Multiple Phosphorylated Variants of the High Molecular Mass Subunit of Neurofilaments in Axons of Retinal Cell Neurons: Characterization and Evidence for Their Differential Association with Stationary and Moving Neurofilaments

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Abstract. The 200-kD subunit of neurofilaments (NF-H) functions as a cross-bridge between neurofilaments and the neuronal cytoskeleton. In this study, four phosphorylated NF-H variants were identified as major constituents of axons from a single neuron type, the retinal ganglion cell, and were shown to have characteristics with different functional implications.

We resolved four major Coomassie Blue-stained proteins with apparent molecular masses of 197, 200, 205, and 210 kD on high resolution one-dimensional SDS-polyacrylamide gels of mouse optic axons (optic nerve and optic tract). Proteins with the same electrophoretic mobilities were radiolabeled within retinal ganglion cells in vivo after injecting mice intravitreally with [35S]methionine or [3H]proline. Extraction of the radiolabeled protein fraction with 1% Triton X-100 distinguished four insoluble polypeptides (P197, P200, P205, P210) with expected characteristics of NF-H from two soluble neuronal polypeptides (S197, S200) with few properties of neurofilament proteins. The four Triton-insoluble polypeptides displayed greater than 90% structural homology by two-dimensional α-chymotryptic iodopeptide map analysis and cross-reacted with four different monoclonal and polyclonal antibodies to NF-H by immunoblot analysis. Each of these four polypeptides advanced along axons primarily in the Group V (SCa) phase of axoplasmic transport. By contrast, the two Triton-soluble polypeptides displayed only a minor degree of α-chymotryptic peptide homology with the Triton-insoluble NF-H forms, did not cross-react with NF-H antibodies, and moved primarily in the Group IV (SCb) wave of axoplasmic transport. The four NF-H variants were generated by phosphorylation of a single polypeptide. Each of these polypeptides incorporated 32P when retinal ganglion cells were radiolabeled in vivo with [32P]orthophosphate and each cross-reacted with monoclonal antibodies specifically directed against phosphorylated epitopes on NF-H. When dephosphorylated in vitro with alkaline phosphatase, the four variants disappeared, giving rise to a single polypeptide with the same apparent molecular mass (160 kD) as newly synthesized, unmodified NF-H.

The NF-H variants distributed differently along optic axons. P197 predominated at proximal axonal levels; P200 displayed a relatively uniform distribution; and P205 and P210 became increasingly prominent at more distal axonal levels, paralleling the distribution of the stationary neurofilament network. Semiquantitative analyses of pulse-radiolabeled NF-H variants between 3 and 60 d after synthesis showed that P197 was the principal NF-H variant composing neurofilaments in the axonally transported wave as this wave advanced along axons at the optic nerve level. Long after the neurofilament transport wave had reached nerve terminals (60 d after injection), P210 and P205 remained prominently radiolabeled along axons, indicating that these variants were the major NF-H constituents of neurofilaments associated with the stationary cytoskeleton.

On the basis of these data, we propose that phosphorylation regulates the specific association of neurofilaments with different domains of the neuronal cytoskeleton. Changes in the phosphorylation state of NF-H during axoplasmic transport may represent one of the mechanisms that triggers the integration of moving neurofilaments into the stationary cytoskeleton.

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Cytoskeletal proteins in many tissues exhibit structural microheterogeneity. Isoforms of tubulin (6, 10, 46, 49, 68), actin (50), microtubule-associated proteins (23, 51, 68, 70), and other cytoskeletal proteins (13, 22, 39, 40, 54, 90), generated by genetic or posttranslational mechanisms, may display selectivity for a particular tissue or cell type or may appear at a specific stage of cellular development. In addition, multiple structural variants of a protein are sometimes observed within the same differentiated cell (23, 40, 80, 90). Although their presence within single cells could reflect the transition of a polypeptide from a precursor form to a fully functional state, variants arising by posttranscriptional or posttranslational processing may also amplify the range of functions that a single gene product serves in different sites within the cell.

Proteins made up of neurofilaments, the intermediate filaments of neurons, are extensively modified after synthesis (3, 30, 31, 59, 63, 64, 80, 81), and the complexity of these posttranslational processing events (1, 18, 60, 61, 66, 69, 84) holds important clues to the function of these cytoskeletal elements. Neurofilaments comprise three subunits (26, 78, 83, 85), referred to as high, middle, and low molecular mass neurofilament proteins (NF-H, NF-M, and NF-L, respectively), to denote their relative molecular sizes (160-210, 140-160, and 70 kD). Microheterogeneity of the neurofilament triplet proteins is generated by limited proteolysis (58, 63), phosphorylation (4, 18, 20, 30, 41, 61, 66, 69), and dephosphorylation (60). The NF-H and NF-M subunits, in particular, are among the most highly phosphorylated proteins in the brain (8, 29, 30), and phosphorylated forms of NF-H and NF-M display different physicochemical properties from those of their unmodified counterparts (30, 32). Since most newly synthesized neurofilament proteins are destined for posttranslational modification and export into axons, it is unclear whether relatively unmodified neurofilament proteins are transient precursors to more mature polypeptides or represent functional constituents of the perikaryal cytoskeleton. Microdifferentiation of the neurofilament network has been suggested by the segregation in some neurons of relatively poorly phosphorylated neurofilaments within neuronal perikarya and extensively phosphorylated neurofilaments within axons (1, 2, 18, 19, 42, 60, 69, 80).

