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Peripherin assembles into homopolymers in SW13 cells

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SUMMARY

The properties of full-length and mutant peripherins were studied in intermediate filament-less SW13 cells to define regions of peripherin that are essential for initiation of filament assembly. A full-length rat *peripherin* gene transfected into SW13 cells resulted in filament formation, consistent with the close structural relationship of peripherin to other type III intermediate filament proteins that readily form homopolymers. Translation of full-length rat peripherin is initiated predominantly at the second of two in-frame AUGs. Deletions within the amino terminus of wild-type peripherin abolished its ability to form filaments in SW13 cells. In contrast, deletion of the entire carboxyl-terminal tail of peripherin did not affect its ability to form filamentous arrays in transfected SW13 cells. These results indicate that, of the intermediate filament proteins that are expressed in mature neurons, only peripherin and α-internexin are capable of making homopolymer intermediate filaments. In addition, mutations of the carboxyl tail of peripherin generally do not interfere with filament network formation.

Key words: peripherin, intermediate filament, homopolymer, cytoskeletal assembly

INTRODUCTION

All intermediate filament (IF) proteins consist of a globular amino-terminal head domain, a central α-helical rod domain, and a carboxyl-terminal tail domain. The rod domain is highly conserved among all IF proteins, but the non-helical end domains are variable in size and sequence (Steinert and Roop, 1988). Sequence homologies in each domain have been used to classify IF proteins into five types (Steinert and Roop, 1988), which also differ in cell type distribution and homopolymer-forming ability. Type I and type II cytokeratins form obligate heteropolymers; type III proteins, thus far, have been found to make homopolymers; type IV neurofilament proteins, except for α-internexin, cannot form homopolymers; and type V lamin protein assembly properties are not yet fully understood (for review, see Fuchs and Weber, 1994). Although peripherin is a type III IF protein (as are vimentin, desmin, and glial fibrillary acidic protein (GFAP) and, thus, would be expected to form a polymer without the aid of another IF subunit protein, this has not been confirmed in vivo. There are no cell lines that express only peripherin; PC12 cells, in which peripherin generally has been studied, also express the type IV IF proteins α-internexin, NF-L, NF-M and NF-H, some of which have been shown to be coassembled with peripherin (Parysek et al., 1991). Transfection of wild-type IF genes into the normally filament-less SW13 cells has been shown to be a reliable system to determine homopolymer-forming ability of a protein (Ching and Liem, 1993; Lee et al., 1993). Thus, we studied the homopolymer-forming properties of peripherin in an 'in vivo' cytoplasmic environment, in SW13 cells.

In addition, we investigated the regions of peripherin involved in filament assembly by analyzing the effect of deleting various portions of peripherin. Previous in vivo studies have shown that an intact rod domain is indispensable for IF formation by all IF proteins tested (for example, see Albers and Fuchs, 1987; Lu and Lane, 1990). The rod region alone, however, is not sufficient to form IF in vivo (Eckelt et al., 1992; McCormick et al., 1993; Raats et al., 1991, 1992).

The N-terminal domain of all IF proteins has been proven to be absolutely required for filament assembly. Removal of part of the vimentin head by Ca^{2+}-activated proteinase (Traub and Vorgias, 1983) or the amino-terminal 67 amino acids of desmin (Kaufmann et al., 1985) totally abolished their ability to assemble in vitro. Similarly, assembly studies in vivo in vimentin-free MCF-7 cells showed that deletion of small or large domains of the amino terminus resulted in the inability of desmin, vimentin, or glial fibrillary acidic protein (GFAP) to form filaments (Chen and Liem, 1994; Herrmann et al., 1992; Raats et al., 1991, 1992).

