

Associations Between Intermediate Filament Proteins Expressed in Cultured Dorsal Root Ganglion Neurons

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The developmental profile of the neurofilament (NF) triplet proteins, α -internexin and peripherin in cultured dorsal root ganglion neurons from gestation day 15 rat embryos was determined by Western blot analysis. At the outset (day 0 in culture), the neurons contained mostly α -internexin. A significant increase in peripherin levels was seen at days 1–2, in the mid-sized (NFM) and low molecular weight (NFL) NF subunits at days 2–3, and in the high molecular weight (NFH) NF subunit at days 5–6. Immunofluorescence microscopy showed that the five intermediate filament proteins were co-localized in all neuronal cell bodies and neurites. Analysis of Triton X-100 extracts from okadaic acid-treated dorsal root ganglion cultures revealed that peripherin and α -internexin followed the same fragmentation pattern observed with NFs. Interactions between the various neuronal intermediate filament proteins in these extracts were assessed by immunoprecipitation under native conditions using antibodies specific for the individual proteins. Co-immunoprecipitation of NFH with NFL, NFM with NFL, NFM with α -internexin, and α -internexin with peripherin demonstrated that the intermediate filament cytoskeleton in cultured sensory neurons is a highly integrated structure. *J. Neurosci. Res.* 47:300–310, 1997. © 1997 Wiley-Liss, Inc.

Key words: neurofilaments; peripherin; α -internexin; okadaic acid

INTRODUCTION

Neurofilaments (NFs) were long considered to be the major type of intermediate filament (IF) expressed in mature neurons (Shaw et al., 1981; Trojanowski et al., 1986). However, neurons have more recently been shown to express two additional IF proteins, peripherin (Portier et al., 1984; Leonard et al., 1988; Parysek et al., 1988) and α -internexin (Fliegner et al., 1990). The expression of peripherin and α -internexin overlaps with that of NF proteins during development and in a subset of adult neurons (reviewed by Nixon and Shea, 1992).

Mammalian NFs are composed of three phosphoprotein subunits with apparent molecular masses of about 68 KDa (NFL), 150 KDa (NFM), and 200 KDa (NFH), as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Hoffman and Lasek, 1975; Julien and Mushynski, 1982). As members of the IF family of proteins, NF subunits display a characteristic domain organization, consisting of an amino-terminal head and a carboxy-terminal tail flanking a highly conserved α -helical rod domain (Geisler et al., 1983).

The occurrence of a rod domain in each NF subunit suggested that the individual proteins might be capable of forming filaments, since IF assembly begins with a coiled-coil dimerization step involving this domain (for a review, see Fuchs and Weber, 1994). However, only NFL is capable of forming substantial, 10-nm-wide homopolymeric filaments in vitro (Geisler and Weber, 1981), and in vivo studies have shown that NFs are obligate heteropolymers whose formation requires NFL together with NFM and/or NFH (Ching and Liem, 1993; Lee et al., 1993; Ohara et al., 1993; Nagakawa et al., 1995).

The finding that NFs are obligate heteropolymers has invited comparison with keratin IFs, which have similar properties. However, keratin IFs are stoichiometric heteropolymers requiring the participation of an acidic and a basic type keratin in coiled-coil dimerization (Fuchs and Weber, 1994). The conditions for NF assembly are less rigid, requiring NFL together with a substoichiometric amount of either NFM or NFH (Lee et al., 1993). There is evidence that heteromeric interactions between NF subunits occur during early stages of NF assembly, at the dimer or tetramer level (Carden and Eagles, 1986; Cohlberg et al., 1995). However, the formation of NFs in cells expressing NFM or NFH at one-tenth the level of NFL (Lee et al., 1993) indicates that

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homopolymeric stretches can be accommodated within the NF structure.

NF assembly and structure may be more complex in neurons that also express peripherin and α -internexin. Transfection studies with an IF-deficient cell line have shown that α -internexin and peripherin can form homopolymeric filaments (Ching and Liem, 1993; Cui et al., 1995). α -Internexin can also co-assemble with each of the three NF subunits in such transfected cells (Ching and Liem, 1993) as well as *in vitro* (Balin and Miller, 1995), and peripherin was shown to co-localize with NF triplet proteins in a subset of neuronal IFs in the sciatic nerve (Parysek et al., 1991). However, little is known about the extent or nature of such interactions in neurons.

Dorsal root ganglia (DRGs) in adult rats contain two distinct types of neurons differing in size and IF content. The small neurons express peripherin, while the large neurons express NFs (Goldstein et al., 1991). On the other hand, embryonic DRGs *in vivo* or cultures of embryonic day 15 (E15) DRG neurons contain a single neuronal type expressing both peripherin and NFs (Goldstein et al., 1996). α -Internexin is also found in embryonic DRG neurons *in vivo* (Fliegner et al., 1994), although its expression declines postnatally (Chiu et al., 1989; Kaplan et al., 1990).

