

Neuronal Intermediate Filament Protein α -Internexin Facilitates Axonal Neurite Elongation in Neuroblastoma Cells

Thomas B. Shea* and Mary Lou Beermann

Department of Biological Sciences, Center for Cellular Neurobiology and Neurodegeneration Research, University of Massachusetts at Lowell, Lowell

We examined the localization and role of α -IN vs. other neuronal intermediate filaments before and during differentiation. Vimentin but not α -IN localized within filopodia-like neurites of undifferentiated cells. During differentiation, α -IN immunoreactivity accumulated within axonal neurites following vimentin but, as previously describe in neurons in situ, before the appearance of NF-L. We therefore manipulated α -IN synthesis, accumulation, and function in attempts to determine whether or not this intermediate filament species played a role in axonal development. Intracellular delivery of anti- α -IN antisense oligonucleotides and antibodies was permissive for neuritogenesis, yet compromised neurite elongation; this effect was further reflected in diminished levels of stabilized axonal microtubules. These data suggest that α -IN plays a role in the development of neuronal polarity. Relatively more α -IN than NF-L accumulated within the plastic axonal neurites induced following serum-deprivation, while stable, dbcAMP-induced neurites treatment contained equivalent levels of each. Protease inhibition increased NF-L and NF-H but not α -IN immunoreactivity within serum-deprived neurites, suggesting that proteolysis restricts NF-L accumulation pending neurite stabilization. To test the possibility that NF-H accumulation is dependent upon NF-L and cannot be mediated by α -IN, we examined levels of NF-H co-precipitated from cells with α -IN and NF-L. Virtually all newly synthesized NF-H co-precipitated with NF-L, while only a small percentage co-precipitated with α -IN. Finally, NF-H or NF-M were absent from the axon hillock or perikaryal area at the base of neurites, where α -IN immunoreactivity is prominent. These data extend earlier cell-free demonstrations that NF-H preferentially associates with NF-L rather than α -IN. *Cell Motil. Cytoskeleton* 43:322–333, 1999. © 1999 Wiley-Liss, Inc.

Key words: differentiation; cytoskeleton; neurofilament; vimentin; 66 kDa neurofilament subunit; NF-66

INTRODUCTION

Following synaptogenesis, the extensively cross-linked infrastructure of an axon may remain basically unaltered throughout the lifetime of the neuron. Accordingly, the assembly and stabilization of the axonal cytoskeleton is a key event in the establishment of mature neuronal morphology, and, in turn, the orderly development of the nervous system and maintenance of its functionality. An accompanying challenge is for a neuron to effect the orderly transition of its axonal cytoskeleton from the presumably flexible structure of a rapidly

growing neurite to the stabilized latticework characteristic of mature axons.

The sequential expression and deposition within axons of several neuronal intermediate filament (IF)

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*Correspondence to: T.B. Shea, Center for Cellular Neurobiology and Neurodegeneration Research, Department of Biological Sciences, University of Massachusetts at Lowell, Lowell, MA 01854.
E-mail: Thomas_Shea@uml.edu

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species accompany nervous system development. These include vimentin (Vm) [Bignami et al., 1982; Cochar and Paulin, 1984], α -internexin (α -IN) [Kaplan et al., 1990] (also independently described as a "66kd neurofilament subunit" or NF-66) [Chiu et al., 1989], peripherin (in certain neurons) [Parysek and Goldman, 1987; Portier et al., 1984], and the neurofilament (NF) triplet [Carden et al., 1987; Shaw and Weber, 1982; for review of sequential neuronal IF expression, see Nixon and Shea, 1992]. Despite advances in understanding of IFs in non-neuronal cells [e.g., for review, see Skalli and Goldman, 1991], the respective roles of neuronal IFs in the establishment and maintenance of the axonal cytoskeleton remains controversial.

The hierarchical expression, post-translational modification, and axonal transport of neuronal IFs is to a large extent recapitulated during differentiation and axonal neurite elaboration of certain neurons and neuroblastoma in culture. Such culture systems, therefore, provide models for studies on neuronal IF function and interaction with other cytoskeletal elements. Intracellular delivery of antisense oligonucleotides and specific antibodies have demonstrated a novel role for Vm in neurite initiation in neuroblastoma and hippocampal neurons in culture. Specifically, Vm facilitates the initial outgrowth of filopodia-like neurites that will ultimately give rise to axonal neurites [Shea, 1990; Shea et al., 1993a; Boyne et al., 1996]. Similar approaches demonstrate that, by contrast, NFs do not participate in either the initiation or elongation of axonal neurites; rather, such studies have provided experimental evidence for the long-purported role of phospho-NFs in stabilization of axonal neurites [Shea and Beermann, 1994].

Studies to date are consistent with the interpretation that role(s) of α -IN may be distinct, although possibly overlapping, with that of other neuronal IFs, particularly with regard to development. α IN mRNA [Fleigner et al., 1994; Chan and Chiu, 1995] and protein [Chien et al., 1998; Kaplan et al., 1990; Galinovic-Schwartz et al., 1991] accumulate in a variety of CNS regions following the decline of Vm, yet prior to the appearance of NF-L. These findings suggest that α -IN may play a unique role in axonal development, including the possibility of providing a scaffold to facilitate subsequent NF deposition within the axonal cytoskeleton [Balin and Miller, 1995; Fleigner et al., 1994] and in some cases replacing NFs entirely [Chien et al., 1996; Chiu et al., 1989]. Herein, we examine the localization of α -IN before and during differentiation, and probe the putative functions of α -IN by intracellular delivery of an α -IN-specific antibody and antisense oligonucleotides, using a neuroblastoma cell line that expresses Vm, α -IN, and NFs.

MATERIALS AND METHODS

Cell Culture, Differentiation, and Radiolabeling

Mouse NB2a/d1 neuroblastoma cells were cultured in DMEM supplemented with 10% calf serum and 2 mM glutamine in multi-chamber glass culture slides (for immunocytochemistry) or 10 cm² Petri dishes (for radiolabeling and immunoblot analyses). To induce differentiation and the outgrowth of axonal neurites, cultures either were either deprived of serum, or received 1 mM dibutyl cyclic AMP (dbcAMP) in the presence of serum [Shea et al., 1985, 1989]. Additional cultures received the protease inhibitor C1 (1 mM; also known as "AllNaI") [Shea et al., 1995] simultaneously with serum-deprivation. Cultures were radiolabeled by aliquoting 1 mCi ³⁵S-methionine into 3 ml of methionine-free medium for 2 hr; these cultures were pre-incubated in methionine-free medium for 15 min prior to radiolabeling [Shea et al., 1990].