The importance of the carboxyl terminus in assembly has been more controversial. In vitro reconstitution studies with the type III IF protein vimentin have shown that proteolytic removal of a portion or all of the tail domain does not prevent oligomerization of subunits to produce IF (Eckelt et al., 1992; Kaufmann et al., 1985; McCormick et al., 1993; Shoeman et al., 1990). In contrast, deletion of the tail domain of the type III IF protein GFAP rendered it assembly incompetent in vitro (Quinlan et al., 1989). Further, other in vitro studies have shown that fragments of the tail, prepared as synthetic peptides, or antibodies to the

tail interfere with assembly of 'normal' IFs (Birkenberger and Ip, 1990; Hatzfeld and Weber, 1992; Kouklis et al., 1991, 1992). In vivo studies are similarly controversial: most studies have found that the C-terminal domain is required for assembly of vimentin, desmin, or GFAP filaments in filament-less cell lines (Chen and Liem, 1994; McCormick et al., 1993; Raats et al., 1991, 1992), but a recent report (Rogers et al., 1995) suggests otherwise. Expression of mutant desmin, missing amino acids 415 to 469 (the whole tail), resulted only in desmin aggregates, but no filamentous structure, in vimentin filament-less MCF-7 cells (Raats et al., 1991). Similar types of aggregates are seen in SW13 cells transfected with a 'tail-less' GFAP gene (Chen and Liem, 1994). Transfection of a carboxyl tail-deleted vimentin gene resulted in a less 'severe' phenotype, a fibrous network that concentrated at the periphery of cells and had a tendency to collapse (McCormick et al., 1993). In contrast, Rogers et al. (1995) found that deletion of the tail of vimentin or desmin did not abolish filament network assembly in SW13 cells. We have further investigated this issue by examining the requirement for the C-terminal tail in peripherin filament assembly.

Given that peripherin appears to be the only IF protein expressed in certain kinds of neurons (Brody et al., 1989; Parysek and Goldman, 1988; Troy et al., 1990), peripherin is likely to make homopolymeric IF networks in those neurons. Thus, we were particularly interested in determining, in fact, whether peripherin had homopolymer-forming ability in cells. Further, we wished to determine the segments of peripherin that are most sensitive to modification relative to IF-forming properties. These results have implications for the types of IF network defects potentially seen in the neurons of humans with *peripherin* gene mutations.

MATERIALS AND METHODS

Plasmid construction

The complete rat *peripherin* gene (Belecky-Adams et al., 1993), containing 900 base pairs noncoding sequence and flanking sequence at the 3′ end of rat *peripherin* gene were used to prepare all constructs. The wild-type *peripherin* gene plasmid, pMT/Perwt (Fig. 1A) was prepared by flanking the *peripherin* gene at the cap site (Thompson and Ziff, 1989) (retaining 37 base pairs of 5′ non-coding sequence) with the mouse metallothionein-I promoter (Wang et al., 1994). For the preparation of the amino-terminal-truncated rat *peripherin* gene, the wild-type gene was digested with *Bam*HI and *Xho*I, which deleted 5′ non-coding sequence and nucleotides of exon 1 that encode the N-terminal 78 amino acids. This fragment was subcloned into vector pcDNA I NEO (Invitrogen, CA), which placed the truncated gene under control of the CMV promoter (pCMV/Per N78, Fig. 1B). The carboxyl-terminal truncation which removed the terminal 65 amino acids, was accomplishing by ligating a fragment of the *peripherin* gene from +1 to the end of exon 6 that was synthesized by PCR to the same 900 base pairs of 3′ noncoding and flanking sequence of the rat *peripherin* gene included in all other constructs (pMT/Per C65, Fig. 1C). The ligation was facilitated by generating a *Stu*I site at the end of exon 6 by PCR, and linking it to the *Stu*I site in the *peripherin* gene that lies just 3′ to the stop codon of the wild-type gene. To prepare mutant *peripherin* gene constructs with point mutations at the putative first or/and second ATG, a pair of primers with desired alternative sequences were made for each mutation. The first ATG was mutated to GTG with primer 1 (5′ GCTGAGTGCCATC 3′), and the second ATG was mutated to GCG with primer 2 (5′ AGCGCGAGCCATCA 3′), resulting in pMT/Per-ATG(1) (Fig. 1D), pMT/Per-ATG(2) (Fig. 1E) and pMT/Per-ATG (1,2) (Fig. 1F). The underlined codons are the mutated ATG codons. The mutations were introduced by site-directed mutagenesis by PCR (Bowman et al., 1990). The plasmid pMT/Per-myc was made by replacing the promoter of pPERIPH/PERIPH-MYC, described by Belecky-Adams et al. (1993), with the mouse metallothionein-I promoter (Wang et al., 1994). The sequence of gene fragments with engineered mutations was confirmed using a double-stranded sequencing protocol (Hsiao, 1991). Each plasmid described above also was used for making stable cell lines by inserting a neomycin or puromycin marker gene into the plasmid.

