ASSOCIATION OF AN AXONALLY TRANSPORTED POLYPEPTIDE (H) WITH 100-Å FILAMENTS

Use of Immunoaffinity Electron Microscope Grids

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ABSTRACT

Polypeptide H (mol wt 195,000) is axonally transported in rabbit retinal ganglion cells at a velocity of 0.7–1.1 mm/d, i.e., in the most slowly moving of the five transport groups described in these neurons. To identify the organelle with which H is associated, we purified H, prepared antibodies directed against it, and adsorbed the antibodies onto Formvar-coated electron microscope grids. When the resulting "immuno-affinity grids" were incubated with extracts of spinal cord and then examined in the electron microscope, they contained as many as 100 times more 100-Å filaments than did grids coated similarly with nonimmune IgG. The ability of the anti-H IgG to specifically adsorb filaments to grids was completely blocked by incubating the IgG with polypeptide H. The 100-Å filaments adsorbed to anti-H immunoaffinity grids could be specifically decorated by incubating them with anti-H IgG. These observations demonstrate that H antigens (and most likely H itself) are associated with 100-Å neurofilaments. In addition, they suggest that the use of immunoaffinity grids may be a useful approach for determining the organelle associations of polypeptides.

The availability of high-resolution techniques (e.g., SDS polyacrylamide gel electrophoresis [SDS-PAGE] and isoelectric focusing) for separating proteins has made it possible to study the synthesis, turnover, and transport of individual proteins without regard to their identity or function. However, once a protein with interesting behavior has been singled out by such techniques, it is often difficult to establish its identity and function. Here we outline a systematic approach for determining the organelle association of a polypeptide known only by its electrophoretic mobility. The strategy is as follows: An electron microscope grid is coated with an antibody directed against the protein of interest, and the resulting "immunoaffinity grid" is used to adsorb specifically organelles containing the antigen. The organelle is then identified by electron microscopy. This strategy may prove to be generally useful for determining the organelle association of some proteins.

In this communication, we describe the use of immunoaffinity grids to show that polypeptide H, an axonally transported polypeptide (15, 16), is associated with the intermediate filaments of nervous tissue. Polypeptide H (mol wt 195,000) is

synthesized in the cell bodies of neurons; in the retinal ganglion cells of rabbits, we have observed its subsequent transport down the axons at a velocity of 0.7–1.1 mm/d (16). At least two other polypeptides, 45 (mol wt 145,000) and 46 (mol wt 73,000), appear to be transported at this same velocity; at least four groups of proteins are transported more rapidly in rabbit retinal ganglion cells (16, 17). One characteristic of polypeptide H that is important for the conclusion of our experiments is that it is subject to a genetic polymorphism in rabbits (15). Two forms (H1 and H2) of the polypeptide can be differentiated by electrophoresis; they appear to differ in molecular weight by 10,000, with H1 having the higher molecular weight (195,000). Different inbred strains of rabbits contain only H1 or H2, whereas different individuals from outbred strains contain only H1, both H1 and H2, or, very rarely, only H2.

MATERIALS AND METHODS

Purification of H

Approximately 5 g of rabbit spinal cord (freshly dissected, or frozen) was homogenized in 30 ml of TED buffer (10 mM Tris, 5 mM EDTA, 2 mM dithiothreitol [DTT]), containing phenyl-methylsulfonyl fluoride (PMSF, 1 mM), and diluted to 360 ml with the same buffer lacking PMSF. The homogenate was sonicated for 5 min, stirred for 1 h at 4°C, centrifuged for 1 h at 100,000 g, and H was precipitated from the supernate by the addition of 0.32 g/ml of $(NH_4)_2SO_4$. The precipitate was sedimented by centrifugation for 1 h at 100,000 g and then resuspended in TED buffer (5 ml) containing 10 mM DTT and 3% SDS. The suspension was heated at 90°C for 5 min, centrifuged at 100,000 g for 45 min, and the supernate was subjected to gel filtration chromatography on a column (2.5 × 120 cm) of Sepharose-6B equilibrated with TED buffer containing SDS (1%). The eluant fractions containing H (identified by analytical SDS-PAGE) were combined and dialyzed against a solution of EDTA (5 mM) and DTT (0.1 mM) and precipitated with 20% TCA; the precipitate was collected by centrifugation and washed with diethyl ether to remove residual TCA. The precipitate was resuspended in TED (1 ml) containing 1.5% SDS and 10 mM DTT. The solution was incubated at 90°C for 5 min, centrifuged at 100,000 g for 45 min at 15°C, and subjected to preparative SDS-PAGE on a gel with a diameter of 1.5 cm in an apparatus from Savant Instruments, Inc. (Hicksville, N. Y.). The running gel (4 cm high) contained 4.5% acrylamide, 0.135% bis-acrylamide, 8 M urea, whereas the stacking gel (1.2 cm high) contained 3% arylamide, 0.09% bis-acrylamide, 8 M urea. The buffer systems were those of Laemmli (6), and the SDS was sequanol grade from Pierce Chemical Co. (Rockford, Ill.). The fractions containing only H (as judged by analytical SDS-PAGE) were combined and concentrated by pressure filtration (XM100 filter, Amicon Corp., Scientific Sys. Div., Lexington, Mass.) and dialyzed against a solution of SDS (0.1%) and DTT (0.1 mM). The resulting preparation was emulsified with an equal volume of Freund's adjuvant (complete for the first injection, incomplete for subsequent injections) and injected subcutaneously into a