Treatment With Antisense Oligonucleotides

Sense- and antisense-oriented cDNA oligonucleotides were synthesized on an ABI DNA synthesizer (model 381B) using an standard phosphoramidite chemistry followed by partial purification as described previously [Shea et al., 1991, 1993a]. Oligonucleotides were generated beginning 12 bases upstream from the initiation codon and continuing to 9 bases downstream according the α -IN sequence of Fleigner et al. [1990; see also Chan and Chiu, 1995, 1996] as follows: sense orientation, written as 5'-3', GGC CCC GGC ACC ATG AGC TTC GCA; antisense orientation, written as 3'-5', TGC GAA GCT CAT GGT GCC GGG GCC (the initiation codon and its reciprocal is underscored). For immunocytochemical analyses following oligonucleotide treatment, cells were plated in 16-well Lab-Tek chamber slides in DMEM containing 10% serum. Twenty-four hours later, this medium was replaced with medium containing 150 μ l of the same medium containing 12.5 μ M sense or anti-sense oligonucleotides. Cultures were incubated for a total of 48 hr with or without oligonucleotides, followed by 4-hr treatment with dbcAMP to induce the outgrowth of axonal neurites [Shea et al., 1985, 1989]. Medium was replaced at 24-hr intervals along with oligonucleotides where appropriate. Oligonucleotide-treated and untreated control cultures were fixed and processed for immunocytochemistry and neurite outgrowth was quantified.

Microinjection of Antiserum

A rabbit polyclonal antiserum ("N1," generous gift of Dr. F.-C. Chiu), generated against the 66-kDa neuronal intermediate filament protein, was diluted 1:30 and 1:60 in phosphate-buffered saline (PBS; pH 7.4), mixed with fluorescein-conjugated dextran tracer (to identify injected

cells), and microinjected essentially as described previously [Cressman and Shea, 1995]. Additional cultures were injected with tracer in PBS alone. Cultures were incubated for 4 or 24 hr in the absence of serum prior to microinjection. Following injection, they were incubated an additional 4 hr in the absence of serum, fixed for 15 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and stored in PBS.

Immunocytochemistry and Immunoblot Analyses

Fixed cultures were extracted for 5 min with 1% Triton-X-100 in 50 mM Tris-HCl (pH 6.8). The order of extraction and fixation was reversed with no obvious alteration in immunocytochemical distribution of any neuronal IFs; however, fixation prior to extraction significantly improved continued adherence of cells to the slide during immunocytochemical procedures. Cultures were then incubated overnight at 4°C in 1:100–1,000 dilutions in PBS of N1 antisera, anti-NF-L antiserum L3 prepared in this laboratory [Shea and Beermann, 1994], a polyclonal antibody directed against Vm [Shea et al., 1993a], and monoclonal antibodies directed against phosphorylated (SMI-31) or non-phosphorylated (SMI-32) NF epitopes. Cultures were rinsed 3 × 5 min in PBS, incubated for 2 hr in a 1:1000 dilution of peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, rinsed 3 × 5 min in PBS, and visualized with diaminobenzidine in the presence of hydrogen peroxide by standard methods [e.g., Shea et al., 1989]. Processed cultures were coverslipped over a drop of PBS:glycerol (1:1). Additional unextracted cultures were immunostained with a monoclonal antibody, 6-11B-1 (generous gift of Dr. G. Piperno) directed against acetylated α -tubulin and previously utilized in these cells [Shea et al., 1990; Shea, 1998]. Immunocytochemical images are presented in reverse-contrast to improve clarity.

For immunoblot analyses, cultures were rinsed in PBS, scraped from the plate in 1% Triton-X-100 in 50 mM Tris-HCl containing 5 mM EDTA, 2 mM PMSF, and 50 μ g/ml leupeptin [Shea et al., 1993a]. Triton-insoluble cytoskeletons were sedimented at 13,000g for 15 min in a microfuge and resuspended in the same buffer. In some experiments, differentiated cultures were fractionated to yield fractions enriched for axonal neurites and perikarya, respectively, and Triton-insoluble cytoskeletons were then generated separately from axonal neurites and perikarya as described [Shea et al., 1993b]. Aliquots of cytoskeletons of entire cultures (200 μ g) or of enriched axonal neurites (20 μ g) were subjected to SDS-gel electrophoresis, and transferred to nitrocellulose. Nitrocellulose replicas were sequentially reacted with N1 and peroxidase-conjugated goat anti-rabbit IgG and visualized with diaminobenzidine by standard methods as described [e.g., Shea et al., 1989].

Immunoprecipitation

Equivalent aliquots (200 μ g) of cytoskeletal proteins derived from oligonucleotide-treated and untreated cultures were resuspended with vortexing in 100 μ l in 50 mM Tris-HCl containing 5 mM EDTA, 2 mM PMSF, and 50 μ g/ml leupeptin with and without 1% SDS, then diluted with 4 volumes of the same buffer containing 1% Triton X-100. Samples were incubated overnight at 4°C with 1:150 dilutions of N1, L3, or a polyclonal antisera (H3) generated in this laboratory against 200 kDa NF-H [Shea and Beermann, 1994]. Samples were incubated with 10 mg of protein A-Sepharose (Sigma, St. Louis, MO) at room temperature for 2 hr, centrifuged (1,000g for 30 sec, which was sufficient to sediment Sepharose beads but insufficient to precipitate the entire cytoskeleton). This centrifugation and resuspension via vortexing was repeated 4×, followed by centrifugation and resuspended in the same buffer without detergents 2×. Following the final centrifugation, immunoprecipitated material was eluted from Sepharose beads by vortexing in 100 μ l of 2× Laemmli treatment buffer containing 0.1% DTT and boiling for 1 min. The Sepharose beads were pelleted, and the resulting supernatant, containing the eluted, immunoprecipitated material, was immediately subjected to SDS-gel electrophoresis. Gels were Coomassie-stained, dried, and incubated for 1–2 weeks against Kodak X-Omat X-ray film to generate autoradiographs. Multiple autoradiographs of varying incubation were generated to insure linearity of exposure.

