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A neurotoxic peripherin splice variant in a mouse model of ALS

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Peripherin, a neuronal intermediate filament (nIF) protein found associated with pathological aggregates in motor neurons of patients with amyotrophic lateral sclerosis (ALS) and of transgenic mice overexpressing mutant superoxide dismutase-1 ($SOD1^{G37R}$), induces the selective degeneration of motor neurons when overexpressed in transgenic mice. Mouse peripherin is unique compared with other nIF proteins in that three peripherin isoforms are generated by alternative splicing. Here, the properties of the peripherin splice variants Per 58, Per 56, and Per 61 have been investigated in transfected cell lines, in primary motor neurons, and in transgenic mice overexpressing peripherin or overexpressing $SOD1^{G37R}$. Of the three isoforms, Per 61 proved to be distinctly neurotoxic, being assembly incompetent and inducing degeneration of motor neurons in culture. Using isoform-specific antibodies, Per 61 expression was detected in motor neurons of $SOD1^{G37R}$ transgenic mice but not of control or peripherin transgenic mice. The Per 61 antibody also selectively labeled motor neurons and axonal spheroids in two cases of familial ALS and immunoprecipitated a higher molecular mass peripherin species from disease tissue. This evidence suggests that expression of neurotoxic splice variants of peripherin may contribute to the neurodegenerative mechanism in ALS.

Introduction

Amyotrophic lateral sclerosis (ALS)* is an adult onset neurological disorder that affects primarily motor neurons of the brain stem and spinal cord, resulting in paralysis and death within 2–5 yr. Although most cases of ALS are sporadic, 15–20% are familial and, of these, 1–2% are caused by mutations within the gene encoding the metalloenzyme Cu/Zn superoxide dismutase-1 (*SOD1*; Rosen et al., 1993). The mechanism by which expression of mutant SOD1 induces motor neuron degeneration remains elusive.

An ultrastructural hallmark of diseased motor neurons in ALS is the presence of perikaryal and axonal aggregates composed of the neuronal intermediate filaments (nIFs), neurofilaments, and peripherin (Carpenter, 1968; Corbo and Hays, 1992; Migheli et al., 1993). Neurofilaments are formed by the coassembly of three type IV nIF proteins, neurofilament light subunit (NF-L; 68–70 kD), neurofilament medium subunit (NF-M; 150–155 kD); and neurofilament heavy subunit (NF-H; 200–210 kD), and are widely expressed throughout the central and peripheral nervous systems (Julien and Mushynski, 1998). Peripherin is an ~58-kD type III nIF protein that is capable of self-assembly and is expressed mostly in the peripheral nervous system and only at low levels in defined neuronal populations of the central nervous system (Portier et al., 1983a, 1993; Parysek and Goldman, 1988; Brody et al., 1989; Escurat et al., 1990). However, after neuronal injury, peripherin expression is increased; e.g., in large dorsal root ganglion neurons and spinal motor neurons after sciatic nerve crush (Troy et al., 1990; Wong and Oblinger, 1990), and also in neuronal populations

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*Abbreviations used in this paper: ALS, amyotrophic lateral sclerosis; DAB, 3,4-diaminobenzidine; IF, intermediate filament; NF-H, neurofilament heavy subunit; NF-L, neurofilament light subunit; NF-M, neurofilament medium subunit; nIF, neuronal IF; PVDF, polyvinyldifluoride; SOD1, superoxide dismutase-1.

Key words: peripherin; isoforms; splicing; SOD1; ALS

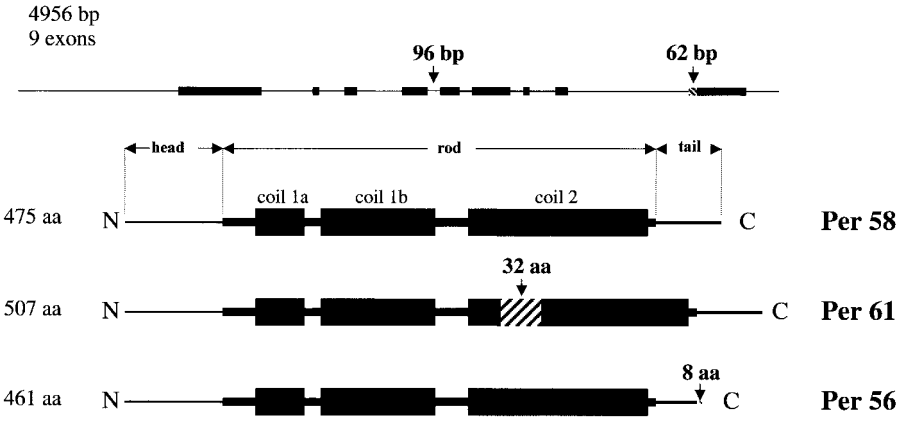

Figure 1. **Alternative splicing of the mouse peripherin gene.** The peripherin gene contains nine exons, with the intron-exon location categorizing it as a type III intermediate filament protein (IFP). As with other IFPs, peripherin has an α-helical rod domain flanked by non–α-helical NH_2-terminal head and COOH-terminal tail domains. The predominant isoform, Per 58, is encoded by all nine exons of the peripherin gene to generate a protein of 475 aa, using the nomenclature of Landon et al. (2000) in which the first of three potential ATG initiation codons is used as the start site. The retention of intron 4, which is 96 bp in length, generates Per 61, corresponding to a 32-aa insertion in coil 2 of the α-helical rod domain. Isoform Per 56 is generated by the use of a cryptic acceptor site at the beginning of exon 9, leading to a deletion of 62 bp, a change in reading frame, and a replacement of the COOH-terminal 21 aa with a unique 8-aa sequence.

of the brain after lesion injury or cerebral ischemia (Beaulieu et al., 2002). This increase in peripherin expression has been suggested to facilitate neuronal regeneration (Troy et al., 1990; Wong and Oblinger, 1990).

To study the potential contribution of abnormalities in nIF expression to motor neuron degeneration in ALS, numerous transgenic mouse models have been developed in which the different nIF genes have been overexpressed or knocked out (for review see Cleveland and Rothstein, 2001; Julien, 2001). Of all these models involving wild-type nIF proteins, only the peripherin transgenic mouse develops a motor neuron degeneration mimicking several aspects of ALS, including the formation of peripherin immunoreactive aggregates and the selective loss of motor neurons (Beaulieu et al., 1999a). These findings indicate that in addition to its possible involvement in regeneration, peripherin also has toxic properties that can lead to neuronal degeneration.

Peripherin is unique compared with other nIF proteins in that three mRNA alternative splice variants and their corresponding translation products have been identified in a mouse neuroblastoma cell line (Landon et al., 1989, 2000). Per 58, the naming reflective of its molecular mass in kiloDaltons on SDS–polyacrylamide gels, corresponds to the peripherin species first identified in mouse neuroblastoma and rat PC12 cells (Portier et al., 1983a), and is encoded by all nine exons of the peripherin gene. Per 61 is identical to Per 58 except for the insertion of a 32–amino acid sequence in coil 2 of the α-helical rod domain, signifying the inclusion of 96 bp of intron 4. Per 56 is generated by the use of a cryptic acceptor site at the beginning of exon 9, resulting in a frameshift and replacement of the COOH-terminal 21 amino acids of Per 58 with a unique 8–amino acid sequence. Why these different peripherin isoforms exist is unknown, and the expression of Per 56 and Per 61 at the protein level has not previously been demonstrated in vivo. However, it is possible that they have different functional properties such that their differential expression may induce distinct consequences to the neuron in which they are expressed.

Here, we have investigated the properties of Per 58, Per 56, and Per 61 in cell lines and in primary motor neurons. We show that each peripherin isoform has different assembly characteristics and that, more importantly, Per 61 is neurotoxic when expressed in motor neurons in primary culture. Using isoform-specific antibodies, we show that Per 61 is expressed in motor neurons of mutant $SOD1^{G37R}$ transgenic mice but not of wild-type control or peripherin transgenic mice, demonstrating that the disease mechanism of mutant $SOD1^{G37R}$ includes expression of a neurotoxic splice variant of peripherin. The Per 61 antibody also labeled pathological lesions in the lumbar spinal cord of ALS cases but not of control. Moreover, the Per 61 antibody immunoprecipitated a higher molecular mass peripherin species, suggesting that expression of alternatively spliced variants of peripherin may contribute to the neurodegenerative mechanism in ALS.