goat. The goat received four injections (~100 μg each) over a period of 6 mo; it was bled 10 d after the last injection.

Purification of IgG

IgG was partially purified from the anti-H serum by precipitating it three times with $(NH_4)_2SO_4$ (40% of saturation at 4°C). The resulting preparation was dialyzed against phosphate-buffered saline (PBS, 0.15 M NaCl, 0.01 M NaH_2PO_4, pH 7), and further purified by affinity chromatography on a column of Sepharose-4B to which proteins (including H) from the $(NH_4)_2SO_4$ step of the H purification (see above) had been coupled by the procedure of Parikh et al. (8). The specific antibodies were eluted from the column with a solution of glycine (0.2 M) and NaCl (0.15 M), pH 2.4.

Nonimmune IgG was purified from the serum of a nonimmunized goat by $(NH_4)_2SO_4$ precipitation (45% of saturation at 4°C) followed by chromatography on DEAE cellulose (Cellex D, from Bio-Rad Laboratories, Richmond, Calif.).

Immunoaffinity Grid Preparation

Copper electron microscope grids (400 mesh) coated with Formvar (0.4% in ethylene dichloride) were floated on drops of PBS containing either anti-H or nonimmune IgG for 4.3 h at room temperature. The grids were rinsed in one drop of PBS and then incubated in a solution (2.5% wt/vol) of polyglutamic acid (Sigma Chemical Co., St. Louis, Mo.) for 50 min at 4°C. The grids were rinsed in two drops of PBS, and washed by floatation in 3 ml of PBS for 1.6 h at 4°C. The coated grids were floated on drops of cell fractions for 30 min at room temperature, then rinsed in nine drops of PBS and one drop of H_2O at 4°C, and negatively stained with unbuffered uranyl acetate (1%). After each rinse, the liquid was drawn off the grid by touching it to the torn edge of a piece of filter paper (Whatman No. 1).

Filament Preparation

Freshly dissected segments of rabbit spinal cord were incubated with a 100-fold excess of a solution containing EDTA (2.5 mM), EGTA (2.5 mM), and Na_2HPO_4 (1 mM), pH 7, for 2 h at room temperature as described by Schlaepfer (10). The swollen tissue was removed from the solution and homogenized with a loose-fitting pestle of a Dounce homogenizer (Kontes Co., Vineland, N. J.). NaCl (1 M) was added to the disrupted tissue to give a final concentration of 0.15 M, and the tissue homogenate was centrifuged at 12,000 rpm for 30 min in the SS-34 rotor of a Sorvall RC2B centrifuge (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) (10). The supernate was applied to a discontinuous sucrose gradient containing layers (3.3 ml each) of 2, 1.5, and 1.0 M sucrose in a solution containing NaCl (0.15 M), EDTA (2.5 mM), and EGTA (2.5 mM). The gradients were centrifuged for 3 h at 35,000 rpm in an SW 40.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The material at the 1- to 1.5-M interface served as a source of filaments.

RESULTS

Purification of H

When rabbit spinal cord was homogenized in a low ionic strength medium, approximately one-half of the H was extracted. In cases where the remaining particulate fraction was reextracted, additional H was solubilized, suggesting that soluble

and insoluble H are not separate species but can be interconverted. Fig. 1 shows the progressive purification of H by gel filtration chromatography and preparative SDS-PAGE. The gel filtration served to increase the relative amounts of proteins of high molecular weights, thereby allowing more H to be applied to the preparative gel; the preparative gel had a limited capacity (~1 mg) for total protein. Fig. 1b shows that preparative SDS-PAGE separated H from polypeptide 45 and all other stained polypeptides. The yield of H was ~100-200 μg from 3 g of spinal cord.

