

# Slow Posttranslational Modification of a Neurofilament Protein

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**ABSTRACT** The synthesis and subsequent modification of neurofilament (NF) polypeptides has been examined in pulse-chase experiments, using cultured chick spinal cord neurons. Fluorography of the [<sup>35</sup>S]methionine-labeled cytoskeletal proteins, after separation by two-dimensional gel electrophoresis, revealed that (a) the mid-size chicken NF protein, NF-M160, is synthesized as a smaller and more basic precursor, NF-M130; (b) beginning ~8 h after translation, NF-M130 slowly and continuously becomes larger and more acidic, attaining the size and charge of NF-M160 16 or more h later, and undergoing no further change in mobility for many days thereafter; and (c) in contrast, the low molecular weight NF protein, NF-L, is synthesized as such, and undergoes no subsequent change in apparent size or charge.

Additional experiments provided evidence that the conversion of NF-M130 to NF-M160 is due, at least in part, to phosphorylation: (a) Incubation of similar cultures in <sup>32</sup>PO<sub>4</sub> resulted in incorporation into NF-M160 and transitional forms, but not into NF-M130. (b) An antiserum to NF-M160 was found by immunoblot analysis to bind strongly to untreated NF-M160, but poorly to phosphatase-treated NF-M160, and not at all to NF-M130. It has already been demonstrated (Bennett, G. S., S. J. Tapscott, C. DiLullo, and H. Holtzer, 1984, *Brain Res.*, 304:291–302) that this anti-NF-M160 fails to stain the soma of motor neurons in sections of chick spinal cord, but detects an increasing gradient of immunoreactivity in the proximal axons.

These results, together with the known kinetics of axoplasmic transport of NF, suggest that the mid-size chicken NF protein is synthesized as NF-M130 and is extensively modified, at least in part by phosphorylation, to become NF-M160 during transport along proximal neurites. Once maximally modified, NF-M160 undergoes no further net change during transport along distal neurites.

A number of studies indirectly indicate that one or more of the three neurofilament (NF)<sup>1</sup> proteins can undergo posttranslational processing that may occur during axoplasmic transport and/or be restricted to only certain parts of a neuron. For example, Nixon et al. (14) report three distinct NF-M size variants in mouse optic nerve, one of which is present in distal but not proximal segments. They emphasize proteolytic processing during transport as a possible mechanism for generating this component. Others, using an immunochemical approach, have obtained several polyclonal (4, 6, 9, 17) and monoclonal (19) antibodies against NF-M and NF-H that bind to axons but not to some neuronal soma or dendrites.

Of particular interest is the demonstration by Sternberger and Sternberger (19) that a set of such monoclonal antibodies apparently distinguishes phosphorylated from nonphosphorylated NF-H, suggesting that in some neurons NF-H is phosphorylated only in the axon. However, there is as yet no direct evidence to support the existence of such localized processing.

We have been examining the synthesis of the NF proteins in cultured avian spinal cord neurons. In the course of these studies, we detected an unusually slow, large, and continuous change in the electrophoretic mobility of one NF protein. The mid-sized chicken NF protein (3) (with a molecular weight of 160,000 and so termed NF-M160) is synthesized as a 130,000-mol-wt precursor (NF-M130), which, after a delay of several hours, slowly undergoes a substantial increase in apparent size and decrease in isoelectric point, to arrive at its definitive

<sup>1</sup> Abbreviations used in this paper: NF, neurofilament; NF-L, NF-M, and NF-H, the low, mid, and high molecular weight neurofilament proteins, respectively.

size and charge ~24–48 h later. No detectable change in size or charge occurred thereafter. In this communication, we describe these experiments and others which together provide evidence that this posttranslational modification is at least partially due to phosphorylation during transport in the proximal neurite.

## MATERIALS AND METHODS

**Cell Culture:** Spinal cord cells from 6-d-old chick embryos were dissociated by trypsinization and plated at  $2.5 \times 10^6$  cells per 35-mm collagen-coated plastic culture dish in medium consisting of 80% minimum essential medium (Gibco Laboratories, Grand Island, NY), 10% horse serum, 10% embryo extract, and antibiotics. Cytosine arabinoside was added at day 1 or 2 for 2 or 3 d to deplete (but not eliminate) dividing (nonneuronal) cells. Cultures were maintained thereafter in medium with reduced embryo extract content (2%).