Evidence that NF-H and NF-M subunits continue to be modified (58, 60, 63, 66) as they advance along axons emphasizes that, even within the axon itself, the cytoskeleton is regionally specialized (6, 58). Functional as well as structural microdifferentiation of the axonal cytoskeleton is supported by observations that neurofilament subunits in central axons compose neurofilaments of two types (62). Neurofilaments in the process of translocation within the slowest phase of axoplasmic transport contribute to a lattice of neurofilaments that is part of the stationary cytoskeletal network along axons (59, 62). Stationary neurofilaments represent the predominant pool of neurofilaments in mature retinal ganglion cell neurons, and this network is particularly prominent in distal axonal regions (59, 62).

The existence of a stationary neurofilamentous cytoskeleton as well as moving neurofilaments implies that the interactions of these cytoskeletal elements during axoplasmic transport must be highly dynamic and tightly regulated. Posttranslational modifications of neurofilament proteins could coordinate these interactions by altering the affinity of neurofilaments for other cytoskeletal structures (28, 47, 53, 55, 74). Modifications of the high molecular mass neurofilament subunit may be particularly important for this process since NF-H subunits are believed to form cross-bridges that interconnect neurofilaments and possibly link these elements to other cytoskeletal components and membrane-bound organelles (25, 75, 88). Phosphorylation, for example, is added primarily to the hypervariable carboxy-terminal end of NF-H which protrudes from the neurofilament core and presumably interacts with other cytoskeletal proteins (8, 16, 32).

To further examine the cellular roles of NF-H and the significance of NF-H phosphorylation in regulating neurofilament organization and function, we used a combination of biochemical, cell biological, and immunological approaches to identify major forms of NF-H in retinal ganglion cells and to characterize certain properties of these variants. Earlier studies showed that NF-H migrates on two-dimensional gels as a diffuse, sometimes discontinuous spot suggesting the presence of multiple microheterogeneous forms (7, 12, 20). Recently, monoclonal antibodies were used to identify several differentially phosphorylated states of NF-H by immunoblot and immunocytochemical analysis (20, 42). In the present investigation, we used a range of criteria to identify different forms of NF-H in a single class of central neurons. In view of the complex dynamics of cytoskeletal elements during axoplasmic transport and maintenance of the axonal cytoskeleton, we focused on the microheterogeneity of NF-H specifically within axons. The results of this study establish the existence of multiple forms of NF-H, the probable mechanism for their generation, and possible functional correlates of this structural microheterogeneity.

Materials and Methods

Animals and Tissue Preparation

10-14-wk-old male or female mice of the C57BL/6J inbred strain were used in all experimental studies. Mouse breeding and maintenance and tissue dissections have been previously described (56, 63). The primary optic pathway, termed optic axons, consisted of (a) the optic nerve severed at the sceral surface of the eye, (b) the optic chiasm, and (c) part of the optic tract extending to, but not including, terminals in the lateral geniculate nucleus. The entire dissected segment was 15 mm long. In some studies, optic pathways were cut into consecutive 1.1-mm segments. Proteins soluble in 1% Triton X-100 solutions and Triton-insoluble cytoskeletal proteins were prepared from optic pathways by the method of Chiu and Norton (9) modified to include a 100,000 g final centrifugation instead of the usual 30,000 g centrifugation.

Tissue Preparation and One-dimensional SDS-PAGE

One-dimensional SDS-PAGE was performed by the procedure of Laemmli (36) using 320-mm slab gels containing 5-15 or 3-7% polyacrylamide gradients. Samples for electrophoresis were prepared under conditions designed to minimize proteolysis and protein loss. Samples were stored at -70°C in the presence of 0.5% BSA and 0.002% bromphenol blue. To analyze unfractonated optic pathways in a single gel lane, a 2-3-ng wet weight of tissue (one optic pathway) was thawed in 150 µl of ice-cold Laemmli buffer (62.5 mM Tris HCl, pH 6.8, 10% glycerol, and 0.002% bromphenol blue) within a small glass homogenizer. After homogenization, the samples were incubated on ice for 10 min with 2 µg each of RNase and DNase. A volume of 10% SDS (40 µl) and 2-mercaptoethanol (7 µl) was then added and the samples were boiled for 10 min, cooled, and loaded on gels. In some cases proteins in polyacrylamide slices

1. Abbreviations used in this paper: NF-H, high molecular mass (200-kD) neurofilament protein; NF-M, middle molecular mass neurofilament protein; NF-L, low molecular mass neurofilament protein.
excised from SDS gels were subjected to a second electrophoresis. These slices were first equilibrated to pH 6.8 in several changes of a Tris-HCl buffer (62.5 mM, pH 6.8) containing 0.1% SDS. The slices were then homogenized and prepared as described above for tissue homogenates, but the RNase and DNase incubation step was omitted. Proteins on gels were stained with 0.1% Coomassie Brilliant Blue (Schwarz-Mann, Cambridge, MA) and destained as previously described (7).

