Neurite Outgrowth in Peripherin-depleted PC12 Cells

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Abstract. Peripherin is the major neuronal intermediate filament (IF) protein in PC12 cells and both its synthesis and amount increase during nerve growth factor (NGF) promoted neuronal differentiation. To address the question of the biological function of peripherin in neurite initiation we have used an antisense oligonucleotide complementary to the 5′ region of peripherin mRNA to specifically inhibit its transcription. The oligonucleotide blocks both the synthesis of peripherin and its increase in response to NGF. Peripherin was found to be a stable protein with a cellular half-life of ~7 d. 6 wk of incubation with the oligonucleotide decreases peripherin to 11% of the level in naive control cells and to 3% of that in NGF-treated control cells. Despite the depletion, NGF elicits apparently normal neurite outgrowth from the oligonucleotide-treated cells. As evaluated by EM, there are few IFs in these cells, either in the cell bodies or neurites. There is no compensatory increase in NF-M, NF-L, or vimentin levels as a result of the inhibition of peripherin synthesis. These findings suggest that peripherin is not required for neurite formation, but is necessary for the formation of a cellular IF network which could be involved in process stability. They also demonstrate the utility of antisense oligonucleotides for the study of proteins with long half-lives.

WHILE at least six neuron-specific intermediate filament (IF)[1] proteins have been identified (Fliegner and Liem, 1991), little is definitively known about their function. The best studied of these is the neurofilament (NF), which is composed of three proteins: NF-L, NF-M, and NF-H (Liem et al., 1978; Fliegner and Liem, 1991). The relative levels of NF proteins in neurons correlate well with axonal diameter, suggesting that neurofilaments play a major role in the regulation of axon caliber (Hoffman et al., 1985, 1987). Another member of the IF family is peripherin, a 57-kD protein which forms IFs in neurons of the peripheral nervous system, midbrain, and cranial nerves (Portier et al., 1983/84*b*; Leonard et al., 1988; Parysek and Goldman, 1988). In contrast to the NFs, which belong to the type IV class of IFs, peripherin belongs to the type III class (Leonard et al., 1988; Parysek et al. 1988; Thompson and Ziff, 1989). Developmentally, peripherin appears at approximately the same time as the neurofilament proteins (Escurat et al., 1990; Gorham et al., 1990; Troy et al., 1990*a*) and is expressed after neuronal proliferation and migration have ceased. In some cases peripherin and the neurofilament triplet proteins coexist in the same neuron while in other cases they appear to exist independently (Ferri et al., 1990; Troy et al., 1990*a*).

Axotomy studies suggest that different functions may be served by peripherin and the NFs. Regenerating dorsal root ganglion neurons (Oblinger et al., 1989) and spinal motor neurons (Troy et al., 1990*b*) show increases in peripherin mRNA and protein shortly after axotomy, with a return to normal levels by completion of regeneration. These increases occur at the same time that the levels of the NFs and their mRNAs are decreasing. The regenerating axon is thinner than the normal axon and only regains its full diameter after regeneration is complete and NF levels have increased (Hoffman et al., 1985). Such observations support a role for NFs, but not peripherin, in regulating axonal caliber and suggest a possible role for peripherin in neurite growth and regeneration.

Peripherin is the major neuronal IF in clonal rat PC12 pheochromocytoma cells (Aletta et al., 1988; Leonard et al., 1988; Parysek and Goldman, 1987). PC12 cells elaborate long, branching neurites in response to treatment with NGF (Greene and Tischler, 1976) and EM studies reveal these to contain IFs (Tischler and Greene, 1978; Parysek and Goldman, 1987). In parallel with process formation, NGF treatment leads to increases in peripherin mRNA (Leonard et al., 1988) and protein (Aletta et al., 1988; Portier et al., 1983/84*a*; Parysek and Goldman, 1987).

Antisense oligonucleotides represent a powerful tool for specifically inhibiting mRNA transcription. For instance, in PC12 cells, exposure to tubulin mRNA antisense oligonucleotides leads to tubulin depletion and inhibition of neurite outgrowth (Teichman-Weinberg et al., 1988). In primary neuronal cultures, antisense oligonucleotide to tau, a microtubule-associated protein, inhibits tau transcription and blocks axon formation (Caceres and Kosik, 1990).

In this report we have used antisense oligonucleotides to

1. *Abbreviations used in this paper*: HS, horse serum; IF, intermediate filament; NF, neurofilament; NGF, nerve growth factor.

