A Role for Intermediate Filaments in Determining and Maintaining the Shape of Nerve Cells

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Abbreviations used in this paper:  IF, intermediate filaments; NF, neurofilaments; MT, microtubules; siRNA, small interfering ribonucleic acid; DM, differentiation medium; CM, complete medium

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Short Title:  Nerve Cell Shape Requires Peripherin

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ABSTRACT

To date, the functions of most neural intermediate filament (IF) proteins have remained elusive. Peripherin is a Type III intermediate filament (IF) protein that is expressed in developing and differentiated neurons of the peripheral and enteric nervous systems. It is also the major IF protein expressed in PC12 cells, a widely used model for studies of peripheral neurons. Dramatic increases in peripherin expression have been shown to coincide with the initiation and outgrowth of axons during development and regeneration, suggesting that peripherin plays an important role in axon formation. Recently, small interfering RNAs (siRNAs) have provided efficient ways to deplete specific proteins within mammalian cells. In this study, it has been found that peripherin-siRNA depletes peripherin and inhibits the initiation, extension and maintenance of neurites in PC12 cells. Furthermore, the results of these experiments demonstrate that peripherin IF are critical determinants of the overall shape and architecture of neurons.
INTRODUCTION

The cytoskeleton of vertebrate cells consists of three major types of protein networks: intermediate filaments (IF), microfilaments (MF) and microtubules (MT). Intermediate filaments are the most diverse of the three as they are encoded by more than 65 genes, making the IF superfamily one of the 100 largest in the human genome (Hesse et al., 2001). These genes are developmentally regulated, resulting in the cell-type specific expression of IF. This is clearly evident in the nervous system, where at least seven different IF proteins are expressed, ranging from the complex neurofilament (NF) heteropolymers composed of the Type IV triplet proteins NF-L, NF-M and NF-H, to the simpler homopolymers of the Type III IF protein, peripherin (Leung et al., 1998). Peripherin forms the major IF system of peripheral and enteric neurons, and it is abundantly expressed in PC12 cells, a widely used model for studies of peripheral neurons (Leonard et al., 1988; Parysek and Goldman, 1987; Portier et al., 1983a; Portier et al., 1983b).

One of the hallmarks of mature or terminally differentiated neurons is their remarkable shape, highlighted by extremely long cytoplasmic processes such as axons. The initiation, extension and maintenance of axons involve the coordinated interactions of different cytoskeletal proteins (Dickson, 2002; Mueller, 1999). To date, only MT and MF have been considered essential for growth cone activity and axon outgrowth (Letourneau, 1996). In contrast, the contribution of neural IF to these processes has not been defined.

The expression patterns of neural IF are correlated with different phases of axonal development. Type III IF, such as peripherin and vimentin, are present throughout the early stages of axon outgrowth, and later Type IV NF triplet proteins are expressed as axons reach maturity (Cochard and Paulin, 1984; Troy et al., 1990a). In mature neurons, NF appear to be
major determinants of axon caliber, and thus conduction velocity (Hoffman et al., 1987; Lasek et al., 1983). However, little is known about the functions of the type III IF in either developing or mature neurons. Based on their presence in early development, it is possible that Type III IF play a role in the initiation, outgrowth and maintenance of axonal structure and shape in both cultured neurons, as well as in neurons in situ. For example, in the case of PC12 cells, it has been shown that peripherin expression is significantly increased following the induction of neurite outgrowth by nerve growth factor (NGF; Aletta et al., 1988; Aletta et al., 1989; Leonard et al., 1987). This is accompanied by the rapid formation of motile non-filamentous peripherin particles and short filaments (or squiggles; Prahlad et al., 1998). These two structural forms, thought to be precursors to long IF, are found in all regions of growing neurites, including the central and peripheral domains of growth cones (Helfand et al., 2003). Peripherin expression has also been correlated with the initiation and outgrowth of axons that take place within the developing nervous systems of vertebrate animals (Gervasi et al., 2000; Troy et al., 1990b; Undamatla and Szaro, 2001). Injured peripheral neurons in situ also show significant increases in peripherin IF expression during axonal regeneration, while at the same time NF protein expression decreases (Oblinger et al., 1989b). The resulting regenerating peripherin-rich axons are of notably smaller diameter relative to mature axons. Once outgrowth is completed, NF triplet proteins return to their normal levels as peripherin expression is down regulated and the mature axon caliber is re-established (Hoffman et al., 1985). Based upon these observations, it appears likely that peripherin is involved in the initiation and outgrowth of axons.

Recently, the use of small interfering RNA (siRNA) to silence the expression of gene products has produced important new insights into the functions of specific targeted proteins in mammalian cells (Elbashir et al., 2001; Shi, 2003). In this study, we use siRNA to determine the
effects of peripherin IF depletion on various parameters related to the shape and form of PC12 cells including the initiation, outgrowth and maintenance of neurites.
MATERIALS AND METHODS

Cell Culture

Stock cultures of rat PC12 cells were maintained in Complete Medium (CM: DMEM (GIBCO) containing 10% calf serum, 1mM sodium pyruvate, and 50 units penicillin and 50mg/ml streptomycin) at 37°C as previously described (Helfand et al., 2003). For some siRNA studies, cells from stock cultures were transferred to laminin (Roche) coated coverslips (Fisher) and grown in the presence of Differentiation Medium [DM; DMEM containing 5% calf serum, 1mM sodium pyruvate, 50 units penicillin and 50mg/ml streptomycin and 30ng/ml of nerve growth factor (NGF, Roche; see Helfand et al., 2003 for details)].