**Radiolabeling of Proteins in Retinal Ganglion Cells In Vivo**

Mice were injected intravitreally with 15-25 μCi of [2,3-3H]proline (sp act, 30-50 Ci/mmol), 75 μCi of [35S]methionine (sp act, 400 Ci/mmol), or 30 μCi of [32P]orthophosphate (sp act, 1,000 Ci/mmol) (New England Nuclear, Cambridge, MA) and were killed at 1-60 d after injection as previously described (56, 63). After SDS-PAGE, radiolabeled proteins were detected by autoradiography. Proteins on gels were homogenized and prepared as described above for tissue homogenates, but slices were first equilibrated to pH 6.8 in several changes of a Tris-HCl buffer (62.5 mM, pH 6.8) containing 0.1% SDS. The slices were then incubated for 60 min at 37°C in 0.5 ml of a Hepes medium (25 mM Hepes, 5 mM KCl, 110 mM NaCl, 50 mM glucose, pH 7.4) containing 75 μCi of [35S]methionine (sp act, 400 Ci/mmol). The medium was then replaced by 1.0 ml of the same medium with a radioisotopic amino acid containing protein synthesis inhibitors (0.5 mM cycloheximide [Sigma Chemical Co., St. Louis, MO] and 0.3 mg/ml chloramphenicol [Sigma Chemical Co.]). Incubation in this medium was continued at 37°C for an additional 5 min. This was repeated once to remove unincorporated radioactivity, and the tissue was then frozen at -70°C until analyzed (57).

**Radiolabeling of Gial Cell Proteins of the Optic Pathway In Situ**

Intact, unlabeled optic pathways, freshly dissected from mice, were incubated for 60 min at 37°C in 0.5 ml of a Hepes medium (25 mM Hepes, 5 mM KCl, 110 mM NaCl, 50 mM glucose, pH 7.4), containing 10 μg of α-chymotrypsin. The digests were lyophilized, redissolved in 10-20 ml of Buffer 1 (acetic acid/formic acid/H2O, 15:5:80), spotted onto cellulose-coated TLC plates, and subjected to electrophoresis in Buffer 1 at 1 kV for 30 min and then chromatographed in butanol/pyridine/acetic acid/H2O (32.5:25:5:20) as described previously (15). The radioactive peptides were visualized by autoradiography.

**Alkaline Phosphatase Digestion of Cytoskeletal Proteins**

Cytoskeletal protein fractions were incubated at 37°C with Escherichia coli alkaline phosphatase (48 U/mg protein; Sigma Chemical Co., St. Louis, MO) in 0.05 M Tris containing 100 mM NaCl, 0.5 mM PMSF, 100 μg/ml leupeptin, and 0.1% aprotinin. After a 5-h incubation, the cytoskeletal proteins were pelleted by centrifugation and prepared for SDS-PAGE as previously described.

**Immunostaining with Monoclonal and Polyclonal Antibodies**

Optic pathway proteins on gels after SDS-PAGE will be used to denote individual protein forms in these studies. On SDS gels containing a 5-15% acrylamide gradient, proteins in the 195-210 kD range, referred to hereafter as the 200-kD proteins, appeared as a diffuse single band or doublet (Fig. 1, lane b). This region, however, could be resolved on 3-7% acrylamide gradient gels into four Coomassie Blue-stained bands displaying apparent molecular masses of 210, 205, 200, and 197 kD (Fig. 1, lane a). Since the patterns revealed by Coomassie Blue staining reflect the protein pools in optic glial cells as well as optic axons, we labeled proteins in optic axons selectively by injecting mice intravitreally with [35S]methionine and analyzed them after 4 d (Fig. 1, lanes c and d). Four radiolabeled proteins (Fig. 1, lane d; Fig. 2, lanes c and e) were resolved that corresponded in electrophoretic migration to the four 200-kD proteins visualized by Coomassie Blue staining. For reasons discussed later, the level of radioactivity associated with the 210- and 197-kD proteins depended on the postinjection in-
optic axons obtained from mice 2 or 5 d after intravitreal injection of [35S]methionine were partitioned into Triton-soluble and -insoluble fractions. The position of fodrin (235 kD) and NF-M (145 kD), respectively. To examine the structural homology among 200-kD proteins, we injected mice intravitreally with [35S]methionine digestion. Of the 34 peptides clearly discernible on the peptide map of P210, 33 were present in P205, 32 were shared by P200, and 30 were present in P197. The P205 and P200 proteins shared two to five additional peptides not detectable on the map of P210. In contrast to the high degree of structural homology among Triton-insoluble 200-kD proteins, only 10 of the peptides common to each of these polypeptides had possible counterparts on the maps of the Triton-soluble proteins (Fig. 4). An additional 22–40 peptides on maps of S200 and S197 were not shared by the insoluble 200-kD proteins. Considerable homology (70–80%) was observed, however, between the two Triton-soluble proteins (Fig. 4).

**Structural Relatedness of 200-kD Proteins**

To examine the structural homology among 200-kD proteins, the Triton-soluble and -insoluble forms of these polypeptides were excised from gels, iodinated with 125I, and subjected to two-dimensional peptide map analysis after a-chymotrypsin digestion. Of the 34 peptides clearly discernible on the peptide map of P210, 33 were present in P205, 32 were shared by P200, and 30 were present in P197. The P205 and P200 proteins shared two to five additional peptides not detectable on the map of P210. In contrast to the high degree of structural homology among Triton-insoluble 200-kD proteins, only 10 of the peptides common to each of these polypeptides had possible counterparts on the maps of the Triton-soluble proteins (Fig. 4). An additional 22–40 peptides on maps of S200 and S197 were not shared by the insoluble 200-kD proteins. Considerable homology (70–80%) was observed, however, between the two Triton-soluble proteins (Fig. 4).