It was important to establish that the polypeptide isolated by the procedure was in fact the axonally transported polypeptide H and contained no other polypeptides that coelectrophoresed with H. Because H can be recognized by its strain-dependent electrophoretic mobility, we performed the purification procedure on the spinal cords of
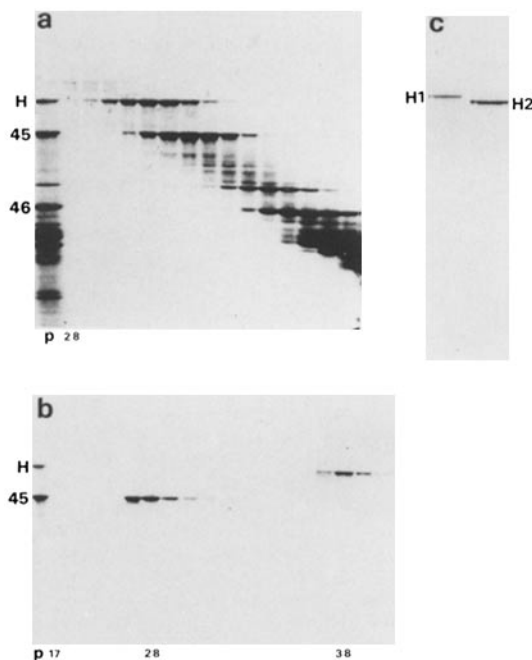

both H1- and H2-containing rabbits. Fig. 1 c shows that the resulting purified polypeptides had the strain-dependent mobility expected for H. Furthermore, the lack of staining material at the H1 position in the H2 preparation and vice versa argues strongly that there was no significant amount of protein other than H that coelectrophoresed with either H1 or H2.

Anti-H Immunoaffinity Grids

To determine which intracellular structure contains H antigens, we prepared immunoaffinity electron microscope grids by incubating Formvar-coated grids with anti-H IgG, and subsequently with polyglutamic acid. The rationale for the incubation with polyglutamic acid was that it might serve to reduce nonspecific adsorption sites on the Formvar. Control grids were prepared in an identical manner, except that an equivalent concentration of nonimmune IgG replaced the anti-H IgG. When the resulting grids were incubated with crude homogenates or partially purified fractions of rabbit spinal cord, and subsequently examined in the electron microscope, it was apparent that the anti-H coated grids contained a much higher concentration of filamentous material than did the control grids (Fig. 2). The filaments were the only structure which appeared in greater numbers on the grids coated with anti-H IgG than on the grids coated with nonimmune IgG. Because the anti-H grids differed from the controls only in the specificity of their IgG coatings, the increased adsorption of filaments to the anti-H grids was most likely the result of a specific antibody-mediated binding of H antigens contained in the filaments.

Table I shows estimates of the number of fila-

FIGURE 1   Purification of polypeptide H. a and b show the polypeptides in fractions (sequential fractions in a, fractions 17, 20, 23, 25, 27, followed by sequential fractions in b) from the gel filtration and electrophoresis columns, respectively. p indicates a sample of the material that was applied to the column or gel. c shows the final purified preparation of H from a rabbit that had only H1 (New Zealand White strain) on the left, and from a rabbit that had only H2 (strain X/J) on the right. The latter rabbit was obtained from The Jackson Laboratory (Bar Harbor, Maine). The samples in c were electrophoresed side by side on a slab gel.

TABLE I

Adsorption of Filaments onto Immunoaffinity Grids *

| | Filaments | | |
IgG concn	Anti-H IgG	Nonimmune IgG	Ratio anti-H:nonimmune
μg/ml	no./μm² × 10⁻³		
7	313 ± 305	125 ± 179	2.5
21	645 ± 197	179 ± 107	3.6
63	914 ± 179	27 ± 60	34.0
189	662 ± 224	18 ± 38	37.0
567	860 ± 242	8.9 ± 29	97.0