Digitization and Image Analyses

Images were captured with a Sony CCD, digitized via direct video input into a Power PC Macintosh 7100AV, and stored as PICT files. Densitometric analyses were carried out on stored images via NIH Image software. All images were captured and processed under identical conditions and within a linear range. Following automated background subtraction, levels of α -IN and NF-L were determined by encircling axonal neurites and, separately, perikarya and minor neurites in 50–100 cells in multiple cultures under each condition; perikarya and minor neurites were considered as one field for these purposes. For comparison or relative levels of α -IN and NF-L within axonal neurites, the total densitometric values for each IF within the entire cell (axonal neurites plus perikaryal/minor neurite field) were normalized. Ratios of the relative amount of each neuronal IF within axonal neurites vs. the amount within perikarya/minor neurite fields were then calculated.

Densitometric analyses of autoradiographs were carried out by encircling bands on digitized images (imported into the above computer via a UMAX flatbed scanner equipped with transparency adaptor) following

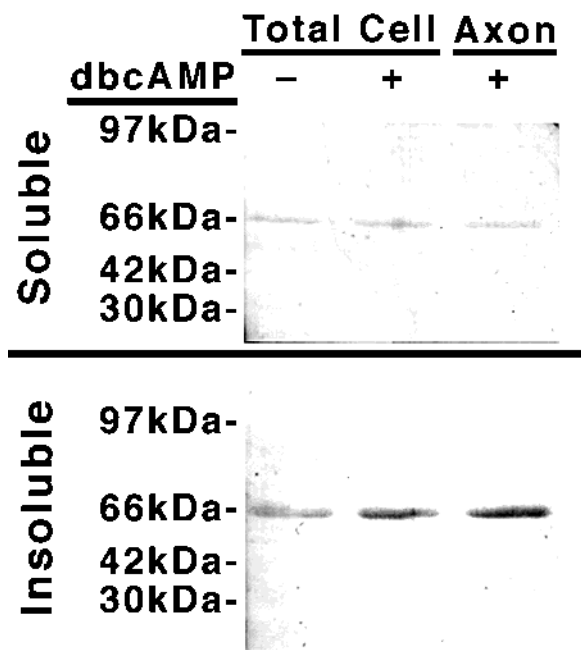


Fig. 1. NB2a/d1 cells constitutively express α -IN. Nitrocellulose replicas of Triton-soluble material and Triton-insoluble cytoskeletons from undifferentiated cells (dbcAMP -) and cells treated with dbcAMP for 3 days (dbcAMP +) immunostained with N1. Note the presence of an immunoreactive species at 66 kDa, the majority of which is recovered within cytoskeletons. Levels of this species increase following differentiation of these cells by serum-deprivation, and significant levels of this species are present within enriched axonal neurite preparations.

background subtraction using NIH Image software. Scanning was carried out within a linear range.

Neurite length was quantified by measuring the contour length of the longest neurite of 50–100 cells in duplicate cultures from separate experiments. This was performed by tracing the longest neurite of each cell with the free-hand line tool in the “neurite length macro” of NIH image software and recording the length in arbitrary units. Values for N1-injected and tracer-injected cells are expressed relative to those obtained for adjacent uninjected cells.

RESULTS

Expression and Localization of α -IN: Comparison to Other Neuronal IFs

Immunoblot analyses of undifferentiated and dbcAMP-differentiated cells with N1 demonstrated the presence of a 66-kDa species within Triton-insoluble cytoskeletons (Fig. 1). Levels of this species increased during differentiation, and preparations enriched for axonal neurites were also enriched in this species. For the remainder of this report, we will refer to this protein as α -IN for simplicity.

In flat, undifferentiated cells lacking filopodia, α -IN was evenly spread throughout the perikaryon (Fig. 2). Undifferentiated cells continuously elaborate and retract short filopodia-like neurites [e.g., Shea et al., 1991]; in cells elaborating filopodia-like neurites at the time of fixation, α -IN exhibited a unique polar distribution within perikarya. α -IN was often distinctly absent or weak within filopodia-like neurites, but was prominent within neurites of ≥ 1 somal diameter, which are present in a minor percentage of cells cultured in the presence of serum (Fig. 2). This distribution markedly differed from that of Vm, which was prominent within filopodia-like neurites. Both α -IN and NF-L were present within axonal neurites elaborated from 1–3 days of dbcAMP treatment. Unlike either Vm or NF-L, α -IN in many cases maintained intense staining within the axon hillock and perikaryon at the base of neurites (Fig. 2). By contrast, during continued dbcAMP treatment, NF-L staining remained evenly distributed throughout the perikaryon, and Vm, also evenly distributed, had declined [e.g., see Shea, 1990]. Notably, extensively phosphorylated NF-H and NF-M epitopes, while prominent within the axonal neurite shaft, were absent from the perikaryal and axon hillock cytoskeletons [see also Shea et al., 1989].

α -IN Facilitates the Development of Neuronal Polarity

Treatment of cultures for 48 hr with antisense-oriented, but not sense-oriented, α -IN oligonucleotides reduced α -IN synthesis by approximately 50%, which was reflected by a reduction in steady-state levels (Fig. 3); the average density of steady-state α -IN levels in sense-treated cells was 137 ± 17 (arbitrary densitometric units), which was reduced to 27 ± 12 by antisense treatment (P value ≤ 0.005). Like untreated and sense-treated cultures, antisense-treated cultures elaborated neurites following serum deprivation. However, while serum-deprived cells receiving sense or no oligonucleotides elaborated long, unbranching, monopolar or bipolar neurites in addition to shorter, web-like neurites [e.g., Shea et al., 1985], antisense-treated, serum-deprived cultures elaborated only shorter web-like networks and did not extend any axonal-like neurite (e.g., Fig. 3). The percentage of cells elaborating unbranching, monopolar, or bipolar axonal neurites in the presence of sense oligonucleotide was $82.5 \pm 3.5\%$ (mean \pm standard error of the mean) while this value was reduced to $59.3 \pm 4.6\%$ in the presence of antisense oligonucleotide ($P \leq 0.01$). These findings indicated that reduction in α -IN synthesis did not prevent neuritogenesis, but compromised the subsequent development of neuronal polarity.