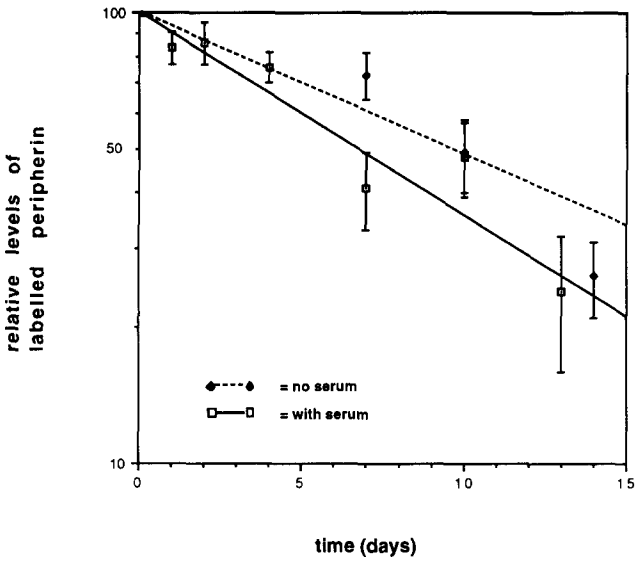

Figure 1. Peripherin half-life in PC12 cells. PC12 cells were labeled with [^{35}S]methionine for 4 h. Cultures were then washed and grown with the indicated nonradioactive medium as described in the methods. At the times indicated, cytoskeletons were extracted and subjected to 6–12% SDS-PAGE (50 μg protein/lane). Autoradiograms were analyzed by densitometry for relative level of labeled peripherin. The peripherin level in cells harvested immediately post-labeling (*day 0*) was assigned a relative value of 100. r^2 = 0.91. Values represent means ± SEM (n = 5).

study the effects of peripherin depletion on responses of PC12 cells to NGF, and in particular, on their capacity to generate neurites.

Materials and Methods

Cell Culture

PC12 cells were grown as previously described (Greene and Tischler, 1976) on rat tail collagen-coated dishes in RPMI 1640 medium containing 5% FCS and 10% horse serum (HS) (JRH Biosciences, Lenexa, KS). For incubation with oligonucleotides, the cells were washed three times with serum-free RPMI 1640 and replated onto fresh collagen-coated dishes in serum-free RPMI 1640 supplemented with 3 μM insulin (Sigma Chemical Co., St. Louis, MO) and 1% BSA. Oligonucleotides were added at the concentrations indicated. Mouse submaxillary nerve growth factor (NGF) was added at a concentration of 50 mg/ml where indicated.

Half-life Studies

Naive PC12 cells (NGF untreated) were labelled with ^{35}S-methionine (ICN Biomedicals, Inc., Irvine, CA; 250 μCi/ml in methionine-free DME for 4 h). Radioactive medium was removed and the cells were washed three times with non-radioactive RPMI 1640. Fresh RPMI 1640 with 5% FCS and 10% HS or with 3 μM insulin, 1% BSA, was added to the dishes. Cells were harvested at time points indicated. Harvesting included washing dishes three times with PBS, then extracting overnight at 4°C with Ca^{2+}-, Mg^{2+}-free PBS buffer containing 1% Triton X-100, 2 mM EGTA, 50 mM NaF, 2 mM PMSF, and Trasylol 20,000 KIU/ml (Aletta et al., 1988). Buffer was removed and the remaining material was scraped up in sample buffer, boiled, and 50 μg protein/sample analyzed by one-dimensional SDS-PAGE (Laemmli, 1970) on 6–12% gradient gels followed by autoradiography. Densitometric scanning of autoradiographs was performed on a MicroScan 1,000 Gel Analyzer (Technology Resources, Inc., Nashville, TN).

Oligonucleotides

Antisense oligonucleotides were synthesized and purified by Operon Technologies, Inc. (Alameda, CA). Four different peripherin antisense oligonucleotides, each 21-bases long, were synthesized. The sequences were inverse complements of nucleotides 35-56(P1), 47-68(P2), 54-75(P3), and 3441-3461(P4). There are two potential ATG translation initiation codons in peripherin (Thompson and Ziff, 1989) at nucleotides 38 and 56. The sequences selected did not correspond to any other sequence in the database (GenBank and EMBL). The sense sequence of nucleotides 35-56(P5) was kindly synthesized and purified by Dr. C. Stein (Columbia University, New York).

Stability of Oligonucleotides in Media

Oligonucleotide P1 was 5′ labeled with [γ-^{32}P]ATP (New England Nuclear, Wilmington, DE) using T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, IN) as described by Sambrook et al. (1989). Labeled oligonucleotide was added to different media and incubated for 24 h at 37°C. Media were : RPMI 1640 with 3 μM insulin, RPMI 1640 with 3 μM insulin + 1% BSA, RPMI 1640 + 5% FCS, RPMI 1640 + 1% FCS, RPMI 1640 + 0.5% FCS, RPMI 1640 + 0.1% FCS. Samples were separated on a 10% acrylamide sequencing gel, and visualized by autoradiography.