Cell culture and transfection

SW13 (cl.2, vimemtin-minus), a human adrenal carcinoma cell line (Sarria et al., 1990, kindly provided by Dr Robert Evans, University of Colorado, Denver, CO) was maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 5% calf serum and 5% fetal bovine serum. Cells were transiently transfected using a slightly modified version (Dush et al., 1988) of the calcium phosphate precipitate method and analyzed 48 hours post transfection. Stable cell lines were obtained by electroporating peripherin plasmids into which a neomycin or puromycin marker gene had been added (Shaw-White et al., 1993). Colonies that were resistant to 200 μg/ml G418 (Geneticin, Gibco BRL, MD) or 2 μg/ml puromycin (Sigma, MO) were picked, and stable cell lines that expressed peripherin were maintained in medium supplemented with G418 or puromycin.

Immunofluorescence labeling

After transient transfection, or after selection of stable transfectants, cultured cells on glass coverslips were rinsed with PBS, fixed in methanol (–20°C for 5 minutes), and rinsed with PBS. The cells were labeled with an affinity-purified rabbit polyclonal antibody against peripherin (1:30; Foley et al., 1994) for 1 hour, followed by incubation with secondary antibody, goat anti-rabbit IgG conjugated with

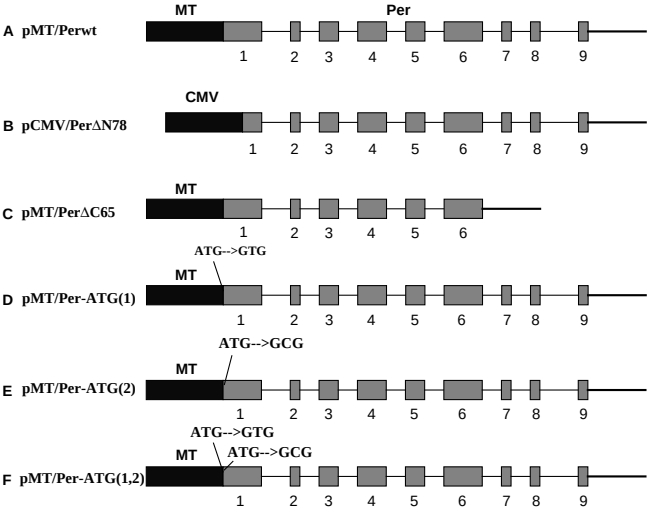

Fig. 1. Schematic diagrams of peripherin transgenes. Wild type (A); N-terminal-truncated mutant missing 78 amino acids (B); C-terminal-truncated mutant missing 65 amino acids (C); full-length peripherin with a point mutation at the first in-frame ATG (ATG to GTG) (D); full-length peripherin with a mutation at the second in-frame ATG (ATG to GCG) (E); full-length peripherin with mutations at both ATGs (F). Peripherin transgenes were driven by the mouse metallothionein-I (MT) or CMV promoter, represented by solid blocks. Rat *peripherin* genes are represented by the numbered hatched boxes, introns are depicted by lines, and the 3′ noncoding and flanking sequences, are identified by a thick line.

fluorescein (1:20 Kirkegaard and Perry, MD) for 1 hour. For double-label immunofluorescence, a mouse vimentin monoclonal antibody (used at 1:50), that detects human, pig, chicken and rat vimentin (V9, Boehringer Mannheim, IN), followed by rhodamine-conjugated goat anti-mouse IgG (1:20, Kirkegaard and Perry, MD) was used. A monoclonal antibody to the human c-myc tag (9E10; Evan et al., 1985) was used for immunofluorescence assays of cells transfected with myc-tagged *peripherin* gene plasmids.

Cell fractionation

To analyze proteins expressed from peripherin plasmids, IF-enriched cytoskeletal preparations (Jones and Goldman, 1985) were made from cultures of stable transfectants. Cytoskeletal pellets and supernatants were analyzed by SDS-PAGE and western blotting by conventional methods (Laemmli, 1970; Towbin et al., 1979).