As with other IF species in these cells [e.g., Shea et al., 1993a], significant reduction of α -IN steady-state levels required at least 48 hr of antisense treatment,

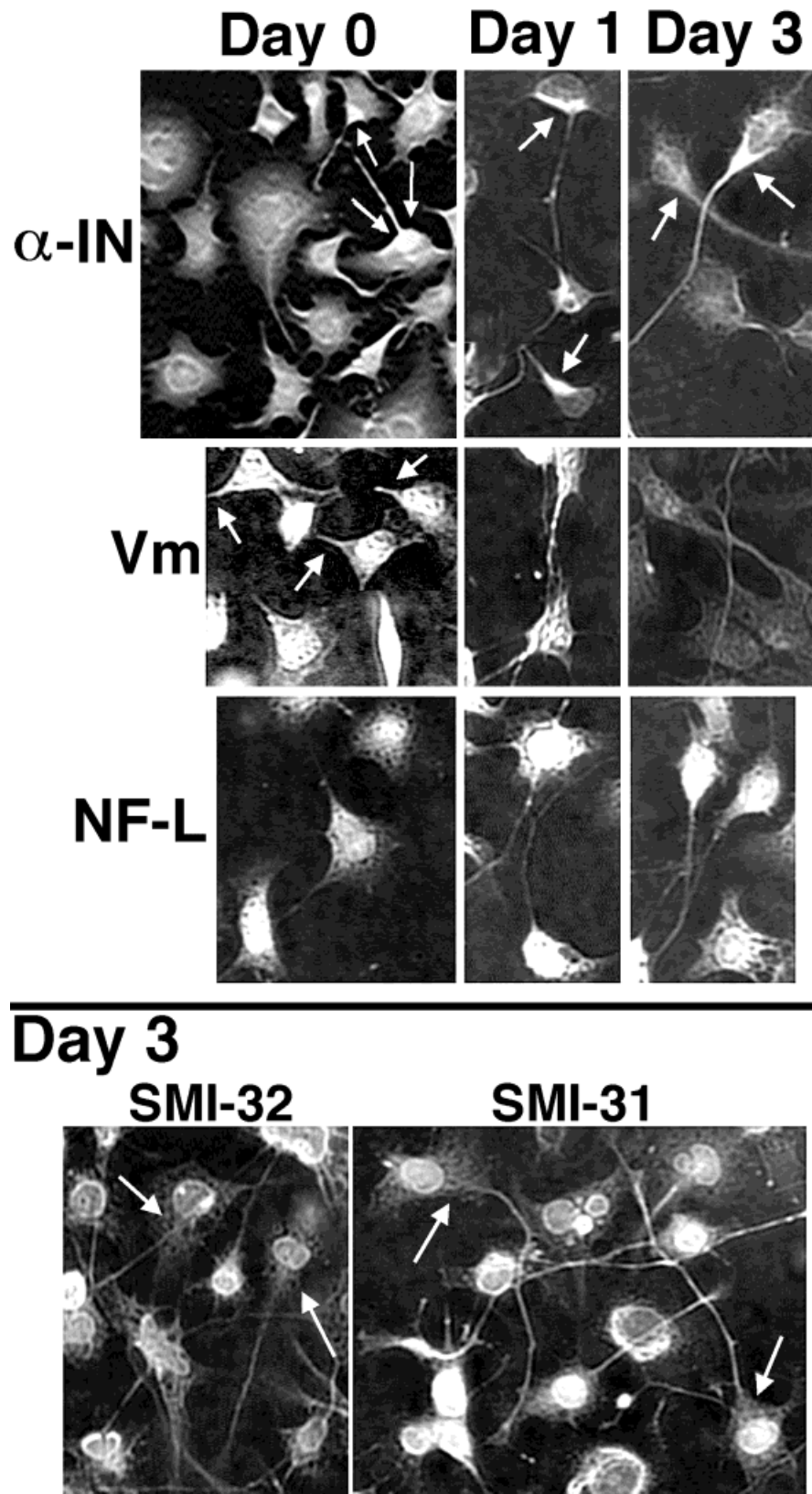


Figure 2.

and serum-deprivation was essential to effect sufficient oligonucleotide uptake (not shown). Since NB2a/d1 cells have completed their most rapid neurite elongation phase within 24 hr of serum deprivation, we were therefore unable to examine effectively the influence of antisense treatment on continued elongation of pre-existing neurites. However, this can be achieved by microinjection of a specific antibody, which acutely interferes with the function of existing levels of the target protein, rather than requiring depletion of steady-state levels prior to functional analysis as is the case with antisense treatment [e.g., Shea and Beermann, 1994; Shea et al., 1993a]. To test the putative role of α -IN in neurite elongation, N1 antibody was, therefore, microinjected into cells previously deprived of serum for 4 and 24 hr; such cells, therefore, had already elaborated neurites. Intracellular

Fig. 2. (on facing page) Distribution of neuronal IFs before and during early differentiation of NB2a/d1 cells. Undifferentiated cells (Day 0) and cells treated with dbcAMP for 1 and 3 days (Day 1 and 3, respectively) were extracted with Triton and immunostained with N1 (to visualize α -IN), anti-Vm, L3 (to visualize NF-L). α -IN is evenly distributed within perikarya of undifferentiated cells lacking neurites. In non-dbcAMP-treated cells that spontaneously elaborate short neurites, α -IN immunoreactivity is concentrated within the perikaryon at the base of neurites (arrows). During differentiation, α -IN remains concentrated within axonal neurites, the axon hillock, and the perikaryal region at the base of axonal neurites (arrows). By contrast, Vm is prominent within filopodia (arrows) and throughout perikarya in Day 0 cells. Vm remains prominent within perikarya and neurites at day 1, and declines during continued differentiation. NF-L is distributed throughout perikarya at all times, but is relatively weak within filopodia of undifferentiated cells. NF-L immunoreactivity is not prominent within neurites at day 1, but increases during continued differentiation (Day 3). Additional extracted Day 3 cultures were immunostained to visualize nonphosphorylated (SMI-32) and phosphorylated (SMI-31) epitopes of NF-M and NF-H. Note that neither of these epitopes are concentrated within the axon hillock or perikaryon at the neurite base (arrows), and that SMI-31 immunoreactivity is instead concentrated within neurites. Cross-reactivity of these epitopes with nuclear lamins results in prominent nuclear labeling [see Shea et al., 1989 and references therein].

Fig. 3. Down-regulation of α -IN compromises axonal neurite development. Treatment of cells with sense and antisense oligonucleotides corresponding to α -IN for 2 h inhibited synthesis of this protein. **Top:** Autoradiographs of material immunoprecipitated from the Triton-soluble fraction of receiving sense, antisense, or no oligonucleotides. The 66 kDa region of the autoradiograph is presented. The accompanying graph presents densitometric analysis of this autoradiograph. Additional cultures were treated with these for 48 h and were deprived of serum for the final 24 h. **Middle** Examination of cultures under phase-contrast microscopy revealed that, while sense-treated cultures elaborated neurites characteristic of dbcAMP-treated cultures in the absence of oligonucleotides, treatment with antisense oligonucleotides instead resulted in the elaboration of multiple shorter neurites. **Bottom** Additional cultures were immunostained with N1 and 6-11B-1 (to visualize acetylated MTs). N1 immunoreactivity confirmed downregulation of steady-state α -IN levels. In addition, antisense oligonucleotide treatment diminished the accumulation of acetylated MTs.