Antibodies

Antibodies used in these studies included rabbit anti-peripherin (Helfand et al., 2003), anti-vimentin 314 (Helfand et al., 2002), and anti-keratin 8 and 18 (Yoon et al., 2001). Mouse monoclonal anti-NF-L (Sigma), anti-NF-M (Sigma), anti-NF-H (Sigma), anti-β-tubulin (TU 27B, provided by Dr. Lester Binder, Northwestern University), anti-human actin (Accurate Chem.), and a rat monoclonal anti-α-tubulin (Serotec) were also employed. In some experiments, rhodamine conjugated or Cy-5 conjugated phalloidin (Molecular Probes) was used to detect actin by fluorescence microscopy. FITC-, lissamine-rhodamine and Cy-5 conjugated goat anti-mouse and anti-rabbit IgG (Molecular Probes) were used for indirect immunofluorescence. Peroxidase-conjugated goat anti-rabbit and anti-mouse IgG (Kirkegaard and Perry) were used for immunoblotting.
Immunofluorescence and Image Analysis

PC12 cells were rinsed in PBS and fixed in either methanol (Mallinckrodt; -20°C) for 4 minutes or 3.7% formaldehyde (Tousimis) at room temperature for 5 minutes. Cells were then processed for indirect immunofluorescence as previously described (Helfand et al., 2003).

Images of fixed, stained preparations were taken with a Zeiss LSM 510 microscope (Carl Zeiss, Inc.) equipped with a 40X oil immersion (1.3 NA, plan-Neofluor lens) and a 100X oil immersion (1.4 NA, plan-apochromatic lens; Helfand et al., 2003).

Half-life Studies

PC12 cells were grown in the presence of either CM or DM for 2 days prior to incubating with methionine and cysteine-free DMEM for 1 hour. All cells were then incubated with [35S] methionine (0.2 mCi/ml in methionine-free DMEM) for 4 hours. Radioactive medium was removed and the cells were washed three times with non-radioactive media, and then fresh CM or DM was added. Cells were harvested at 0, 24, 36 and 48 hours time points. At each time point, cells were washed three times with PBS and collected in lysis buffer. PBS containing cell lysates was then centrifuged at 10,000xg in a tabletop centrifuge (Beckman R centrifuge). The pellet was incubated in IF lysis buffer (Starger and Goldman, 1977; PBS containing 1% Triton X-100, 500mM KCl, 2mM MgCl2, 0.5mg/ml DNase 1, and 2mM PMSF) for 10 minutes at room temperature and centrifuged at 10,000xg (Beckman R centrifuge). The amounts of protein present in the total cell lysate, supernatant and IF-enriched pellet fractions were determined by Bradford (BioRad) analysis. The pellet fractions were sonicated into Laemmli buffer (Laemmli, 1970) and 4µg of protein from each time point was loaded per lane and analyzed by SDS-PAGE.
and immunoblotting. The levels of $^{[35]S}$ methionine incorporated into peripherin were
determined by autoradiography. Densitometric scanning of autoradiographs was performed on a
phosphoimager (Molecular Dynamics Storm 860) and analyzed using the ImageQuant program
(version 5.0).

Small Inhibitory Ribonucleotide (siRNA) Studies

Peripherin-siRNA was synthesized using the Ambion Silencer siRNA Construction Kit
(Catalog #1620). To this end, a target sequence was identified by first scanning the length of the
Rattus norvegicus peripherin cDNA sequence for a series of nucleotides that initiated with two
sequential adenosine residues. The following DNA template and its complementary antisense
sequence were then used as primers to make double stranded peripherin-siRNA:
aagagctacaggagctcaacg (base pairs 325-345; Accession #AF031878). A second peripherin
siRNA (peripherin-siRNA2) using the DNA template aaggacgtggacgacgccacg (base pairs 633-
653) was employed in these studies to ensure that the effects of the first peripherin-siRNA did
not inhibit some unknown unrelated protein. For controls, a Rattus norvegicus neurofilament
light chain subunit (NF-L) siRNA was made by using the base pairs aagccgagctgttggtgctgc (base
pairs 427-447; Accession # NM_031783) and their complementary sequence; and an unrelated
Xenopus laevis lamin B1 (XLB1) siRNA was made using the following 21-mer DNA template
and its complementary antisense sequence: aacaccagatctccaggca (base pairs 342-363;
Accession # S01496). A PubMed Blast search of the rat genome revealed that the peripherin and
neurofilament light chain primers were specific and there was no homologous sequence when the
XLB1 DNA primers were compared to any other rat proteins.
In some experiments, cells containing siRNA were detected by labeling the siRNA with fluorescein (FAM) according to the protocol described by the Silencer™ siRNA Labeling Kit (Ambion; Catalog #1634).

Transfection

Peripherin-siRNA or peripherin-siRNA2, at a concentration between 5-10nM, were introduced into PC12 cells by oligofectamine (Invitrogen) delivery. Transfection was carried out under the following experimental conditions: PC12 cells were maintained in CM for up to 96 hours after transfection; cells were maintained in CM for 72 hours after transfection and then in DM for an additional 24 hours; cells were maintained in DM for up to 96 hours after transfection; cells were grown in DM for 24 hours prior to transfection and then maintained in DM for up to 96 hours. Controls for all experiments consisted of transfecting PC12 cells with *Xenopus laevis* lamin B1 siRNA or NF-L siRNA, at a concentration between 5-10nM, or mock transfecting with only oligofectamine as controls.

Determination of the Effects of siRNA

Immunofluorescence and immunoblotting were used to determine the effects of siRNA. For immunofluorescence studies, cells were fixed and processed at 24, 48, 72 and 96 hours post-transfection as previously described (for details, see Helfand *et al.*, 2003). Phase and fluorescence images were captured simultaneously with the Zeiss LSM 510 confocal microscope. In addition, Z-section series (~0.30 µm/slice) were captured to observe the organization of peripherin in all regions of cells.
For immunoblotting experiments, PC12 cells were plated in CM or DM to a density of 6x10^5 cells/ml in 60mm culture dishes 4 hours prior to transfection. The cells were either transfected with peripherin-siRNA (see above) or mock transfected (controls) using oligofectamine and maintained for periods up to 96 hours. Following trypsinization, cells were collected in either CM or DM and counted using a double Neubauer hemacytometer (Clay Adams). The cell suspension was pelleted on a tabletop centrifuge (200xg, IEC HN-SII), and the medium was decanted. The pellet of cells was immediately sonicated into 200 µl of Laemmli sample buffer (Laemmli, 1970). Samples of each preparation containing the equivalent of ~200,000 cells were analyzed by SDS-PAGE (Laemmli, 1970). The separated proteins were transferred to nitrocellulose for immunoblotting (Towbin et al., 1979). All antibody incubations were carried out in PBS containing 5% non-fat dry milk (Sigma). Immunoblots were analyzed by either visualizing horseradish peroxidase-conjugated antibodies or enhanced chemilumminescence (ECL; Amersham) using radiographic film (Amersham) as previously described (Helfand et al., 2003; Prahlad et al., 1998).