**Differential Axoplasmic Transport of the 200-kD Proteins**

Intravitreal injection of radiolabeled amino acids intensely labels the three neurofilament subunits, which then advance along optic axons in the slowest phase (Group V or SCa) of axoplasmic transport (0.3–0.7 mm/d) (3, 26, 62, 87, 89). To compare the transport characteristics of 200-kD proteins with these established patterns, we injected mice intravitreally with [3H]proline and, after 3 or 7 d, divided the optic pathway into consecutive 1.1-mm segments (Fig. 5). These were then separated into Triton-soluble and -insoluble fractions, subjected to electrophoresis, and quantitated by laser densitometry after fluorography. Changes in the distribution of individual Triton-soluble and -insoluble proteins between 3 and 7 d (Fig. 5) demonstrated the progressive advance of these proteins along optic axons by axoplasmic transport as previously established (62, 63, 65). At 7 d after injection, the transport wave containing P210, P205, P200, and P197, which advanced at 0.3–0.5 mm/d, was clearly separated from the transport wave containing Triton-soluble 200-kD proteins, which advanced at a typical Group IV (SCb) rate (1–2 mm/d) based upon comparisons with other typical Group IV proteins (62). A small proportion of P205 protein also moved at a Group IV rate. An autoradiogram (Fig. 5, inset) depicting the labeling patterns of Triton-insoluble and -soluble 200-kD proteins in the optic nerve and optic tract at 5 d after isotope injection distinguished the P205 variant moving in Group IV from the other Triton-insoluble 200-kD proteins moving in Group V (Fig. 5, lanes a vs. b) and illustrates the appearance of S200 and S197 in the optic tract by 5 d (Fig. 5, lane d) as predicted from a Group IV transport rate (1 mm/day) (Fig. 5, lanes c vs. d).
Figure 3. Two-dimensional peptide map analyses of Triton-insoluble 200-kD proteins from mouse optic axons. Each of the four Triton-insoluble individual 200-kD proteins was excised from SDS gels, iodinated with $^{125}$I, and digested with α-chymotrypsin (see Materials and Methods). Chymotryptic peptides, separated in the first dimension (left to right) by high voltage electrophoresis and in the second dimension (bottom to top) by TLC, were revealed by autoradiography. Homologous configurations of peptides derived from P210, P205, P200, and P197 (a−d, respectively) are indicated on each peptide map.

**Phosphorylation and Dephosphorylation of 200-kD Proteins**

The subunits of all classes of intermediate filaments appear to contain phosphate (83). Intravitreal injection of $^{32}$Porthophosphate in mice has also been shown to label a limited group of axonal proteins, which includes the three neurofilament subunits (61, 65). Among the 200-kD proteins, only the Triton-insoluble group incorporated $^{32}$P (Fig. 6, cf. lanes a and b). In separate experiments, glial proteins were radiolabeled in situ by incubating excised but otherwise intact optic nerves in the presence of $^{32}$Porthophosphate under conditions that preserve the cellular integrity of the tissue (Fig. 6, lanes c and d). Although numerous proteins including α-fodrin (Fig. 6, lane c) and myelin basic protein (not shown) were intensely labeled, no $^{32}$P radioactivity was detected in the 197–210-kD region of gel lanes containing Triton-insoluble proteins (Fig. 6, lane c). Two Triton-soluble proteins in this region of the gel incorporated low levels of radioactivity (Fig. 6, lane d).

To obtain evidence for the in vivo phosphorylation of each of the four 200-kD proteins, we took advantage of a previous
Figure 4. Two-dimensional peptide map analysis of Triton-soluble 200-kD axonal proteins. The \( \alpha \)-chymotryptic peptide maps of the Triton-soluble 200-kD proteins S200 (A) and S197 (B) were prepared from mouse optic axons as described in Fig. 3. Autoradiograms show common configurations of peptides.

**observation** (Fig. 2) that P197 was more prominently radiolabeled at the earliest postinjection timepoint and P205 and P210 were most abundant at longer survival times (also see below). Autoradiograms of 200-kD proteins in optic axons from mice obtained 5 h after injection of \([\text{32P}]\)orthophosphate revealed \( \text{32P} \) radioactivity evenly distributed within the region of the SDS gel that included P197 and P200 (Fig. 6, lane f). By 5 d after injection, radiolabeled forms in optic nerve (Fig. 6, lane g) and optic tract (Fig. 6, lane h) comigrated predominantly in the region of P205 and P210 and included only small proportions of P200 and little, if any, P197. The conclusion that each of the Triton-insoluble 200-kD proteins incorporated \( \text{32P} \) in vivo, suggested by these distribution patterns, was further supported by immunocytochemical studies below.

Certain structural features of neurofilament subunits, including phosphorylation state, may be distinguished by the differential reactivity of the subunit with antibodies directed against specific peptide sequences or epitopes containing or lacking phosphate (80). Individual 200-kD proteins excised from 3–7% polyacrylamide SDS gels were placed in adjacent lanes of a second SDS gel and subjected to electrophoresis again. The proteins were electroblotted onto nitrocellulose and immunostained with NF-H monoclonal antibodies. The four Triton-insoluble polypeptides were detected by immunostaining the transferred proteins with a monoclonal antibody (SM32) that recognizes phosphatase-insensitive (presumably nonphosphorylated) epitopes on neurofilament proteins (Fig. 7 A) (80). SM31 (Fig. 7 B) (80) and R3 (data not shown) (14), two monoclonal antibodies that exclusively recognize phosphorylated epitopes, also immunostained these proteins. In addition, EL, an antiserum which is also specific for phosphorylated epitopes (76) recognized all four forms (Fig. 7 C). The 197-kD variant, however, reacted less strongly compared to the other proteins with SM31 but not with SM32 (Fig. 7 B). This differential staining pattern was reproducibly seen in multiple analyses. In contrast to these results, the Triton-soluble 200-kD polypeptides did not cross-react with these antibodies (Fig. 7, lanes e and f).