The co-assembly of different IF proteins is often assessed by methods such as immunofluorescence microscopy (Ching and Liem, 1993; Lee et al., 1993) or immunoelectron microscopy (Balin et al., 1991; Parysek et al., 1991; Balin and Miller, 1995), which provide little insight into the types of interactions involved. The availability of a method known to cause the fragmentation of NFs in cultured DRG neurons has enabled us to take a different approach to study interactions between the various neuronal IF proteins. Indeed, treating DRG neurons with okadaic acid (OA), a potent inhibitor of protein phosphatase-2A and protein phosphatase-1 (Cohen et al., 1990), causes NFs to fragment (Sacher et al., 1992, 1994). In this report we show that peripherin and α -internexin co-localize with NFs in cultured E15 DRG neurons and undergo OA-induced fragmentation similar to that of NFs. In such OA-treated cultures, we have characterized the associations between the various neuronal IF proteins in Triton X-100 (Triton)-soluble oligomers by immunoprecipitation analysis. Our results indicate that α -internexin is a key element in the integration of the various neuronal IF proteins through its predominant association with NFM and peripherin.

MATERIALS AND METHODS

Materials

OA was from LC Services (Woburn, MA). Specific, phosphorylation-independent monoclonal antibodies (Abs)

against NFL (NR4), NFM (NN18), and NFH (N52) were from Sigma Chemical Co. (St. Louis, MO). Monoclonal Abs against peripherin (MAB1527) and α -internexin (MAB1525) were from Chemicon International, Temecula, CA, while the one against vimentin (V9) was from Boehringer Mannheim (Montreal, Canada). Enhanced chemiluminescence reagents were from NEN (Mississauga, Canada). Neuronal IF proteins were purified by preparative SDS-PAGE as described (Julien and Mushynski, 1982). The NF proteins and α -internexin were from adult rat spinal cord, while peripherin was from PC12 cells.

Cell Culture and Developmental Profile of Neuronal IF Proteins

Rat DRGs were dissected, dispersed, and maintained in defined medium as previously described (Sacher et al., 1992). For developmental analysis of neuronal IF protein expression, cells were plated on 12-well tissue culture dishes and allowed to attach to the collagen substrate for 1 hr. The medium was then drained and replaced, and the first plated sample (time = 0 hr) was harvested in sample buffer (2% SDS, 62.5 mM TrisHCl, pH 6.8, 5% (v/v) β -mercaptoethanol, 10% (v/v) glycerol) (Laemmli, 1970). An untrypsinized sample and a trypsinized but unplated sample were also kept for analysis. Standardization of the samples was achieved by harvesting in equal volumes of sample buffer and by loading identical volumes on the gels. This is a suitable method since E15 neurons are post-mitotic and do not die to any significant degree during the first 30 to 40 days in culture (E.A., unpublished observation). For separate analysis of neurites and neuronal cell bodies, the cells that would normally be used for culture in a dispersed configuration were concentrated down to a very small volume (10 μ l), plated at the center of a 35-mm dish, and allowed to attach for 0.5–1.0 hr at 37°C. The dishes were then flooded with medium, and the resulting localized cultures consisted of a central neuronal cell body mass eventually surrounded by a halo of neurites.

OA Treatment and Time-Course Analysis

Twenty-five to 32-day-old cultures were treated with 1 μ M OA for 0, 1, 2, and 4 hr. Neuronal cell bodies and neurites were physically separated for analysis using a punch with a diameter equal to that of the cell body mass. Samples were harvested in cytoskeleton extraction buffer (CSK buffer: 1% Triton, 100 mM NaCl, 50 mM TrisHCl, pH 7.5, 50 mM NaF, 2 mM EDTA, 2 mM levamisole, 1 mM phenylmethylsulfonyl fluoride), vortexed for 30 sec, and centrifuged at 13,000g for 15 min. The resulting pellets (13K pellet) were dissolved in SDS-Sample buffer, and the 13,000g supernatants (13K

supernatant) were further centrifuged at 100,000g in a Beckman airfuge for 5 min. The resulting pellets (100K pellet) and supernatants (100K supernatant) were dissolved in sample buffer or by adding one-half volume of 3× SDS-sample buffer, respectively. The 13K and 100K pellets and the 100K supernatants were in identical final volumes, and equal volumes were analyzed by SDS-PAGE and Western blotting.

Immunoprecipitation

13K supernatants from cultures treated with OA for 4 hr were immunoprecipitated for 1 hr at 4°C with one of the monoclonal Abs against either NFL, NFM, NFH, α -internexin, or peripherin bound to anti-mouse IgG1 Abs crosslinked to agarose beads (Sigma Chemical Co., St. Louis, MO). After centrifugation, the pelleted beads were washed repeatedly with CSK buffer. The immunoprecipitated proteins were solubilized by boiling for 5 min in SDS sample buffer and analyzed by SDS-PAGE and Western blotting.

SDS-PAGE and Western Blotting

Electrophoresis of the samples on 6% SDS-polyacrylamide gels was performed as described (Laemmli, 1970). The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Inc.) in a buffer containing 48 mM Tris and 39 mM Glycine. The protein bands were then detected with the Abs mentioned in the figure legends and visualized by enhanced chemiluminescence as described by the manufacturer.

