Specific Disruption of a Schwann Cell Dystrophin-Related Protein Complex in a Demyelinating Neuropathy

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Summary

Dystroglycan-dystrophin complexes are believed to have structural and signaling functions by linking extracellular matrix proteins to the cytoskeleton and cortical signaling molecules. Here we characterize a dystroglycan-dystrophin-related protein 2 (DRP2) complex at the surface of myelin-forming Schwann cells. The complex is clustered by the interaction of DRP2 with L-periaxin, a homodimeric PDZ domain-containing protein. In the absence of L-periaxin, DRP2 is mislocalized and depleted, although other dystrophin family proteins are unaffected. Disruption of the DRP2-dystroglycan complex is followed by hypermyelination and destabilization of the Schwann cell-axon unit in $Prx^{-/-}$ mice. Hence, the DRP2-dystroglycan complex likely has a distinct function in the terminal stages of PNS myelinogenesis, possibly in the regulation of myelin thickness.

Introduction

The dystrophin-glycoprotein complex (DGC) has been well characterized in skeletal muscle where it links extracellular proteins, such as the laminins of the basal lamina, to dystrophin and the cortical actin cytoskeleton by virtue of their interactions with α- and β-dystroglycan, respectively (Ahn and Kunkel, 1993; Gee et al., 1993; Ibraghimov-Beskrovnaya et al., 1992; Yamada et al., 1996a, 1996b). Mutations in components of the DGC disrupt the complex and cause several types of muscular dystrophy (reviewed in Straub and Campbell, 1997), possibly due to a lack of mechanical buffering against the shearing forces of contraction (Pasternak et al., 1995; Petrof et al., 1993; Stevenson et al., 1997).

Dystrophin-dystroglycan complexes are also widely distributed in the vertebrate nervous system. In the peripheral nervous system (PNS), Schwann cells express dystroglycan and a truncated form of dystrophin, Dp116 (Matsumura et al., 1993, 1997a, 1997c; Yamada et al., 1994). DGCs in the PNS have been implicated in mediating the responsiveness of Schwann cells to extracellular signals from laminin-2, because contact with the basal lamina modulates the expression of genes required for myelination (Bunge et al., 1986, 1990; Carey et al., 1986; Eldridge et al., 1989). A second gene of the dystrophin family encoding utrophin (dystrophin-related protein 1) is also expressed in Schwann cells (Love et al., 1989;

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Matsumura et al., 1993). Recently, a third member of the dystrophin family, dystrophin-related protein 2 (DRP2), has been identified, the transcript of which is reported to be enriched in the CNS (Dixon et al., 1997; Roberts et al., 1996; Roberts and Sheng, 2000). Nevertheless, thus far, DRP2 has not been shown to be a component of a DGC.

In recent years, the signaling role of DGCs has been increasingly recognized with the identification of the syntrophins, PDZ domain-containing proteins that interact with dystrophin (Ahn et al., 1996; Ahn and Kunkel, 1993; Froehner et al., 1997). PDZ domains are protein-interaction motifs implicated in the assembly of macromolecular-signaling complexes (Kornau et al., 1997; Tsunoda et al., 1997). We have characterized the PDZ domain-containing protein L-periaxin as a candidate member of a signaling complex at the Schwann cell plasma membrane (Gillespie et al., 1994; Scherer et al., 1995; Dytrych et al., 1998). Mice that lack a functional Prx gene ensheath and myelinate peripheral nerve axons in an apparently normal manner, but the sheath destabilizes and the mice develop a severe demyelinating neuropathy (Gillespie et al., 2000). These data suggest that the periaxins play an essential role in stabilizing the Schwann cell-axon unit in the myelinated fibers of the vertebrate PNS. This view is supported by the recent discovery of autosomal recessive forms of Charcot-Marie-Tooth (CMT) disease caused by mutations in the human PRX gene (Boerkoel et al., 2001; Guilbot et al., 2001).

