrifuged at 10,000 rpm for 15 min. The pellet of axoplasm was homogenized in solution B and then layered on top of an equal volume of solution C and centrifuged at 10,000 rpm for 15 min. The pellet was retained, and the layer at the interface was rehomogenized; this step repeated three to four times to maximize the yield. The pellets were finally homogenized in solution B and centrifuged on a discontinuous sucrose density gradient of 1.0, 1.5, and 2.0 M sucrose in 0.03 M phosphate buffer, 0.01 M mercaptoethanol, pH 6.5 at 20,000 rpm for 60 min. The in-
terface between 1.5 and 2.0 M sucrose contained the highly purified filaments.

Human neurofilaments (NF) were prepared from a brain obtained at autopsy approximately 6 hr after death from a patient who died of nonneurological disease. The human NF preparations were of the same degree of purity as the bovine. Sciatic nerve NF were obtained by a modified procedure in which a Sorvall omni-mixer was used to homogenize the nerve. The preparations were contaminated with collagen and cell debris, and the gel patterns showed only about 40% of the total protein comigrating with bovine NF. Only the major band eluted from gels was used for the sciatic nerve studies.

GFA was isolated from bovine brain by the method of Dahl and Bignami (25). The tissue was homogenized in 0.05 M sodium phosphate buffer, pH 8.0 (weight/volume, 1:4) and after centrifugation at 12,000 × g for 20 min, the supernatant was added to hydroxylapatite and stirred for 15 min. The hydroxylapatite was repeatedly washed with 0.05 M sodium phosphate buffer pH 8.0 till all unabsorbed protein was removed. GFA was eluted with 0.01 M potassium phosphate buffer pH 8.0; eluates were pooled and precipitated by adding ammonium sulfate to a final concentration of 30%. A short time interval between the homogenization of the partially thawed white matter and the second washing of the hydroxylapatite in sodium phosphate was critical in order to avoid degradation.

Tubulin was prepared from bovine brain by the method of Shelanski et al (27). Samples were used within a week of preparation whenever possible. All storage was at −25°C.

Sodium Dodecyl Sulfate (NaDodSO4)-Urea Gel Electrophoresis. Samples containing 0.1% NaDodSO4, 0.12 M mercaptoethanol, 8 M urea, 30 mM Tris-HCl, and bromophenol blue were immersed in a boiling water bath for 3 min. Electrophoresis on 7% acrylamide gels was performed according to Shelanski (28). Thyroglobulin, bovine serum albumin, actin, and tubulin were used as molecular weight standards.

Iodination and Peptide Mapping. After staining with Coomassie brilliant blue, bands containing 20 μg of protein were cut from the gels, eluted with a solution containing 0.05 M sodium phosphate pH 7.5, 0.1% NaDodSO4, and 1 mM phenylmethylsulfonylfluoride at 37°C for 2 days. Samples were lyophilized and then iodinated according to Bray and Brownlee (29). After the termination of iodination, the mixtures were dialyzed—first against methanol, then against deionized water. The dialyzed solutions were lyophilized and the residues were resuspended in a minimum volume of 0.1 M ammonium bicarbonate. The iodinated proteins were digested with L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone (TPCK)-treated trypsin (Worthington) (1:50 weight/weight) for 18 hrs at 37°C. The digested mixtures were lyophilized several times to remove traces of ammonium bicarbonate. The residues were resuspended in 0.1 ml of deionized water and centrifuged to remove insoluble particles. Aliquots of the supernatant were spotted on silica gel G thin-layer chromatography plate in a system of n-propanol-ammonium hydroxide (7:3, vol/vol). The chromatogram was subjected to electrophoresis in the second dimension at 1000 V for 1½ hr in a buffer containing 10% pyridine, 3% acetic acid pH 6.5. Detection was by exposure to x-ray film. One-dimensional maps were made in the chromatographic direction only, and multiple samples were spotted on the same plate.

Preparation of Antisera and Immunodiffusion Studies. Antiserum to NF was raised in rabbits in our laboratories. The major neurofilament band in acrylamide slab gels was cut out with a clean razor blade, chopped into small pieces, and homogenized in phosphate-buffered saline. Approximately 400 μg of protein in 2 ml of buffer was emulsified with complete Freund’s adjuvant and injected subcutaneously. Four weeks later a booster of 200 μg of protein was injected in a similar manner. The rabbit was bled a week later from the marginal ear vein, and the serum was collected by centrifugation. Antiserum to GFA was raised in rabbits using protein preparations isolated from plaques of multiple sclerosis or purified from bovine brain as the antigens. Antitubulin antibody was the gift of Dr. Gerald Fuller and Bill Brinkley (30). Muscle actin was provided by Dr. T. D. Pollard. Immunodiffusion was performed on modified Ouchterlony plates containing 1% agar, 0.86% NaCl, 0.1% NaDodSO4, and 0.5% Triton X-100.