Results

Assembly properties of peripherin isoforms in transfected SW13 vim(−) cells

Alternative splicing of the mouse peripherin gene transcript generates at least three peripherin isoforms, Per 58, Per 61, and Per 56 (Fig. 1; Landon et al., 1989, 2000). The specific assembly characteristics of the peripherin isoforms have not been investigated previously. Here, the assembly properties of the individual peripherin isoforms were analyzed in SW13 vim(−) cells, an adrenal carcinoma cell line that lacks an endogenous cytoplasmic intermediate filament (IF) network (Sarria et al., 1994). Ectopic expression of cDNAs encoding the different peripherin isoforms generated proteins of the expected molecular masses in cell extracts analyzed by SDS-PAGE, i.e., 58 kD for Per 58; 56 kD for Per 56; and 61 kD for Per 61 (Fig. 2 A). Immunocytochemical analysis of the cytoplasmic distribution of the expressed peripherin isoforms revealed that both Per 56 and Per 58 were capable of self-assembly, forming IF networks, whereas Per 61 was assembly incompetent, forming aggregates (Fig. 2 B). Furthermore, although Per 56 and Per 58 were capable of coassembly into a filamentous network, expression of Per 61 with either Per 56 or Per 58 induced aggregation (unpublished data).

As peripherin and neurofilaments are coexpressed in several neuronal cell types both normally and after injury, it is important to investigate the potential interactions between

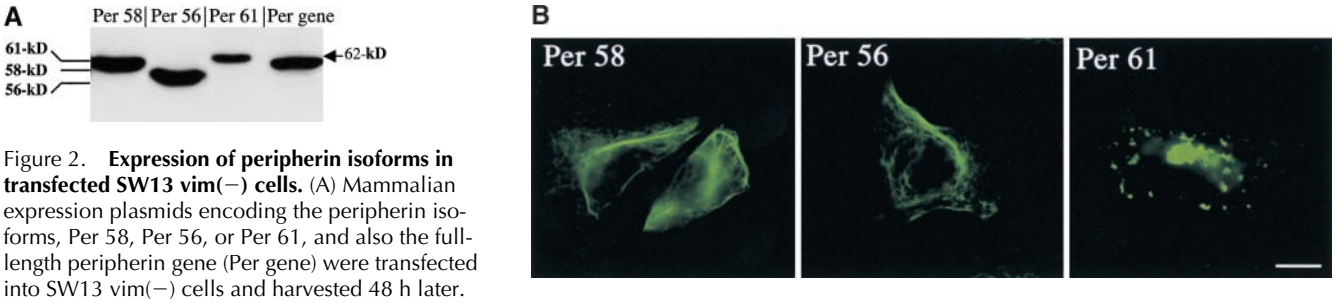

Figure 2. **Expression of peripherin isoforms in transfected SW13 vim(−) cells.** (A) Mammalian expression plasmids encoding the peripherin isoforms, Per 58, Per 56, or Per 61, and also the full-length peripherin gene (Per gene) were transfected into SW13 vim(−) cells and harvested 48 h later. Equal protein loadings (10 μg) of harvested cells were resolved on 7.5% (wt/vol) SDS–polyacrylamide gels, and then transferred to PVDF membrane and probed with a monoclonal antibody recognizing peripherin (MAB1527). For Per 61, immunoprecipitation using peripherin polyclonal antiserum (AB1530) was used to obtain sufficient quantities for detection on immunoblots. The immunoblot shows that expression of the plasmids encoding Per 58, Per 56, and Per 61, as indicated, generate proteins of the expected molecular masses with the position of the 62-kD marker shown (arrow). The major peripherin species expressed from the full-length peripherin gene (Per gene) corresponds to the molecular mass of isoform Per 58. (B) SW13 vim(−) cells transfected with the individual peripherin isoform expression plasmids were labeled immunocytochemically with monoclonal antibody to peripherin (MAB1527) to ascertain the cytoplasmic distribution of the expressed proteins. From the figure it can be seen that both Per 58 and Per 56 were capable of self-assembly to form an IF network that was distributed evenly throughout the cytoplasm. These results are similar to those obtained with the full-length mouse peripherin gene (Beaulieu et al., 1999b). In contrast, Per 61 was assembly incompetent, forming aggregates of uneven size and distribution. Bar, 10 μm.

these filament types especially in relation to aggregate formation, which is associated with numerous pathological conditions including ALS. We have shown previously, in cotransfection studies in SW13 vim(−) cells using the mouse peripherin gene, that peripherin is not only assembly competent but can also coassemble with NF-L (Beaulieu et al., 1999b). However, coexpression of NF-H or NF-M with peripherin causes disruption of filament assembly (Beaulieu et

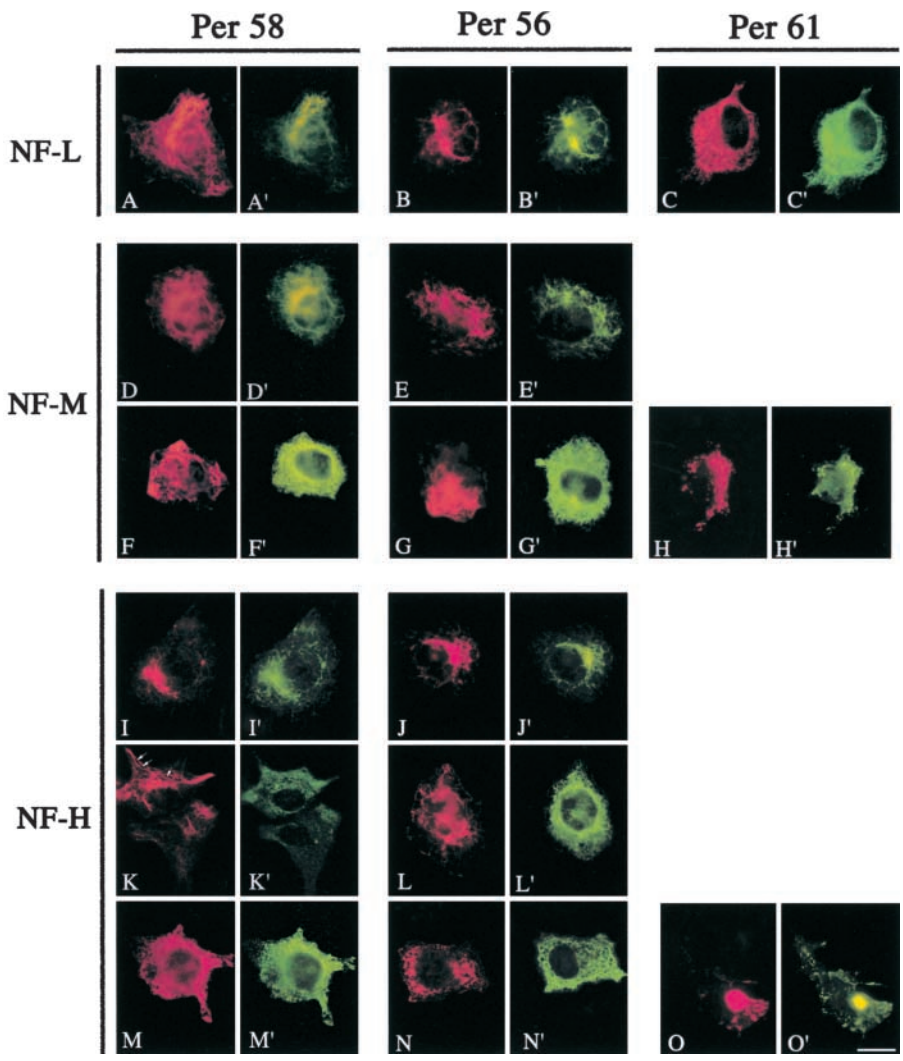

Figure 3. **Assembly characteristics of peripherin isoforms in transfected SW13 vim(−) cells.** SW13 vim(−) cells were transfected with expression plasmids encoding the different peripherin isoforms together with plasmids encoding NF-L, NF-M, or NF-H. Assembly properties of the coexpressed proteins were analyzed by double immunofluorescence labeling using monoclonal antibody to peripherin (MAB1527; red) and polyclonal antiserum (green) to NF-L (AB1983), NF-M (AB1981), and NF-H (AB 1982) in the respective cotransfected cells. Isoforms Per 58 and Per 56 were capable of coassembly with all three neurofilament subunits (A, A′; B, B′; D, D′; E, E′; I, I′; and J, J′), whereas isoform Per 61 was incapable of coassembly into an IF network with any of the neurofilament subunits (C, C′; H, H′; and O, O′). Additional phenotypes were observed in cells cotransfected with Per 58 or Per 56 and NF-M or NF-H. These were filamentous bundles of peripherin in the presence of nonassembled NF-M (F, F′ and G, G′) or NF-H (K, K′ and L, L′; arrows in K indicate filaments) and nonassembled Per 58 or Per 56 in the presence of nonassembled NF-H (M, M′ and N, N′). Bar, 14 μm.