Immunofluorescence

Twenty-five to 32-day-old dispersed sister cultures grown on collagen-coated glass slides were rinsed with phosphate-buffered saline (PBS: NaCl, 137 mM; KCl, 2.7 mM; Na₂HPO₄, 10 mM; KH₂PO₄, 1.8 mM; pH 7.4) and fixed with methanol for 20 min at -20°C. Following rehydration, the samples were incubated for 30 min at 37°C with blocking buffer (PBS with 10% goat serum and 0.3% Triton) and stained for 1 hr at 37°C with a cocktail containing a rabbit polyclonal anti-NFM Ab (1:100) and one of the commercial monoclonal Abs (1:100 to 1:500) against the individual neuronal IF proteins diluted in blocking buffer. The slides were then rinsed 5 to 6 times for 5 min with PBS containing 0.3% Triton and incubated with Texas red- or DTAF-conjugated species-specific secondary Abs (1:100) and rinsed under the same conditions as used with the primary Abs. Slides were then photographed using black-and-white KODAK TMAX400 and a Zeiss microscope with the camera set on automatic exposure. Typical exposures were from 15 to 25 sec for

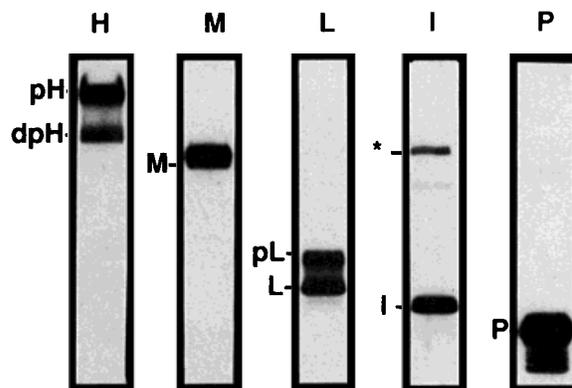


Fig. 1. Verification of the specificity of the monoclonal Abs against the individual neuronal IF proteins by Western blot analysis. DRG samples containing the various phosphovariants of NFL and NFH were resolved by SDS-PAGE, and individual lanes were developed with one of the monoclonal Abs. The letters H, M, L, I, and P at the top of the individual lanes refer to the monoclonal Abs against NFH, NFM, NFL, α -internexin, and peripherin, respectively. pH and dpH, hyper- and hypophosphorylated forms of NFH, respectively; M, NFM; pL and L, hyperphosphorylated and normal NFL, respectively; I, α -internexin; P, peripherin; *, a cross-reactive band of unknown origin.

the texas-red filter and 25 to 60 seconds for the DTAF filter.

RESULTS

The specificity of the monoclonal Abs used in this study was verified by testing them individually on a Western blot containing all of the neuronal IF proteins, including phosphovariants of NFH and NFL. The results in Figure 1 indicate that the individual Abs showed no appreciable cross-reactivity with the other IF proteins. Note that since anti- α -internexin also labeled an unknown Triton-soluble band (*) migrating just above NFM, only the bottom half of Western blots was probed with this antibody.

Available *in vivo* and *in vitro* data relating to neuronal IF expression in adult and embryonic rat DRGs (Chiu et al., 1989; Kaplan et al., 1990; Goldstein et al., 1991, 1996; Fliegner et al., 1994) suggested that cultures of E15 DRG neurons would express peripherin and α -internexin along with the NF triplet proteins. The results in Figure 2 indicate that before trypsinization, E15 DRG neurons contained mainly α -internexin with traces of peripherin and NFL (Fig. 2, lane B). Trypsinization alone had little impact on the profile of neuronal IF proteins (Fig. 2, lane T). One hour after plating, the harvested samples had lesser amounts of these proteins, due largely to the observed failure of some neurons to attach to the collagen substrate. There was an ordered

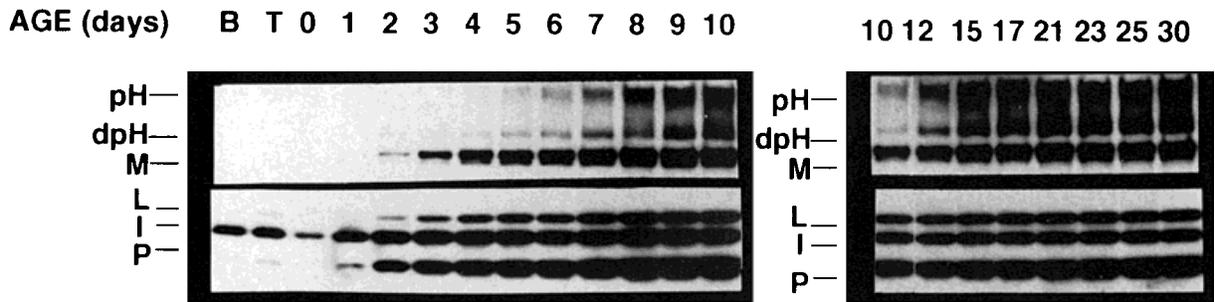


Fig. 2. Western blot analysis of the developmental profile of neuronal IF proteins in cultured E15 DRG neurons. DRGs were cultured as described in Materials and Methods and harvested in equal volumes of sample buffer at the times (in days) indicated at the top of each lane. In the panel on the right, sample volumes were one-fourth of those in the left-hand panel to permit visualization of the entire range of samples with a

single film exposure. The day 10 sample was reloaded in the right panel at one-fourth the volume for comparison. The upper part of the blot was probed with monoclonal Abs against NFH and NFM, while the lower part was probed with monoclonal Abs against NFL, α -internexin, and peripherin. IF protein bands are designated as described in Figure 1. B and T, before and after trypsin, respectively.

increase in the levels of neuronal IF proteins. The neurons contained mainly α -internexin at the outset (day 0). An increase in peripherin levels was seen at days 1–2, in NFL and NFM at days 2–3, and in NFH at days 5–6. Beyond day 17 the levels of all five IF proteins changed very gradually.