Live Cell Imaging

PC12 cells were plated onto gridded coverslips (Bellco) and transfected with FAM labeled-peripherin siRNA (see above) for 72 hours and then exposed to NGF for 24 hours. Fluorescence and phase-contrast images of these cells were acquired simultaneously using the Zeiss LSM 510 microscope. Transfected cells were identified based upon the presence of the labeled siRNA. Images of transfected cells were captured every 10 seconds for time periods up to 10 minutes. After observation, cells were formaldehyde fixed and stained with peripherin antibody (see above) to verify peripherin depletion in transfected cells.
**Morphometric Measurements and Statistical Analysis**

Two observers made all of the morphometric measurements of PC12 cells independently using the Zeiss LSM 510 imaging software (Helfand et al., 2003). Phase contrast and fluorescence images were used to determine the overall shape of cells by measuring the cellular perimeter, cellular area, form factor, cellular convex hull, process domain and process index as previously described for nerve cells (see Table 1; Kawa et al., 1998; Lepekhin et al., 2001; Soll et al., 1988). The form factor is defined as \( \frac{4\pi \times \text{cellular area}}{\text{perimeter}^2} \) (Soll et al., 1988). Thus, perfectly round cells have a form factor of 1, whereas stellate cells have lower form factor values (Soll et al., 1988). The cellular convex hull delineates the boundaries of a cell by connecting the tips of processes (for an example see Table 1, a, b; Kawa et al., 1998). After establishing the convex hull, the process index and process domains were determined (for an example, see Table 1, a, b). The process index is related to the number of cellular processes (e.g., neurites) and is defined as the number of areas contained outside of the cell contour but within the cellular convex hull (Kawa et al., 1998). It has been demonstrated that the process domain correlates with process length and is defined as the difference between the areas of the cellular convex hull and the cellular area (Kawa et al., 1998). All data were subsequently analyzed by Microsoft Excel and a Student’s T test (unpaired, two-tailed) was used to determine any significant differences (p<0.005) between the parameters measured in the peripherin siRNA transfected and control or mock transfected cells.

More detailed analyses of PC12 cell morphology were made (for an example, see Table 2,a): cell body length (defined as the length [\( \mu \text{m} \)] of the longest longitudinal axis of the cell body that does not extend into the cytoplasmic processes), number of processes (defined as any cytoplasmic protrusion from the cell body that is \( \geq 2 \mu \text{m} \) in length), length [\( \mu \text{m} \)] of processes,
number of neurites (defined for NGF-treated cells as any cytoplasmic process greater or equal to one cell body length), and length (µm) of neurites. All of these data were analyzed as described above, except that significance was defined as p<0.002.
RESULTS

Peripherin siRNA Decreases the Level of Peripherin Expression

When non-differentiated PC12 cells are grown in the absence of nerve growth factor (NGF-free culture medium [CM]; see Materials and Methods), they appear fibroblastic in shape due to the presence of a few short cytoplasmic processes (Figure 1a). Under these culture conditions virtually all cells (99.6%; 498/500) contain peripherin structures, mainly in the form of long IF, with lesser amounts of short IF (squiggles) and some non-filamentous precursors (particles) distributed throughout the cytoplasm (Figure 1a and inset; Helfand et al., 2003; Parysek and Goldman, 1987). A subpopulation of cells also express NF-M (~33% [99/300]; Figure 2a-f; (Parysek et al., 1991), and a very small number of cells (~3% [9/300]; data not shown; Parysek et al., 1991) express low levels of NF-L. When expressed, the NF-M is associated with a subset of peripherin IF as determined by double label immunofluorescence (see Figure 2a-c; Beaulieu et al., 1999b; Parysek and Goldman, 1987). No other IF proteins (vimentin, NF-H, or keratin) could be detected by immunofluorescence (data not shown).

Non-differentiated PC12 cells were transfected with peripherin- siRNA (see Materials and Methods). These preparations were fixed and processed for immunofluorescence with peripherin antibody 24, 48, 72 and 96 hours later (see Figure 1c-i). After ~48 hours, ~14% (27/200) of the cells exhibited an obvious decrease in the number of long IF structures (see Figure 1f, h). In these same cells there was an apparent increase in the relative number of particles and squiggles (see for example, Figure 1f, h). At 72 hours, ~42% (82/220) of cells displayed reduced levels of peripherin: of these, ~17% contained only particles and squiggles (partially silenced) and ~83% showed no detectable peripherin (extensively silenced). At ~96 hours post-transfection, the number of cells showing decreased peripherin expression had risen
to ~61% (121/220); ~20% of these were partially silenced (as seen in Figure 1f, h) and ~80% were extensively silenced (as seen in Figure 1c-e; g, i). Identical results were obtained using a second peripherin siRNA (peripherin-siRNA2; as described in Materials and Methods; data not shown). In contrast, control cells transfected with *Xenopus laevis* lamin B1 siRNA, NF-L siRNA, or mock transfected (see Materials and Methods) showed no detectable alterations in the peripherin IF network at any of the time points (data not shown). Under all of these transfection conditions, including peripherin-siRNA, there were no obvious changes in the actin or MT staining patterns as detected by fluorescence microscopy (for example, see Figure 1f-i). It is also important to note that similar results were obtained using either non-labeled or fluorescein-labeled siRNAs (Silencer™ siRNA Labeling Kit; Ambion).

The medium molecular weight NF subunit (NF-M) requires the presence of NF-L or a Type III IF protein, such as peripherin, to co-assemble into IF (Beaulieu *et al*., 2000; Parysek *et al*., 1991). Therefore, another way to confirm peripherin depletion was to monitor the immunofluorescence pattern of NF-M in peripherin silenced cells. In the vast majority of these cells observed at 96 hours after transfection, NF-M formed punctate non-filamentous structures distributed throughout the cytoplasm (~98%; 196/200; Figure 2d-f). Therefore, NF-M is unable to assemble filamentous structures in the absence of peripherin IF.