al., 1999b). We compared the coassembly properties of each of the peripherin isoforms with the individual neurofilament subunits, NF-L, NF-M, and NF-H. Per 56 and 58 were capable of coassembly with all three neurofilament subunits (Fig. 3, A, A′; B, B′; D, D′; E, E′; I, I′; and J, J′). However, there were additional phenotypes observed in which Per 56 or Per 58 formed filamentous bundles in the presence of nonfilamentous NF-M (Fig. 3, F, F′; and G, G′). This was also observed in cells cotransfected with Per 56 or Per 58 and NF-H (Fig. 3, K, K′; and L, L′). A further phenotype observed in Per 56/NF-H– or Per 58/NF-H–cotransfected cells was the presence of punctate peripherin together with punctate NF-H (Fig. 3, M, M′; and N, N′). As the SW13 vim(−) cells used for this study were a clonal cell line used within two to seven passages, we concluded that these differences were attributable to differences in the relative expression levels of peripherin and/or the neurofilament subunits.

Per 61 was incompetent for IF assembly with all three neurofilament subunits (Fig. 3, C, C′; H, H′; and O, O′). Of note was the difference in cytoplasmic distribution of Per 61 in SW13 vim(−) cells cotransfected with NF-L compared with those transfected with Per 61 alone or together with NF-M or NF-H (compare Fig. 2 and Fig. 3, H and O, with Fig. 3 C). In Per 61/NF-L–cotransfected cells, the distribution of Per 61 was more granular and appeared more evenly dispersed throughout the cytoplasm in contrast to a more speckled phenotype in cells transfected with Per 61 alone. We also observed that coexpression with NF-L appeared to "stabilize" Per 61, with a greater number of cells appearing transfected after immunocytochemical labeling and a higher yield of Per 61 apparent on immunoblots (unpublished data).

Expression of Per 61 is neurotoxic to motor neurons in culture

Overexpression of peripherin in transgenic mice leads to late onset and selective degeneration of motor neurons (Beaulieu et al., 1999a). Furthermore, we have recently shown, using intranuclear microinjection studies, that overexpression of peripherin induces death of motor neurons in culture (Robertson et al., 2001). To directly test the effect of individual peripherin isoform expression on motor neuron viability, expression plasmids encoding each peripherin isoform were microinjected into motor neuron nuclei in primary culture (Durham et al., 1997). Motor neurons in dissociated spinal cord cultures do not express peripherin (Robertson et al., 2001); therefore, the expression of the peripherin isoforms individually microinjected into motor neurons could be detected with commercially available antibody to peripherin (MAB1527). The viability curve in Fig. 4 A shows that Per 61 is extremely toxic to motor neurons. Almost all of the motor neurons were dead after only 7-d expression of the Per 61 plasmid. In contrast, the effect of Per 58 and Per 56 expression on viability of motor neurons was not significantly different from control, in which the expression plasmid (pRcCMV) alone was microinjected. Immunocytochemical analyses of microinjected motor neurons with antibodies recognizing peripherin or NF-L revealed that both Per 58 and Per 56 could integrate normally into the existing nIF network (Fig. 4, B and C), whereas Per 61 formed aggregates similar to those observed in SW13 vim(−) cells transfected with Per 61 expression plasmid (compare Fig. 4 C with Fig. 2 B). In addition, motor neurons expressing Per 61 appeared shrunken with few neuritic processes, changes consistent with neurotoxicity and neuronal death (Fig. 4 B). These findings show that expression of Per 61, and not Per 58 or Per 56, is neurotoxic when expressed in motor neurons in culture.

Expression of Per 56 but not Per 61 in motor neurons of peripherin transgenic mice

Peripherin aggregates are a feature of transgenic mice overexpressing peripherin and of transgenic mice overexpressing mutant SOD1^{G37R} (Tu et al., 1996; Julien and Beaulieu, 2000). To analyze the contribution of Per 56 and Per 61 to the formation of these aggregates, synthetic peptides corresponding to the unique sequences in Per 61 and Per 56 were used to generate isoform-specific antibodies. As there is no unique sequence in Per 58, it was not possible to generate an antiserum specifically recognizing this isoform. Therefore, we could not establish if there was a specific increase in Per 58 expression in motor neurons of peripherin or SOD1^{G37R} transgenic mice using this approach. The Per 56 and Per 61 antibodies were purified by affinity chromatography and their specificity was tested by immunocytochemistry (Fig. 5, A–F) and immunoblotting (Fig. 5 G) using SW13 vim(−) cells expressing either Per 56, Per 58, or Per 61. Immunocytochemical labeling of the transfected SW13 vim(−) cells with anti–Per 56 and anti–Per 61 revealed that both antisera were specific for their respective immunogens (Fig. 5, D and E). There was no cross-reactivity of anti–Per 61 with either Per 58 or Per 56 (Fig. 5, A and C) and conversely anti–Per 56 did not detect Per 61 or Per 58 (Fig. 5, B and F). These findings were also confirmed immunocytochemically in similar experiments performed on microinjected motor neurons (unpublished data). Immunoblotting with polyclonal peripherin antiserum (AB1530) established the expression of the different peripherin isoforms in cell extracts of Per 58–transfected (Fig. 5 G, lane 1), Per 56–transfected (Fig. 5 G, lane 2), and Per 61–transfected (Fig. 5 G, lane 3) SW13 vim (−) cells. Probing of the same samples with anti–Per 61 or anti–Per 56 confirmed the specificity of the isoform-specific antisera (Fig. 5 G).

Transgenic mice overexpressing the mouse peripherin gene develop a motor neuropathy with features mimicking some aspects of ALS, specifically the loss of motor neurons in the spinal cord, precipitated by the formation of peripherin aggregates both in motor axons and perikarya (Beaulieu et al., 1999a). Lumbar spinal cord sections from two different lines of peripherin transgenic mice, Per and Per;LKO together with wild-type mice (C57Bl6), were labeled with anti–Per 56 and anti–Per 61. Motor neuron death induced by overexpression of peripherin is exacerbated by the loss of NF-L in Per;LKO transgenic mice (Beaulieu et al., 1999a) and lack of NF-L may affect peripherin isoform expression.