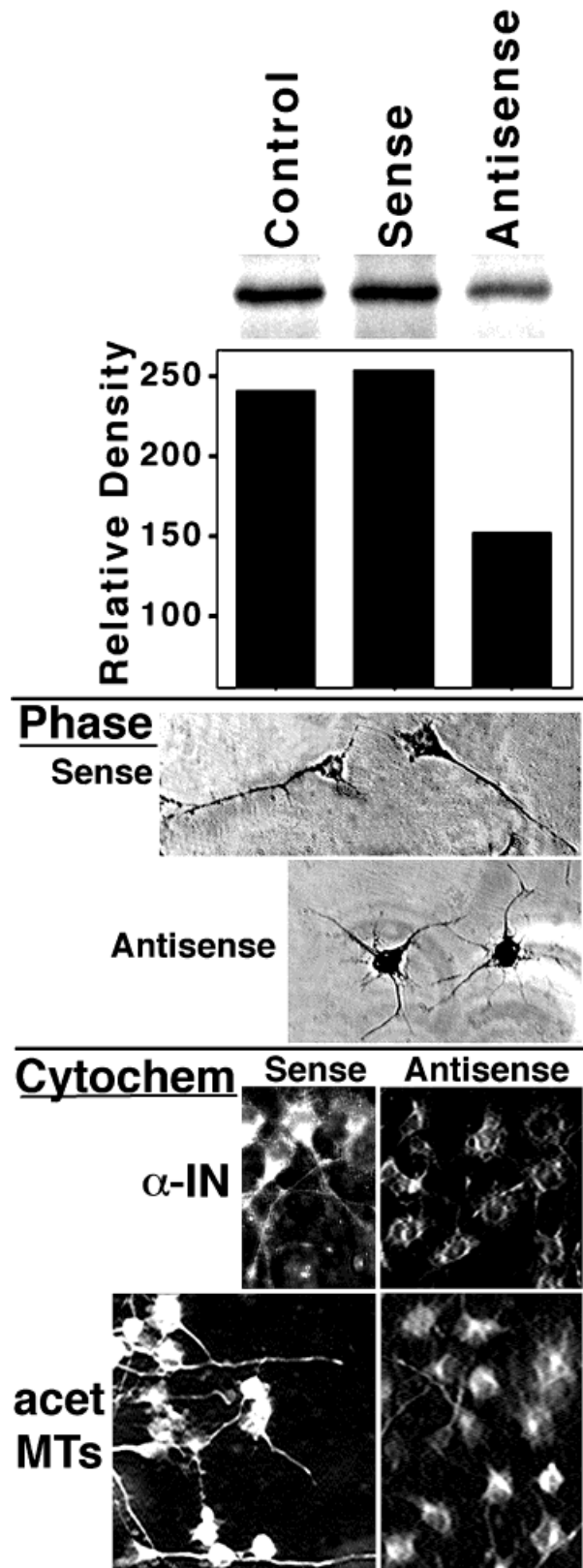


Figure 3.

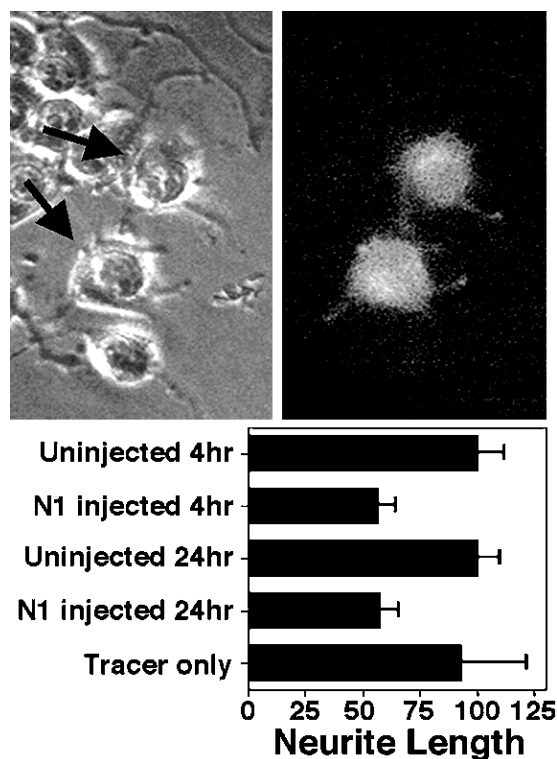


Fig. 4. Intracellular delivery of anti- α -IN antibodies inhibits axonal neurite elongation. Cells deprived of serum for 4 or 24 h were microinjected with N1 mixed with fluorescein-conjugated dextran (as tracer to locate injected cells) or tracer alone. Cells were cultured for an additional 4 h, then fixed and examined under phase-contrast and fluorescein optics. Shown are phase contrast and corresponding fluorescein image of cells injected with N1 4 h after serum deprivation. Continued elongation of neurites was diminished in N1-injected cells (arrows in phase image; fluorescein image confirms injection of cells). The accompanying graph presents the relative neurite length (mean \pm standard deviation presented in arbitrary units) compiled from 50 or more cells in multiple cultures.

delivery of N1 compromised the continued elaboration of these neurites (Fig. 4).

α -IN Downregulation Interferes With Accumulation of Stabilized Tubulin

Acetylation of α -tubulin is a polymer-specific post-translational modification that denotes relatively slowly turning-over microtubules (MTs) [e.g., Baas, 1997, and references therein]. While acetylation itself does not confer stability to MTs, acetylated MTs include the population of MTs that are relatively stabilized as compared to the entire complement of MTs, and are prominent within axons/axonal neurites in situ and in culture [Baas and Black, 1990; Black and Keyser, 1987; Cambrey-Deakin and Burgoyne, 1998; Shea, 1999]. Immunostaining of NB2a/d1 cells with a monoclonal antibody (6-11B-1) specific for acetylated α -tubulin has previously demonstrated accumulation of acetylated MTs within

axonal neurites, and that this accumulation is temporally correlated with stabilization of axonal MTs in these cells [see Shea, 1998, and references within]. Downregulation of α -IN with antisense α -IN oligonucleotides as described above also diminished acetylated MT immunoreactivity (Fig. 3). No corresponding reduction in total α -tubulin immunoreactivity (visualized with a commercial polyclonal antibody directed against all forms of α -tubulin) was observed (not shown).