RESULTS

As shown in Fig. 1a, the NF preparation separated into several bands on NaDodSO4-urea gel electrophoresis. The slowest migrating band was designated band 1; the next compo-
neurofilament, band 2, etc. The major component, band 7, had a molecular weight of 54,000. This band represented over 90% of the total protein by densitometry. This estimate was in good agreement with morphological examination at the electron microscopic level. Tubulin preparations showed the usual major bands, as well as the high molecular weight proteins (31) and trace bands at 68,000 and 70,000. A single major band was seen in both the actin and GFA preparations. The GFA and NF migrated at the same position on the gel (Fig. 1b) and slightly behind the more rapidly moving tubulin band. This would correspond to a molecular weight of about 54,000.

Immunological Specificity of the Antiserum to NF. In regular Ouchterlony plates, 0.1% NaDodSO₄ caused formation of false precipitin lines with normal rabbit serum. However, with the addition of NaDodSO₄ to the agar itself, this artifact can be eliminated. The addition of 0.5% Triton X-100 to the plates containing 0.1% NaDodSO₄ not only prevents the formation of the artifact, but also improves the image of the precipitin line.

In modified Ouchterlony plates, which contained 0.1% NaDodSO₄, 1% agar, and 0.5% Triton X-100, the NaDodSO₄-solubilized NF preparation formed a precipitin line with antiserum to NF, but no precipitin lines were formed with normal rabbit serum, antisera to nonfibrous proteins (e.g., bungarotoxin antiserum, ovalbumin antiserum), or with 0.1% NaDodSO₄ alone or with bovine serum albumin in 0.1% NaDodSO₄ (Fig. 2a).

Neurofilaments isolated from human brains or rabbit sciatic nerve were shown to crossreact with antiserum to calf NF (Fig. 2b). The immunological identity of the components of the calf NF preparation was also tested. As indicated by immunodiffusion, only component 4 (molecular weight ≥ 145,000) and the major component (band 7) were immunologically active with an immunodiffusion pattern of complete identity (Fig. 2c).

Neurofilaments and GFA crossreacted with antiserum to GFA, showing a line of identity (see Fig. 3a). Immunization with NF followed by a booster injection at 4 weeks resulted in the production of antiserum to NF that crossreacted with GFA with no visible spurring at the intersection of the precipitin lines (Fig. 3b). Neither the anti-NF nor the anti-GFA crossreacted with actin, total tubulin, α-tubulin, or β-tubulin over the wide range of antibody and antigen concentrations assayed. The anti-tubulin crossreacted with tubulin from a variety of species but not with NF, GFA, or actin. Preincubation of anti-NF and anti-GFA with tubulin did not inhibit their reactivity with NF and GFA.

Incubation of anti-NF with intact, nondenatured neurofilaments and subsequent centrifugation to remove the filaments resulted in the removal of anti-NF activity from solution.

FIG. 2. (a) Ouchterlony plate showing reaction between neurofilament protein (center well) and antiserum against neurofilament (well 1). Other wells contain: 0.1% NaDodSO₄ (2), bovine serum albumin in 0.1% NaDodSO₄ (3), normal rabbit serum (4), anti-bungarotoxin (5), and anti-ovalbumin (6). (b) Reaction between anti-neurofilament antiserum (center well) and human neurofilaments (2) and rabbit sciatic nerve neurofilaments (3). Calf neurofilament is in wells 1 and 4. (c) Reaction between anti-neurofilaments and components of the neurofilament preparation is in well 1; band 4 is in well 2 and band 7 (major component) in well 3.

FIG. 3. (a) Antiserum to GFA (center well); GFA (well 1) and NF (wells 2 and 3), showing crossreaction with no evidence of spurring. Coomassie blue stain. (b) Central well contains anti-neurofilament antiserum which crossreacts with NF (well 1) and GFA (well 2).
Peptide Mapping. Iodinated peptides were prepared from bands eluted from acrylamide gels. Chromatographic examination of these samples revealed marked differences among the peptide maps of NF major band, α-tubulin, β-tubulin, and actin. On the other hand, the one-dimensional maps of GFA and NF showed very marked similarity, though differences do exist (Fig. 4). This similarity is retained in two-dimensional maps as well (Fig. 5). The protein from band 4 differs little, if at all, from the major filament band. Preliminary studies of NF peptides show similar patterns in this protein from mouse, rabbit, and calf.