There was a small amount of labeling of motor neurons with anti–Per 56 in wild-type tissue and more intense labeling in both lines of peripherin transgenic mice (Fig. 6). In the Per;LKO transgenic mouse tissue, there was a greater propensity for peripherin aggregation compared with the

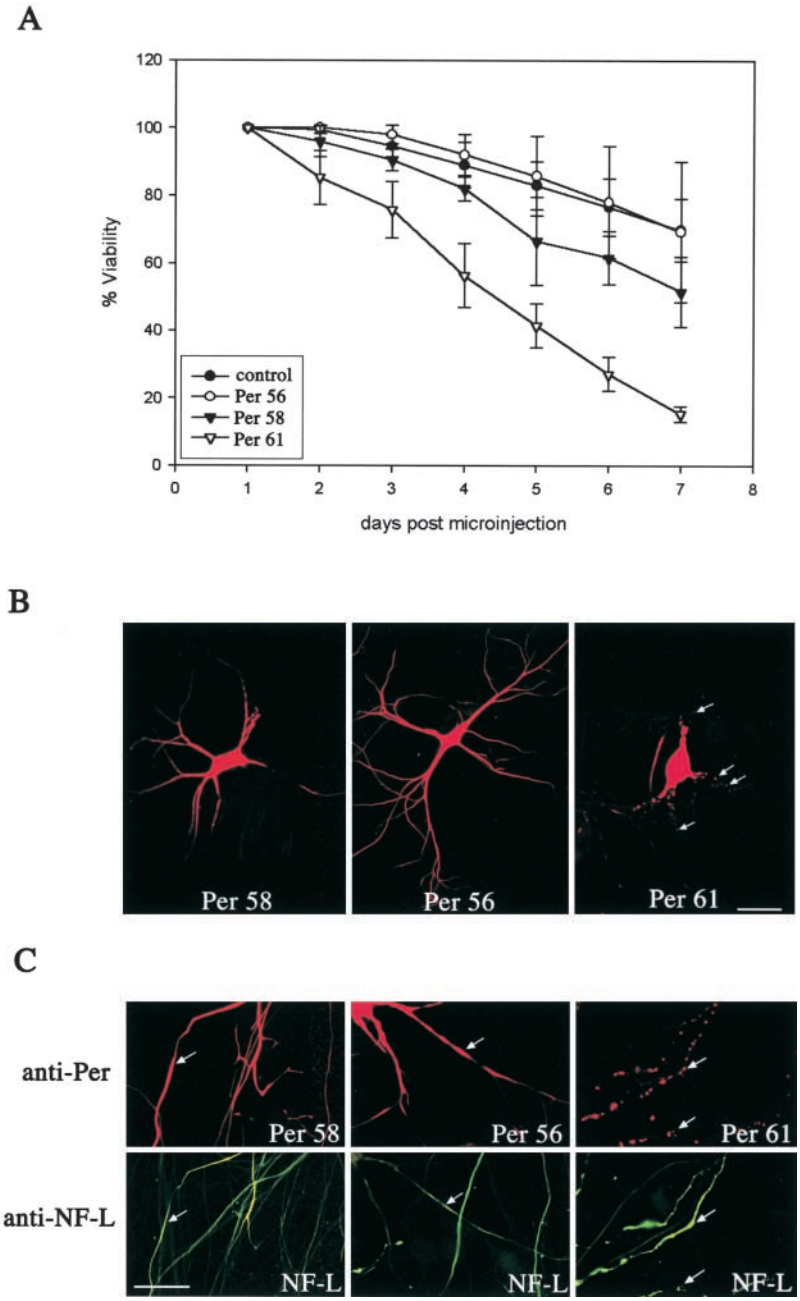

Figure 4. **Per 61 induces death of motor neurons in culture.** (A) Plasmid expression vectors encoding the individual peripherin isoforms, Per 58, Per 56, Per 61, or vector alone (pRcCMV; control) were microinjected along with dextran-FITC into the nuclei of motor neurons in dissociated spinal cord cultures. Microinjected motor neurons, containing dextran-FITC, were identified by epifluorescence microscopy. The number of viable motor neurons were counted each day for 7 d and the results from each microinjection experiment compared. The chart shows the composite of three separate experiments performed for each peripherin isoform. Per 61 was extremely neurotoxic with >90% of the microinjected motor neurons dead within 7 d of expression. The effects of Per 56 and Per 58 on motor neuron viability were not significantly different from control. (B) Motor neurons microinjected with either Per 58, Per 56, or Per 61 were labeled by indirect immunofluorescence with antibody recognizing peripherin (MAB1527). It is noteworthy that motor neurons in dissociated spinal cord cultures do not express detectable levels of peripherin and therefore expression of the peripherin isoforms in microinjected motor neurons could easily be detected using peripherin antibody. The results show the comparative cytoplasmic distribution of the peripherin isoforms after 3 d expression. Both Per 58 and Per 56 integrated normally into the existing cytoplasmic network with even distribution throughout the perikarya and neuritic processes. Apparent varicosities in peripherin labeling in the Per 58 expressing motor neuron are due to crossing of neurites from nonmicroinjected neurons that block the peripherin signal. In contrast, Per 61 did not integrate into the existing IF network, but instead formed aggregates that were also present in neurites (arrows). Bar, 50 μm. (C) Double immunofluorescence labeling of microinjected motor neurons with monoclonal antibody to peripherin (MAB1527; red) and polyclonal antiserum to NF-L (AB1983; green) shows that both Per 58 and Per 56 colocalize with NF-L, whereas Per 61 disrupts the existing IF network (arrows). Bar, 30 μm.

corresponding peripherin labeling in Per transgenic mice (Fig. 6, compare C with E), as had been observed previously with peripherin polyclonal antiserum, AB1530 (Beaulieu et al., 1999b). In contrast, there was no detectable labeling of either wild-type or peripherin transgenic mouse motor neurons (Per and Per;LKO) with antibody recognizing Per 61 (Fig. 6, D and F).

Expression of Per 61 in motor neurons of transgenic mice expressing mSOD1^{G37R}

Transgenic mice overexpressing by 6-fold (L29) or 12-fold (L42) the G37R mutant of the *SOD1* gene linked to human ALS develop a motor neuron-like disease (Wong et al., 1995). The onset and severity of disease is increased in L42 compared with L29, reflective of the increased SOD1^{G37R} transgene copy number (Wong et al., 1995). Labeling of lumbar spinal cord sections from L29 and L42 transgenic mice with anti–Per 56 showed a similar labeling to that obtained in Per transgenic mice, with a specific and intense labeling of motor neurons (Fig. 7, A and C). However, most interestingly, in contrast to our findings in motor neurons of wild-type and peripherin transgenic mice, Per 61 immunoreactivity was detected in motor neurons of both L29 and L42 SOD1^{G37R} transgenic mice showing the presence of aggregates not only in perikarya but also in proximal neurites. This labeling correlated with disease onset, only rarely being observed in motor neurons of presymptomatic mice. Other smaller aggregates, similar to those described previously using polyclonal peripherin antibody (Julien and Beaulieu, 2000), were also labeled (Fig. 7, B and D, arrowheads). Competition with the synthetic peptides used to raise the antibodies showed the specificity of this labeling (Fig. 7, E and F). Moreover, RT-PCR of RNA extracted from spinal

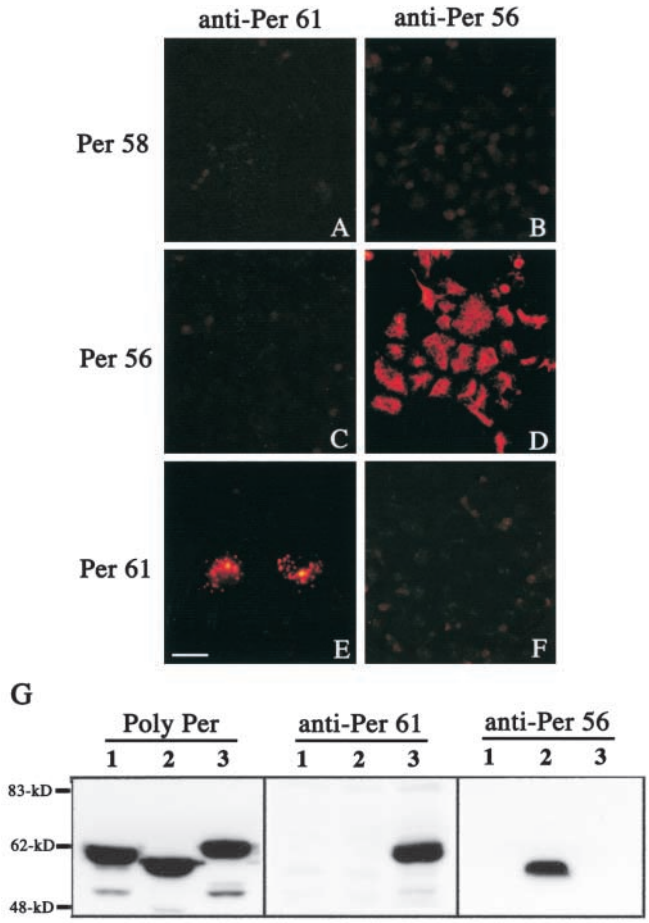

Figure 5. **Characterization of specific antisera to peripherin isoforms.** (A–F) SW13 vim(−) cells transfected with plasmid expression vectors encoding Per 58, Per 56, or Per 61 were labeled by indirect immunofluorescence with anti–Per 56 or anti–Per 61. There was no labeling of Per 58 by either antibody (A and B). Both anti–Per 56 and anti–Per 61 were specific for their respective proteins, Per 56 appearing filamentous (D) and Per 61 speckled (E). Bars: (A–D and F) 20 μm; (E) 30 μm. (G) Immunblots of cell extracts from NIH 3T3 cells transfected with Per 58 (lane 1), Per 56 (lane 2), or Per 61 (lane 3) were probed with polyclonal antiserum to peripherin (Poly Per; AB1530); anti–Per 61 and anti–Per 56. The results obtained confirmed the specificity of the Per 56 and Per 61 antisera.