PAGE and Western Immunoblotting

Cultures were washed free of media with Ca^{2+}-, Mg^{2+}-free PBS and lysed in sample buffer for one-dimensional SDS-PAGE. Material from equal numbers of cells (300,000 cells/lane; 50–100 μg protein) were electrophoresed on 6–12% gradient gels, and protein was blotted onto nitrocellulose. The nitrocellulose was blocked with 5% milk and then incubated with a mixture of anti-peripherin at 1/2,000 and anti-actin (gift of J. C. Bulinski, Columbia University) at 1/10,000, or with a mixture of anti-NF-L at 1/1000, anti-NF-M at 1/500, anti-vimentin at 1/100 (gifts of R. Liem, Columbia University) and anti-actin at 1/10,000. After being washed with PBS, filters were incubated with ^{125}I-protein A, washed, dried, and autoradiographed (Georgieff et al., 1991).

Quantification of Data

Autoradiograms were scanned with a densitometer and a calculation of the ratio of peripherin to actin was made for each sample. All calculations were based on these ratios.

Cell and neurite size and number were measured from light micrographs using Bioquant System IV software (R&M Biometrics, Inc., Nashville, TN).

Electron Microscopy

Cells were grown in serum-free medium with NGF as described above, in the presence or absence of oligonucleotide P1 (0.2 μM) for 40 d. They were then fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in PBS for 30 min, fixed with 1% osmium tetroxide (Electron Microscopy Sciences) for 20 min, dehydrated through alcohols, and infiltrated with Epon 812 (SPI Supplies, West Chester, PA) overnight. The cultures were embedded in Epon, polymerized at 60°C for 24 h, cooled, and the plastic was broken away. Cell areas from the Epon-embedded monolayer were chosen and re-embedded in an Epon capsule. Thin sections (60–90 nm) were cut with an MT5000 microtome, stained with uranyl acetate and lead citrate and examined with a JEOL 100S electron microscope (100S; JEOL USA, Crawford, NJ).

Results

Peripherin Half-life in PC12 Cells

The half-life of peripherin in living PC12 cells was determined to estimate the time that would be required for its depletion. PC12 cells, without NGF, were labeled with [^{35}S]methionine for 4 h and then maintained for a period of up to 14 d. After various times, cell cytoskeletons were prepared and analyzed by SDS-PAGE and autoradiography. The 57-kD band in these preparations is composed solely of peripherin and is free of contamination by vimentin or other proteins (Aletta et al., 1988). These studies showed that peripherin levels fell to 50% of their control values in ~7 d

Table 1. Sequences of the Antisense Oligonucleotides Used for Inhibition Experiments

Oligo	5'nt	Sequence	3'nt
P1	35	GCTGGCGGAAGATGGCATTCA	56
P2	47	GTGATGGCTCATGCTGGCGGA	68
P3	54	CGACGAGTGATGGCTCATGCT	75
P4	3,441	GTAGCTGTGAATAGAAGACTT	3,461

The sequence positions are taken from the nucleotide sequence data published by Thompson and Ziff (1989).

when cells were grown with serum, and in ~10 d when grown without serum (Fig. 1). For similar studies with PC12 cells treated with NGF for 2 wk, the half-life of peripherin was also found to be ~7 d.