**Incorporation of [ $^{35}$ S]Methionine and [ $^{32}$ P]Phosphate:** [ $^{35}$ S]methionine (>800 Ci/mmol; Amersham Corp., Arlington Heights, IL) was added at 100  $\mu$ Ci per dish in 1 ml of complete culture medium (for 24 h continuous labeling) or of methionine-free minimum essential medium (for 3-h pulse). In pulse-chase experiments, dishes were rinsed three times with complete culture medium at the end of the 3-h pulse and further incubated in complete medium for times from 5 h to 8 d. Incorporation of [ $^{32}$ P]orthophosphate (carrier-free, as disodium phosphate, New England Nuclear, Boston, MA) was carried out in phosphate-free minimum essential medium (Flow Laboratories, Inc., McLean, VA) for 2.5 h, using 100  $\mu$ Ci/ml/dish. Cultures were labeled at various times between 5 and 21 d in vitro with similar results.

At the time of sacrifice, cultures were washed three times with PBS at 0°C, then scraped and homogenized in 2 ml/dish of PBS containing 0.5% Triton X-100, 3 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, and 100  $\mu$ g/ml leupeptin. In the case of  $^{32}$ P-labeled cultures, 25 and 50 mM NaF was added to the PBS and PBS-Triton solutions, respectively. Homogenates were stored at -70°C for later analysis.

**Analysis of [ $^{35}$ S]- and [ $^{32}$ P]-labeled Culture Proteins:** The Triton-insoluble material (cytoskeletal fraction) was separated from the soluble fraction by centrifugation at  $5,000 \times g$  for 10 min, extracted with acetone and then with chloroform:methanol (2:1) at 0°C and solubilized in electrophoresis sample buffer (12). The intermediate filament proteins of many different types of cells are essentially completely recovered in the detergent-insoluble fraction (e.g., reference 7), and this was found to be true of NF in neuronal cultures as well (see Results).

The Triton-soluble and -insoluble proteins, both with and without added unlabeled NF standard, were separated by one-dimensional SDS PAGE (10% acrylamide; Tris-glycine buffer system [12]) and by two-dimensional isoelectric focusing/SDS PAGE. The latter was carried out essentially according to the methods of O'Farrell (15), using 4 parts pH 4–6 and 1 part pH 3–10 ampholytes (Bio-Rad Laboratories, Richmond, CA) for the focusing gels, and 10% acrylamide for the second dimension (5). Samples containing  $1\text{--}2 \times 10^5$  cpm were mixed with an equal volume of lysis buffer (15) for application to the isoelectric focusing gels (5). The stained and destained gels were impregnated with ENHANCE (New England Nuclear;  $^{35}$ S only), dried and exposed to Kodak XAR-5 (for  $^{35}$ S) or Dupont Cronex 4 (for  $^{32}$ P) film for 4–7 d.

**Bulk NF Preparation:** The high salt and detergent-insoluble fraction (23) of whole adult chicken brain was used as the standard source of NF proteins for co-migration with labeled culture proteins, and for immunoblot analyses. The final suspension was precipitated with acetone, extracted with chloroform:methanol (2:1), and solubilized in electrophoresis sample buffer (5).

**Immunoblots:** The NF fraction was separated on one- and two-dimensional gels as above, and transferred to nitrocellulose electrophoretically (20). Incubation of the nitrocellulose strips in rabbit antiserum, goat anti-rabbit IgG, peroxidase-anti-peroxidase, and development with diaminobenzidine and  $\text{H}_2\text{O}_2$  was exactly as previously described (5). The rabbit anti-NF-M160 has been characterized (4), and shown on one-dimensional immunoblots to react only with NF-M160. Treatment of transferred proteins with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) (type VII-T; 100  $\mu$ g/ml) was carried out at 37°C for 2.5 h in 0.1 M Tris, pH 8.0, containing 1 mM phenylmethylsulfonyl fluoride (19).