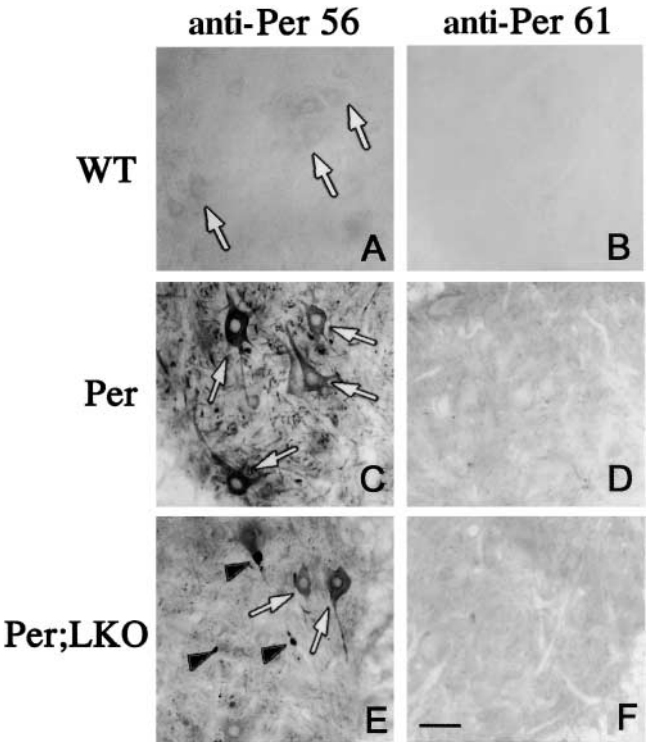

Figure 6. **Expression of Per 56 in motor neurons of peripherin transgenic mice.** (A–F) Lumbar spinal cord sections from wild-type (WT; A and B) and Per (C and D) and Per;LKO (E and F) transgenic mice were labeled immunocytochemically with anti–Per 56 and anti–Per 61. Per 56 expression was detected mildly in motor neurons of C57Bl6 mice (A, arrows) and strongly in both Per (C) and Per; LKO (E) transgenic mice, forming aggregates in the latter (arrowheads). In contrast, no labeling of motor neurons was detected with anti–Per 61 in spinal cord sections from C57Bl6 mice (B) or in either of the peripherin transgenic mice (D and F). Bar, 60 μm.

cord showed the presence of the mRNA for Per 61 in $SOD1^{G37R}$ transgenic mice (Fig. 7 G). These results show that, in addition to Per 56, there is expression of Per 61 in motor neurons of $SOD1^{G37R}$ transgenic mice.

Selective anti–Per 61 labeling of motor neurons in ALS lumbar spinal cord

Although splice variants of peripherin have not been identified in human, the synthetic peptide used to generate the Per 61 antibody spans a region of intron 4 conserved at the nucleotide level between mouse, rat, and human (Foley et al., 1994). Using the Per 61 antibody, we have labeled pathological lesions in the lumbar spinal cord of two out of three familial ALS cases with no labeling detected in two control cases. The Per 61 labeling was intense and correlated with the occurrence of peripherin abnormalities (as revealed with peripherin antibody). Fig. 8 shows the lumbar spinal cord sections from a familial ALS case labeled with antibody to peripherin (Fig. 8 A), with Per 61 antibody (Fig. 8, B and C), and sequential double labeling with Per 61 (3,4-diaminobenzidine [DAB]; brown) and antibody to peripherin (Fig. 8 D, alkaline phosphatase, pink). Peripherin immunoreactivity localized mainly to the motor neuron perikaryon and to spheroid structures (Fig. 8, black arrows) consistent with previous reports of peripherin abnormalities in cases of ALS (Fig. 8 A; Corbo and Hays, 1992; Migheli et al., 1993; Strong, 2001). Per 61 immunoreactivity was also predominantly localized to motor neuron perikarya and less frequently to axonal spheroids (Fig. 8, B and C). Other similar structures were also present in both peripherin-labeled and Per 61–labeled tissue (Fig. 8, A and B, white arrows). Per 61 immunoreactivity appeared as speckles, as has been observed in all the other experimental paradigms tested (this is clearly apparent in the axonal spheroid shown in the inset of Fig. 8 B, white arrow). In double labeling experiments, Per 61 and peripherin immunoreactivity could be localized in many cases to the same motor neuron (Fig. 8 D).

Anti–Per 61 immunoprecipitates an abnormal peripherin species from ALS lumbar spinal cord tissue

Per 61 antibody was used in an immunoprecipitation assay to verify that this antibody was indeed recognizing human peripherin. Polyclonal peripherin antibody (AB1530) was

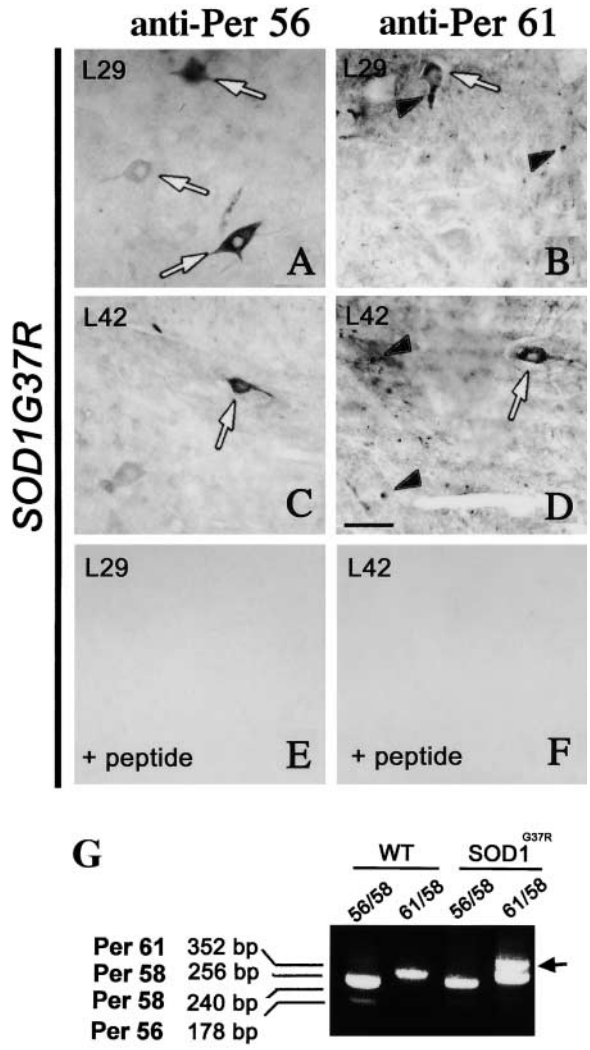

Figure 7. **Expression of Per 61 in motor neurons of mutant SOD1^{G37R} transgenic mice.** (A–F) Lumbar spinal cord sections from L29 (A and B) or L42 (C and D) mutant SOD1^{G37R} transgenic mice were labeled immunocytochemically with anti–Per 56 and anti–Per 61. Per 56 expression was detected in motor neurons of both L29 and L42 mutant SOD1^{G37R} transgenic mice (A and C, white arrows). Expression of Per 61 was also detected (B and D) with anti–Per 61 labeling aggregates in motor neuron perikarya and proximal axons (white arrows), in addition to smaller inclusions located in the surrounding neuronal tissue (arrowheads). E and F show ablation of the Per 56 or Per 61 immunoreactivity in the presence of the respective immunogenic peptides. Bar, 60 μm. (G) RT-PCR of total RNA extracted from wild-type (WT) or mutant SOD1^{G37R} (L29; endstage) spinal cord using primers 56/58 to detect Per 56 (178 bp) and primers 61/58 to detect Per 61 (352 bp). Note the Per 61 PCR product apparent in the RNA sample derived from SOD1^{G37R} spinal cord (arrow).