* Immunoaffinity grids prepared with the indicated concentrations of IgG were incubated with sucrose gradient-purified filaments. The filament numbers were estimated from direct observation of 10 fields on each grid. Each field was 11 μm² magnified 61,000 times.
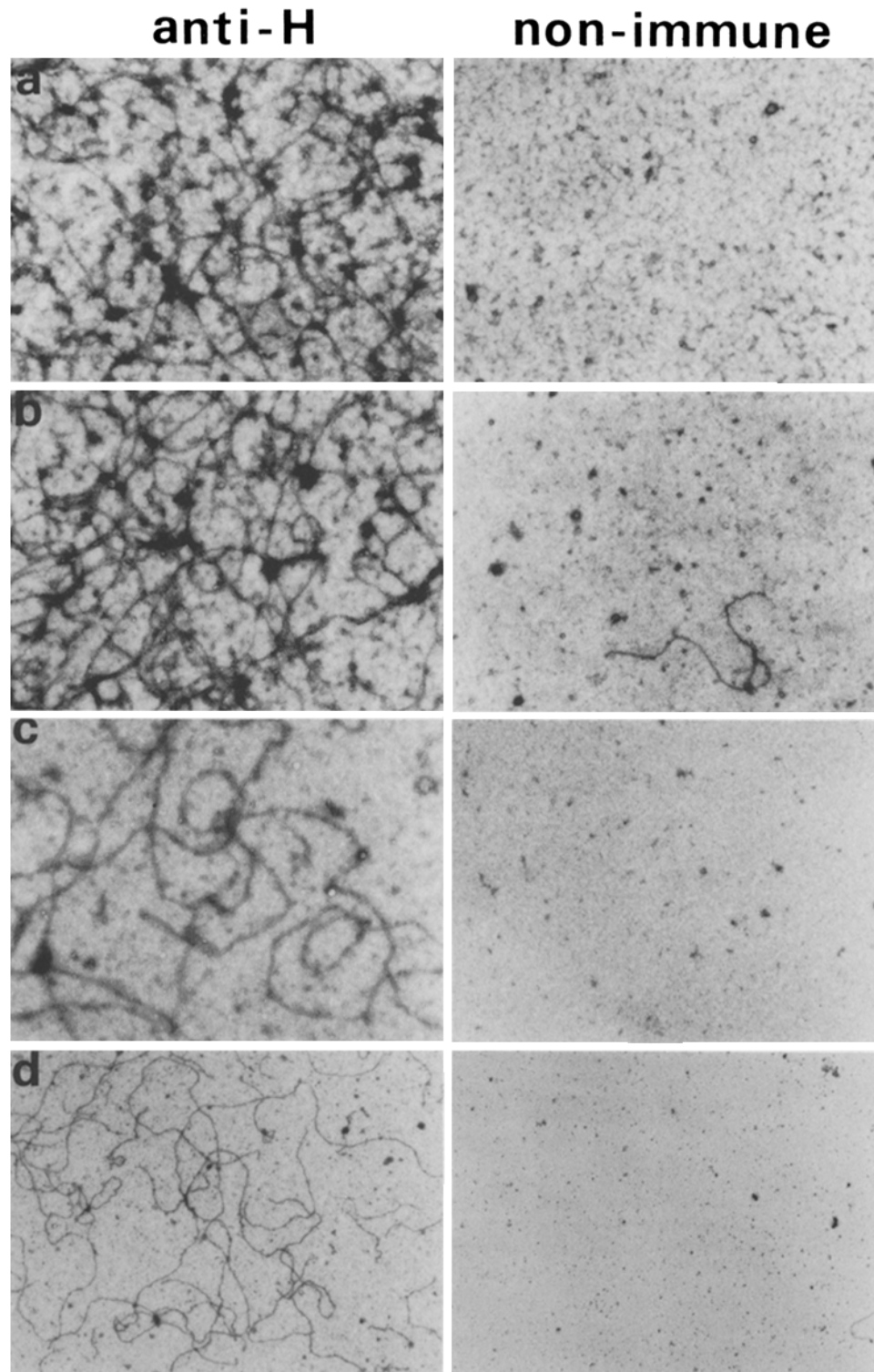
FIGURE 2   Adsorption of filaments from various fractions of spinal cord. The left and right columns show immunoaffinity grids prepared with 567 μg/ml of anti-H IgG and nonimmune IgG, respectively. (a) Whole homogenate of spinal cord. (b) Supernate of a 3.5×10^5 $g_{avg} \times$ min centrifugation of spinal cord extract prepared according to the method of Schlaepfer (10). (c and d) Sucrose gradient fraction of spinal cord prepared as described in Materials and Methods. (a–c) × 25,000; (d) × 7,100.

ments adsorbed on grids coated with increasing concentrations of anti-H and nonimmune IgG. It is apparent that the number of adsorbed filaments increased with increasing concentrations of anti-H IgG, as might be expected if the filament binding were mediated by the antibody. On the other hand, the number of filaments bound to the nonimmune IgG-coated grids decreased with increasing IgG concentration. This latter effect most likely represents the blocking of the remaining nonspecific adsorption sites on the Formvar by the nonimmune IgG. At the highest IgG concentration used (567 μg/ml), nearly 100 times more filaments bound to the anti-H-coated grid than to the control.