The majority of phosphate groups on NF-H can be removed in vitro by alkaline phosphatase treatment (8, 31, 65). We used this strategy to examine whether the Triton-insoluble 200-kD proteins were all phosphorylated or included a population of unphosphorylated NF-H variants. Axonal cytoskeletal proteins before and after incubation with *E. coli* alkaline phosphatase for 5 h at 37°C were compared by Coomassie Blue staining and immunoblot analysis using the SM132 and SM131 antibodies (Fig. 8). Dephosphorylation was associated with the complete disappearance of the four Triton-insoluble 200-kD proteins from their typical migration positions on gels (Fig. 8, lanes a and c) and appearance of a major immunoreactive protein at 160 kD (Fig. 8, lanes b and d), where newly synthesized unmodified NF-H subunits comigrate (18, 61, 69). The phosphorylated and dephosphorylated NF-H subunits displayed a high degree of structural homology when two-dimensional iodopeptide maps were analyzed after digestion with \( \alpha \)-chymotrypsin: more than 95% of the major peptides were shared in common (Fig. 9).
Non-Uniform Distribution of 200-kD Proteins along Optic Axons

Neurofilaments are twice as numerous at distal levels of mouse optic axons than at proximal levels. To investigate the relationship of the individual Triton-insoluble 200-kD proteins (NF-H variants) to steady-state pools of neurofilaments, we examined the axonal distributions of these variants by measuring the total content of each variant in proximal, middle, and distal levels of the optic nerve and optic tract by laser densitometry after the proteins were separated by SDS-PAGE and stained with Coomassie Blue. The content of the 210- and 205-kD polypeptides, like that of NF-M and NF-L subunits, increased markedly from proximal to distal levels of optic axons (Fig. 10). The P200 variant, by contrast, displayed a relatively uniform distribution. Finally, P197 was the most prominent variant in proximal segments but was the least prominent variant at distal levels of optic axons (Fig. 10).

Association of 200-kD Proteins with the Stationary Neurofilament Network along Axons

Previous studies demonstrate that neurofilament proteins are associated with at least two types of neurofilaments in optic axons: those undergoing continuous slow axoplasmic transport and those that are stationary and comprise the predominant neurofilament network along optic axons (62). To seek differences in the association of individual Triton-insoluble proteins (solid lines) P210 (●), P205 (■), P200 (▲), and P197 (○), and two Triton-soluble 200-kD proteins (dotted lines), S200 (□) and S197 (○), was determined on fluorograms by laser densitometry after the proteins were separated by SDS-PAGE (3–7% polyacrylamide gradient). The relative distribution of each radiolabeled polypeptide along optic axons at 7 d after injection is illustrated. The optic chiasm begins after segment 5. The peaks of the transport wave for the labeled Triton-insoluble proteins (solid arrow) and Triton-soluble proteins (dotted arrow) at 3 d after injection are indicated to illustrate the movement of the wave during this time interval. (Inset) Autoradiogram from a single SDS gel (3–7% polyacrylamide gradient) containing radiolabeled proteins in Triton-insoluble (lanes a and b) and Triton-soluble (lanes c and d) fractions of optic nerve (lanes a and c) and optic tract (lanes b and d) 5 d after mice were injected intravitreally with [3H]proline. Protein loads on gels are the same as those in Fig. 2.

Figure 7. Immunoblot analysis of 200-kD proteins with anti–NF-H monoclonal antibodies directed against phosphatase-insensitive epitope(s) (SMI 32; A) or phosphatase-sensitive epitope(s) (SMI 31; B), and a polyclonal antiserum directed against phosphatase-sensitive epitope(s) on NF-H (7, 76) (EL; C). Triton-insoluble proteins from optic axons were separated by SDS-PAGE (3–7% polyacrylamide gradient). Individual bands corresponding to the four Triton-insoluble proteins P210 (lane e), P205 (lane b), P200 (lane c), and P197 (lane d), and two Triton-soluble proteins, S200 (lane e) and S197 (lane f), were separated by SDS-PAGE (3–7% polyacrylamide gradient), excised from the gel, subjected again to electrophoresis in adjacent lanes of a second gel, and then subjected to Western blot analysis. An amount of each polypeptide derived from an equivalent amount of fresh tissue (4 mg) was loaded into each lane.
Figure 8. SDS-PAGE analysis of NF-H subunits before and after neurofilaments in Triton-insoluble fractions of optic axons were dephosphorylated by incubation for 5 h in vitro with E. coli alkaline phosphatase. A combination of SMI 31 and SMI 32 antibodies was used in Western blot analysis to detect NF-H before (lane a) or after (lane b) dephosphorylation. Equal amounts of protein (120 μg) were applied to each lane. For comparison, the same SDS gel, after electroblotting, was stained with Coomassie Blue to demonstrate the remaining protein corresponding to NF-H before (lane c) and after (lane d) dephosphorylation.