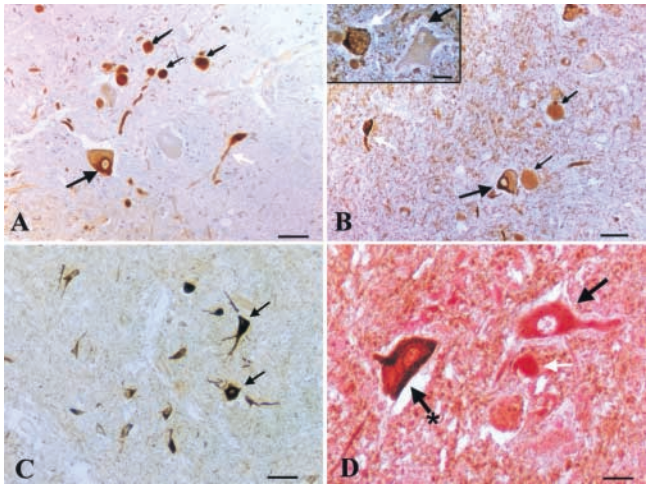

Figure 8. **Selective anti–Per 61 labeling of motor neurons in ALS lumbar spinal cord.** Lumbar spinal cord sections from a familial ALS case labeled with antibody to peripherin (A); anti–Per 61 (B and C); and double labeled with anti–Per 61 (DAB, brown) and antibody to peripherin (D, alkaline phosphatase, pink). Peripherin antibody, as shown in A, labeled predominantly motor neuron perikaryon (large black arrow) and spheroidal structures (small black arrows). Neuritic processes were also labeled (white arrow). Per 61 specifically labeled motor neuron perikarya (B, large black arrows, and C, black arrows) and neuritic processes (B, white arrow). Spheroids were clearly apparent from the DAB labeling (B, small black arrows). The inset of B shows the speckled labeling in an axonal spheroid (indicated by white arrow) consistent with previous observations of Per 61 immunoreactivity, next to an unlabeled motor neuron perikaryon (inset, black arrow). The intensity of the Per 61 immunoreactivity in motor neuron perikarya is clearly apparent (C, black arrows). No labeling of control tissue was observed (not depicted). Per 61 immunoreactivity was colocalized with peripherin immunoreactivity in some (D, black arrow with asterisk), but not all motor neuron perikarya (D, black arrow). White arrow in D indicates small speroids. Bars: (A and B) 30 μm; (C) 70 μm; and (D) 12 μm.

also used to determine whether the increased peripherin immunoreactivity detected in two out of three familial ALS cases tested correlated with an increased expression of peripherin. Lysates of lumbar spinal cord from the same ALS cases used to generate the immunohistochemical data (Fig. 8) was used for the immunoprecipitation assays. Motor neurons in these cases had peripherin abnormalities and were labeled with Per 61 antibody. Control lumbar spinal cord tissue, in which there was no Per 61 immunoreactivity detected immunohistochemically, was also tested for comparison. Immunoblots of the resultant anti–Per 61 and anti-peripherin immunoprecipitates were probed with monoclonal peripherin antibody. In initial experiments, the RIPA-insoluble fraction was exclusively used for immunoprecipitation assays, as peripherin is normally localized to this fraction. Although we detected an overall increase in peripherin expression in ALS lumbar spinal cord tissue compared with control tissue (Fig. 9), we did not detect a Per 61 species, probing the blots with either monoclonal peripherin or anti–Per 61. However, in experiments using transfected SW13 vim(−) cells, we found that Per 61 partitioned largely to the soluble fraction of cells lysed in RIPA buffer (unpublished data), which is consistent with defects in the assembly of Per 61 to form a detergent-insoluble IF network. Consequently, the RIPA-soluble fraction was used for the anti–Per 61 immunoprecipitation assays. Results show that the Per 61 antibody immunoprecipitated a species that was detected on immunoblots by the monoclonal peripherin antibody. Moreover, this Per 61 species migrated at a slower rate than the regular human peripherin (Fig. 9), which is consistent with a predicted increase in molecular mass because of an abnormal splicing event.

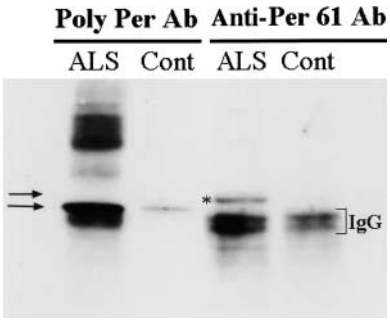

Figure 9. **Anti–Per 61 immunoprecipitation of higher molecular mass peripherin species.** Anti–Per 61 and polyclonal peripherin (Poly Per) immunoprecipitates from ALS and control lumbar spinal cord tissue were resolved on a 10% SDS–polyacrylamide gel and transferred to PVDF membrane by electroblotting. The membrane was then immunoblotted with monoclonal peripherin antibody to detect peripherin species immunoprecipitated by Poly Per and anti–Per 61. Increased peripherin expression was detected in the pellet fraction of ALS tissue immunoprecipitated with Poly Per (compare first two lanes). Anti–Per 61 immunoprecipitated a species from the supernatant fraction of ALS tissue that was recognized by the monoclonal peripherin antibody (lane 3, asterisk). This peripherin species had a retarded migration, indicative of an increased molecular mass (compare arrows indicating the migration position of regular peripherin [lower arrow] and Per 61 peripherin [higher arrow]). Contaminating IgG from the immunoprecipitation assay is indicated.

Discussion

Here, we have demonstrated for the first time the in vivo expression of alternatively spliced variants of peripherin both normally and in a pathogenic setting. We have shown expression of Per 61, an isoform demonstrated to be neurotoxic, in motor neurons of two lines of transgenic mice expressing mutant SOD1^{G37R}, but not in control mice or peripherin transgenic mice, demonstrating that the disease mechanism of SOD1^{G37R} includes expression of a neurotoxic splice variant of peripherin. Furthermore, Per 61 immunoreactivity was detected in motor neurons of lumbar spinal cord from two out of three familial ALS cases, the Per 61 antibody immunoprecipitating a peripherin species that had a higher molecular mass than regular peripherin on SDS–polyacrylamide gels. These findings suggest that aberrant splicing of the peripherin gene transcript can occur in ALS.

Three peripherin isoforms, Per 58, Per 56, and Per 61, were first identified at both the mRNA and protein levels in a mouse neuroblastoma cell line (Landon et al., 1989, 2000). Per 58 is identical to the peripherin species described by Portier and colleagues who first identified this type III IF protein in mouse neuroblastoma and rat PC12 cells (Portier et al., 1983a,b). Per 58 is encoded by all nine exons of the peripherin gene and is the most predominant species detected on immunoblots of neuronal tissue extracts. Per 61 is generated by the retention of intron 4 of the peripherin gene introducing a 32–amino acid insertion within coil 2 of the conserved α-helical rod domain of Per 61. This insertion contains three α-helix disrupting proline residues, thereby potentially disrupting the filament forming capacity of Per 61. Per 56 is generated by the use of a cryptic acceptor site at the beginning of exon 9, resulting in a peripherin species in which the COOH-terminal 21 amino acids of Per 58 are replaced with a unique 8–amino acid sequence (Landon et al., 1989). Per 56 has lost the COOH-terminal tyrosine of Per 58, which has been shown to be phosphorylated in several systems (Angelastro et al., 1998). Although the purpose of this COOH-terminal tyrosine phosphorylation of peripherin is unknown, its absence in Per 56 may have functional consequences.