To address the question of whether the neuronal IF proteins co-localize in the same neurons, dispersed cultures grown on collagen-coated glass slides for twenty five days were double stained with a rabbit polyclonal anti-NFM Ab and a monoclonal Ab against one of the neuronal IF protein. The DTAF- and Texas red-conjugated secondary Abs showed no detectable species cross-reactivity (data not shown). The fluorescence micrographs in Figure 3 show that monoclonal Abs against the individual neuronal IF proteins each stained the same neurites (A' to E') and neuronal cell bodies (F') as the polyclonal anti-NFM Ab (A to F), indicating that all neuronal IF proteins are completely co-localized in these cultured DRG neurons. However, differences in staining intensities of some neurites were observed, indicating that the relative proportions of different IF proteins in these processes can vary. Neuronal cell bodies were also stained by antibodies against all of the neuronal IF proteins; hence only the results for anti-NFM and anti- α -internexin are shown in Figure 3.

As a first step toward determining whether the various neuronal IF proteins are associated or form independent structures, we tested whether OA treatment would fragment assembled peripherin and α -internexin in the same way as it does NFs (Sacher et al., 1994). Localized DRG cultures were treated with 1 μ M OA for 1–4 hr, the cell bodies and neurites were harvested separately in CSK buffer, and various fractions were obtained by differential centrifugation (see Materials and Methods). The 13K and 100K pellets and 100K superna-

tant were resolved by SDS-PAGE, and Western blots were probed with Abs against NFH, NFM, NFL, α -internexin, and peripherin (Fig. 4). In the untreated perikaryal sample (0 hr), most of the NFH was hypophosphorylated, while axonal NFH was exclusively hyperphosphorylated as can be expected from *in vivo* observations (Sternberger and Sternberger, 1983). A continuous reduction in the gel electrophoretic mobility of perikaryal NFH (dpH) was seen over the 4-hr course of OA treatment, reflecting its increased phosphorylation in the presence of the phosphatase inhibitor. The perikaryal compartment also differed from the axonal compartment in that the untreated sample contained a greater proportion of Triton-soluble NFH, NFL, and peripherin, perhaps reflecting the presence of newly synthesized, unassembled subunits. The amounts of all five neuronal IF proteins in the 100K pellet from cell bodies and neurites reached a maximum at 2 hr and then leveled off. In contrast, IF protein levels in the 100K supernatant increased continuously over the 4-hr time-course, indicating that neuronal IFs in OA-treated neurons were first fragmented into large, 13,000g-soluble oligomers and then underwent further fragmentation to a point where they were soluble at 100,000g. This sequence is essentially the reverse of that which occurs during incorporation of newly synthesized NF subunits into the cytoskeleton (Shea et al., 1988).

Estimates based on the Western blot in Figure 4 indicate that roughly two-thirds of the neuronal IF proteins ended up in the 13K supernatant after 4 hr of OA treatment. A hyperphosphorylated form of NFL (pL), with a markedly reduced mobility, appeared in cell bodies and neurites at this time and was found predominantly in the 100K supernatant.

The finding that α -internexin and peripherin were also rendered Triton soluble by OA treatment suggested that they might be coassembled with NFs. To test this