**Immunohistochemistry:** Cultures were established as above, except for plating at  $5 \times 10^5$  cells/ml in 35-mm culture dishes containing squares of Aclar (Allied Chemical Corp., Waltham, MA). Fixation and incubations for indirect immunofluorescence were as previously described (4).

## RESULTS

When cultures were exposed to [ $^{35}$ S]methionine continuously for 24 h, fluorography of two-dimensional gels containing the Triton-insoluble proteins revealed (Fig. 1b) substantial label incorporated into the 70,000-mol-wt band; the position of the low molecular weight chicken NF protein (NF-L) (3). However, radiolabeled NF-M160 was barely detectable and it merged with an oblique, increasingly radioactive streak ending in a band of smaller size ( $M_r$  130,000) and more basic pI. The latter band did not correspond to any obvious component in the NF standard, although careful inspection of Coomassie Blue-stained two-dimensional gels containing maximal loads of the standard alone revealed trace amounts of both the 130,000-mol-wt polypeptide and the streak bridging it with NF-M160 (Fig. 1a). The high molecular weight chicken NF ( $M_r$  180,000–205,000) (3, 8) is present in very low concentrations relative to NF-L and NF-M160 (Fig. 1a and reference 8), and was not included in the present analysis.

If, instead of continuous labeling, cultures were exposed to a 3-h pulse of [ $^{35}$ S]methionine and then either sacrificed

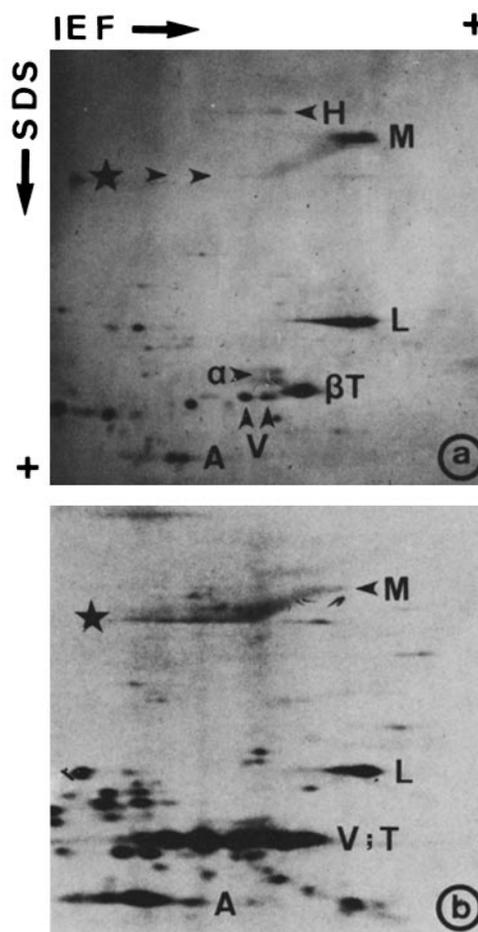
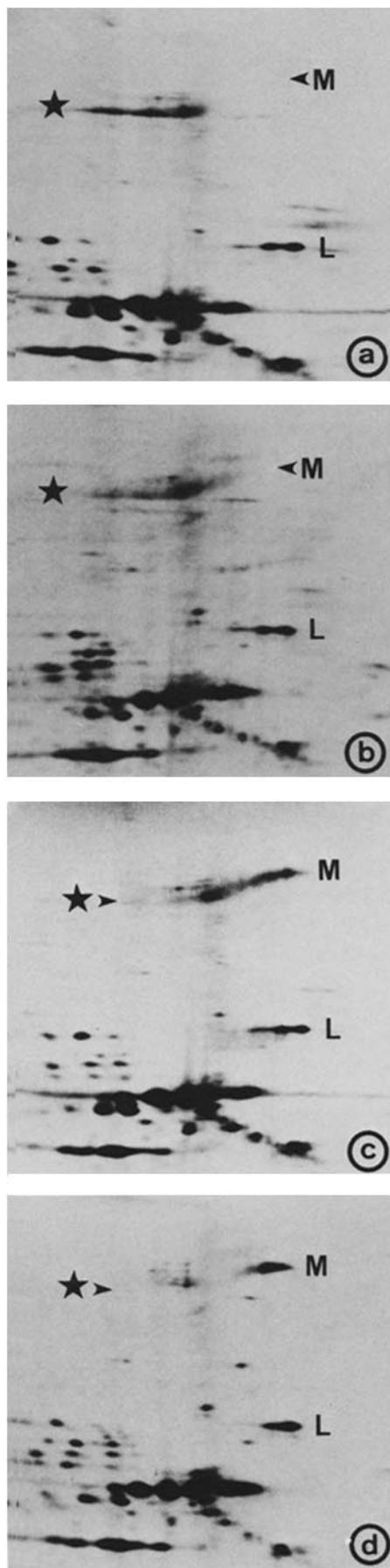