Cytoplasmic aggregates comprised of nIFs are a characteristic feature of degenerating motor neurons in ALS and of mouse models of ALS, including peripherin transgenic mice and mice expressing mutant SOD1^{G37R}. How these aggregates are formed is not known but may be related to the assembly properties of the individual IF subunits or to the interactions between different IF types, such as neurofilaments and peripherin. This is especially relevant when it is considered that peripherin expression is increased after injury in certain neuronal populations that normally only express neurofilaments (Beaulieu et al., 2002). Moreover, the differential expression of peripherin splice variants, particularly Per 61, may influence aggregate formation. In previous work from this and other laboratories, peripherin generated from ectopic expression of the peripherin gene in SW13 vim(−) cells showed that peripherin was capable of self-assembly to form a filamentous network and could coassemble with the neurofilament subunits to varying degrees, being capable of coassembly with NF-L, but not with NF-M or NF-H (Cui et al., 1995; Beaulieu et al., 1999b). The contribution of peripherin isoforms to filament assembly was not considered in these previous studies. In this regard, we compared the assembly properties of the peripherin isoforms, Per 58, Per 56, and Per 61 either alone or coexpressed with the neurofilament subunits in transfected SW13 vim(−) cells and in microinjected motor neurons in primary culture. Both Per 58 and Per 56 were competent for self-assembly to form filamentous networks in SW13 vim(−) cells and were capable of coassembly with the neurofilament subunits. Moreover, Per 58 and Per 56 were assembly competent when expressed in microinjected motor neurons in primary culture, apparently interacting normally with the endogenous neurofilament network. In contrast, Per 61 was assembly incompetent, either when expressed alone or together with the neurofilament subunits in SW13 vim(−) cells. In a further study using SW13 cells stably expressing Per 58 and Per 56, introduction of Per 61 by ectopic expression by transfection of the Per 61 cDNA induced aggregation of both Per 58 and Per 56 (unpublished results). Furthermore, Per 61 was assembly incompetent in microinjected motor neurons, forming aggregates in the perikaryon and neuritic processes, disrupting the endogenous neurofilament network and inducing cell death. Expression of Per 58 and Per 56 had no significant effect on the viability of motor neurons compared with control populations microinjected with vector alone. These findings demonstrate that Per 61 expression is toxic to motor neurons and that death correlates with the formation of aggregates.

Using the isoform-specific antisera, expression of Per 56 and Per 61 was investigated in motor neurons of lumbar spinal cord sections from two lines of peripherin transgenic mice and from two lines of transgenic mice expressing mu-

tant SOD1^{G37R}. Low levels of Per 56 expression was detected in motor neurons of wild-type (C57Bl6) mice, whereas increased labeling was detected in motor neurons of both peripherin and mutant SOD1^{G37R} transgenic mice. Expression of Per 61 was not detected in motor neurons of wild-type or peripherin transgenic mice but did occur in motor neurons of SOD^{G37R} transgenic mice, demonstrating for the first time differential expression of peripherin isoforms in a disease paradigm of ALS. The demonstrated neurotoxicity associated with Per 61 expression in motor neurons in primary culture suggests that abnormal Per 61 expression may contribute to motor neuron degeneration in vivo.

The synthetic peptide used to generate the Per 61 antibody spans a region of intron 4 that is conserved at the nucleotide level between mouse, rat, and human (Foley et al., 1994). This suggested that the epitope recognized by anti–Per 61 in mouse may exist as an equivalent peripherin isoform in human. Using this antibody, we labeled pathological lesions in two out of three familial ALS cases with no labeling detected in control cases. This immunoreactivity correlated with the presence of peripherin abnormalities, including labeling of motor neuron perikarya and axonal spheroids, and could be colocalized to the same motor neurons. This finding suggested that a similar abnormal splice variant of peripherin was expressed in motor neurons in ALS. However, from the only known gene sequence, intron 4 of human peripherin is 91 bp long, whereas in mouse it is 96 bp (Foley et al., 1994). This would mean that a complete retention of intron 4 in human, as occurs in mouse to generate Per 61, would lead to a frameshift, formation of a premature stop codon, and generation of a truncated peripherin species. To test this possibility, we performed immunoprecipitation assays using the Per 61 antibody and also commercially available peripherin polyclonal antiserum. Using this approach, we made three important findings. First, the increased peripherin immunoreactivity observed in the ALS cases tested was because of an increased expression of peripherin. This is of interest when it is considered that peripherin expression can be induced in motor neurons after neuronal injury (Troy et al., 1990), suggesting that increased peripherin expression in ALS may be because of an injurious event. Second, the Per 61 antibody immunoprecipitated a species from ALS pathological tissue that was recognized by monoclonal peripherin antibody on immunoblots. Third, this immunoprecipitated species ran at a higher molecular mass than regular peripherin on SDS–polyacrylamide gels, which is consistent with what would be expected if a splicing abnormality were to occur. Indeed, there is evidence from ESTs that it is possible to have read through into intron 4 of the human peripherin gene (GenBank/EMBL/DDBJ accession no. BE786797). Although we have not as yet identified the specific splicing event that would account for the Per 61 species, we have detected it in ALS tissue. This does provide additional support that such abnormal splicing events can occur.

Expression of alternatively spliced variants has been described for other IF proteins, including nuclear lamins (Fisher et al., 1986; McKeon et al., 1986), glial fibrillary acidic protein (Zelenika et al., 1995), and human synemin (Titeux et al., 2001). Moreover, an alternative splice variant of desmin is causative of some forms of cardiac and skeletal myopathies (Park et al., 2000). Interestingly, most disease-causing mutations of IF proteins are located within the conserved α-helical rod domain, as has been most clearly demonstrated for the keratins in numerous skin-blistering diseases (Fuchs, 1994) and for desmin in several desmin-related myopathies (Carlsson and Thornell, 2001). Per 61 has many similarities to these previous findings, with a disrupted α-helical rod domain having a dominant negative effect on peripherin IF assembly and inducing motor neuron death.

Our findings demonstrate for the first time the in vivo expression of alternatively spliced variants of peripherin both normally and in a pathogenic setting. The expression of Per 61 in motor neurons of transgenic mice expressing mutant SOD1^{G37R}, but not of control mice or of peripherin transgenic mice, shows that the disease mechanism of SOD1^{G37R} includes expression of a neurotoxic splice variant of peripherin. The detection of Per 61 specifically in motor neurons and axonal spheroids in spinal cord of ALS cases suggests that abnormal expression of alternatively spliced variants of peripherin warrants further investigation.

Materials and methods

Transient transfections

A human adrenal carcinoma cell line, SW13 vim(−), that lacks cytoplasmic IFs, was derived by dilutional cloning of SW13 cells obtained from the American Type Culture Collection. The calcium phosphate transfection procedure was used as previously described (Beaulieu et al., 1999b). The mouse peripherin isoform cDNAs, Per 56, Per 58, and Per 61 (provided by Dr. Francoise Landon, CNRS UMR 7000, Paris, France [Landon et al., 1989, 2000]), were subcloned into the BamH1-EcoRV site of pRcCMV (Invitrogen). Ectopic expression of each peripherin isoform in SW13 vim(−) cells was detected using peripherin monoclonal antibody (MAB1527; CHEMICON International, Inc.) and isoform-specific rabbit polyclonal antibodies that were raised to synthetic peptides corresponding to the unique sequences within Per 61 (EWRCASQPGLSATAQ) and Per 56 (CLLRPQEL). Each antiserum was purified by column affinity chromatography using the respective antigenic peptide linked to Sepharose beads. The subcloning of the neurofilament cDNAs has been described previously (Beaulieu et al., 1999b). The yield of Per 61 from cell lysates of transfected SW13 vim(−) cells was low, reflecting the instability of this protein; therefore, an immunoprecipitation protocol similar to that described in Lindenbaum et al. (1987) was used to obtain sufficient quantities of Per 61 for immunoblotting. For later immunoblotting experiments, Per 61 was obtained directly from cell lysates of transfected NIH3T3 cells, which have an endogenous vimentin network that stabilizes Per 61, as was initially observed for NF-L.