Fig. 3 shows a higher magnification of a typical filament on an anti-H immunoaffinity grid. Its diameter (90–100 Å) indicates that the specifically adsorbed filaments belong to the class of intermediate (100 Å) filaments of nervous tissue.

Filaments Postdecorated with Anti-H IgG

Schlaepfer has shown that intermediate filaments can be decorated with specific antifilament antibodies in a manner that can be observed by electron microscopy (9); the decoration results in an apparent increase in the filament diameter as well as an increase in contrast. When intermediate filaments adsorbed to Formvar-coated grids were incubated with anti-H IgG, the filaments became decorated, as shown in Fig. 4. The decoration was specific for anti-H IgG; it did not occur when filaments on grids were incubated with nonim-

mune IgG (Fig. 4). Furthermore, the filaments adsorbed both to anti-H immunoaffinity grids and to nonimmune-coated grids became decorated when postincubated with anti-H IgG (Fig. 5). These observations serve three purposes: first, they provide additional evidence that H antigens are contained in the intermediate filaments; second, they serve to rule out the possibility that the difference between anti-H and control immunoaffinity grids might be in the visibility (because of decoration by the antibody on the grid) of the filaments rather than in the number adsorbed. If this had been the case, the difference in the number of filaments (Fig. 5) would have been eliminated by the postdecoration of both the immune and control grids; and third, the increased contrast of the decorated filaments allowed them to be observed at lower magnifications, where they could be counted more accurately, because their ends were more often included in the field of observation.

Fig. 6 shows the amount of filamentous material adsorbed to grids prepared by incubating them with different concentrations of immune or nonimmune IgG, and postdecorating them with anti-H IgG. The filaments were quantitated both by counting them (Fig. 6, upper curve) and determining their total length (Fig. 6, lower curve) from photographs of randomly chosen fields. As was suggested by Table I, it is apparent from Fig. 6 that the number of adsorbed filaments increased with increasing concentrations of anti-H IgG, and decreased with increasing concentrations of nonimmune IgG. In the case of the grids coated with
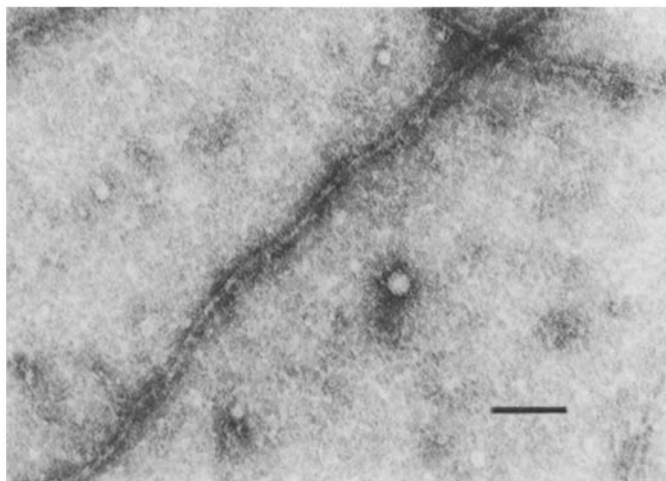

FIGURE 3   A typical filament from the immunoaffinity grid shown in Fig. 1 c. Bar, 100 nm. × 97,000.
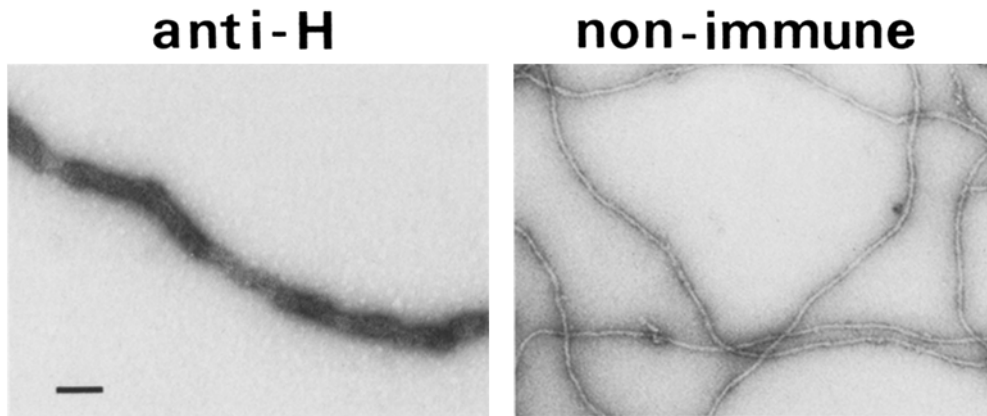
anti-H non-immune