Peripherin silenced undifferentiated cells were morphologically distinct from non-transfected cells on the same coverslips. These cells appeared flatter and had fewer processes as determined by combined fluorescence and phase contrast microscopy. In order to more accurately assess the effects of peripherin-siRNA on PC12 cell morphology, the cellular perimeter, mean cell area, form factor, process domain and process index were determined (see Table 1 a, b; Materials and Methods). In particular, the form factor, process index and process
domain values reflect the roundness of cells, number of processes, and process length, respectively (Kawa et al., 1998; Soll et al., 1988). The resulting data revealed that the overall shape of peripherin silenced cells and control cells (i.e. NF-L siRNA and mock transfected) was different as reflected by their increased form factor values and decreased process index and process domain values (Table 1). Therefore, peripherin depleted cells contained fewer and shorter processes relative to controls.

A more detailed analysis of the morphological features of these cells was carried out by determining the average number and length of cytoplasmic processes as well as the average length of cell bodies (see Table 2; for definition see Materials and Methods and in Table 2, a). The average number (2.67 ± 1.77; n=50) and length (9.91 ± 9.08µm; n=50) of cytoplasmic processes was determined in mock transfected control cells (Table 2). In contrast, silenced cells had significantly (Student’s T test; p<0.002) fewer (1.13 ± 1.98; n=50) and shorter (6.52 ± 5.56µm; n=50) cytoplasmic processes (Table 2). In addition, the average cell body length was significantly increased (20.22 ± 10.77µm; n=50) in peripherin depleted cells compared to controls (13.9 ± 6.03µm; n=50) producing a more flattened configuration (see Figure 1c,g,i and Table 2). In contrast, no significant differences in either the length of cell processes or cell bodies were observed between NF-L siRNA, mock, and Xenopus laevis B1 siRNA transfected control groups (Table 2; data not shown).

Peripherin is Required for Neurite Initiation and Outgrowth

We previously determined that ~75-80% of the outgrowth of PC12 cell neurites takes place within 24-48 hours following exposure to NGF (Figure 1b; 48 hours; Helfand et al., 2003). However, the most significant effects of peripherin-siRNA were not observed until ~72-96 hours
post-transfection (see above). Therefore, to determine whether peripherin is required for neurite initiation and outgrowth, undifferentiated PC12 cells were transfected with peripherin siRNA and maintained in CM for ~72 hours. At this time, the medium was replaced with DM containing NGF. After 24 hours (~96 hours post-transfection), cells were fixed and processed for immunofluorescence. Under these conditions, ~55% (109/200) of the cells showed extensive silencing of peripherin. The morphological features of these cells were indistinguishable from silenced undifferentiated cells (see Figure 1e, g, i). The majority (~90%) contained no recognizable peripherin structures (extensively silenced) and others contained a few peripherin particles and squiggles (partially silenced). All of the cells devoid of detectable peripherin were fibroblastic in shape (Figure 3a-f). Furthermore, there were significant (p<0.005) increases in the form factor and corresponding decreases in the process index and the process domain, suggesting that they were more spheroidal and had fewer and shorter processes (Table 1; see Materials and Methods). The average number (0.17 ± 0.52; n=50) and length (7.16 ± 17.06µm; n=50) of neurites was significantly decreased (p<0.002) compared to the average number (2.2 ± 2.6; n=75) and length (28.32 ± 22.89µm; n=75) of neurites in control cells (Table 2). Thus, in the absence of peripherin there is a 13 fold reduction in the number of neurites and an ~4 fold reduction in the length of any residual neuritic processes (see Table 2, number and length of neurites). The average cell body length of peripherin-siRNA treated cells (35.51 ± 13.62µm) was significantly greater than control cells (16.39 ± 7.88µm; Figure 3d-f; Table 2), demonstrating that these cells were more extensively flattened. Furthermore, the overall distributions of actin (Figure 3a-c) and MT (data not shown) in peripherin silenced cells were similar to those in non-differentiated cells grown in CM (compare with Figure 1g,i). Taken
together, the results of these experiments suggest that peripherin is required for both the initiation and outgrowth of neurites in PC12 cells.

Live cell observations of peripherin-silenced cells were made to examine any differences in the initiation of growth cones between peripherin depleted and control cells. To this end, PC12 cells were transfected with fluorescein labeled peripherin-siRNA, grown for 72 hours in CM, challenged with NGF and then observed live with phase contrast microscopy at different time periods up to 24 hours. During this period, the silenced cells were easily distinguished from those not transfected with siRNA by their fluorescence and overall morphology. Differences between the silenced and non-silenced cells became evident after 2-4 hours and up to 24 hours after the addition of NGF (DM). Peripherin silenced cells were not able to extend neuritic processes (for example, Video Supplement 1). However, extensive membrane ruffling was seen on silenced cells within 2 hours after the addition of NGF. These ruffling regions were localized in several broad regions at the cell perimeter, but none had the typical fan-shaped appearance of growth cones. Even after 24 hours, this ruffling activity was typically seen in several regions of the cell surface with no dominant region taking on either the role or the morphology of a typical growth cone. In contrast, peripherin containing cells exhibited well-defined growth cones that were actively protruding at the leading edges of extending neurites within a few hours after the addition of NGF (for example, see Video Supplement 1).