FIGURE 1 (a) Coomassie Blue-stained two-dimensional gel of the NF fraction of whole adult chicken brain (50  $\mu$ g total protein). (b) Fluorograph of a two-dimensional gel containing detergent-insoluble proteins from a culture that had been incubated with [ $^{35}$ S]methionine continuously for 24 h. (H, M, and L) NF-H, NF-M160, and NF-L, respectively; (V) vimentin; ( $\alpha$  and  $\beta$ )  $\alpha$ - and  $\beta$ -tubulin; (A) actin; (\*) presumptive NF-M160 precursor, NF-M130. The actin, vimentin, and tubulin spots in b are overexposed and therefore appear poorly resolved.



immediately or chased for 5 h in unlabeled medium and then sacrificed (Fig. 2a), no incorporation at all into NF-M160 could be detected, although NF-L again was heavily labeled. Additionally, the 130,000-mol-wt band was now sharp and discrete in size, although it extended over several tenths of a pH unit. An equally broad range of pI values was obtained after a 1-h pulse. The absence of radiolabeled NF-M160 from the Triton-insoluble fraction was not due to its failure to pellet with the cytoskeletal proteins, because no labeled NF-M160 (or NF-L) could be detected on fluorographs of the Triton-insoluble fraction.

Longer chase times revealed that the 130,000-mol-wt component was gradually being converted into NF-M160. After 8 h (Fig. 2b), the 130,000-mol-wt band began to spread towards larger size and lower pI. By 21 h, small amounts of label could be detected at the position of the NF-M160 standard, and by 48 h (Fig. 2c) the NF-M160 spot was substantially labeled, with virtually no label remaining at the 130,000-mol-wt position. At both 21 and 48 h, however, some label remained in the oblique streak. Only after 4 d (Fig. 2d) was there no longer detectable radioactivity in the streak. For at least the next four d (8-d chase, the longest examined), no further change in size or charge occurred in the NF-M160-associated label. At no point in these pulse-chase experiments was there any change in the position of label associated with NF-L or with vimentin (contributed by the remaining non-neuronal cells) or, indeed, with any other component of the detergent-insoluble fraction.

These results suggested that the 160,000-mol-wt NF-M polypeptide present in bulk NF preparations was not synthesized as such, but rather as a 130,000-mol-wt precursor (~30,000 less) which was subsequently modified in some way that increases its apparent size. However, isolation and analysis of most intermediate filament proteins, including the NF polypeptides, often results in the presence of proteolytic degradation products. Conceivably, newly synthesized NF-M160 may be unusually susceptible to proteolysis and may become more resistant with time. Thus, it is possible that the large difference in apparent size between newly synthesized and previously accumulated NF-M160 is an artifact generated upon disruption of the cells, despite the use of several protease inhibitors. This was rendered unlikely by sacrificing cultures at the end of a 3-h pulse of [<sup>35</sup>S]methionine by flooding the dish with acetone or 10% trichloroacetic acid at 0°C, and immediately solubilizing the entire precipitate by boiling in electrophoresis sample buffer, thus minimizing opportunities for artifactual degradation. Fluorographs of two-dimensional gels containing these total cellular proteins produced results identical to those obtained after detergent extraction: a radiolabeled band identical in charge and size to NF-M130.