Intranuclear microinjection of cultured motor neurons

Dissociated spinal cord cultures from E13 CD1 mice were prepared as described previously (Durham et al., 1997; Roy et al., 1998). These cultures contain numerous cell types including motor and DRG neurons, astrocytes, and microglia that can be identified morphologically and immunocytochemically using cell-specific markers (Durham et al., 1997; Robertson et al., 2001). The pRcCMV expression vectors (100 ng/μl) incorporating Per 56, Per 58, or Per 61 were microinjected into motor neuronal nuclei along with the fluorescent marker, 70-kD dextran-FITC (15 mg/ml; Molecular Probes) according to the method of Durham et al. (1997). Injected motor neurons were identified by the presence of dextran-FITC visualized by epifluorescence microscopy; morphology was evaluated by phase microscopy. Viability was assessed daily by counting the number of motor neurons containing the marker. The number of viable neurons counted on each day was normalized to the number present on day 1 after microinjection. Experiments were performed in triplicate cultures, with 50–100 motor neurons on each coverslip surviving the injection.

Immunocytochemistry of cultured cells

SW13 vim(−) cells grown on glass coverslips were fixed in methanol for 5 min at −20°C and blocked for 30 min in 3% (wt/vol) BSA, 0.1% Triton

X-100 in PBS. For the motor neuron intranuclear microinjection studies, the fixation method of Roy et al. (1998), was used. Immunocytochemistry was performed using antibodies recognizing peripherin (monoclonal MAB1527; polyclonal AB1530) and polyclonal antibodies to NF-L (AB1983), NF-M (AB1981), and NF-H (AB1982); all were purchased from CHEMICON International, Inc. and used at 1:1,000 diluted in blocking solution. The generated rabbit polyclonal peripherin isoform-specific antiserum to Per 56 and Per 61 were both used at 1:500. Antibody distribution was visualized by epifluorescence microscopy after incubation with secondary antibodies, anti–mouse/anti–rabbit IgG conjugated to Alexa Fluor 488 (green) or Alexa Fluor 594 (red) (diluted 1:250; Molecular Probes).

Immunoblotting

Cells were harvested in 62.5 mM Tris, pH 6.8, containing 2% SDS and 10% glycerol, and assayed for total protein using the bichinconinic acid assay (Sigma-Aldrich). Loadings of 10–15 μg of protein were routinely analyzed on 7.5% (wt/vol) SDS–polyacrylamide gels and then blotted to the polyvinyldiflouride (PVDF) membrane. For immunoblotting, membranes were incubated with monoclonal antibodies recognizing peripherin (MAB1527, 1:5,000; anti–Per 56 and anti–Per 61, 1:500) or actin (1:10,000; Roche Diagnostics) and antibody binding revealed with the ECL detection system (NEN Life Science Products).

Transgenic mice

The mice used in this study have been characterized previously and included C57Bl6 (wild-type, $n = 3$); heterozygous transgenic mice overexpressing peripherin under control of the peripherin gene promoter in a wild-type background (Per; $n = 2$, age 7 mo; and $n = 1$, age 23 mo) or NF-L knockout background (Per;LKO; $n = 3$, age 7 mo; Beaulieu et al., 1999a); and two lines of transgenic mice expressing G37R mutant SOD1, line 42 (L42; $n = 2$, age 3 mo; $n = 2$, age 4 mo; and $n = 2$, age 5.5 mo), which has an aggressive phenotype with death occurring at 6–7 mo of age; and line 29 (L29; $n = 2$, age 4 mo; $n = 2$, age 6 mo; $n = 4$, age 10.5–12 mo), which has a milder phenotype, reflective of the lower copy number of integrated mutant *SOD1* transgenes (Wong et al., 1995). All transgenic mice were selected to include ages in which motor neuron degeneration is apparent (Wong et al., 1995; Beaulieu et al., 1999a). For immunohistochemical analysis of transgenic mouse tissue sections, the method of Nguyen et al. (2001) was used. For RT-PCR, spinal cords were removed from mice that had been killed by CO_2 and total RNA extracted using TRIzol Reagent (Invitrogen). Total RNA was estimated at OD 260 and the reverse transcriptase reaction was performed using Superscript II RNase H-Reverse transcriptase (Invitrogen). PCR was performed using 50 p.m. of both sense and antisense primers, 2 U Taq DNA polymerase (Invitrogen) and otherwise standard conditions for 35 cycles: 95°C for 30 s; 55°C for 1.5 min; and 72°C for 1 min. The sense and antisense primers flanked the unique regions in Per 61 (61/58 primer pair) at nucleotide positions 2210–2233 and 2857–2881, respectively, and Per 56 (56/58 primer pair) at nucleotide positions 3637–3661 and 4567–4592, respectively. (Primer sequences: 61/58, TCCCGCCTAGAACTGGAGCGCAAG and TGGCGGCGTCCGACAGGTCAGCAT; 56/58, TGCCTGAGATGGAGCCTCTCCAGGA and GCATGCAGAGCAGGACTGGATACAG). The expected sizes of the PCR products using the 61/58 primer pair are 256 bp for Per 58 and 352 bp for Per 61. Correspondingly, the expected sizes of the PCR products using the 56/58 primer pair are 240 bp for Per 58 and 178 bp for Per 56 (Landon et al., 2000).

Human ALS cases

Three patients with familial ALS (46-yr-old female, 58-yr-old male, and 61-yr-old male) were followed in the Motor Neuron Diseases Clinic at London Health Sciences Center. The diagnosis of ALS was confirmed at autopsy. It was unknown if these patients carried mutations in the *SOD1* gene. The two normal control cases (58-yr-old male and 75-yr-old female) were selected on the basis of the absence of any neurologic deficits or neuropathologic abnormalities at autopsy. Paraffin sections of lumbar spinal cord from the ALS and control cases were rehydrated in a series of graded ethanol washes and H_2O and blocked for 1 h in 0.2% (wt/vol) Triton X-100, 5% (wt/vol) BSA in PBS. Endogenous peroxidase activity was blocked with 3% (vol/vol) hydrogen peroxide in PBS for 5 min followed by three washes for 3 min in PBS. Peripherin antibody (AB1530; 1:1,000) and Per 61 antibody (1:500) were diluted in 0.05% (wt/vol) Triton X-100, 5% BSA in PBS. Antibody incubations were performed overnight at 4°C, and the antigen–antibody complex was detected using the Vectastain Elite ABC Kit (Vector Laboratories), with substrate developed using DAB and hydrogen peroxide. For double labeling with Per 61 and peripherin, the labeling was done sequentially, first with Per 61 antibody (DAB), and then with peripherin antibody, using alkaline phosphatase as the substrate.

For immunoprecipitation with Per 61 antibody, 200 mg of lumbar spinal cord from ALS or control cases was homogenized in 2 ml RIPA buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate) containing 0.1% SDS and protease inhibitor cocktail (Sigma-Aldrich). Homogenates were split into two 1-ml samples and centrifuged at high speed in an Eppendorf microcentrifuge at 4°C for 10 min. Supernatants were removed and kept at 4°C. Pellets were solubilized in 1% SDS, boiled for 5 min, and made to 0.1% SDS with RIPA buffer. The supernatant and pellet fractions were preimmune absorbed for 45 min at 4°C with 50 μl protein A–Sepharose and 2 μl Per 61 preimmune serum. Per 61 and peripherin polyclonal antiserum were cross-linked to 100 μl of protein A–Sepharose using disuccinimidyl suberate (Seize™ X protein A Immunoprecipitation Kit; Pierce Chemical Co.). The Sepharose-linked antibodies were divided in two, and 50 μl of each was added to the supernatant or pellet fractions. Samples were rotated at 4°C for 2–3 h. The immunoprecipitates were pelleted using a microcentrifuge and washed five times with RIPA buffer containing protease inhibitors. Protein recognized by the Per 61 antibody or peripherin antibody were released from the protein A–Sepharose by boiling in Laemmli sample buffer. Immunoprecipitated protein, separated from the protein A–Sepharose by centrifugation, was loaded onto 10% SDS–polyacrylamide gels and blotted to PVDF membrane. For immunoblotting, membranes were incubated with peripherin monoclonal antibody (MAB1527; CHEMICON International, Inc.) and antibody binding revealed using the ECL detection system (NEN Life Science Products).

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