Immunoblot Analyses of the Effects of Peripherin siRNA

Immunoblot analyses of whole cell lysates were performed to further characterize the effects of peripherin-siRNA. To this end, PC12 cells were transfected with peripherin-siRNA and, in parallel cultures, mock transfected. After transfection, the cells were maintained in either
CM or DM. In control cells maintained in CM, the levels of peripherin in total cell lysates remained relatively constant for 24-96 hours (Figure 4a; CM Mock). The overall levels of peripherin in siRNA transfected cells grown in CM appeared unaltered for ~72 hours and then decreased to ~20-40% (n=5) of their original levels by 96 hours (Figure 4a; peripherin-siRNA). It should be noted that the amount of peripherin present in mock transfected cells grown in the presence of NGF increased significantly between ~48-96 hours (Figure 4b; DM Mock; also see (Leonard et al., 1987 and Aletta et al., 1988). In comparison, by 96 hours the amount of peripherin present in silenced cells grown in DM decreased to ~10-40% of the levels detected at 48 hours after transfection with siRNA (n=5; Figure 4b; peripherin siRNA). Peripherin-siRNA inhibited the increase in peripherin expression induced by NGF as compared to controls (Figure 4b; compare 72-96 hours DM, mock with DM, peripherin-siRNA). The results obtained in both CM and DM demonstrate that the effects of peripherin-siRNA are not obvious until ~72-96 hours post-transfection which is consistent with the immunofluorescence results (see above and Figure 1). It should be noted that complete peripherin silencing was never observed by immunoblotting, which is supported by the finding that ~40% of the cells do not appear to be transfected with peripherin-siRNA as determined by immunofluorescence (see above). The levels of actin (Figure 4a,b) and tubulin (data not shown) remained relatively constant under all experimental conditions, confirming the specificity of the effects of peripherin-siRNA. In addition, cells transfected with *Xenopus laevis* lamin B1 siRNA or mock transfected (Figure 4c,d) showed no differences in peripherin expression in either CM or DM for periods up to 96 hours, further confirming the specific effects of peripherin-siRNA.

In addition to peripherin and NF-M, PC12 cells have been reported to express low levels of other types of IF proteins (Franke *et al*., 1986; Parysek and Goldman, 1987). Therefore, it
was of interest to determine whether the expression of these other proteins was altered in silenced and control cells. Immunoblot analyses revealed no differences in the overall levels of vimentin, NF-L, NF-M, NF-H, or keratin under any of the experimental conditions examined (data not shown; see Materials and Methods). Therefore, there is no obvious compensatory expression of other IF proteins upon the loss of peripherin.

*Peripherin is Also Required for the Maintenance of Neurites*

Peripherin appears to be the major cytoskeletal component of differentiated peripheral neurons (Aletta *et al.*, 1989). Therefore, it was also of interest to determine whether it is required for the maintenance of neurites, once they are formed. In a series of experiments, undifferentiated cells were transfected with fluorescein-labeled peripherin-siRNA (see Materials and Methods) and then immediately placed in NGF-containing medium (DM) for up to 96 hours. The results showed that the majority of peripherin-siRNA containing cells extended neurites that were morphologically indistinguishable from control cells at ~24 hours post-transfection (Figure 5 a, Table 1 and Table 2). This was expected as the effects of siRNA are not obvious until ~72-96 hours post-transfection (see above). However, by ~48 hours, some cells containing peripherin-siRNA appeared to possess shorter neurites relative to controls, suggesting a slower rate of neurite outgrowth (Figure 5 h). This observation was also supported by a small increase in the form factor, indicating a tendency towards a more rounded contour (Figure 5 g and Table 1). In addition, many transfected cells displayed dramatically decreased peripherin staining within their cell bodies, while many of their neurites retained peripherin (Figure 5 b). After ~96 hours, however, most peripherin silenced cells contained no identifiable peripherin structures and they appeared fibroblastic (Figure 5 c). This tendency towards a more fibroblastic
shape was also supported by an increase in the form factor and a decrease in the process index and process domain values (Table 1). In mock transfected control cells after 96 hours, the average cell body length was $18.95 \pm 6.56 \mu m$ (n= 50), and the average number and length of neurites were $2.24 \pm 1.1$ and $62.77 \pm 41.4 \mu m$ (n=50), respectively (Table 2). These values were not significantly different from the values obtained from NF-L siRNA transfected cells that had an average cell body length of $23.88 \pm 8.02 \mu m$ (n= 20), and an average number and length of neurites that were $3.50 \pm 2.36$ and $54.94.77 \pm 12.99 \mu m$ (n=20; Table 2). In contrast, direct measurements of peripherin silenced cells showed a significant increase in the average cell body length ($34.82 \pm 14.65 \mu m$; n= 50), while both the number ($0.17 \pm 0.42$; n=50) and length ($6.89 \pm 15.72 \mu m$; n=50) of neurites decreased significantly (Table 2). This emphasizes the dramatic effects of peripherin silencing on the shape and form of differentiated PC12 cells.

To further confirm whether PC12 cells require peripherin for the maintenance of neurites, differentiated cells (treated with NGF for 24 hours) were transfected with peripherin-siRNA and maintained in DM for up to an additional 72 hours. Under these culture conditions, ~10-20% of the cells were transfected with peripherin-siRNA. None of these showed obvious changes in their immunofluorescence patterns at 24 hours post-transfection (Figure 5 d). After ~48 hours, some cells contained only peripherin particles and squiggles and most of these had only short cytoplasmic processes with no obvious neurites (Figure 5 e). Approximately 72 hours after transfection, the average number ($0.33 \pm 0.61$; n= 50) and length ($9.48 \pm 16.33 \mu m$; n= 50) of neurites in silenced cells decreased significantly compared to the average number ($2.46 \pm 1.57$; n= 50) and length ($55.18 \pm 45.5 \mu m$; n= 50) of neurites in mock transfected cells (Figure 5 f, j; Table 2). In addition, the average cell body length ($31.12 \pm 13.4 \mu m$; n= 50) of silenced cells was significantly increased relative to controls ($17.16 \pm 6.25 \mu m$; n =50; Table 2). These changes in
morphology were reflected as a significant increase in the form factor and corresponding decreases in both the process index and process domains (Figure 5 i and Table 1). The results conclusively demonstrate that peripherin is required for the maintenance of neurites.