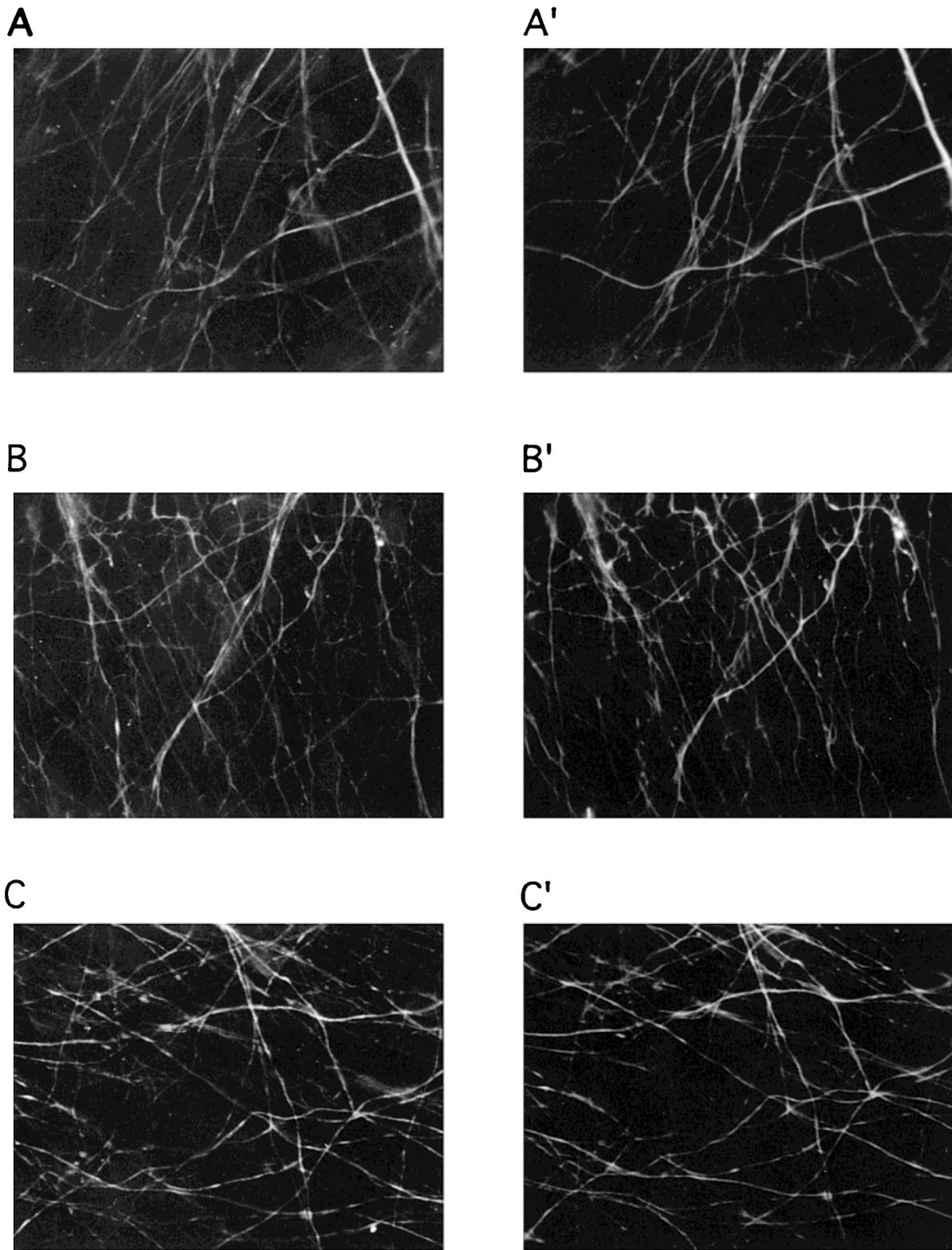
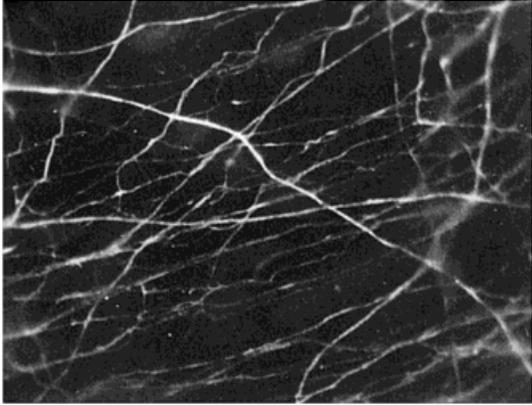


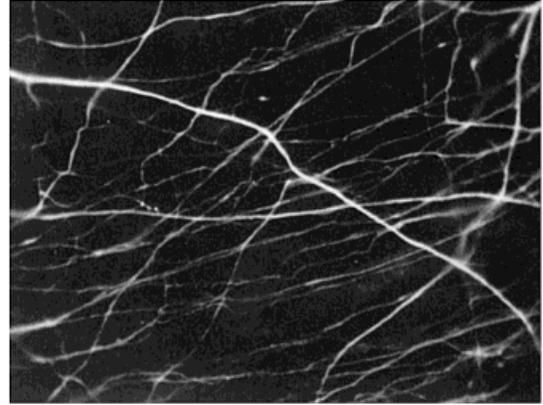
Fig. 3. Co-localization of neuronal IF proteins in cultured E15 DRG neurons by immunofluorescence microscopy. Cells were plated on collagen-coated glass slides, fixed, and stained as described in Materials and Methods. **A–F**: Sister cultures stained with a rabbit polyclonal anti-NFM Ab. **A'–F'**: Costain-

ing with monoclonal Abs specific for NFH (A'), NFM (B'), NFL (C'), peripherin (D'), and α -internexin (E',F'). Panels A, A' to E, E' show staining of neurites, while F and F' show cell body staining.

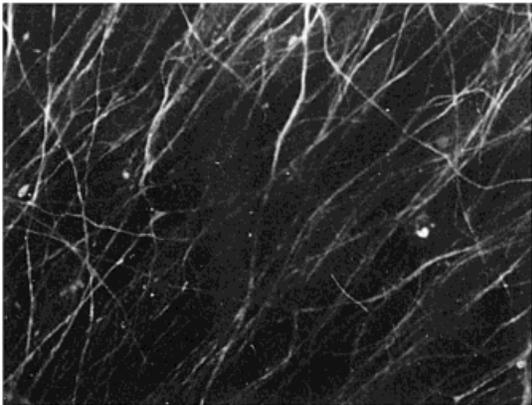
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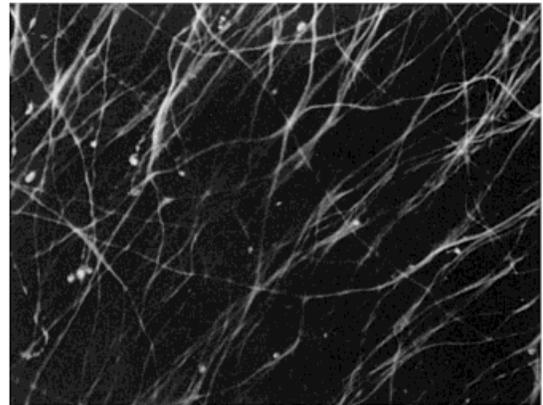
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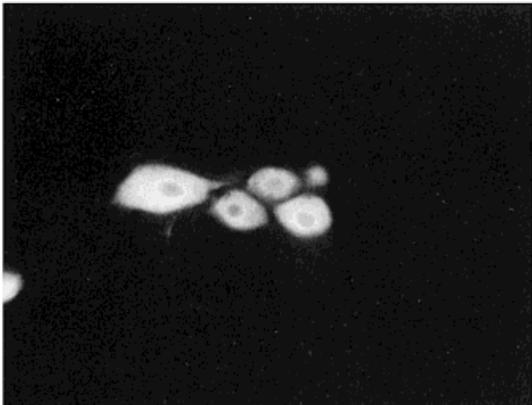
E