The fact that the NF polypeptides contain large amounts of phosphate (10, 11, 13, 16, 18) suggested the possibility that the conversion from NF-M130 to NF-M160 entails phosphorylation. When cultures were incubated in [<sup>32</sup>P]phosphate,

FIGURE 2 Fluorographs of two-dimensional gels containing the detergent-insoluble proteins from cultures that had been exposed to a 3-h pulse of [<sup>35</sup>S]methionine and chased for 5 (a), 8 (b), 48 (c), and 96 (d) h. (M, L, and \*) NF-M160, NF-L, and presumptive NF-M160 precursor, NF-M130, respectively.

both NF-M160 and NF-L (and vimentin) were heavily labeled (Fig. 3). Additionally, less label was incorporated into the oblique streak between NF-M160 and the acidic end of NF-M130, but none was detected in most of the broad pH range over which NF-M130 extends. Thus, it appears that NF-M160 and the intermediate forms, but not NF-M130, contain exchangeable phosphate.

We have previously reported (4) that an antiserum against chicken NF-M160 fails to bind to the soma of motor neurons in frozen sections of chick spinal cord, but detects an increasing gradient of NF-M160 immunoreactivity in the first several hundred micrometers of the axons of these neurons. In cultures of chick spinal cord neurons, this antiserum stains neurites brightly, but does not stain most neuronal soma (Fig. 4). Using the procedure developed by Sternberger and Sternberger (19), we examined the effect of treating chicken NF-M160 with alkaline phosphatase, after transfer to nitrocellulose, on its ability to bind anti-NF-M160 (Fig. 5). Phosphatase

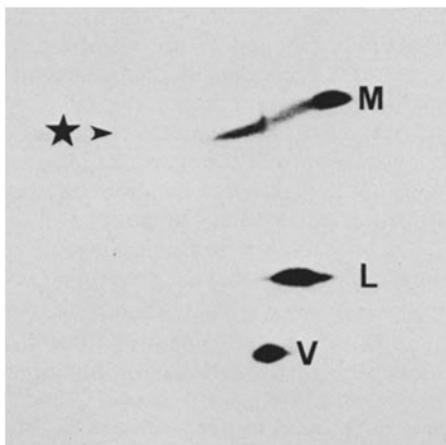


FIGURE 3 Autoradiograph of a two-dimensional gel containing the detergent-insoluble proteins of a culture incubated in [ $^{32}$ P]phosphate for 2.5 h. (M, L, V, and \*) NF-M160, NF-L, vimentin, and NF-M130, respectively.

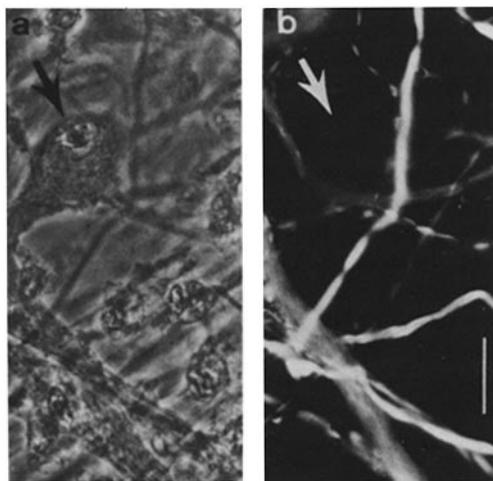


FIGURE 4 Phase-contrast (a) and fluorescence (b) micrographs of a single field in a spinal cord culture stained with anti-NF-M160. The arrow indicates a large neuronal soma that is not stained by the antibody. Bar, 20  $\mu$ m.  $\times 500$ .

treatment completely eliminated binding of this antibody to the same amount of NF-M160 (0.6  $\mu$ g) that strongly bound the antibody on the untreated strip, and only slight binding was obtained with greater amounts. Although smaller loads were not run in this experiment, an intense band can still be obtained on untreated strips with at least a threefold further reduction in protein loaded (see reference 5, Fig. 14). The great diminution in the ability of NF-M160 to bind anti-NF-M160 upon removal of phosphate suggests that this anti-NF-M160 is directed against a phosphate-dependent antigenic site(s) on NF-M160.