**The Half-life of Peripherin in Non-Differentiated and Differentiated PC12 Cells**

Immunofluorescence observations showed significant reductions in peripherin silenced cells as early as 48 hours after transfection, which is consistent with the turnover rates that have been determined for other Type III IF proteins (McTavish et al., 1983). This is in stark contrast to other results suggesting that the half-life for peripherin is 7-10 days (Troy et al., 1992). Based upon this discrepancy, we felt that it was important to measure the half-life of peripherin in our PC12 cell line by pulse labeling with $[^{35}\text{S}]-$ methionine for 4 hours, followed by transfer to non-radioactive medium for different time periods (see Materials and Methods). These experiments were carried out on cells grown in either CM or DM for 48 hours (see Materials and Methods). Following labeling, IF-enriched cytoskeletons were prepared and analyzed by SDS-PAGE, immunoblotting and autoradiography at 0, 24, 36, and 48 hours. The results of two separate sets of experiments demonstrated that the total amounts of peripherin present in PC12 IF-enriched cytoskeletal preparations were similar at each time point (Figure 6 a; compare lanes 3 and 4; 7 and 8). However, the levels of $[^{35}\text{S}]-$ methionine-labeled peripherin fell to 50% of their control values within ~33 hours in CM and ~43 hours in DM (Figure 6a: lanes 1 and 2; 5 and 6; Figure 6 b). These results are consistent with the time required for peripherin depletion using siRNA (~72-96 hours; see above).
DISCUSSION

Different cell types are frequently distinguished by their shapes. This is best exemplified in nerve cells, which are remarkably asymmetric due to the presence of long processes such as axons and dendrites. The use of siRNAs to silence the expression of specific proteins in mammalian cells has allowed us to directly address the function of IF in the initiation, outgrowth and maintenance of neurites. The results of this study clearly demonstrate a significant role for neural IF in both the formation and maintenance of neuritic processes, and thus cell shape. Peripherin-siRNA dramatically decreased the amount of endogenous peripherin in PC12 cells and coincidentally inhibited their ability to extend and maintain neurites. This was demonstrated by the finding that the average length of processes after peripherin silencing was similar to that observed in non-differentiated cells. In contrast, peripherin positive cells extended neurites that were at least 4-5 times longer (Table 2). These observations are also supported by the findings that there are dramatic increases in peripherin expression during the initiation and outgrowth of axons that take place in early developing nervous systems of mammals, amphibians and fish (Gervasi et al., 2000; Troy et al., 1990b; Undamatla and Szaro, 2001). Similarly, peripherin expression increases significantly during NGF-induced neurite outgrowth in PC12 cells (Aletta et al., 1988; Parysek and Goldman, 1987; Troy et al., 1990a). The levels of peripherin are also up-regulated ~2-3 fold during the regrowth of axons following axotomy of mature dorsal root ganglion axons, while the levels of NF triplet proteins dramatically decrease (Hoffman and Lasek, 1980; Oblinger et al., 1989a; Oblinger et al., 1989b).

The inhibition of peripherin expression using the siRNA approach has numerous advantages over other methods used to inhibit the production of specific proteins in vivo. In this regard, it has been found that siRNA is much more stable and effective in inhibiting expression
over longer time periods under normal growth conditions than is the case for antisense probes (Bertrand et al., 2002). For example, in earlier studies, it was found that PC12 cells had to be grown in serum-free medium for 28 days in order to attain significant decreases in peripherin expression using the antisense approach (Troy et al., 1992). The half-life for peripherin under these growth conditions was found to be between 7-10 days or almost 6 times greater than that reported in this study. Although we cannot explain these discrepancies, it is conceivable that growth in serum-free medium for prolonged periods may have adversely affected the normal expression pattern of peripherin.

The PC12 cell line is an excellent model for studying the function of neural IF in the initiation, outgrowth, and maintenance of neurites, as well as in the overall shape determination of nerve cells (Greene and Tischler, 1976). This is attributable to the finding that all of the cells express one major IF protein, peripherin, which assembles into homopolymer IF in both undifferentiated and differentiated states. Another important property of PC12 cells for these studies is based upon the finding that the expression levels of other IF proteins, such as vimentin and NF, are not detectably increased following the addition of NGF to undifferentiated cells. Even more importantly, the expression of these other IF proteins is not altered to compensate for the dramatic decrease in peripherin expression induced by siRNA. This was best exemplified in the subpopulation of PC12 cells that normally express both peripherin and NF-M. This latter NF protein cannot assemble into IF on its own, but it can co-assemble with Type III IF such as peripherin (Beaulieu et al., 1999a; Parysek et al., 1991; see Figure 2a-c). Our findings that NF-M only formed non-filamentous aggregates in peripherin silenced cells confirmed that there was no compensatory up-regulation of either Type III of type IV (NF) proteins (see Figure 2d-f).
Further support for the importance of IF in the determination of cell shape in the nervous system comes from studies of astrocytes. Reactive astrocytes extend long cytoplasmic processes containing three IF proteins; the Type III glial fibrillary acidic protein (GFAP) and vimentin, and the Type IV protein nestin (Eliasson et al., 1999). Astrocytes from vimentin-null mice contain GFAP IF networks, and there is no obvious alteration in their shape. The same is true for the astrocytes obtained from GFAP-null mice which express vimentin IF (Lepekhin et al., 2001; Pekny et al., 1998). However, astrocytes obtained from mice that are null for both GFAP and vimentin, do not contain endogenous IF, and they approximate a rounded cellular morphology devoid of long cytoplasmic processes. Interestingly, mice null for either vimentin or GFAP can form glial scars in response to brain injury, while double knock-out (GFAP-/-, vimentin-/-) mice cannot heal brain injuries resulting in severe intracranial hemorrhaging (Pekny et al., 1999). Taken together, it appears that some degree of functional overlap exists between vimentin and GFAP, as vimentin appears to be capable of compensating for the loss of GFAP and vice versa. This functional compensation is important to keep in mind when interpreting the results obtained from IF null mice. For example, a recent study reported that peripherin null mice exhibit significant increases in the vimentin as well as the Type IV homopolymer IF protein, α-internexin (Lariviere et al., 2002).

The results of the peripherin-siRNA experiments clearly add a new dimension to the many studies documenting the importance of actin and microtubules (MT) in the structure and function of growth cones and axons, as well as the overall shape of nerve cells (for review, Letourneau, 1996). In further support of the roles of the different structural forms of IF in these activities, it has recently been demonstrated that IF and their constituent proteins are present throughout all regions of nerve cells, including actin-rich growth cones (Chan et al., 2003;
Helfand et al., 2003). Our results strongly suggest that peripherin performs essential functions within these regions, as peripherin depleted cells cannot form growth cones nor can they extend or maintain neuritic processes (Video Supplement 1).