E'



F



F'



Fig. 3 continued.

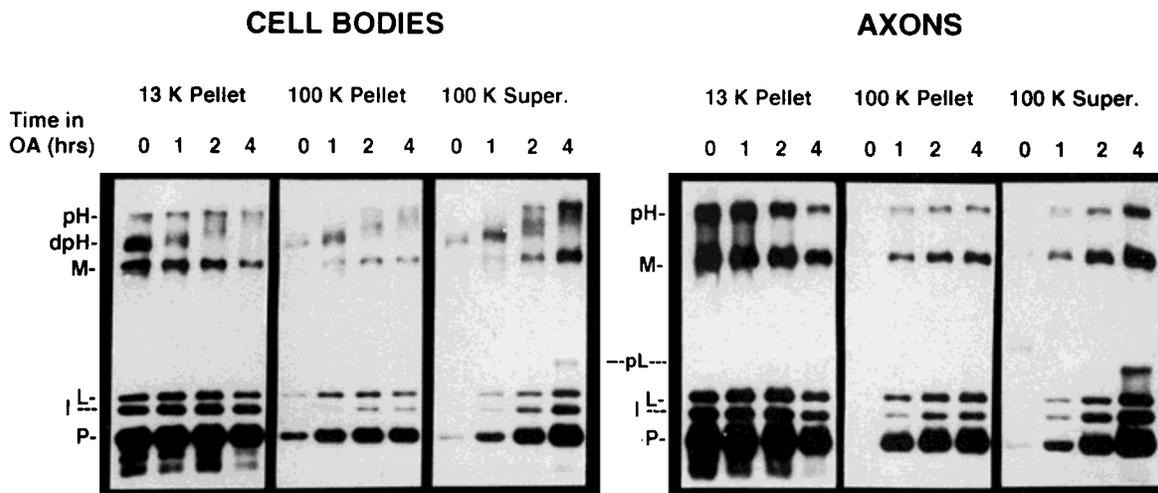


Fig. 4. Western blot analysis of the time-course of neuronal IF fragmentation in OA-treated neurons. Localized DRG cultures were treated with 1 μ M OA for 0, 1, 2, or 4 hr. The cell bodies and neurites were separately harvested in CSK buffer and centrifuged at 13,000g for 15 min. The pellets (13K pellet) were dissolved in SDS-sample buffer, and the supernatants were

centrifuged at 100,000g for 5 min, yielding 100,000g-insoluble (100K pellet) and -soluble (100K super) fractions. Pellets and supernatants were resolved by SDS-PAGE and analyzed by Western blotting as described in the legend to Figure 2. IF protein bands are designated as described in the legend to Figure 1.

possibility, Triton-soluble IF oligomers produced in DRG neurons treated with OA for 4 hr were further analyzed by immunoprecipitation of the 13K supernatants from cell bodies and neurites with Abs against individual IF proteins. Immunoprecipitates were then analyzed by Western blotting using a cocktail of Abs against all five neuronal IF proteins as probes to determine whether any co-immunoprecipitation was taking place (Fig. 5).

There are three separate controls for the immunoprecipitation data shown in Figure 5. The first (Fig. 5, 50% yield) provides a 50% recovery index as it represents the IF proteins in half of the amount of 13K supernatant used for each immunoprecipitation. The second control (Fig. 5, No Abs) shows that trace amounts of only NFM and α -internexin bound to the anti-mouse IgG1-agarose beads in the absence of an anti-IF antibody. The final control (Fig. 5, 2 \times dilution) shows successive twofold dilutions of equimolar amounts of individually purified neuronal IF proteins. The strongest signal was obtained with the anti- α -internexin Ab, the weakest with anti-peripherin and anti-NFL, while Abs against NFM and NFH gave more or less equivalent signals.