RESULTS

Wild type peripherin forms a filamentous network in the absence of preexisting IF

The human adrenal carcinoma cell line, SW13, lacks cytoplasmic IF and thus is suited for assaying the homopolymer-forming properties of peripherin. To assess whether peripherin alone can assemble into a filamentous network, plasmid pMT/Perwt (Fig. 1A), encoding wild-type peripherin, was introduced into SW13 cells by calcium phosphate-mediated transfection. After 48 hours, cells were fixed and examined for peripherin by fluorescence immunohistochemistry. About 5% of the cells were labeled, revealing a prominent filamentous array (Fig. 2A). To exclude the possibility that these filamentous networks were formed with the help of endogenous vimentin in SW13 revertant cells, since less than 5% of the SW13 cells revert back to their original state and synthesize vimentin, double-labeling was performed to visualize peripherin and vimentin simultaneously. Almost every cell containing a peripherin network did not contain a vimentin network (Fig. 2B, vimentin double-label of cell shown in Fig. 2A). To demonstrate that the peripherin filaments persist, twelve independent isolates of SW13 cells transfected with plasmid pMT/Perwt containing a puromycin marker gene were selected and analyzed for their capacity to express and organize peripherin into a filamentous array. In these lines, the filamentous architecture indicated that wild-type peripherin can polymer-