Differential Deposition of α -IN and NF-L Following Serum Deprivation or dbcAMP Treatment

We next compared the relative extent of α -IN and NF-L deposited within Triton-insoluble cytoskeleton of axonal neurites elaborated by dbcAMP treatment with those elaborated following serum-deprivation. Like dbcAMP treatment, serum deprivation induces rapid elaboration of axonal neurites; however, these neurites remain very plastic as evidenced by their rapid retraction following addition of serum to the culture even after 24 h of serum deprivation (Fig. 5) [see also Shea et al., 1991]. Neurites elaborated in response to dbcAMP treatment do not exhibit such plasticity or retraction, but rather exhibit continuous outgrowth over 3–7 days (not shown) [Shea and Beermann, 1994].

Both α -IN and NF-L accumulated within neurites induced by either treatment. However, a relatively greater percentage of the total α -IN immunoreactivity within the cell (i.e., perikaryon + neurite) accumulated within axonal neurites induced following serum-deprivation as compared to NF-L immunoreactivity (Fig. 6A,B). This difference was evident by 24 h and was more pronounced by 48 hr (Fig. 6A,B). One interpretation of these findings is that α -IN is selectively incorporated within neurites induced by serum-deprivation, while NF-L is restricted from incorporating into serum-deprived, but not dbcAMP-induced, axonal neurites. In this regard, under both conditions of differentiation, the majority of immunoreactivity towards both α -IN and NF-L are observed within perikarya, indicating that axonal levels are controlled by mechanism(s) other than bulk synthesis levels [see also Shea, 1994a, 1995].

Deposition of NF-L Within Axonal Neurites Induced by Serum Deprivation Is Regulated by Proteolysis

The protease inhibitor C1 was added to cultures simultaneously with serum deprivation, which enhances neurite outgrowth under these conditions [Shea et al., 1991; Shea, 1994a]. We then examined whether or not this treatment altered the steady-state levels of α -IN or NF-L within neurites. Axonal NF-L immunoreactivity was

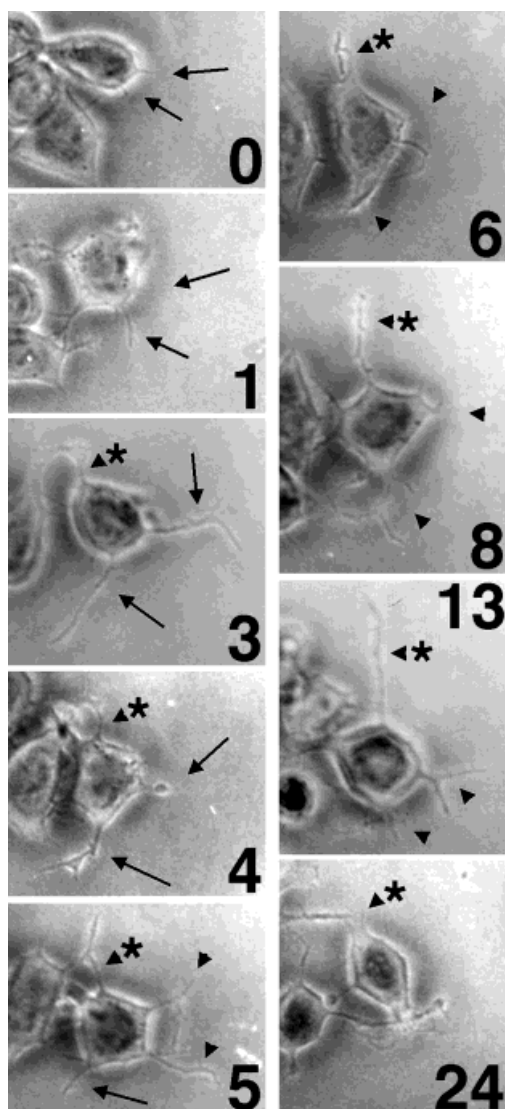


Fig. 5. Neurites induced by serum deprivation are highly plastic. Cultures were deprived of serum and individual cells were photographed repeatedly over 24 h. Note that filopodia-like neurites (*arrows*) elaborated within 1 h of serum deprivation have but undergone retraction by 4–6 h. Additional putative neurites, elaborated at 5–6 h (*arrowheads*) also undergo eventual retraction. An additional filopodia-like neurite, first observed at 3 h (*arrowhead with asterisk*) continues to elongate until 13 h, but has undergone a dramatic shift in direction by 24 h.

increased by 2.37-fold by C1, while that of α -IN was essentially unchanged (Fig. 6A,C).

NF-H Preferentially Associates With NF-L Vs. α -IN Within Cells

Assembly-competent NF-H is present within NB2a/d1 perikarya prior to and during differentiation [e.g., Shea et al., 1990; Shea, 1994b], yet does not accumulate within axonal neurites until 2–3 days of

elaboration [Shea et al., 1989; Shea and Beermann, 1994]. However, accumulation of phospho-NF-H within axonal cytoskeletons of serum-deprived NB2a/d1 cells is increased by treatment with C1 (Fig. 6; see also Shea, 1994a). Taken together with the above increase in axonal NF-L following C1 treatment, these findings suggested that the characteristic accumulation of NF-H within axonal cytoskeletons is dependent upon NF-L and cannot be mediated by α -IN. To test this possibility, we immunoprecipitated α -IN and NF-L with specific antisera and examined the levels of co-precipitated NF-H in a manner similarly utilized by Athlan and colleagues [Athlan and Mushynski, 1997; Athlan et al., 1997]. NF-L co-precipitated newly synthesized NF-H to an extent comparable to that directly immunoprecipitated by an anti-NF-H antibody (Fig. 7). In contrast, N1 precipitated only a small percentage of NF-H at any time (Fig. 7). These data indicate that, at least in these cells, NF-H does not associate even transiently to any major extent with α -IN. Co-precipitation was not derived from cross-reactivity of either N1 or L3 with NF-H, since inclusion of 1% SDS during resuspension and homogenization of cytoskeletons completely prevented co-precipitation of NF-H by either N1 or L3 (not shown).