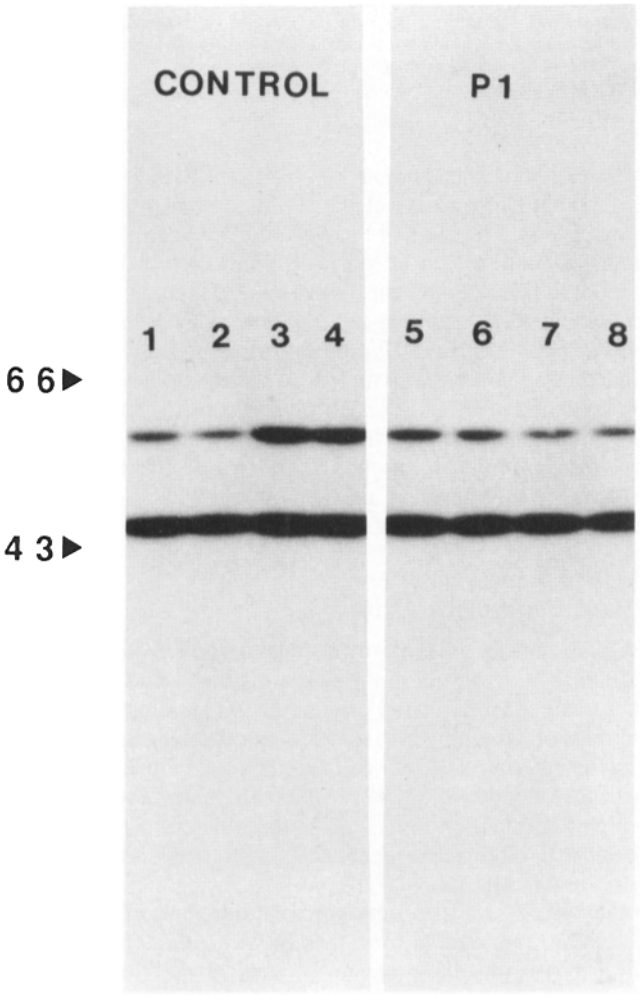

Figure 2. Effect of P1 antisense oligonucleotides on the induction of peripherin by NGF. Cells (300,000/lane) were analyzed by Western immunoblotting using anti-peripherin (1:2,000) (*upper bands*) and anti-actin (1:10,000) (*lower bands*). (*Control*) Naive PC12 cells (lanes *1* and *2*); PC12 cells treated with NGF (50 ng/ml) for 2 d (lanes *3* and *4*). (P1) PC12 cells treated with P1 (0.2 μM) for 2 d (lanes *5* and *6*); PC12 cells treated with both P1 (0.2 μM) and NGF (50 ng/ml) for 2 d (lanes *7* and *8*). All cultures were grown in serum-free medium, as described in the methods. Positions of 66- and 43-kD markers are shown on the left.

Table II. Effect of Oligonucleotides on the NGF-induced Peripherin Increase in PC12 Cells

Oligo	Percent increase in peripherin	*n*
Control	80 ± 10	12
P1	0 ± 3	15
P2	65 ± 5	14
P3	100 ± 11	9
P4	80 ± 10	9
P5	80 ± 9	6

P5 is the sense sequence of P1. Cells were grown for 2 d in serum-free medium supplemented with insulin and BSA, with or without NGF (50 ng/ml) and with or without the indicated oligonucleotide (0.2 μM). Protein from equal numbers of cells was analyzed by western immunoblotting and percent increase in peripherin is expressed as the change in peripherin level after treatment with NGF as compared to peripherin levels in NGF-untreated cultures. *n* = number of independent samples analyzed.

Evaluation of Peripherin Antisense Oligonucleotides

Four 21-base peripherin antisense oligonucleotides were synthesized. One was complementary to the 3' coding region (P4) of peripherin mRNA. The other three were complementary to the 5' region (P1-P3). Peripherin has two potential ATG translation initiation codons, at 38 and 56 nucleotides, respectively, 3' to the cap site (Thompson and Ziff, 1989). Both are in frame, although it has been suggested that the second ATG has a preferred sequence context for initiation (Thompson and Ziff, 1989). The oligonucleotides were designed to span this region, with two including only the second ATG site (P2 and P3), and the third encompassing the first and ending just before the beginning of the second site (P1) (Table I).

The four oligonucleotides were initially screened for ability to inhibit the NGF-induced increase in PC12 cell peripherin protein levels (Portier et al., 1983/84*a*; Aletta et al., 1988). When PC12 cells were treated for 2 d with NGF there was a consistent increase in peripherin levels (Fig. 2). This response was completely inhibited by oligonucleotide P1 (Fig. 2 and Table II). As shown in Table II, antisense oligonucleotides P3 and P4 had no effect on the NGF-induced increase in peripherin protein levels, while P2 partially inhibited this response. The sense oligonucleotide complementary to P1 had no effect on the peripherin increase (Table II). All oligonucleotides were used at a concentration of 0.2 μM and media were changed daily. All further experiments were done with the P1 oligonucleotide.

Degradation of oligonucleotide P1 was assessed by incubating ^{32}P-labeled P1 with different media. Addition of as little as 0.1% FCS to the medium resulted in ~30% more degradation of the oligonucleotide over 24 h (data not shown). For this reason, experiments were carried out with serum-free medium supplemented with 1% BSA and 3 μM insulin. The latter medium has been shown to promote long-term survival of PC12 cells (Rukenstein et al., 1991).

Dose–response experiments revealed that optimal results were obtained using concentrations of P1 antisense oligonucleotide ranging from 0.2–0.5 μM; a decrease in effect was observed above 1 μM.

To assess the effect of the antisense oligonucleotide on peripherin protein synthesis, cells were pretreated with 0.2 μM P1 for 5 d and then labeled with [^{35}S]methionine for 4 h. Under these conditions, peripherin protein synthesis was