The relative increases and decreases in the expression patterns of different IF proteins during the development and the regeneration of axons may reflect the unique biochemical, mechanical and motile properties of individual types of IF. With respect to motility, it has been demonstrated that peripherin and NF containing short filaments (or squiggles) move along MT tracks at rates up to 1-2\(\mu\)m/sec in association with the molecular motors, kinesin and cytoplasmic dynein (Helfand et al., 2003; Helfand et al., 2002; Prahlad et al., 2000; Shah et al., 2000; Wang et al., 2000; Yabe et al., 2000; Yabe et al., 1999). From these observations, it appears that the mechanisms governing the intracellular transport of short NF and peripherin squiggles are not fundamentally different from each other. However, the overall distances traveled by these two types of IF are significantly different. These differences are related to the amount of time that these two types of IF spend moving relative to the time they spend in a stationary phase (i.e., their “pause times”). For short NF, the pause times are \(\sim\)4 times longer than their peripherin counterparts, and this explains why the net translocation of NF is much slower than peripherin IF (Wang et al., 2000).

The slower movements of NF may be related to their structure and biochemical properties. As mentioned above, Type IV NF proteins are composed of three subunits, two of which (NF-H and NF-M) possess extremely long highly charged C-terminal domains thought to be involved in the crossbridging of NF to other NF, as well as to other cytoskeletal components, such as MT (Miyasaka et al., 1993; Nakagawa et al., 1995). It has been proposed that these crossbridges account for the increased resistance of NF to mechanical strain (Leterrier et al.,
1996; Nakagawa et al., 1995). In contrast, type III IF (e.g. peripherin) have much shorter C-termi
mini and are less resistance to mechanical strain (Leterrier et al., 1996). Based on these
observations, it is interesting to speculate that the functional significance of the developmentally
regulated changes in the expression of different types of neural IF may be related to their specific
motile and mechanical properties. For example, during early development, nerve cells require a
great deal of plasticity as they undergo the rapid outgrowth and dramatic cell shape changes that
typify the transition from immature into mature neurons. Therefore, the early expression of
homopolymer Type III IF, such as those containing peripherin or vimentin, could provide the
cytoskeletal flexibility needed during early development and during the regeneration of injured
neurons. In contrast, the overall slower moving, more strain resistant NF may provide the
increased stability necessary to maintain the shape and mechanical integrity of terminally
differentiated adult neurons.
ACKNOWLEDGEMENTS

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REFERENCES


Figure Legends.

Figure 1. Characterization of Peripherin in PC12 Cells Before and After Treatment with siRNA.

Undifferentiated PC12 cells (i.e. grown in CM) fixed and processed for immunofluorescence. (a) Peripherin (green) is present in ~100% of the cells. Inset shows a region of similar cell demonstrating the presence of particles and squiggles. (b) Following exposure to NGF for 48 hours, long neurites are present and the fluorescence intensity detected following processing for peripherin appears to be greatly increased relative to undifferentiated cells grown in CM. This latter observation most likely reflects the increased expression of peripherin.

(c-e) PC12 cells were transfected with fluorescein-labeled peripherin-siRNA and fixed and stained with peripherin antibody after 96 hours. Using confocal microscopy, 20-40µm Z-section images at 0.3µm intervals, were captured to monitor the distribution of peripherin (green). (d, e) depict all images in a Z-stack series. (c) A phase contrast image of the same cells demonstrates that peripherin depleted cells (see *) spread more extensively over the coverslip (compare with the two non-transfected peripherin containing cells that typically possess numerous cytoplasmic processes in the same microscopic field). Silenced cells contain the fluorescent siRNA (blue; see Materials and Methods).

Undifferentiated PC12 cells were transfected with peripherin-siRNA and fixed and processed for double immunofluorescence at 72 hours (f, h) and 96 hours (g, i) post-transfection; (f, g) peripherin (green) and actin (red); (h, i) peripherin (green) and tubulin (red). At 72 hours, particles and squiggles are evident in many cells (partially silenced); while at 96 hours, the majority of transfected cells contain no identifiable peripherin structures (extensively silenced;
g,i). Peripherin siRNA had no obvious effects on the distributions of actin or tubulin (f-i). Images (a-i) were captured with a Zeiss LSM510 confocal microscope. Size bars = 10µm.

**Figure 2. Peripherin Depletion Alters the Distribution of NF-M.**

(a-c) Low magnification view of undifferentiated PC12 cells (grown in CM) double labeled with peripherin (green; a) and NF-M (red; b) antibodies demonstrate that ~30% of cells express NF-M. (d-f) Higher magnification of these NF-M expressing cells reveals that NF-M associates with a subset of peripherin filamentous structures (f; yellow). (g-i) In cells observed ~72 hours after exposure to peripherin-siRNA (i, blue), peripherin levels decrease and NF-M forms non-filamentous punctate structures throughout the cytoplasm. Note the partial reduction in peripherin expression in the cell (*) that does not express NF-M. Size bars a-c, g-i = 10µm; Size bar d-f = 5µm.

**Figure 3. Peripherin is Required for Neurite Initiation and Outgrowth.**

(a-f) PC12 cells were transfected with peripherin-siRNA, maintained in CM for 72 hours, followed by exposure to NGF for 24 hours. (a) Peripherin depleted cells (see *) appeared fibroblastic in shape and were not able to form neurites. (b, c) The actin (red) networks in these cells were similar to those observed in undifferentiated cells grown in CM (see Figure 1f, g). Note that non-silenced cells (green) are capable of forming neurites. (d-f) Higher magnification view of an extensively silenced cell demonstrates the more extensively spread morphology and a lack of neurites. The presence of fluorophore-labeled peripherin-siRNA is indicated in f (blue). For comparison, a peripherin (green) filled neuritic process of an unsilenced cell is shown to the left. Size bars = 10µm.
**Figure 4. Immunoblot Analyses of Peripherin-siRNA Depletion.**

PC12 were transfected with peripherin-siRNA or mock transfected (mock) and then grown in either CM or DM for 24, 48, 72 or 96 hours. (a, b) Samples of the total protein obtained from ~200,000 cells were analyzed by SDS-PAGE and immunoblotting with peripherin and actin antibodies. (a; mock) The amounts of the ~57kDa peripherin protein present in mock transfected cells grown in CM remained relatively constant during the time course of the experiment. (a; peripherin-siRNA) However, peripherin-siRNA treated cells maintained in CM contained ~80% less peripherin by 96 hours post-transfection. (b; mock) Increased amounts of peripherin were observed between ~48-96 hours in mock transfected cells grown in DM. (b; peripherin-siRNA) Exposure to peripherin-siRNA significantly decreased the levels of peripherin by ~90%. There were no observable effects on the levels of actin under any of these conditions (a, b).