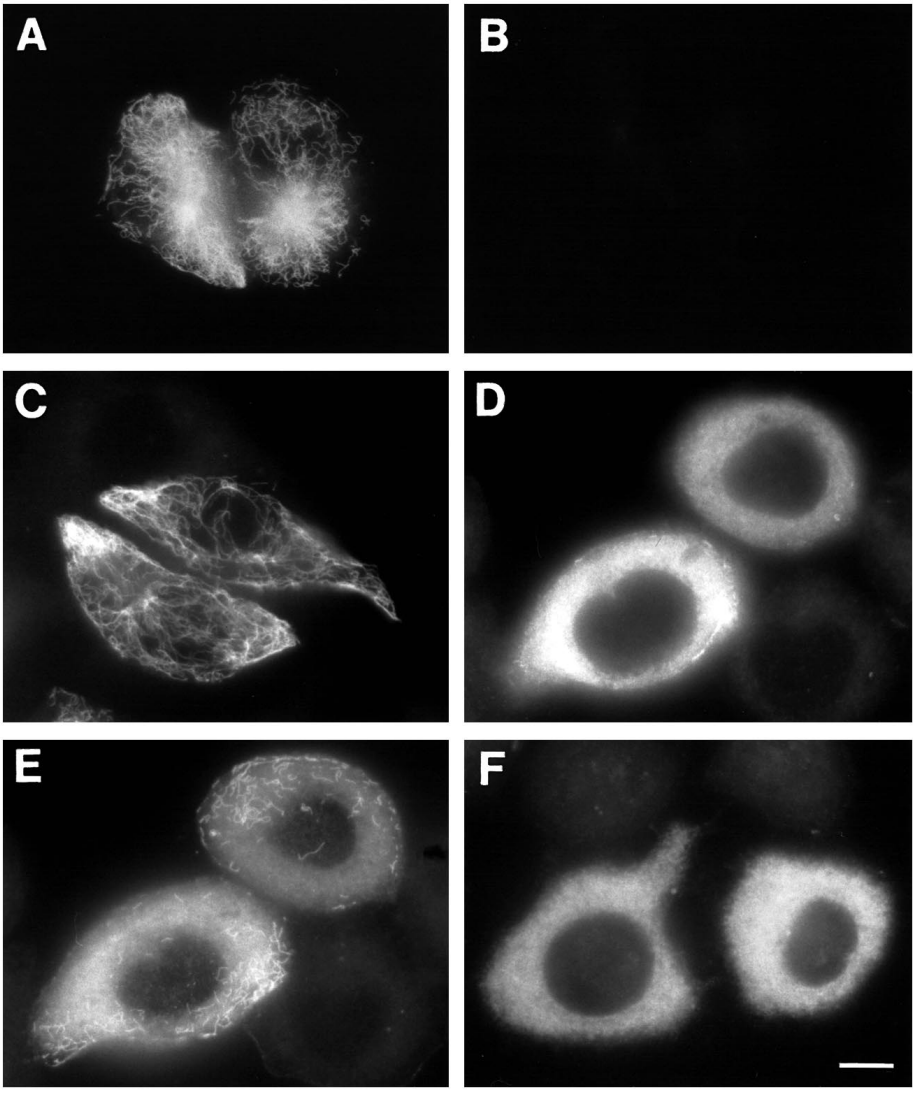

Fig. 2. Immunofluorescence with peripherin antibody on stably transfected SW13 cells expressing pMT/Perwt (A); pMT/Per-ATG(1) (C); pMT/Per-ATG(2) (D,E), and pMT/Per-ATG (1,2) (F). The cells shown in A were double-labeled with vimentin antibody (B). Only cells transfected with a *peripherin* gene in which the 2nd in-frame ATG is intact (not mutant) (A,C) contain a filamentous cytoplasmic network. Bar, 10 μm.

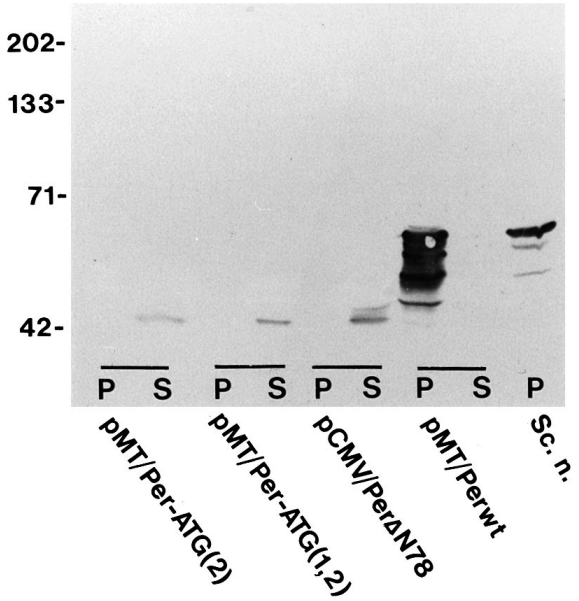

Fig. 3. A western blot of pellets (P) and supernatants (S) derived from cytoskeletal preparations of SW13 cell cultures stably transfected with the indicated plasmids supports the conclusion that the 2nd ATG is used as the primary translation initiation codon. Expression of a peripherin transgene in which the 2nd ATG is mutant (pMT/Per-ATG (2), gives rise to synthesis of a soluble 45 kDa protein, the size predicted of peripherin translated from the third in-frame ATG. This protein species is identical to that expressed from a transgene in which both the first and second ATG are mutant (pMT/Per-ATG (1,2)) or one in which all sequences prior to the third ATG are removed (pCMV/per 78). SW13 cells transfected with the wild-type *peripherin* gene (pMT/perWT) express a full-length insoluble protein that is identical in size to native peripherin derived from rat sciatic nerve (Sc.n). The blot was probed with affinity-purified peripherin antibody.

ize autonomously in the absence of preexisting IFs and that these filaments can persist stably for multiple passages.

The second AUG in peripherin mRNA is the likely primary translation initiation site