The anti-NFH immunoprecipitates shown in Figure 5 contained very low amounts of the other neuronal IF proteins, indicating that there was free NFH present in the extracts. However, there were large amounts of NFH in the anti-NFL immunoprecipitates, suggesting the presence of NFH/NFL hetero-oligomers that either contained substoichiometric amounts of NFL or could not be immunoprecipitated by anti-NFH. The next panel (anti-

NFM) shows that significant amounts of NFL, α -internexin, and peripherin, as well as small amounts of NFH, co-immunoprecipitated with NFM. The highest level of co-immunoprecipitation was obtained with the anti-NFL Ab, which brought down large amounts of the NF triplet proteins and lesser amounts of α -internexin and peripherin. Anti- α -internexin co-immunoprecipitated NFM and peripherin along with α -internexin. The anti-peripherin immunoprecipitates contained peripherin and α -internexin along with lower amounts of NFM. Only one-fifth of the latter immunoprecipitate was loaded on the gel due to the large amounts of peripherin in our DRG cultures and the particular effectiveness of the anti-peripherin Ab for immunoprecipitation.

To further confirm that co-immunoprecipitation of neuronal IF proteins was not due to the fortuitous association of IF proteins in general, the Western blots were stripped and reprobed with an anti-vimentin Ab. The latter showed that there was virtually no vimentin in any of the immunoprecipitates although an appreciable amount could be seen in the initial Triton-soluble extracts (Fig. 5, bottom panel).

DISCUSSION

In this report, we show that cultured E15 DRG neurons from rat embryos resemble their *in vivo* counterparts in certain aspects of their developmental expression of neuronal IF proteins. Examination of Figure 2 indicated that α -internexin was the predominant species in

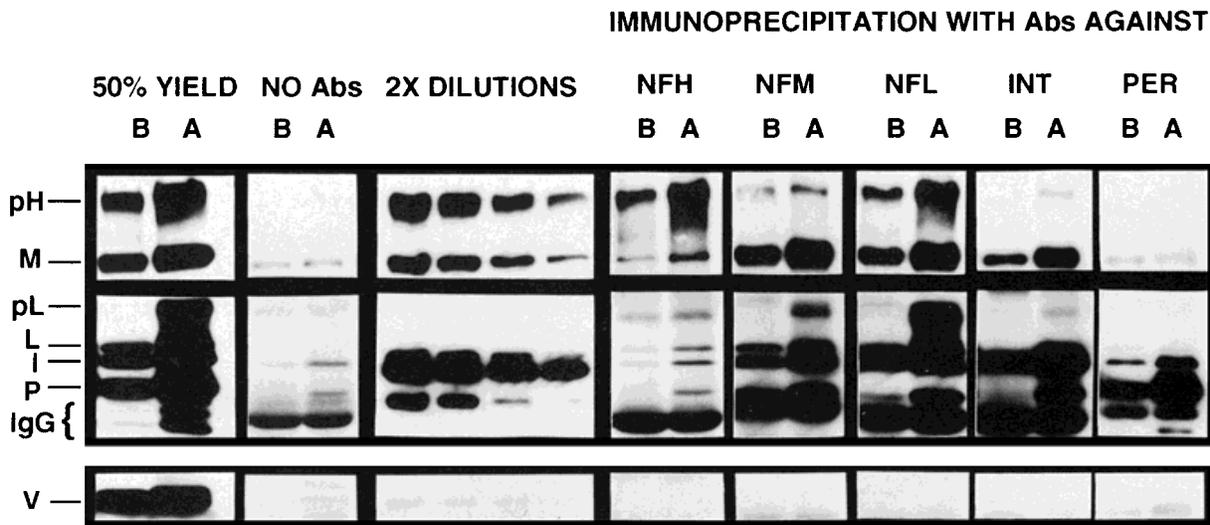


Fig. 5. Western blot analysis of immunoprecipitates from the 13,000g Triton-soluble fractions of cell bodies and neurites of OA-treated neurons. Localized DRG cultures were treated with 1 μ M OA for 4 hr; the cell bodies and neurites were separately harvested in CSK buffer and centrifuged at 13,000g for 15 min. The supernatants were immunoprecipitated as described in Materials and Methods. The panel designations at the top include the following: 50% YIELD, each lane contains one-half of the amount of 13K supernatant fraction used for immunoprecipitation; NO Abs, control with no immunoprecipitating antibody; 2 \times DILUTIONS, successive twofold dilutions of equimolar amounts of all five neuronal IF proteins. NFH, NFM,

NFL, INT, and PER refer to immunoprecipitates obtained with anti-NFH, anti-NFM, anti-NFL, anti- α -internexin, and anti-peripherin, respectively. B and A refer to the cell body and neurite fractions, respectively. Western blots were probed as described in the legend to Figure 2. IF protein bands are designated as described in the legend to Figure 1. Western blots of the various samples and immunoprecipitates were stripped by extraction with 2% SDS, 0.7% β -mercaptoethanol, at 56°C for 30 min and reprobed with an anti-vimentin antibody. The lowest panel shows the vimentin (V) band in these samples. The major band just under peripherin is the IgG heavy chain (IgG).

freshly dissected DRGs and for the first 2 days after plating, consistent with its early expression *in vivo* (Fliegner et al., 1994). The early increase in peripherin levels contradicted reports of its tightly coordinated expression with NFL in DRGs (Escurat et al., 1990). On the other hand, the observed co-expression of NFL and NFM concurs with the results of previous studies (Carden et al., 1987), as does the delay in onset of NFH expression (Shaw and Weber, 1982; Pachter and Liem, 1984; Lindenbaum et al., 1988).