200-kD proteins (NF-H variants) with these two types of neurofilaments, we compared the proportions of each radiolabeled variant as radiolabeled neurofilament proteins were initially entering axons in the advancing wavefront of slow axoplasmic transport and at stages during and after the integration of transported neurofilaments into the stationary cytoskeleton (Fig. 11). Mice injected intravitreally with [35S]methionine were analyzed at timepoints when (a) labeled neurofilaments were predominantly in a moving phase (3 d), (b) both moving and stationary neurofilaments were well radiolabeled (15 d), or (c) labeled stationary neurofilaments predominated along optic axons (60 d). These timepoints were based on the results of previous studies (62). The optic axon segments, dissected from mice at these three timepoints, were fractionated and the Triton-insoluble fraction was analyzed by SDS-PAGE. Densitometric scans of the autoradiographs from multiple gels demonstrated that, at 3 d after injection, polypeptides migrating within the 195–205-kD region of the gel were the most intensely labeled NF-H variants (Fig. 11). By 60 d after injection, labeling in the 195–197-kD region was markedly diminished; however, P210 was intensely labeled, and P205 also displayed increased radioactivity. At the 15-d postinjection timepoint, when both moving and stationary neurofilaments were radiolabeled, the radioactivity displayed a pattern intermediate between the 3- and 60-d timepoints.

Discussion

Multiple Structural Variants of NF-H in Axons

In this study, we have identified a set of four 197–210-kD polypeptides as variants of the NF-H subunit of neurofilaments. The property of Triton X-100 insolubility, a previously established characteristic of axonal neurofilaments (51), effectively distinguished all neurofilament-related 200-
kD proteins in optic axons from unrelated proteins with similar apparent molecular masses. Each of the four Triton-insoluble 200-kD proteins was synthesized in neurons (Figs. 1 and 2), advanced along axons predominantly or exclusively in the Group V (SCa) phase of axoplasmic transport (3, 89) (Fig. 5), and incorporated phosphate groups in vivo (30) (Fig. 6). In addition, each of the four proteins cross-reacted with antibodies to an epitope common to all classes of intermediate filament proteins (72) (data not shown) and with a set of monoclonal and polyclonal antibodies against NF-H (Fig. 7). Finally, the four polypeptides displayed more than 90% homology by two-dimensional peptide map analysis after &alpha;-chymotryptic digestion (Fig. 3). The extremely close structural homology between these NF-H variants suggests that relatively small modifications that influence the charge or conformation of the protein accounted for apparent molecular mass differences on SDS gels. Although closely related, the individual NF-H variants could be clearly distinguished from each other by differences in their electrophoretic mobilities, their relative specific radioactivities at different times after pulse-labeling with [35S]methionine in vivo, and their regional distribution along axons.

Two Triton-soluble 200-kD proteins, by contrast, satisfied few if any conventional criteria for neurofilament protein identification. Although they displayed apparent molecular masses roughly similar to those of certain NF-H variants, they were transported more rapidly than neurofilament proteins Group IV or SCb transport phase (Fig. 5), did not incorporate phosphate in vivo (Fig. 6), and were not recognized by antibodies to NF-H (Fig. 7) or to the general class of intermediate filaments (72). They displayed some structural similarities to each other but only limited peptide homology with the NF-H variants (Fig. 4). Despite some structural similarities with NF-H, the Triton-soluble 200-kD proteins do not represent nonphosphorylated forms of NF-H, since unmodified NF-H subunits, as well as enzymatically dephosphorylated NF-H subunits, display an apparent molecular mass of 160 kD and retain more than 90% of peptide homology with extensively modified, insoluble forms of NF-H (Fig. 9). The high degree of homology between iodopeptide maps of phosphorylated and dephosphorylated forms of NF-H is not unexpected since many of the phosphate-rich chymotryptic peptides are unlabeled or weakly labeled by 125I (Sihag, R. K., and R. A. Nixon, unpublished observations), suggesting, therefore, that they either lack iodination sites or that phosphorylation blocks iodination of potential sites on these peptides. It is also difficult to account for S200 and S197 as soluble proteolytic derivatives of NF-H variants since they contained no label after [32P]orthophosphate administration. The heavily phosphorylated region of NF-H is at least 65 kD in size (16), and its removal should result in large molecular mass changes. This conclusion is further supported by the observation that each of the Triton-soluble proteins displays many &alpha;-chymotrypsin peptides that are not shared by any of the NF-H variants (Fig. 4).

One or both of the 200-kD soluble polypeptides may correspond to brain myosin (48), which migrated to an apparent molecular mass in the 197-200-kD range (Lewis, S. E., and R. A. Nixon, unpublished observations). In addition, we have observed that several [35S]methionine-labeled axonal polypeptides with similar apparent molecular masses coassemble with microtubules, suggesting that one or both may be microtubule-associated proteins (Nixon, R. A., and I. Fischer, unpublished observations). A group of similarly sized microtubule-associated proteins has been observed in rat liver (35).
**Microheterogeneous Variants of NF-H Are Phosphoproteins**

Previous studies suggest several possible mechanisms to account for NF-H microheterogeneity, which was initially noted in early two-dimensional gel analyses of axonal neurofilament proteins (5, 12). The existence in rabbits of two NF-H isoforms differing in apparent molecular mass by 10,000 Da is attributed to genetic polymorphism (86, 87). This phenomenon has not been observed in other species, including mice. Other examples of neurofilament protein microheterogeneity reflect modifications to the protein after it is synthesized. Limited proteolysis during axoplasmic transport, for example, generates major NF-M variants of lower apparent molecular mass that display a non-uniform distribution along optic axons (63, 67). These limited proteolytic modifications of NF-M within axons are distinct from the degradation of neurofilament polypeptides that reach nerve terminals (37, 58). In similar extensive studies of NF-H, we were unable to demonstrate interconversion of NF-H variants by limited proteolysis in vivo or in vitro (Lewis, S. E., and R. A. Nixon, unpublished observations).