Based on the molecular mass of rat peripherin and the prediction that type III IF protein amino termini are of similar length, there are two potential in-frame translation start sites in exon 1 of the rat *peripherin* gene. The first and second AUG codons are separated by 15 nucleotides. To establish which of these serve as the predominant initiation site, the first and the second AUG codons of the rat *peripherin* gene were mutated by site-directed mutagenesis, either singly or in combination. When the first AUG was changed to GUG, and the mutant plasmid pMT/Per-ATG(1) was transfected into SW13 cells, immunofluorescence revealed a filamentous network (Fig. 2C) identical to wild-type peripherin in transfected SW13 cells. When the second AUG was mutated to GCG (pMT/Per-ATG (2)), however, both diffuse and filamentous labeling was seen in the same SW13 cells (Fig. 2D,E). Optical sectioning of these cells revealed that the diffuse labeling was distributed throughout the cell (Fig. 2D), but a few filamentous fragments lie only on the cell surface closest to the coverslip (Fig. 2E). These results were consistent with the idea that two types of peripherin were produced in cells transfected with pMT/Per-ATG(2): one capable of forming filaments and the other not. The most plausible explanation for these results is that polypeptide derived by initiation at the first AUG gave rise to filaments, and that the diffusely distributed protein represented truncated polypeptide generated from the next downstream (third) AUG. A third AUG codon is 234 nucleotides, encoding 78 amino acids, downstream from the first AUG and has a relatively more favored consensus sequence than that of the first AUG. To test the proposition that the third AUG is functional and that mutant protein initiated at this codon is not assembly-competent, SW13 cells were transfected either with a *peripherin* gene in which both the first and second AUG were mutated (pMT/Per-ATG(1,2) or with a gene encoding mutant peripherin that lacks 78 amino-terminal amino acids (pCMV/Per N78). When immunolabeled for peripherin, cells transfected with either construct displayed only diffuse labeling patterns (Fig. 2F, pMT/Per-ATG(1,2) (Fig. 3A, pCMV/Per N78). Transient and stable transfections yielded identical results. This hypothesis was corroborated by western blot which showed that the predominant protein expressed in SW13 cells transfected with pMT/Per-ATG(2) was a soluble 45 kDa protein, the size predicted by translation initiation at the third AUG. In addition, this protein was identical in size to the major protein expressed from pMT/per-ATG(1,2) and pCMV/Per N78, plasmids in which the third AUG is the first intact AUG (Fig. 3). Together, these results suggest that the second AUG serves as the predominant translation start site and that the first AUG may act as a translation start codon, but it is not efficient. Further, the diffuse labeling pattern of peripherin that lacks an amino terminus (pCMV/per N78) (Fig. 4A) indicates that the amino-terminal portion of peripherin is essential for homopolymer filament formation.

Effects of modification in carboxyl terminus of peripherin on assembly

To study whether the carboxyl terminus of peripherin is required for filament formation, we modified the C terminus or deleted the entire tail domain (65 amino acids). Surprisingly, in the majority of SW13 cells transfected with the plasmid encoding tail-less peripherin, filament networks were formed (Fig. 4B). In a minority of cells (3/57, or 5.2%), diffuse or punctate labeling patterns were present (Fig. 4C). Several potential causes for this minor variation in phenotype could include differences among cells in level of transgene expression, stage of cell-cycle, or expression of undefined components necessary for IF assembly. To test whether modification of the carboxyl tail had a negative effect on filament formation, the filament-forming ability of a myc-tagged peripherin, in which the ultimate carboxyl-terminal 18 amino acids of peripherin were replaced by a c-myc peptide tag (18 amino acid peptide), was tested in SW13 cells. Expression of the plasmid pMT/Per-myc resulted in the formation of a filamentous network in SW13 cells that appeared, by immunofluorescence, to be identical to that of wild-type peripherin (Fig. 3D). Thus, unlike perturbations of the N terminus, deletion or mutation of the C terminus of peripherin is compatible with filament formation.

DISCUSSION

Peripherin is a neuron-specific IF protein that, along with the type IV neurofilament proteins NF-L, NF-M, NF-H, and α-