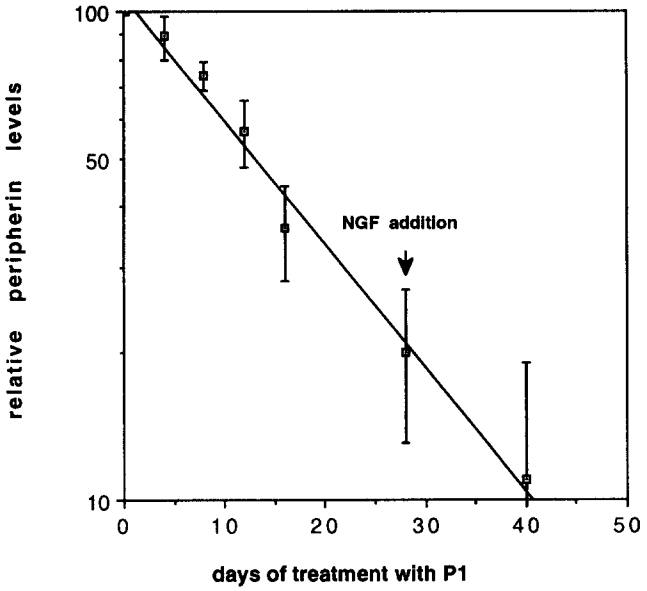

Figure 3. Depletion of peripherin in PC12 cells using antisense oligonucleotide P1. Cells were grown in the presence of oligonucleotide P1 (0.2 μM) for 28 d in serum-free medium. At 28 d cells were replated and grown with NGF (50 ng/ml) in the continued presence of P1 (0.2 μM). At times indicated, cells (300,000/lane) were analyzed by Western immunoblotting using anti-peripherin (1:2,000) and anti-actin (1:10,000). Data are presented as percent of peripherin in naive cells and are normalized to levels of actin. $r^2 = 0.99$. Values represent means ± SEM ($n = 8$).

inhibited by 90% ± 5%. Peripherin half-life in the presence of P1, evaluated by prelabeling with [^{35}S]methionine and then culturing in the presence of the oligonucleotide, was found to be 10 d, the same as in control serum-free cells.

Depletion of Peripherin by Exposure to Antisense Oligonucleotide P1

Cells were incubated with antisense oligonucleotide for up to 28 days before the addition of NGF. Attempts to carry PC12 cells in serum-free medium with insulin and BSA without NGF for periods longer than this were confounded by increasing variability in cell survival. This was true for both control and oligonucleotide-treated cultures. At 28 d the peripherin level was 20% of that in control cells (Fig. 3). The addition of NGF at this point did not increase the peripherin content of the oligonucleotide-treated cells; instead the peripherin level continued to decrease. After 40-d incubation with the antisense oligonucleotide (28 d without NGF and 12 d with NGF) peripherin protein (expressed per cell) was reduced to 11% of the level in naive cells (Fig. 3). However, when compared to control cells which had been subjected to the same regimen of 28 d without and 12 d with NGF, but in the absence of oligonucleotide, the peripherin levels were only 3 to 4% of control values. This is a result of an approximately threefold increase in peripherin in control cells treated with NGF. A similar degree of depletion was obtained with cells simultaneously treated with antisense oligonucleotide and NGF throughout the experiment.

Although peripherin is by far the major IF protein in PC12 cells (Aletta et al., 1988, 1989), other IFs have been detected

Table III. Quantification of Neurite Number and Length in Cells Treated with and without P1 Oligonucleotide

Days in culture	Cell clump diameter	Number neurites/clump	Neurite length
	(μm)		(μm ± SE)
15:			
control	210	39 ± 10	224 ± 87
P1	210	39 ± 10	245 ± 54
18:			
control	80	15 ± 5	191 ± 90
P1	80	15 ± 5	167 ± 85
18:			
control	130	22 ± 3	122 ± 60
P1	130	22 ± 3	122 ± 48
29:			
control	66	12 ± 2	155 ± 78
P1	66	12 ± 2	125 ± 40

Cells were grown for the times indicated in the absence (*control*) or presence (*P1*) of oligonucleotide P1 (0.2 μM). All cells were treated with NGF 2 wk before analysis. Neurites from 10 different cell clumps (chosen at random, but of equal diameter) at each condition indicated were counted and measured using Bioquant System IV. Average fiber bundle diameter was 2 μm for both control and P1 treated cells at 18 d of treatment.

immunologically including the NF triplet (Lee et al., 1982; Lee, 1985; Lindenbaum et al., 1988) and vimentin (Lee and Page, 1984). The work of Parysek and Goldman (1987) indicates that the "vimentin" previously detected in PC12 cells may have been peripherin. Although the sequence of oligonucleotide P1 was chosen to avoid homology with messages encoding other known IF proteins, the possibility remained that there could be coordinate or reciprocal regulation of different IFs in PC12 cells. To ascertain if this were indeed the case, we measured levels of vimentin, NF-M, and NF-L by Western immunoblotting in NGF-treated control and P1-treated cells. There were no detectable effects of peripherin depletion on the levels of these proteins.

NGF-induced Neurite Formation by Peripherin-depleted Cells

PC12 cells were treated for 28 d with antisense oligonucleotide P1 followed by 12 d of treatment with P1 and NGF, and then analyzed by light and EM. At the light microscopic level it was not possible to distinguish the peripherin-depleted cells from controls. Both cell types had similar numbers of neurites and the latter were of comparable diameter and length (Table III and Fig. 4). As time in serum-free media increased, clump size decreased in both control and experimental cells.