DISCUSSION

Immunocytochemical comparison of neuronal IF distribution before and during NB2a/d1 differentiation demonstrated a unique temporal and spatial distribution of α -IN as compared to other neuronal IFs. Neither α -IN nor NF-L were localized within the filopodia-like neurites elaborated by undifferentiated cells, in which Vm was prominent. Similarly, neither α -IN nor NF-L were selectively located within neurites following 1 day of dbcAMP treatment, at which point Vm is at the peak of its transient developmental increase in newly elaborated axonal neurites [Shea, 1990]. However, α -IN, unlike either Vm or NF-L, was selectively concentrated within the axon hillock and perikaryon at the base of axonal neurites. Both α -IN and NF-L accumulated within axonal neurites during continued differentiation, while Vm declined [see also Shea, 1990; Shea et al., 1993a]. Throughout continued differentiation, α -IN maintained its concentration within the axon hillock, while NF-L instead remained evenly dispersed throughout the perikaryon.

Inhibition of α -IN expression and function did not prevent neuritogenesis but inhibited continued outgrowth of existing neurites. By contrast, similar studies indicated that Vm was required for neuritogenesis but not continued elongation [Shea et al., 1993a; Boyne et al., 1996], while NFs were neither involved in neurite initiation nor continued elongation, but instead stabilized axonal neurites following their initial rapid growth phase [Shea and

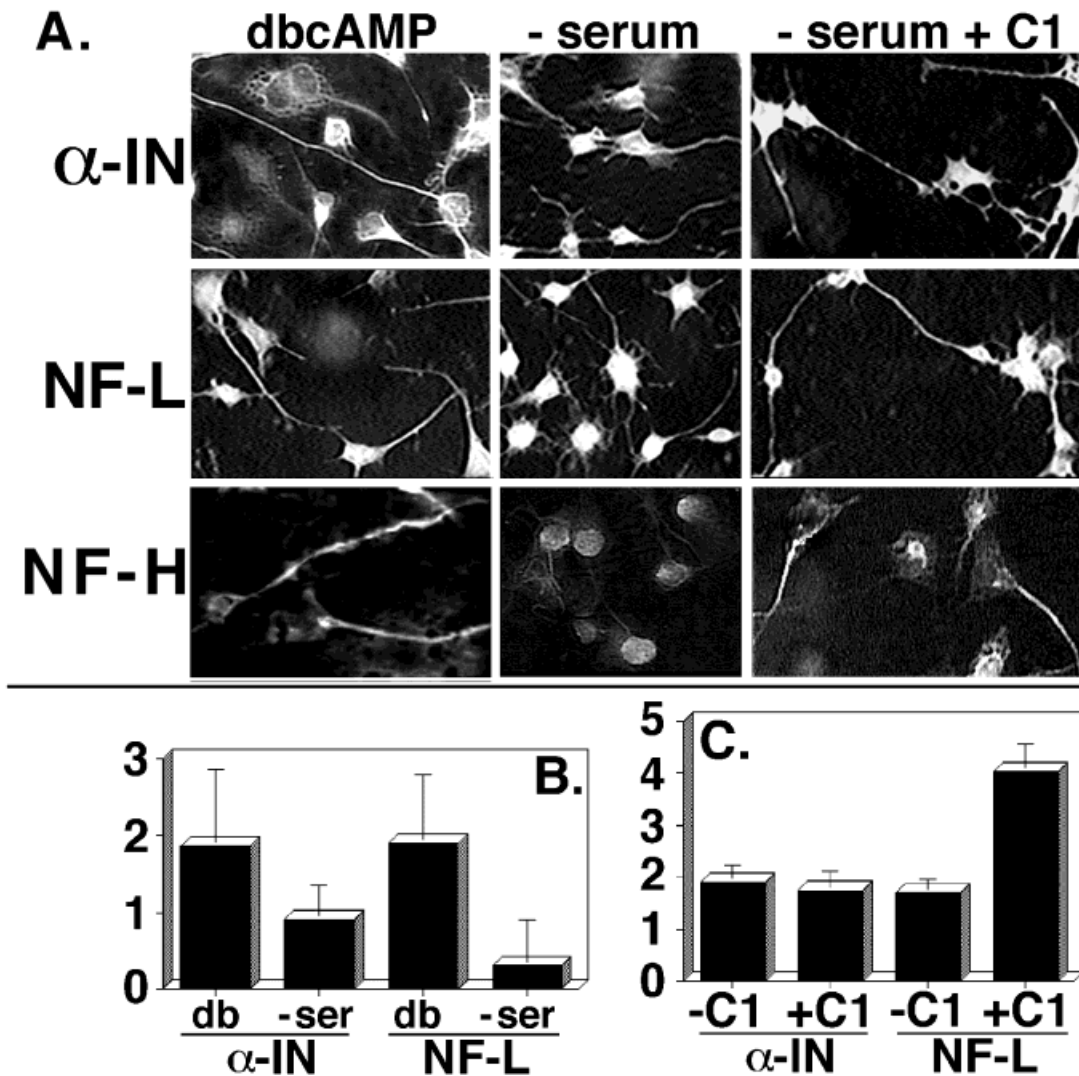


Fig. 6. Differential accumulation of neuronal IFs within neurites induced under different conditions. **A:** α -IN, NF-L, and extensively phosphorylated NF-H (RT97) immunoreactivity in cells treated for 24 h with dbcAMP, or deprived of serum for 24 h in the presence and absence of the protease inhibitor C1. Note that α -IN immunoreactivity is similar under all conditions. NF-L and NF-H immunoreactivity, by contrast, is apparently weaker in neurites induced by serum deprivation vs. dbcAMP treatment, and is increased by C1. The accompanying graphs present comparative analysis of the ratios of α -IN and NF-L immunoreactivity within axonal neurites. **B:** Comparison of these

ratios in cells either treated with dbcAMP or deprived of serum for 24 h. While both α -IN and NF-L are present to a similar extent within axonal neurites of dbcAMP-treated cells, neurites induced by serum-deprivation contain relatively less NF-L than α -IN. **C:** Comparison of these levels in neurites elaborated during 24 h of serum-deprivation in the presence or absence of C1. For these analyses, the ratio of α -IN and NF-L in axonal neurites serum-deprived cultures was first normalized. Note that α -IN levels are unchanged by C1, while NF-L levels underwent a dramatic increase.