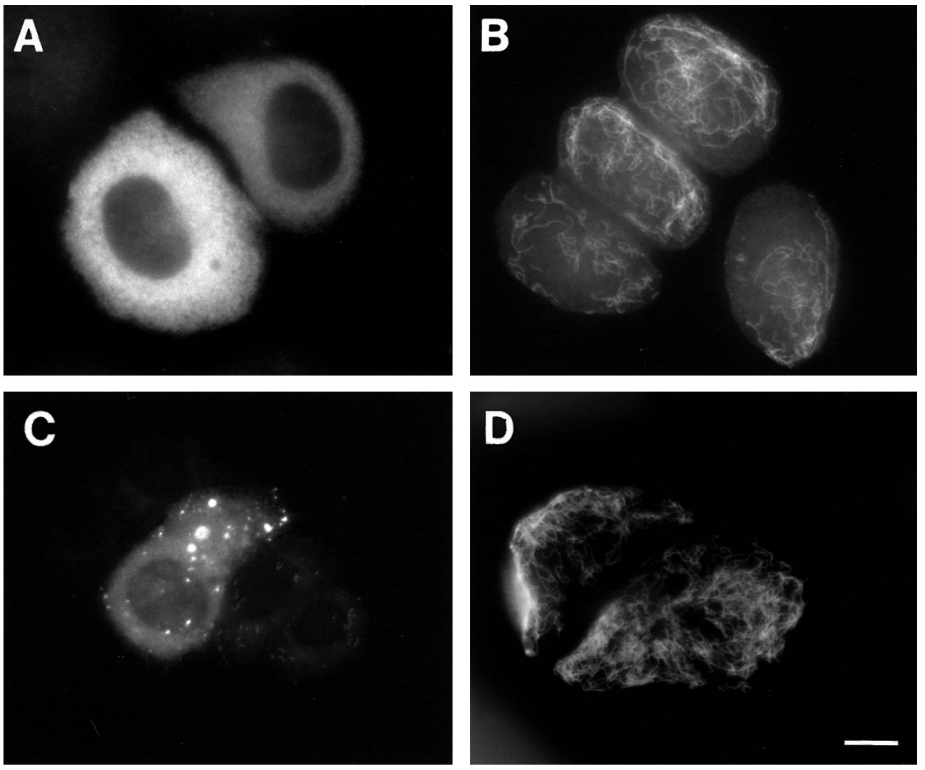

Fig. 4. Immunofluorescence of SW13 cells transfected with pCMV/Per N78 shows that expression of peripherin missing the amino terminus results in diffuse peripherin fluorescence (A). In contrast, loss of the carboxyl terminus, as shown in cells transfected with pMT/Per C65 (B), does not prevent the formation of peripherin-labeled filaments in the majority of cells. A minority of cells in the culture did not show filamentous peripherin label (C). Transfections with pMT/Per-myc, in which the 18 terminal amino acids of peripherin were replaced by a myc tag, however, resulted in filamentous peripherin label in all cells (D). Bar, 10 μm.

internexin, is expressed in the adult nervous system. The association between peripherin and the other IF proteins expressed in the mature nervous system is limited, however, because the exon-intron structure of the *peripherin* gene is distinct from those encoding the type IV neurofilament proteins. In addition, the amino acid sequence of the amino head and carboxyl tail regions of peripherin show little similarity with the NF-triplet proteins or α-internexin (Liem, 1990). Rather, both properties are most similar to those of other type III IF proteins (Leonard et al., 1988; Thompson and Ziff, 1989) such as vimentin, desmin, and GFAP. Since other type III IF proteins readily form homopolymers in cells that lack pre-existing type III IF (Raats et al., 1991; Sarria et al., 1990), it has been believed that peripherin can form homopolymers, but, there was no direct in vivo evidence to support this. Our results clearly demonstrate that peripherin forms a homopolymer in vivo. The assembly of wild-type peripherin in SW13 cells, cells that express no other type III IF proteins or NF proteins, suggests that peripherin also may form homopolymers in neurons. Thus, neurons that appear to express only peripherin (Parysek et al., 1991; Troy et al., 1990) are likely to be capable of forming homopolymeric neuronal IF containing peripherin.

We also used mutational analyses to determine whether the amino and carboxyl-terminal tails of peripherin were required for assembly into filamentous networks. Deletion of nucleotides encoding the amino terminus of peripherin, or mutation of the putative translation start site of the *peripherin* gene, completely abolished its ability to assemble into filamentous structures. This property of peripherin is similar to that of all other IF proteins that have been analyzed (see references cited in Introduction). Peripherin that lacks the entire carboxyl-terminal tail region, however, forms filamentous networks. This finding differs from most other studies on type III IF proteins. For example, expression of tail-less desmin or tail-less GFAP in type III IF-less cells resulted in a dotted, rather than filamentous labeling pattern (Raats et al., 1992). Expression of vimentin lacking the entire carboxyl tail in SW13 cells gave rise to a fibrous labeling pattern that was limited to the cell periphery (McCormick et al., 1993). In contrast, Rogers et al. (1995) have recently reported that deletion of the C-terminal tail of either desmin or vimentin does not affect network assembly in SW13 cells. Our observation that tail-less peripherin polymerizes into a filamentous network that resembles a wild-type IF network, therefore, is similar to the findings of Rogers et al. on desmin and vimentin. Although the C terminus of peripherin is not required for polymerization, this region of the molecule is as highly conserved among different species as the remainder of the protein, implying selective pressure for a phenotype. Either the assembly of these mutant subunits is subtly aberrant, in ways that are not detectable by light microscopy, or this portion of the molecule subserves some function other than assembly.

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