In order to elucidate the pathophysiology of both the human disease and the mouse model, we undertook to identify the putative-signaling complex in which the periaxins participate. Here we show that L-periaxin is linked to a previously unknown dystroglycan complex by the dystrophin-related protein DRP2. L-periaxin dimerizes at its PDZ domain, which probably contributes to the distinctive clustering of the complex in the Schwann cell plasma membrane. Without L-periaxin, the complex is mislocalized and DRP2 is unstable, in striking parallel to the aberrant DGCs in some of the muscular dystrophies. It seems likely that the characterization of other components of this dystroglycan complex will identify candidate genes for other autosomal recessive forms of CMT.

Results

Interaction and Colocalization of L-Periaxin with DRP2

To identify proteins that interact with L-periaxin, we screened a sciatic nerve cDNA library by the yeast two-hybrid system using as bait the N terminus of the protein (aa 2–459), which includes the PDZ domain and basic domain (Figure 1C) (Dytrych et al., 1998; Sherman and Brophy, 2000). Seven overlapping DRP2 clones were isolated from a total of 1.6×10^6 colonies, two of which were only one and ten amino acids longer, respectively, than clone 39–374 and are not displayed (Figure 1A). No other dystrophin or dystrophin-related cDNAs were found. The full-length cDNA encoding DRP2 was ob-
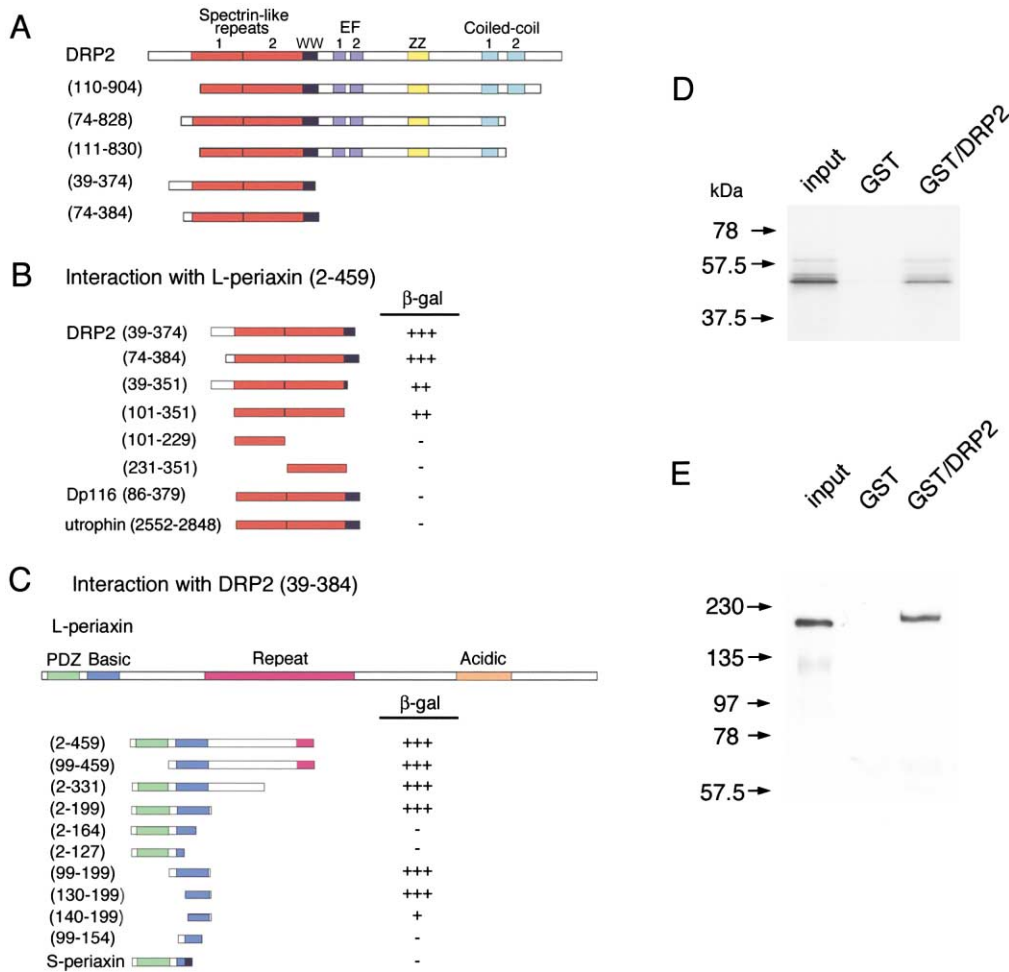
Figure 1. Interaction of L-Periaxin and DRP2

(A) Structure of full-length DRP2 and the different DRP2 clones isolated from the yeast two-hybrid screen using L-periaxin (aa 2–459) fused to the GAL4 DNA binding domain as bait. Numbers in brackets refer to amino acids.