Neurofilament protein microheterogeneity is also generated by protein phosphorylation (4, 18, 20, 30, 41, 69, 80) and dephosphorylation (60). The immunocytochemical detection of phosphorylated neurofilaments predominantly in axons and relatively less phosphorylated or unmodified subunits in neuronal perikarya (2, 41, 42, 80) has been corroborated by biochemical studies showing that newly synthesized NF-H and NF-M are extensively phosphorylated as they enter axons (1, 4, 18, 61). These previous studies of various neuron types have identified two NF-H variants, a relatively unmodified (160-kD) form predominating in perikarya and a phosphorylated (200-kD) form present in axons. The possibility that neurons contain multiple phosphorylated NF-H variants as major constituents of the axonal neurofilament lattice, however, was suggested by observations that each of the individual triplet proteins continues to undergo unique sequences of phosphorylation and dephosphorylation events during axoplasmic transport (60, 65).

Several lines of evidence indicate that microheterogeneity of NF-H subunit structure in optic axons arises by phosphorylation, and possibly dephosphorylation, during axonal transport. NF-H, the most highly phosphorylated subunit, contains between 25 and 50 phosphate groups per molecule (8, 29, 30). Since phosphate addition slows the electrophoretic mobility of many proteins (55), including NF-H (30, 34), varying phosphorylation states can easily account for differences in apparent molecular masses of the NF-H variants. When neurofilaments from mouse optic axons were enzymatically dephosphorylated, the four NF-H variants disappeared, giving rise to a 160-kD polypeptide (Fig. 8). This extensively dephosphorylated form comigrates on SDS gels with newly synthesized (unmodified) NF-H subunits labeled in vivo (61). These observations suggest that the varying molecular masses of the NF-H variants do not reflect size differences but, rather, changes in the electrophoretic mobility of the polypeptide induced by phosphate addition (34). Although it is conceivable that the varying electrophoretic mobilities of NF-H isoforms could reflect different conformational states of identically phosphorylated molecules, other data suggest that NF-H variants differ in net phosphorylation state. Previous studies indicate that phosphorylated NF-H subunits with the slowest electrophoretic mobility on SDS gels display a more acidic charge by IEF than subunits with a faster mobility (20, 82). Furthermore, the conversion of NF-H variants from lower to higher apparent molecular masses during axonal transport in optic axons (Figs. 2 and 6) is associated with a shift in isoelectric point of the NF-H subunit pool toward more acidic pH values, which is consistent with phosphate addition (65).

Available evidence suggests that phosphorylation may serve as a mechanism for coordinating the interactions of neurofilaments with other cytoskeletal elements during axoplasmic transport (65, 68). Neurofilament protein phosphorylation is a dynamic process involving multiple protein kinases (32, 33, 43, 77) and phosphatases (60). Not only do neurofilaments continue to be phosphorylated as they are transported along axons (65) but initially incorporated phosphate groups are also turned over with a specific timing and dependence on location within the axon (60). The rate of phosphate turnover is different for each of the neurofilament triplet proteins (60), and phosphates are removed preferentially from specific sites on at least the NF-L and NF-M subunits. Kinase and phosphatase systems involved in neurofilament phosphorylation, therefore, have the diversity and apparent specificity required of a mechanism for regulating complex protein–protein interactions. In addition to these observations, it is known that phosphates are primarily added to the carboxy-terminal tailpiece of NF-H which protrudes from the neurofilament core and presumably interacts with other cytoskeletal proteins (8, 16, 32). Electron microscopic studies (25, 75, 88) support the function of the peripheral domain of NF-H, in particular, as a cross-link between neurofilaments and microtubules. Other cytoskeletal proteins that may link intermediate filaments with microtubules or with the plasma membrane have also been shown to be phosphoproteins (55). For some of these proteins, phosphate addition or removal from specific sites on the polypeptide markedly changes its binding affinities (23, 28, 47, 53, 73, 74).

**Functional Implications of NF-H Microheterogeneity**

The presence of multiple NF-H variants within axons of a single class of central neurons raises the possibility that these isoforms serve different functions. The suspected role of the NF-H subunits in forming cross-links between cytoskeletal elements (24, 25, 75, 88) is an additional reason to view the structural diversity of NF-H in functional terms. NF-H subunits form 4–6-nm-diam and 20–60-nm-long cross-bridges that link neurofilaments to other neurofilaments and possibly to microtubules and membrane-bound organelles (24). In addition, cross-bridges of different dimensions have been observed between neurofilaments and other organelles (24) although the participation of NF-H has not been firmly established. In developing neurons, the late appearance of phosphorylated forms of NF-H, after that of NF-L and NF-M (13, 71, 87), coincides with the transition from a state in which few interneurofilamentous cross-bridges exist to one in which most axonal neurofilaments are extensively cross-linked. This major reorganization event during development may reflect changes in the functional state of the neurofilamentous cytoskeleton.