As reported previously (Goldstein et al., 1996), E15 DRG neurons fail to differentiate *in vitro* into two distinct phenotypes expressing either peripherin or NFs. In addition to coexpression of peripherin and NF proteins (Goldstein et al., 1996), we have shown that they retain another embryonic feature, the continued expression of α -internexin. Thus, the reciprocal changes in the levels of NFL and α -internexin expression that occur during development (Fliegner et al., 1990) were not seen. These discrepancies may be due to the inability of cultured neurons to establish contact with appropriate target cells, as attempts to normalize IF protein expression in DRG neurons through the addition of skeletal and heart muscle

extracts have met with limited success (Goldstein et al., 1996).

Following the fragmentation pattern of the neuronal IF network in OA-treated cultures provided some insight into the relationship between the different components. In addition to NF proteins, the Triton solubility of peripherin and α -internexin also increased under these conditions. More significantly, the time-course and extent of solubilization were the same for all of these proteins in both the perikaryal and axonal compartments, suggesting that they were interconnected.

OA-induced NF fragmentation is reversible to a point (Sacher et al., 1992) and is likely due to inhibition of protein phosphatase-2A (Cohen et al., 1990; Sacher et al., 1994), an enzyme associated with NFs that has been proposed to play a role in preserving their filamentous structure (Saito et al., 1995). Moreover, NF fragmentation correlates with an increase in the phosphorylation of two protein kinase A sites in the head domain of NFL (Giasson et al., 1996). Head domain phosphorylation may similarly affect α -internexin (Tanaka et al., 1993), but there is a lack of evidence linking peripherin phosphorylation with disassembly (Aletta et al., 1989). Neverthe-

less, it appears that OA shifts the equilibrium between the antagonistic effects of protein phosphatase-2A and protein kinase A (Giasson et al., 1996), apparently magnifying an oligomerization process involved in NF dynamics (Okabe et al., 1993). Thus, IF proteins contained in the oligomeric products of OA-induced disassembly are likely to retain the normal association patterns that exist *in situ*.

The validity of the co-immunoprecipitation paradigm we used to test for possible associations between the various neuronal IF proteins is based upon several criteria. First, the Western blot in Figure 1 indicated that the individual monoclonal Abs we used did not show any cross-reactivity with other IF proteins. Second, the control in Figure 5 carried out in the absence of primary Abs showed that the fragmented IFs remained soluble throughout the immunoprecipitation protocol. Furthermore, reference to the 50% yield index in Figure 5 indicated that in some cases, co-immunoprecipitated species comprised up to half of the starting material and were likely to be representative of the total fraction. Also noteworthy was the lack of vimentin, a component of satellite cells in DRG cultures, in the immunoprecipitates. All three NF subunits have been shown to co-assemble with vimentin in co-transfected non-neuronal cells (Chin and Liem, 1989, 1990; Monteiro and Cleveland, 1989). The failure of vimentin to co-immunoprecipitate with any of the neuronal IF proteins indicated that the associations we observed did not result from interactions taking place in the Triton extracts following cell lysis.

The co-immunoprecipitation data showed several associations between neuronal IF proteins, but it still is not clear whether the interactions are direct or are due to some protein(s) not detected in these experiments. There is also a problem concerning the apparent lack of reciprocity between co-immunoprecipitates obtained with anti-NFL as compared to those obtained with anti-NFH, anti- α -internexin, and anti-peripherin, respectively. A high level of co-immunoprecipitation of all the neuronal IF proteins was effected by anti-NFL, perhaps reflecting its key role in NF assembly (Ohara et al., 1993). On the other hand, only low amounts of NFL were co-immunoprecipitated by anti-NFH, and none was seen in the anti- α -internexin and anti-peripherin immunoprecipitates. These apparent discrepancies can be explained in several ways. In the case of NFH, it is possible that two forms of this subunit are present in the Triton-soluble fraction, one consisting of free NFH and the other comprising NFH associated with NFL in a form that is refractory to immunoprecipitation by anti-NFH. It is also possible that NFH is associated with hetero-oligomers containing substoichiometric amounts of NFL. However, the latter possibility cannot apply to the anti- α -internexin and anti-peripherin immunoprecipitates because they did

not contain NFL. In these two cases it appears that epitope masking may take place in those hetero-oligomers that contain NFL along with α -internexin and/or peripherin. Although the basis for these discrepancies remains to be determined, they do not detract from the validity of our data in demonstrating associations between the various neuronal IF proteins.

The low level of association between NFM and NFH indicated by the co-immunoprecipitation studies suggests that the two subunits may be contained in different subsets of oligomers. This difference may reflect a fundamental aspect of NF organization and could explain the more dynamic nature of NFH in neurons (Takeda et al., 1994).

The present study provides direct evidence for an association between α -internexin, NFM, and peripherin in cultured E15 DRG neurons, indicating that the IF cytoskeleton in these cells is a highly integrated structure. Perhaps the co-assembly of α -internexin and peripherin with NFs produces IFs with a high degree of plasticity required to support neurite outgrowth (Nixon and Shea, 1992). This notion concurs with the presence of high levels of α -internexin in embryonic DRGs (Fliegner et al., 1994) and with the upregulation of peripherin in large NF-containing DRG neurons during axonal regeneration (Oblinger et al., 1989). The elucidation of these and other questions pertaining to the neuronal IF cytoskeleton will no doubt be facilitated by the availability of cultured neurons that co-express all of the major neuronal IF proteins.

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