At the EM level, in contrast to controls, the experimental cells were remarkable for a lack of IF bundles (Fig. 5). At lower power, the experimental cells had grossly normal appearing processes, consistent with their light microscopic appearance. At higher power (Fig. 5), both control and experimental cells contained normal complements of microtubules, coated pits, dense-core vesicles, and ribosomes. The control cells contained prominent bundles of IFs running centrally along the length of their processes as well as abundant short strands of IF in their perikarya (Fig. 5 *a*). These filaments did not appear to form bridges to the microtubules. In the experimental cells (Fig. 5 *b*) there was a complete absence of IF bundles and the cytoplasm appeared less electron

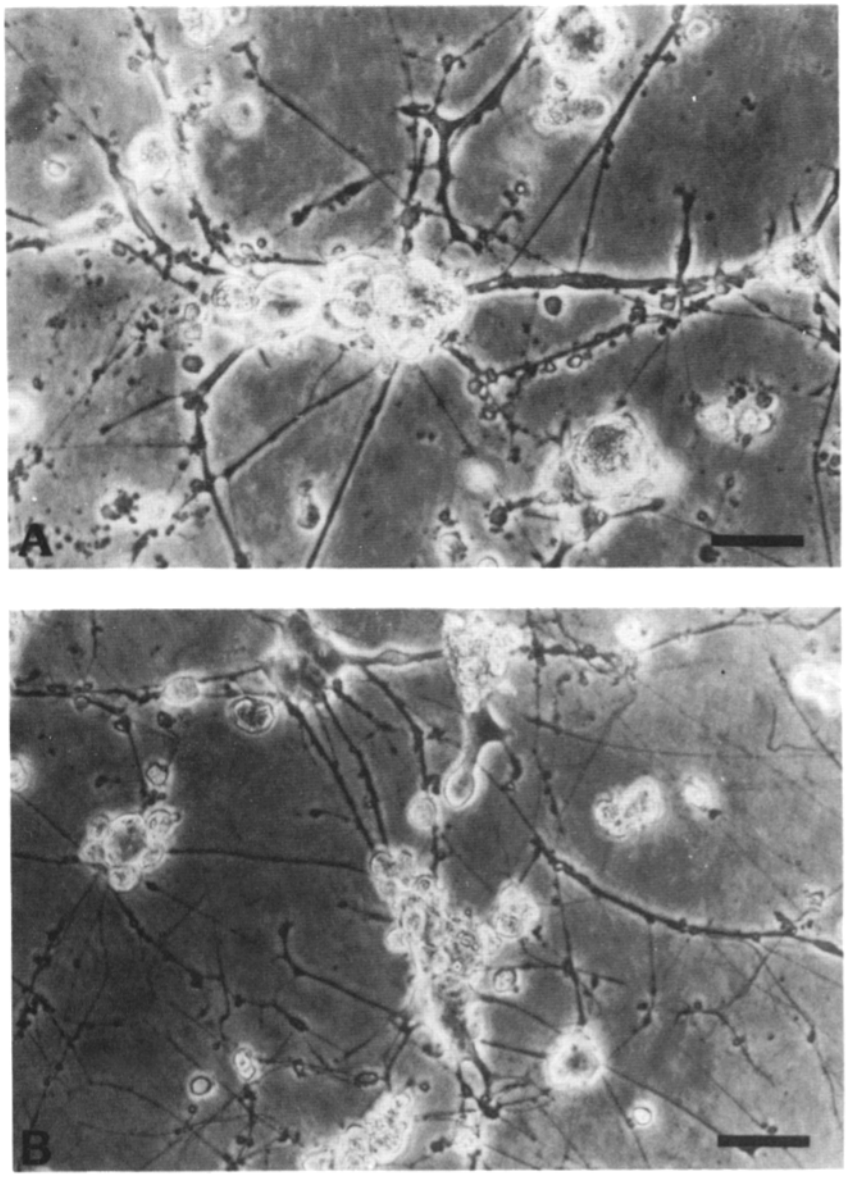

Figure 4. Effect of peripherin depletion on the morphology of NGF-treated PC12 cells. Cells were grown for 29 d, in the absence (*a*) or presence (*b*) of P1 (0.2 μM), with NGF (50 ng/ml) added from days 11–29. Phase contrast optics. Bar, 50 μm.

dense. Rare short strands of IFs could be seen in both the neurites and cell bodies. The protein composition of the filaments was not determined.