FIGURE 4   Decoration of filaments with anti-H. Filaments from a sucrose gradient fraction were adsorbed directly onto Formvar-coated grids. The grids were rinsed in two drops of PBS and incubated for 45 min at 4°C with 25 μl of either anti-H IgG (left) or nonimmune IgG (right), each at ~10 μg/ml. The grids were rinsed and negatively stained as described in Materials and Methods. Bar, 100 nm. × 58,000.
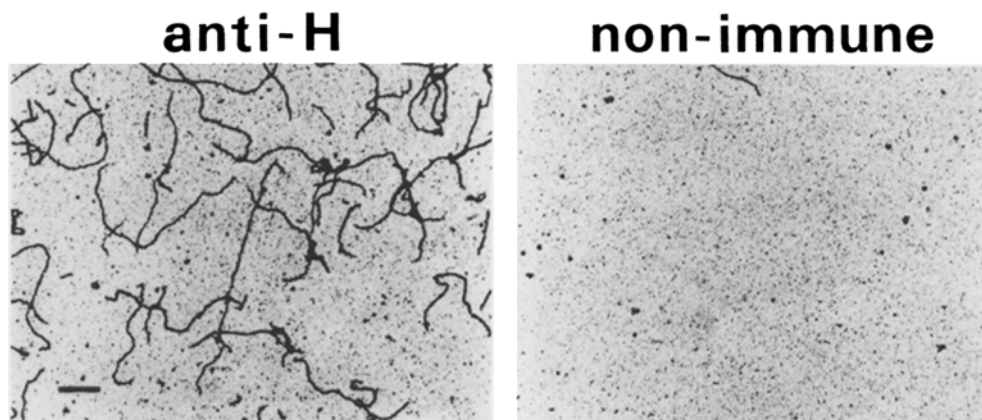
anti-H non-immune

FIGURE 5   Decoration of filaments on immunoaffinity grids with anti-H. Immunoaffinity grids were prepared with 567 μg/ml of anti-H IgG (left) or nonimmune IgG (right). After the grids were incubated with a filament fraction from a sucrose gradient, they were washed, and then both were incubated with anti-H IgG (25 μl, 23 μg/ml) for 38 min at 4°C. The grids were washed again and negatively stained. Bar, 1 μm. × 5,600.

567 μg/ml of IgG, more than 100 times more filamentous material was adsorbed to the immunoaffinity grid than to the control. Fig. 6 also shows the effectiveness of polyglutamic acid in reducing the nonspecific adsorption of filaments to the grid; in the absence of IgG, it reduced nonspecific adsorption by a factor of eight.

Adsorption of Anti-H Activity

If the adsorption of intermediate filaments to immunoaffinity grids is indeed mediated by anti-H antibodies, then preincubation of the anti-H

IgG with H should neutralize the filament-adsorbing capacity of the IgG. Therefore, we incubated an aliquot of anti-H IgG with a macerated piece of polyacrylamide gel containing H; the gel had been fixed (17) and the fixative had been removed by incubation with PBS. When we tested a filtrate of this H-adsorbed IgG for its capacity to mediate filament binding on immunoaffinity grids, its activity had been reduced to the background level (Table II). On the other hand, Table II shows that anti-H IgG that had been incubated with the same weight of gel containing no protein retained its
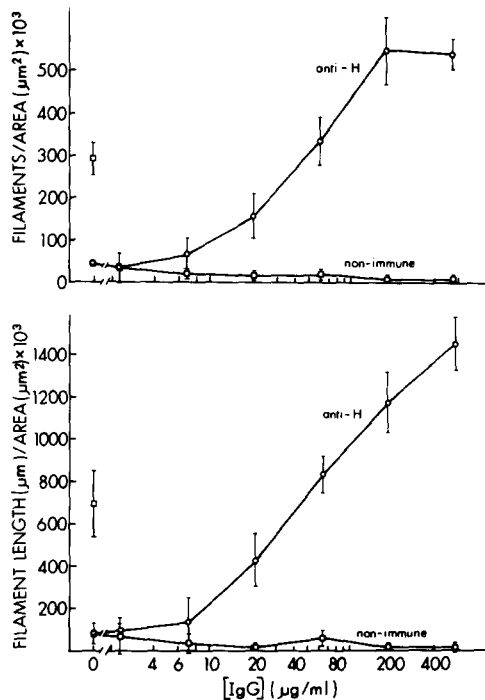
FIGURE 6 Filaments adsorbed to immunoaffinity grids prepared with different concentrations of IgG, and post-decorated with anti-H IgG as described in Fig. 5. Five fields ($\times$ 7,600) on each grid were photographed. To obtain a random sample, the fields were selected at such low beam intensities that the filaments were not visible. In the upper figure, filaments were counted directly from the negatives. In the lower figure, the filament lengths were measured from $\times$ 2.5 enlargements by computerized planimetry. The single square at 0 IgG concentration shows the amount of filaments bound to a grid that was not incubated with polyglutamic acid or IgG.