If NF-M130 is the non- (or partially-) phosphorylated form of NF-M160, and anti-NF-M160 is specific to completely phosphorylated NF-M160, this antiserum should not bind to NF-M130. We examined this with an immunoblot of a two-dimensional gel to which a maximal load of the NF preparation had been applied (same amount as for Coomassie Blue-stained gel in Fig. 1a). When stained with anti-NF-M160 (Fig. 6), an intense spot was obtained at the position of NF-M160, and several spots and streaks of smaller size and more acidic pI were also prominent. The latter correspond to degradation products of NF-M160 (4), which are exaggerated by the large load (at least 150 times that needed to detect NF-M160 (see reference 5, Fig. 15), and by prolonged development in diaminobenzidine to ensure the detection of low levels of binding (the NF-M160 spot appeared in seconds; the others appeared only several minutes later). However, there was no binding at the position of NF-M130 or the oblique streak. This is not likely to be due entirely to insufficient NF-M130 on the blot because this load does result in visible Coomassie Blue stain at the NF-M130 and streak positions, whereas the NF-M160 degradation products, which bind the antibody, are not visible in Coomassie Blue-stained gels (Fig. 1a). Furthermore, identical immunoblots incubated with other antisera prepared against NF-M in different rabbits do result in binding to NF-M130 and the streak as well as to NF-M160 (not shown). Thus, the anti-NF-M160 that does not

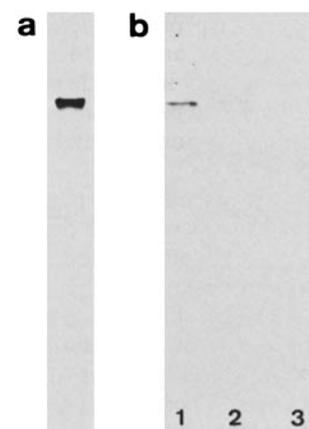


FIGURE 5 Immunoblots of the NF fraction of whole adult chicken brain, separated by SDS PAGE, transferred to nitrocellulose, and stained with anti-NF-M160 (1/500 dilution). Strip a was incubated in alkaline phosphatase (see Materials and Methods) for 2.5 h, whereas strip b was incubated in buffer alone, prior to incubation of both strips in antiserum. All subsequent steps, including photography, were identical for both strips. The amount of total protein loaded in each lane was 0.6  $\mu$ g for strip a and 4 (lane 1), 1 (lane 2), and 0.6 (lane 3)  $\mu$ g for strip b.

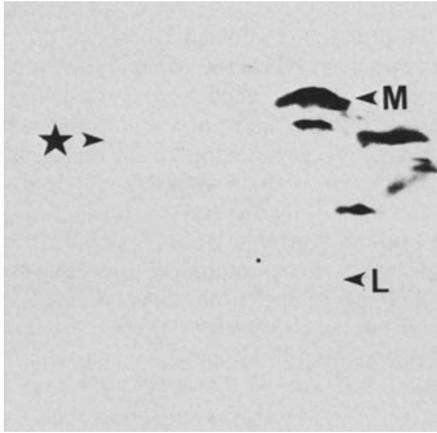


FIGURE 6 Immunoblot of the NF fraction of whole adult chicken brain, separated by two-dimensional gel electrophoresis, transferred to nitrocellulose, and stained with anti-NF-M160 (1/500 dilution). Amount of total protein loaded was 50  $\mu$ g. Compare with Coomassie Blue-stained gel containing the same amount of protein (Fig. 1a). (M and \*) NF-M160 and NF-M130.

bind to phosphatase-treated NF-M160 also does not bind to NF-M130. This result is consistent with the suggestion that NF-M130 is a less-phosphorylated form of NF-M160. The results of the two immunoblot analyses together suggest that the gradient in immunoreactivity revealed by immunohistochemistry (4) is a gradient in the conversion, mainly via phosphorylation, of NF-M130 to NF-M160.