Discussion

We used an antisense oligonucleotide to deplete PC12 cells of peripherin to examine the role of this protein in neuronal differentiation. Peripherin is an NGF-regulated IF that is the major IF in PC12 cells (Parysek and Goldman, 1987; Leonard et al., 1988; Aletta et al., 1988, 1989). Increases in PC12 cell peripherin mRNA and protein are detectable at 2 d of NGF treatment and continue in parallel with process outgrowth (Leonard et al., 1988). Our data indicate that peripherin has a half-life of ~7 d in both naive and NGF-treated cells grown with serum, and of ~10 d in cells grown without serum. This is at the upper end of the range of determined half-lives for a variety of proteins. The majority of rat liver proteins have half-lives ranging from 2–8 d (Glass and Doyle, 1972). For cytoskeletal proteins, the range is also wide: 2 d for actin in fibroblasts (Antecol, 1988), 3.5 d for cytokeratin A, 4.3 d for cytokeratin D (Denk et al., 1987), 8 d for glial fibrillary acidic protein (GFAP) and vimentin in astrocytes (Chiu and Goldman, 1984), and almost 9 wk for GFAP in vivo (DeArmond et al., 1986). Our preliminary experiments (unpublished data) suggest that the half-life of peripherin in cultured newborn rat sympathetic neurons is twice as long as in PC12 cells.

Two possible initiation codons have been identified for peripherin (Thompson and Ziff, 1989). Of the four antisense oligonucleotides that were tested for efficacy in blocking the NGF-induced increase of peripherin, total blockade was achieved only by the oligonucleotide that encompassed the first ATG and that stopped just before the second ATG. Partial inhibition was achieved with an oligonucleotide that started 10 bases after the first ATG and included the second ATG. The oligonucleotide that began at the second ATG was ineffective. If it is assumed that antisense nucleotides work

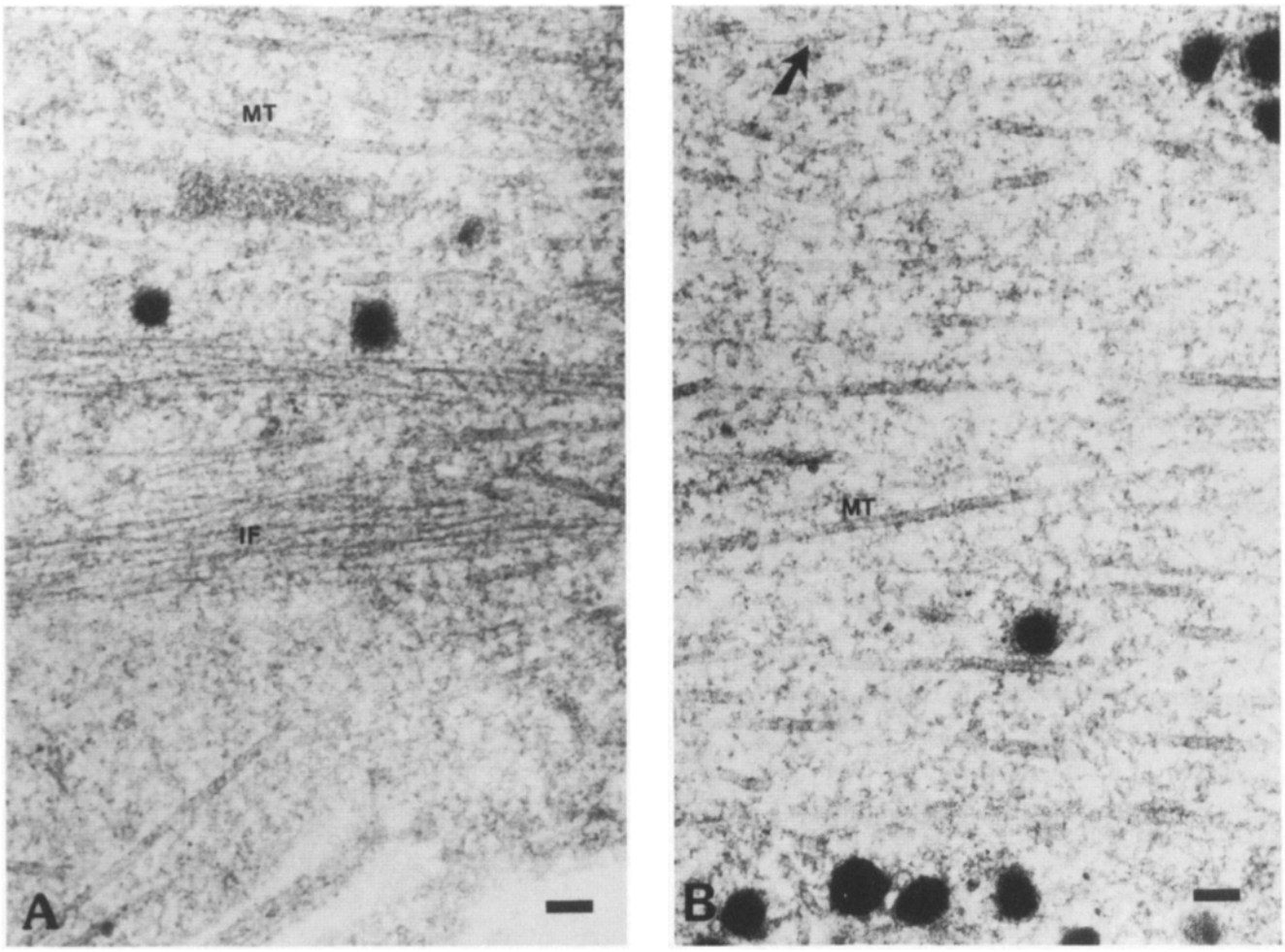

Figure 5. Electron micrographs of PC12 cells grown for 40 d in the absence (*a*) or presence (*b*) of PI (0.2 μM), with NGF (50 ng/ml) added from days 29–40. *IF*, intermediate filament bundles; *MT*, microtubules; *arrow*, indicates possible small strands of IF. Bar, 0.1 μm.