(B) Identification of the DRP2 binding domain for L-periaxin. The strength of interaction between L-periaxin (aa 2–459) and a series of DRP2 constructs in a yeast two-hybrid β-galactosidase assay was assessed semi-quantitatively by the time taken for colonies to turn blue (+++, <30 min; ++, 30–60 min; +, 60–180 min). Interaction of DRP2 with utrophin and Dp116 is compared. Numbers in brackets refer to amino acids.

(C) Identification of the L-periaxin binding domain for DRP2. The strength of interaction between DRP2 (aa 39–384) and a series of L-periaxin deletion constructs in a yeast two-hybrid assay was assessed semi-quantitatively by the time taken for colonies to turn blue. A schematic diagram of the full-length L-periaxin is shown for comparison. Numbers in brackets refer to amino acids.

(D) Interaction of the binding domains of L-periaxin and DRP2 in vitro. GST or a GST-fusion protein of DRP2 (aa 110–384) were incubated with ^{35}S-labeled L-periaxin (aa 2–379) generated by transcription and translation in vitro. Radioactively labeled L-periaxin in the recovered GST and GST-fusion protein fraction was detected after SDS-PAGE and autoradiography. The input lane only contained radioactive L-periaxin.

(E) Biochemical interaction between the L-periaxin binding domain of DRP2 and full-length L-periaxin. GST or a GST-fusion protein of DRP2 (aa 110–384) were incubated with sciatic nerve lysate and any bound L-periaxin was detected by Western blotting using an L-periaxin antibody after SDS-PAGE. The input lane confirms the presence of L-periaxin in the lysate.

tained by screening a rat sciatic nerve cDNA library and by 5′ RACE (see Experimental Procedures and Figure 1A) and the deduced amino acid sequence was identical to that recently reported with the exception of the following four changes: K525Q, R642K, H928L, and S929C (Roberts and Sheng, 2000). To confirm that both L-periaxin and DRP2 are required to activate GAL4 transcription, we established that transformation of yeast cells with L-periaxin and DRP2 cDNAs was necessary both for β-galactosidase (β-gal) activity and for growth of colonies on plates lacking histidine.

Truncation of DRP2 and L-periaxin allowed us to iden-

tify the specific regions responsible for protein-protein interaction by monitoring β-gal activity. It was striking that all of the DRP2 clones from the two-hybrid screen included spectrin-like repeats and at least part of the adjacent WW domain, both of which are conserved in dystrophin and the dystrophin-related proteins (Figure 1A; for review see Winder, 1997). Consistent with these observations, the minimal region required for interaction with L-periaxin comprised the two spectrin-like repeats of the DRP polypeptide (Figure 1B). The WW domain was not essential, although there was some attenuation in the strength of interaction with L-periaxin when this

region was truncated (Figure 1B). In order to determine if the binding of L-periaxin were specific for DRP2, we compared this interaction in a yeast two-hybrid assay with the ability of L-periaxin to associate with the analogous domains of the two other members of the dystrophin family found in Schwann cells, namely Dp116 and utrophin. The equivalent regions of Dp116 and utrophin displayed no propensity to interact with L-periaxin even in the presence of their WW domains (Figure 1B).