DISCUSSION
As shown in the results, the immunoreaction of NF antiserum to NF is specific. Although 0.1% NaDodSO₄ causes the formation of precipitin lines with serum, which is in agreement with Green et al. (32), this artifact is eliminated by the addition of 0.1% NaDodSO₄ and 0.5% Triton X-100 in the agar. There are several other examples in the literature of nonimmune precipitation reactions occurring in double diffusion tests (33, 34). However, we have been unable to find any report that adequately explains the nonimmune precipitation effect of NaDodSO₄.

The demonstration of a similarity in immunological reactivity and peptide maps between the NF and GFA is surprising in view of the differences in their known solubility characteristics and the supposed glial-specificity of the GFA. This similarity might be explained in several ways. One possibility is that GFA may be the soluble precursor of the extremely stable neurofilament. The neuron and glial cell might synthesize the soluble form and then, according to the ionic strength or other factors in the environment, these proteins might assemble to form neurofilaments or glial filaments which could be involved in structural support and intracellular transportation. It could be argued that, in spite of the fact that the neurofilaments are prepared from axon preparations in which there is little astroglial contamination, the final preparation is relatively enriched in the filaments from such contaminants. Some support for this argument can be found in the existence of two morphologically distinct types of filament bundles in the purified preparations (20). These bundles differ primarily in the density of their packing, and one type could possibly be of astroglial origin. Nonetheless, only one major band is found on gel electrophoresis, suggesting biochemical identity of both types of bundles. Filaments prepared from rabbit sciatic nerve also give the same major electrophoretic band and immunological reaction. Since no astroglia exist in the peripheral nerve, this argues strongly against the NF being of predominantly glial origin. It could also be argued that GFA isolated from bovine brain was of axonal rather than glial origin. However, the human GFA preparations used to raise antisera were likely of glial origin, since they were obtained from severely gliosed human tissues and constituted up to 35% of the total water-soluble protein in this material (35). Human and bovine GFA show similar peptide patterns and amino-acid composition (unpublished data).

A second possibility is that GFA and NF are the products of different, closely related genes and the proteins differ primarily in their final assembly state.

The apparent inconsistency between immunodiffusion and immunofluorescence findings is left unresolved by this work and suggests avenues for future research. Extensive experience with the antibody to GFA in immunofluorescent assays have shown this antigen to be limited to glial cells and indeed only to a special class of glia, i.e., the astrocytes (24).

As demonstrated by this study, GFA and NF gave a reaction of complete identity using both GFA and NF antisera.
However, the antigen–antibody reaction occurred under completely different conditions in the immunodiffusion plate and in tissue sections. In the immunodiffusion plate, the precipitin lines occurred in the presence of detergent, while the immunohistochemical localization by immunofluorescence was obtained in air-dried cryostat sections using high dilutions of the antigen. It is thus possible that the immunofluorescence reaction is seen only under special conditions, such as when the protein is in a soluble or even membrane-associated form, as suggested by studies with a glial cell line, rather than assembled into tight bundles of filaments (M. Schachner, T. B. Carnow, A. Bignami, and S. H. Yen, in preparation). In this respect, it is interesting to note that reactive glial cells display bright immunofluorescence as early as 2 days after injury (36), while little immunofluorescence staining was seen in severely gliotic optic nerves 6 months after enucleation though they were still intensely positive against GFA antisera by disc immunodiffusion (unpublished observations). It is possible that with the use of electron microscopic immunohistochemistry, it would be possible to see localization to the neurofilament in the axon as well as the astrocyte. The role that this protein may serve in its soluble and in its particulate form is unknown. The problem of the control of assembly and the reasons that the assembled form, at least in the neuron, is so insoluble will require further study.

The finding that the intermediate filament subunits in neurons and glia are so closely related strongly suggests the possibility that the cytofilaments of other, nonneural, cells will also be found to be similar. The characteristics of these proteins differ very much from those of actin and of tubulin and, therefore, encourages the consideration of intermediate filaments as a separate class of organelles on a biochemical as well as a morphological basis. The physiological role of these filaments is unknown and the extent to which they interact with other filamentous systems in the cell is unexplored.

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