(c, d) As a further control, PC12 cells were transfected with either peripherin siRNA (periph), the unrelated *Xenopus laevis* lamin B1(XLB1), or mock transfected with oligofectamine (mock). Immunoblot analyses of total cell lysates prepared from PC12 cells grown in either CM (c) or DM (d) at 96 hours, revealed that only the peripherin-siRNA was able to decrease the levels of peripherin.

**Figure 5. Peripherin is Required for the Maintenance of Neurites in PC12 cells.**

(a-c) PC12 cells were transfected with labeled-peripherin-siRNA (blue, see arrows; FAM see Materials and Methods) and were immediately placed in DM for up to 96 hours. (a) After 24 hours most transfected PC12 cells contained peripherin and had extended neurites. (b) After ~48 hours, many cells showed decreased amounts of peripherin fluorescence within their cell bodies.
Interestingly, many of these cells retained peripherin structures within their neuritic processes (see silenced cell in b). (c) By ~72-96 hours post-transfection, many silenced cells appeared fibroblastic in shape (for comparison see Figure 1c) and contained no neurites compared to non-transfected cells in the same field of view. (g, h) Analyses of cellular morphology of peripherin-siRNA treated cells demonstrated significant increases in the form factor and significant decreases in neurite length over time. In contrast, the form factor decreased and neurite length increased in mock transfected control cells over time.

(d-f) Non-transfected PC12 cells were exposed to NGF (DM) for 24 hours to induce neurite outgrowth, and then transfected with either labeled peripherin-siRNA (blue; see arrows) or mock transfected. Differentiated transfected cells were maintained in DM for 24, 48 and 72 hours followed by fixation and staining. (d) Twenty-four hours after transfection, PC12 cells displayed peripherin networks and long neurites. (e) At ~48 hours, many cells exhibited a decrease in the total peripherin fluorescence and an increase in the number of peripherin particles throughout the cell body. (f) After ~72 hours, most silenced cells appeared fibroblastic in shape. (i, j) Peripherin silenced cells demonstrated significant increases in their form factor and significant decreases in neurite length during the time course of these experiments (j; note that the average neurite length at 24 hours reflects the exposure to NGF before transfection). In contrast, the form factor decreased and neurite length increased in mock transfected control cells (i, j). Size bars = 10μm.

Figure 6. Determination of Peripherin Half-Life.

Non-differentiated (grown in CM) and differentiated (grown in DM) PC12 cells were pulse-labeled with $^{35}$S-Methionine for 4 hours and then chased with non-radioactive medium for
up to 48 hours. Intermediate filament-enriched cytoskeletal preparations were made at 0, 12, 36 and 48 hour time points and the proteins were separated by SDS-PAGE and analyzed by immunoblotting and autoradiography. The amount of $^{35}$S-Methionine labeled peripherin present in these preparations at the 48 hour time point (a; lanes 2 and 6) was decreased relative to those obtained at the 0 time point (a; lanes 1 and 5). However, the levels of total peripherin protein remained relatively constant between 0 (a; lanes 3 and 7) and 48 hours (a; lanes 4 and 8) as determined by immunoblotting. (b) The amounts of labeled peripherin present at 0 hours were assigned a value of 100 and later time points were expressed as relative levels of this value. The half-life of peripherin contained within PC12 cells grown in CM and DM was found to be ~33 hours and ~43 hours, respectively. Arrows point to peripherin (57kDa).

**Table 1. Morphometric Analyses of Peripherin-siRNA and Mock Transfected Cells: Form Factor, Process Index, and Process Domain.**

The form factor (see Materials and Methods) was determined for both peripherin-siRNA and mock transfected PC12 cells in all experiments. The convex hull (white lines; a, b) was used to determine the process index (the number of areas between neurites and the convex hull; see for example a; numbers 1-7). In addition, the difference between the area contained within the convex hull and the area contained within the cell contour (demarcated by the red line, a; and blue line, b) was used to determine the process domain. Size bars =10µm.

Experimental condition abbreviations: CM96 refers to transfected cells grown in the absence of NGF for 96 hours; CM72/DM24 refers to transfected cells grown in the absence of NGF for 72 hours and then exposed to NGF for 24 hours; DM-24, -48, and -72 refer to transfected cells grown in the presence of NGF for 24, 48 or 72 hours; DM24/DM*-24, -48, -72
refer to cells exposed to NGF for 24 hours, transfected and then maintained in the presence of NGF for an additional 24, 48 or 72 hours.

Table 2. Morphometric Analyses of Peripherin-siRNA and Mock Transfected Cells: Cell Body Length, Number of Processes and Length of Processes.

The longest axis of the cell body was used to determine the average cell body length (see Materials and Methods and yellow line, a). Cytoplasmic processes were defined as any cytoplasmic protrusion from the cell body that was ≥2µm in length. Neurites were defined for NGF-treated cells as any cytoplasmic process that was greater or equal to one cell body length. The process and neurite lengths were measured according to these definitions (for examples, red lines in a). White lines delineate an example of the boundaries that were used to define where a process (neurite) began. In addition, the average numbers of processes and neurites were determined. See Table 1 legend for explanations of experimental conditions. Size bar = 10µm.
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![Images of neuronal structures](image1.png)
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<td>50</td>
<td>31.12</td>
<td>13.4</td>
<td>&lt; 0.001</td>
<td>2</td>
</tr>
<tr>
<td>mock</td>
<td>50</td>
<td>17.16</td>
<td>6.35</td>
<td>&lt; 0.001</td>
<td>3.12</td>
</tr>
</tbody>
</table>
a

CM

DM

b

Relative Levels of $^{35}$S-Met Labeled Peripherin Protein

Time (hours)

DM $t_{1/2} = 42.9$ hours
CM $t_{1/2} = 32.6$ hours

$\Delta$ DM
$\blacktriangle$ CM