## DISCUSSION

The pulse-chase experiments clearly show a large and continuous posttranslational change in the electrophoretic mobility of chicken NF-M. The continuity between NF-M130 and NF-M160 on the fluorographs, the shift with time of label from the position of the former to that of the latter, and the detection of NF-M130 and transitional forms in bulk NF fractions from adult brain, leave little doubt that the NF-M130 polypeptide is the precursor to NF-M160. The presence of a continuum of polypeptides intermediate in size and charge between NF-M130 and NF-M160 suggests that the modification does not occur in a single step. The direction of the change (toward larger apparent size) is not consistent with proteolytic processing, nor do we see evidence of this at longer times after synthesis.

The results of [ $^{32}$ P]phosphate incorporation and immunoblot analyses are consistent with, but do not prove, the suggestion that this posttranslational modification involves, at least in part, phosphorylation. The  $^{32}$ P labeling experiment does not, by itself, permit estimation of the relative degree of phosphorylation of protein at the position of NF-M160 versus that at progressive positions along the oblique streak, and it does not rule out the incorporation, at a level below the limit of detection, of a small amount of phosphate into NF-M130. Similarly, we do not know how much phosphate was removed by the alkaline phosphatase treatment. The phosphate content of the chicken NF polypeptides has not been analyzed, but mammalian NF-M and NF-H are both highly phosphorylated (10, 11, 22), with bovine NF-H containing as many as 104 mol of phosphate per mol of polypeptide (10). The failure of anti-NF-M160 to bind to the intermediate streak (Fig. 6) could indicate that the epitope(s) is phosphorylated late in the progressive processing, or that it is not accessible or in the

proper conformation until phosphorylation is complete. Whether or not the degree of chicken NF-M phosphorylation, and/or anomalous migration in SDS gels, is sufficient to account for an increase in  $M_r$  of 30,000 remains to be determined. We cannot presently rule out the possibility that other, as yet unidentified, groups are added as well. Despite these uncertainties, the results of both types of analysis point strongly towards phosphorylation as the modification responsible for the conversion of NF-M130 to NF-M160, and for the remaining discussion we assume phosphorylation to be the only type of posttranslational processing to occur.

Recently, Wong et al. (22) isolated a less highly phosphorylated, soluble variant of bovine NF-M that does not assemble with the NF-L core. The relationship between this bovine variant and chicken NF-M130 is not clear at present, for their experiments did not demonstrate a precursor relationship of the variant to filament-associated, fully phosphorylated NF-M, and there was no difference between the two in apparent size. We did not find appreciable amounts of NF-M130 in the soluble fraction, although we do not know that NF-M130 is actually associated with filaments.

Interpretation of the delay after synthesis before modification of NF-M130 begins, and the prolonged time over which it occurs, requires consideration of neuronal architecture and the fact that the cellular components are continually moving. The NF proteins move down the neurites in the cytoskeletal compartment at the slowest transport rate (SCa or group V: 0.2–1 mm/day [1, 2]), although the rate may be higher in growing axons (21). The finding that nearly 24 h are required after synthesis, before completely phosphorylated NF-M160 has been produced, suggests that this processing occurs largely during transport along the initial portion of the neurite. Our previous immunohistochemical demonstration of an increasing gradient of NF-M-immunoreactivity progressing outward along the proximal portion of motor axons (4), and the present immunoblot results, are consistent with this localization. The fact that the modification is complete 2–4 d after synthesis, and that no further change occurs for many days thereafter, suggests that transport along the distal neurite occurs without further net change in degree of phosphorylation, and without proteolytic processing.

The spinal cord cultures used in these experiments contain different types of neurons and both axons and dendrites of varying lengths. The present experiments do not reveal whether all NF-M130 is quantitatively converted into NF-M160, or if, instead, as predicted by the immunohistochemical results from several laboratories (4, 6, 9, 17, 19), a fraction is transported without phosphorylation or with a lesser degree of phosphorylation, along some neurites. However, since no NF-M130 is detectable beyond 2 d, and no intermediate forms beyond 4 d, any NF-M130 that has not been completely phosphorylated must be degraded within that time, perhaps by being confined to short processes (dendrites?) and arriving at the terminals within 2–4 d. In addition, our results do not indicate whether or not complete modification can occur only within neurites. Indeed, the fact that anti-NF-M160 does bind to the soma of sensory neurons (4) suggests that the processing in some neurons can be carried out entirely within the soma. Further experiments are required to address these questions.

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