ability to adsorb filaments to grids. These results support the conclusion that the ability of anti-H IgG to mediate filament adsorption to grids is dependent upon its content of anti-H antibodies.

It is interesting to note that when anti-H IgG was incubated with similar gel slices containing polypeptides 45 and 46, the number of filaments on subsequently prepared immunoaffinity grids appeared to be reduced, but to a lesser extent than in the case of preincubation with H (Table II). This observation suggests that some of the antigenic determinants in the H polypeptide may be shared by polypeptides 45 and 46.

DISCUSSION

The foregoing observations serve two purposes. First, they demonstrate the feasibility of immu-

TABLE II

Filaments on Immunoaffinity Grids Prepared with Adsorbed Anti-H IgG

Anti-H IgG adsorbed with:*	Filaments‡
	$\mu m/mm^2$
H	11 ± 7.1
45	38 ± 27
46	148 ± 45
Neutral gel	253 ± 52
Background§	19 ± 15

* Aliquots (1 ml) containing 20 μg of anti-H IgG and 20 μg of nonimmune IgG were incubated with portions (1 g) of macerated polyacrylamide gel containing either H, 45, 46 (approximate relative amounts = 1:2:3, respectively), or no protein (neutral gel). The adsorbed IgG was separated from the gel by filtration through Uni-Pore filters (Bio-Rad Laboratories), and used to prepare immunoaffinity grids; the grids were incubated with filaments purified on a sucrose gradient as described in Materials and Methods. Filaments were postdecorated with anti-H IgG as described in Fig. 5.
‡ Filament length was determined directly on negatives ($\times$3,600) of fields (five per grid) selected without regard to filament content as in Fig. 6.
§ The grid was prepared with 20 μg/ml of nonimmune IgG.

noaffinity grids as a systematic approach for establishing the association between a polypeptide and an organelle. Second, the use of this approach has provided direct evidence that antigenic determinants associated with a 195,000-dalton axonally transported polypeptide are also associated with intermediate filaments of nervous tissue.

The possibility that immunoaffinity grids might be employed to determine the association of a protein with an organelle was suggested to us by a report describing the use of serologically specific electron microscopy as a quantitative assay for plant viruses (2). In this report, carbon-backed Parlodion-coated grids were incubated with antisera against either tobacco mosaic virus or potato virus Y; the resulting grids preferentially adsorbed the appropriate virus as judged by negative staining electron microscopy. Our results indicate that a similar procedure can be applied to subcellular organelles. We found that the number of organelles bound to a Formvar film can be significantly increased when an antibody against antigens in the organelle is first adsorbed to the surface of the Formvar. Three observations indicate that the increase in organelle binding is specifically mediated by the antibody. First, the binding of the organelle

increased with increasing concentrations of the antibody. Second, nonimmune IgG used in place of the antibody did not cause increased binding of the organelle; on the contrary, the nonimmune IgG decreased the binding of the organelle, presumably by occupying the remaining nonspecific adsorption sites on the Formvar. (In the case of the intermediate filaments studied here, we found that nearly 90% of the nonspecific adsorption could be eliminated by incubating the grids with polyglutamic acid.) Third, the increased binding of the organelle was eliminated when the antibody was neutralized by its antigen.