Beermann, 1994]. Taken together, these findings suggest a role for α -IN in supporting the elongation of axonal neurites subsequent to the development of polarity, yet prior to their stabilization. That α -IN plays a supportive role in the accumulation of acetylated MTs within developing axonal neurites is consistent with this possibility, since MT polymerization and stabilization are essential for axonal neuritogenesis in these cells [Shea and Beermann, 1994]. The normal enrichment of α -IN, rather than NF-L, within the relatively plastic neurites induced

by serum-deprivation, and the accumulation of NF-L within these neurites following their stabilization by protease inhibition [e.g., Shea et al., 1991], is also consistent with a role for α -IN in supporting axonal neurite elongation. These results and interpretation are consistent with the previous demonstration that α -IN expression also preceded that of NF-L during neuronal differentiation of an embryonic carcinoma cell line and in cultured dorsal root ganglion neurons, which also suggested a role distinct from that of NFs in neurite

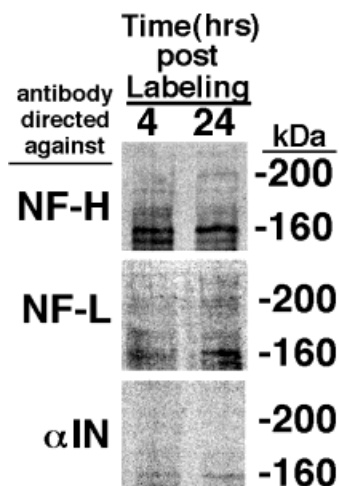


Fig. 7. NF-H preferentially co-assembles with NF-L rather than α -IN within cells. Autoradiographic analysis of NF-H isoforms precipitated from Triton-insoluble cytoskeletons by anti-NF-H antibody H3, and NF-H isoforms co-precipitated with either anti-NF-L antibody L3 or anti- α -IN antibody N1. Only the portion of autoradiographs relevant to NF-H (i.e., the 160–200 kDa region) is presented. Note that NF-L co-precipitates virtually all radiolabeled NF-H by 24 h. α -IN, in contrast, co-precipitates only small levels of NF-H.

outgrowth [Athlan et al., 1997; Chiu et al., 1995]. The ability of α -IN, unlike NFs, to form homopolymers [Ching and Liem, 1993, 1998], may provide for a relatively early role in neurite elongation prior to NF-induced stabilization. Notably, we have not in these analyses examined peripherin [Parysek and Goldman, 1987; Portier et al., 1984], which is also expressed by these cells (data not shown). It remains possible that one or more aspects of neuritogenesis is facilitated by this additional neuronal IF.

Phosphorylated and nonphosphorylated NF-H and NF-M epitopes were not detected within the axon hillock. Since α -IN was concentrated in this area, these findings suggest that these subunits do not preferentially associate with α -IN within cells. In this regard, cell-free analyses demonstrate that α -IN can indeed copolymerize with NF subunits [Athlan and Mushynski, 1997; Balin and Miller, 1995; Chiu, 1991; Ching and Liem, 1998], but that α -IN does not co-assemble as effectively with NF-H and NF-M as does NF-L [Chiu, 1991]. The increase of NF-H within neurites along with that of NF-L following protease inhibition supports the conclusion that NF-H also selectively co-polymerizes with NF-L in intact cells. Our demonstration that NF-H and NF-M do not accumulate within the axon hillock and the neurite base (where α -IN is selectively concentrated) leaves open the possibility that phospho-NFs are excluded from this area by α -IN-mediated displacement of NF-L. One speculation is that this segregation of neuronal IFs maintains a greater degree of plasticity within the axon hillock than may be

found within the axon shaft, which may facilitate the initial sorting of components for various phases of axonal transport. The selective concentration of α -IN in the axon hillock may also provide nucleation sites for NF assembly, as suggested by cell-free studies [Balin and Miller, 1995]. The possibility that α -IN plays a supportive role, yet maintains a degree of plasticity that would be precluded by extensive deposition of phospho-NFs, is consistent with compartmentalization of α -IN within dendrites in cultured hippocampal neurons [Benson et al., 1996].

Studies in transgenic animals lacking cytoskeletal proteins including neuronal IFs have in a number of cases not displayed an overt phenotype, lending the impression that the protein in question lacks an essential function. While this interpretation cannot be discarded, it is in profound discord with the phylogenetic conservation of neuronal IFs. Accordingly, the search for more subtle phenotypes, and identification of compensatory mechanisms, is important for elucidation of the role(s) of neuronal IFs. Culture of neurons, and of cell lines that can be induced to elaborate axonal neurites, are particularly useful for analyses of the consequences of downregulation of cytoskeletal proteins under conditions that may preclude compensatory mechanisms that exist in situ. For example, antisense oligonucleotide and antibody-mediated inhibition of Vm expression and function clearly demonstrate a requirement for Vm for neuritogenesis in culture [Shea et al., 1993a; Boyne et al., 1996], yet a transgenic mouse lacking Vm develops normally [Colucci-Guyon et al., 1994]. Similarly, while inhibition of NF-mediated interactions in culture inhibited axonal neurite stabilization and led to axonal retraction [Shea and Beermann, 1994], apparently normal development was observed in the absence of NFs in a mutant quail [Ohara et al., 1993] and in a transgenic mouse unable to transport NFs into axons [Eyer and Peterson, 1994]. These disparate results are addressed by the demonstration that providing a sufficiently adherent substrate in culture reduces and in some cases eliminates the requirement for cytoskeletal proteins [Lamoureux et al., 1990; Shea et al., 1992; Smalheiser, 1989a,b; Smalheiser and Schwartz, 1987; Smith, 1994], suggesting that neuronal pathfinding mechanisms can compensate for deficiencies in cytoskeletal support during axonal development in situ. Assigning functional roles to neuronal IFs is rendered more complex by their potential to serve overlapping roles. For example, axonal fibers of cerebellar granule cells lack NFs but express α -IN [Chien et al., 1996; Chiu et al., 1989], which may reflect that certain small-caliber axons do not require the degree of stabilization generally attributed to phospho-NFs [for review, see Nixon and Shea, 1992]. While α -IN and NFs may indeed serve overlapping functions, the results of the present study provide experimental evi-

dence, consistent with the developmental precedence of α -IN vs. NFs in situ [Chan and Chiu, 1995; Chien and Liem, 1995; Chien et al., 1996, 1998; Fleigner et al., 1994; Galinovi-Schwartz et al., 1991; Giasson and Mushynski, 1997; Kaplan et al., 1990] and in culture [Athlan et al., 1997], that α -IN plays a unique role in axonal neurite outgrowth as ascertained in this model system.

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NOTE ADDED IN PROOF

As this article went to press, it was demonstrated that overexpression of α -internexin causes abnormal neurofilamentous accumulations and neuronal dysfunction in situ. [Ching et al. 1999. Overexpression of α -internexin causes abnormal neurofilamentous motor coordination deficits in transgenic mice. *J Neurosci* 19:2974–2986.]

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