best in the region of translation initiation, these results suggest that the first ATG can function as the translation initiation codon, either in contrast or in addition to the second, which has been proposed previously as the more likely site for initiation (Thompson and Ziff, 1989). In support of this, a cDNA for rat peripherin has been isolated (S. Chin and R. Liem, personal communication) in which only the first ATG is present. An antisense oligonucleotide to the 3′ coding region had no effect. This is in agreement with reports that antisense oligonucleotides to the 5′ region generally work better than those to the 3′ region (i.e., Teichman-Weinberg et al., 1988).

The antisense oligonucleotide used in these studies was most effective at concentrations of 0.2–0.5 μM. This is in contrast to work using antisense oligonucleotides to other cytoskeletal proteins, such as tubulin in PC12 cells (Teichman-Weinberg et al., 1988) and tau in primary cerebellar cultures (Caceres and Kosik, 1990) in which concentrations of 50 μM produced the best effects. In non-neuronal systems, maximal effects of antisense oligonucleotides have also been obtained at concentrations substantially higher than our optimal concentrations. For example, inhibition of IL-6 in AIDS-KS cells required 15–20 μM oligonucleotide (Miles et al., 1990). All of these systems, as ours, have used serum-free conditions. Although the mechanisms that dictate optimal effective levels of antisense oligonucleotides are unknown, our findings indicate that, at least in some cases, these compounds may be active at lower concentrations than generally used.

Antisense oligonucleotides have previously been used to inhibit synthesis of soluble cytoskeletal proteins with relatively fast turnover times (Teichman-Weinberg et al., 1988; Caceres and Kosik, 1990). Our studies show that they can also be used effectively for more stable proteins provided appropriate culture conditions are used. Under the conditions used, we were able to almost completely inhibit peripherin synthesis and substantially deplete peripherin from the cells. Process outgrowth was unaffected by depletion of peripherin indicating that this protein is not required for neurite initiation, elongation, or maintenance. We also found no increases in NF proteins or vimentin to compensate for the decrease in peripherin, and EM studies showed a complete absence of IF bundles in the oligonucleotide-treated naive or neurite-bearing cells. Interestingly, in preliminary experiments antisense oligonucleotide-induced reduction of peripherin to less than half of control values in cultured newborn rat sympathetic neurons had no effect on morphology or viability.

The present findings leave us with open questions as to the function of peripherin in neuronal morphogenesis. Peripherin is present early in the formation of processes by embryonic peripheral and ventral horn neurons (Escurat et al., 1990; Gorham et al., 1990; Troy et al., 1990*a*). It is the major, and perhaps only, IF in certain ganglion neurons (Parysek and Goldman, 1988; Oblinger et al., 1989; Ferri et al., 1990; Troy et al., 1990*a*) and its expression increases during neural regeneration (Oblinger et al., 1989; Troy et al., 1990*b*). These facts all suggest that it plays an important role in some aspect of neurite growth or stability. On the other hand, cells which have only a small fraction of their normal complement of this protein extend processes at a normal rate, in normal number and with normal morphology. It is, of course, possible that peripherin is present in great excess and that these small amounts are sufficient. However, if the protein is in great excess it is difficult to understand why it increases both in de novo generation of neurites induced by NGF and on neuronal regeneration after axotomy. In contrast to the present observation, Weinstein et al. (1991) used transfection of antisense constructs to deplete GFAP in a human astrocytoma cell line and found that process formation was inhibited. One alternative is that our experiments have not tested the appropriate function. In both neuronal development and neuronal repair, the growing axon is required to thread its way though the complex neuropil to reach its target. This pathfinding may well depend on the erection of a relatively rigid scaffold behind the growing region of the neurite and this rigidity might be imparted by the peripherin filaments. Neurites growing over a tissue culture surface negotiate a relatively simple terrain and might not require this support. At the EM level, the peripherin filaments are bundled closely together and, unlike NFs, do not appear to interact with microtubules to form a cytoplasmic lattice. This core or bundle of filaments might allow the peripherin filaments to provide structural support while allowing dynamic rearrangements of other cytoskeletal elements that are compatible with neurite extension. Similarly, the relatively short processes (<1 mm) generated in culture may not provide an adequate test of the role of peripherin in maintaining the stability of the long projection processes in which this protein is generally localized.

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