An attractive feature of the immunoaffinity grid strategy for determining the association of a protein with an organelle is that, in principle, it requires a minimum number of speculations concerning the nature of the organelle. For example, it was possible to demonstrate the specific adsorption of 100-Å filaments onto anti-H IgG-coated grids even in crude homogenates of nervous tissue; therefore, the nature of the H-containing organelle could be inferred without assumptions regarding how the organelle would respond to fractionation procedures. The technique might also prove useful for examining associations between organelles because it provides a means of concentrating an organelle and its associated structures on an electron microscope grid with minimum preliminary fractionation. The limitation of the immunoaffinity grid approach is that the antigen of interest must be located in an exposed position on an organelle that can be recognized by electron microscopy. Although filaments are probably particularly favorable organelles in these regards, it seems likely that with appropriate variations in the procedure, a variety of organelles would be accessible to this approach.

The subunit composition of the 100-Å neurofilaments has been a subject of considerable controversy. When filaments have been prepared by a procedure designed to purify myelinated axon segments and subsequently liberate their neurofilaments by means of osmotic shock, the resulting preparations have consistently contained a major polypeptide with a mol wt in the range of 49,000–60,000 (1, 3, 5, 12, 14, 18). In contrast, Hoffman and Lasek (4) inferred that three polypeptides (mol wt 200,000, 145,000, and 68,000) that are transported slowly down the axons of the rat sciatic nerve are associated with neurofilaments. Hoffman and Lasek's hypothesis has been supported

by reports that, during experimentally induced neuronal degeneration (11, 13), polypeptides resembling this "slow component triplet" accumulate and disappear in synchrony with the neurofilaments. In addition, recent preparations of intermediate filaments from peripheral and central nervous tissue (7, 10) have been enriched in polypeptides resembling the triplet, and Schlaepfer (9) has reported that antibodies against a 68,000-dalton polypeptide (which may correspond to one of the triplet polypeptides) are reactive with intermediate filaments.

The experiments reported here use two criteria to show that antigens contained in the axonally transported protein H (whose molecular weight [195,000] is similar to one of the triplet polypeptides) are physically associated with intermediate filaments. First, immunoaffinity grids coated with anti-H IgG specifically adsorbed 100-Å filaments, and second, anti-H IgG specifically decorated intermediate filaments. We can be unusually confident in equating the axonally transported polypeptide H (identified as a radioactive electrophoretic band [16]) with the protein used to produce the antibody, as both show the same strain dependence in electrophoretic mobility.

Although the observation that antigens are shared between polypeptide H and intermediate filaments does not necessarily require that H is itself associated with the filaments, the following considerations strongly support this conclusion: The most purified filament fraction used in our experiments contains only three major polypeptides (Fig. 7), one of which can be identified as H by its strain-dependent electrophoretic mobility. While the other two major polypeptides (45 and 46 in Fig. 7) appear to share antigenic determinants with H (reference 7 and Table II), anti-H IgG which had been exhaustively adsorbed with 45 and 46 retained its ability to decorate intermediate filaments and to form filament-specific immunoaffinity grids (data not shown). Thus, the filaments contain antigens unique to H among the major polypeptides in the filament fraction; it therefore seems safe to conclude that H itself is associated with the filaments. The nature of the association between H and the filaments (i.e., whether it is an integral subunit of the filament or a subunit of some structure peripherally attached to the filament) is not yet clear. Our experiments also do not rule out the possibility that H is associated with additional structures not discerned
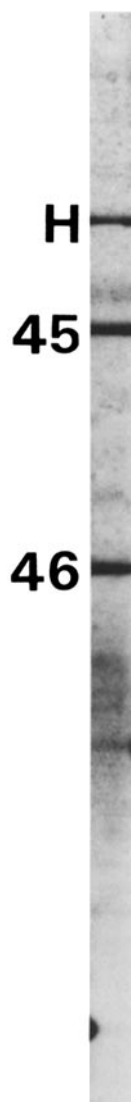
posed by Hoffman and Lasek [4]) that the transport of neurofilaments is a major function of the most slowly moving group of axonally transported proteins. Our preliminary experiments with antibodies directed against polypeptides 45 and 46, the two additional group V polypeptides that resemble the triplet polypeptides in molecular weight, support the conclusion that these polypeptides are also associated with intermediate filaments.

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FIGURE 7 A filament fraction from a sucrose gradient, electrophoresed and stained with Coomassie Blue. The symbols indicate polypeptides discussed in the text.

by electron microscopy.

Although nervous tissue includes filaments from both neurons and glial cells, the H-containing filaments are most likely of neuronal origin because axons, but not glial cells, can be intensely stained with anti-H IgG in sections of nervous tissue by means of indirect immunofluorescence (J. Levine and M. Willard, manuscript in preparation). The conclusion that H is associated with neurofilaments supports the idea (originally pro-

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