Mature retinal ganglion cell neurons contain two distinct axonal pools of neurofilaments which may have different functions (62). One pool, which is conveyed along axons in...
the slowest phase of axoplasmic transport (Group V or SCa),
displays a half-life of 17-20 d. These axonally transported
neurofilaments contribute to a second, larger population that
is stationary and forms a non-uniform neurofilament network
along axons. This network, together with other stationary
cytoskeletal elements (62), comprises a complex that turns
over with a relatively long half-life (>55 d) (62). We have
proposed that moving neurofilaments, unassociated or weak-
ly associated with other cytoskeletal proteins, represent a rel-
ative gately rapid mechanism for modifying axonal geometry to
effect, for example, moderate changes in axonal caliber (27)
or to repair local injury (58). While providing structural sup-
port, moving neurofilaments could also allow the degree of
plasticity required by, for example, a growing axon during
development. By contrast, the stationary neurofilament lat-
tice in mature neurons is well suited as a framework to stabi-
lize a given axonal shape, direction, or spatial orientation,
and to organize and anchor constituents within the axoplasm
(61, 62).

Our results provide evidence for a differential association
of NF-H variants with the moving and stationary neurofila-
ments networks in optic axons. The 210- and 205-kD variants
were observed to be major NF-H constituents of the stan-
dary neurofilamentous cytoskeleton. These NF-H variants ap-
pear to arise from one or more of the lower molecular mass
variants during axoplasmic transport since a shift from pre-
dominantly 197-205-kD forms at 3 d after pulse-radiola-
beling (e.g., Figs. 2 and 11) to predominantly 205-210-kD
forms at 15 d (Fig. 11) occurs without a significant decrease
in total NF-H radioactivity (62). This conversion accords
with previous observations that the phosphorylation state of
NF-H increases during this time interval as axoplasmic
transport proceeds (65). The 197-kD NF-H variant, by con-
trast, appears to be predominantly associated with the popu-
lation of moving neurofilaments. This polypeptide was the
principal NF-H form entering optic axons within 3 to 48 h
after pulse-labeling in vivo (Figs. 6 and 11), and transport
kinetics indicate that most neurofilaments at very proximal
levels of optic axons are moving (62). Estimates of the total
content of the four NF-H variants along axons (Fig. 10), in
addition to showing that each is a major axonal protein, fur-
ther support the distinction between neurofilaments predomi-
nantly composed of 197-kD NF-H subunits and those con-
taining the 205- and 210-kD NF-H forms. The proximal to
distal increase in P210 and P205 content along optic axons
parallels the non-uniform deposition of neurofilaments con-
taining radiolabeled P210 and P205 variants into the station-
ary cytoskeleton (62). If P197 were associated primarily with
moving neurofilaments, its steady-state level along axons can
only be expected to diminish proximally to distally, as we ob-
served, when moving neurofilaments containing P197 vari-
ants become stationary neurofilaments containing P205 and
P210 variants. Finally, P200 was a component of radiola-
beled neurofilaments entering optic axons and those remain-
ing in optic axons at 60 d after [35S]methionine injection.
The smaller proximal to distal differences observed in the
content of this variant along axons are, therefore, consistent
with its apparent association with moving and stationary
neurofilaments.

Changes in the phosphorylation state of NF-H, reflected
by the relative proportions of each NF-H variant, could, in
fact, be viewed as a mechanism to regulate partitioning of
neurofilaments between the moving and stationary neurofila-
ment network. An analogy may be drawn to previously ob-
served effects of phosphorylation on myosin structure (11).
In the unphosphorylated state, myosin head domains are held
down in the myosin filament backbone by head–head or
head–backbone interactions. Phosphorylation inhibits these
interactions causing the head domains to extend outward,
which thereby facilitates cross-bridge formation with actin.
By analogy, relatively dephosphorylated carboxyl-terminal
domains of NF-H (and possibly NF-M) would be expected
to have increased affinity for the filament core and to effect,
therefore, a conformation of the neurofilament that is steri-
cally favorable for its entry into the axon and its initial trans-
port. Indeed, neurofilaments in some neurons appear to
move at faster rates as they initially enter axons than when
they reach more distal levels of the axon. Progressive phos-
phorylation of the carboxy-terminal domains of NF-M and
NF-H would reduce interaction with the filament core, and
the resulting radial extension of these domains would be ex-
pected to retard neurofilament movement and favor inter-
actions with axonal elements, including constituents of the
stationary cytoskeleton. Different conformations of the car-
boxy-terminal domains, represented by the different NF-H
variants, might, therefore, define certain properties of the
cross-bridges such as the spacing, reversibility, and speci-
ficity for moving or stationary elements.

Functional distinctions among different NF-H isoforms
are further suggested by immunoblot analyses with a mono-
clonal antibody that recognizes a phosphatase-sensitive epi-
tope on NF-H (2, 13). This antibody has been previously
shown to immunostain axons only late in neuron develop-
ment, considerably after the NF-H polypeptide appears and
undergoes a certain degree of posttranslational modification
by phosphate (2, 13). We have observed that this antibody
recognizes the 210- and 205-kD NF-H variants and to a much
lesser extent recognizes the 200 kD NF-H variant, but does
not immunostain the 197-kD form (Nixon, R. A., S. E.
Lewis, and D. Dahl, unpublished observations). Thus, the
same NF-H variants that are distinguished by their differen-
tial associations with moving and stationary neurofilament
networks appear also to be distinguished by the timing of
their appearance during development.

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