The DRP2 binding site in L-periaxin was identified as a basic domain of three basic subdomains, BD1 (aa 116–145), BD2 (aa 146–176), and BD3 (aa 176–196), which has been characterized previously as a nuclear localization signal (NLS) (Figure 1C) (Gillespie et al., 1994; Sherman and Brophy, 2000). A construct truncated by the removal of amino acids 130–140 at the N terminus of the basic domain bound considerably less well to DRP2 (Figure 1C). These ten amino acids include the four contiguous lysines of BD1. BD1 has also been shown to be essential for NLS function (Sherman and Brophy, 2000). Indeed, BD1 has substantial NLS activity on its own (Sherman and Brophy, 2000), in contrast to its complete inability to bind DRP2 (Figure 1C). These results point to an essential role for most of the basic domain in DRP2 interaction. Since the PDZ domain of L-periaxin was not required for association with DRP2, it was consistent that S-periaxin, a truncated periaxin isoform of 147 amino acids having the PDZ domain and a unique 20 amino acid C terminus, but lacking the basic domain, did not interact with DRP2 (Figure 1C) (Dytrych et al., 1998). Biochemical support for the interaction between L-periaxin and DRP2 was obtained by showing that a GST-DRP2 fusion protein (aa 110–384 of DRP2) coprecipitated with either a fragment of the L-periaxin protein generated by transcription and translation in vitro (aa 2–379) (Figure 1D) or full-length L-periaxin from a sciatic nerve lysate (Figure 1E).

In order to confirm that the interaction between L-periaxin and DRP2 is physiologically relevant, we examined the subcellular and tissue distribution of DRP2. As has been previously documented, L-periaxin was abundantly expressed in the Schwann cell plasma membrane (Figure 2A) (Dytrych et al., 1998; Gillespie et al., 1994; Scherer et al., 1995). DRP2 was found in the same location and appeared to have a focal distribution in cross section (Figure 2A). The clusters of DRP2 were particularly striking at the surface of teased myelinated fibers (Figure 2B). Strong colocalization of L-periaxin with DRP2 was evident although it did appear that L-periaxin was also present outside of the clusters (Figures 2A and 2B). Like L-periaxin, DRP2 was not detectable in non myelin-forming Schwann cells (data not shown). Immunoelectron microscopy of a section of trigeminal nerve confirmed that DRP2 and L-periaxin were both localized to the plasma membrane at the abaxonal surface of the Schwann cell-axon unit (Figure 2C), and light microscopy of a cross section of trochlear nerve revealed that several of these clusters could be detected at the surface of each Schwann cell in the same plane of the section (Figure 2D). Confocal analysis of a teased sciatic nerve fiber at three longitudinal optical sections, top, middle and bottom, demonstrated the surface nature of these spheroidal DRP2 clusters.

DRP2 has been detected in a variety of tissues by RT-PCR, including brain, colon, testis, ovary, and kidney (Dixon et al., 1997). We found that expression of the DRP2 protein was very low in these tissues by comparison with myelinated peripheral nerve (Figure 3A). Western blotting with an antibody raised against a different DRP2 peptide gave identical results (data not shown). Overexposure of the Western blot did reveal trace levels of DRP2 in other tissues; similarly, using DRP-specific riboprobes, we also detected weak signals by in situ hybridization in brain.

The best characterized dystrophin and dystrophin-related protein complexes with dystroglycan are in skeletal muscle. Hence, it was of interest to compare the expression pattern of DRP2 with other members of such complexes in both peripheral nerve and skeletal muscle. The restriction of DRP2 expression to Schwann cells was in striking contrast to the other two members of the dystrophin family, dystrophin (Dp116 in the PNS) and utrophin, and to β-dystroglycan and α-dystroglycan (Figure 3B). Note that utrophin is also expressed in the perineurium (arrow).

Thus, the results of yeast two-hybrid experiments, biochemical studies, and immunolocalization analysis converge to the conclusion that L-periaxin and DRP2 are present in a complex that is primarily expressed in the plasma membrane of Schwann cells.

L-Periaxin Specifically Interacts with DRP2 and Dystroglycan to Form a Transmembrane Complex

Like dystrophin and utrophin, DRP2 has a potential binding site for β-dystroglycan. However, thus far, it has not been demonstrated that DRP2 interacts with β-dystroglycan. Yeast two-hybrid experiments confirmed that DRP2 could bind directly to the cytoplasmic tail domain of β-dystroglycan (Figure 4A). Further, it has been proposed that not only the WW and EF hand domains but also the ZZ domain at the C terminus of both dystrophin and utrophin are required for optimal interaction with β-dystroglycan (Jung et al., 1995; Suzuki et al., 1994; Tommasi di Vignano et al., 2000). We found that this was also the case for DRP2 (Figure 4A). Hence, it appeared that DRP2 localizes to the Schwann cell plasma membrane by virtue of its binding to β-dystroglycan.

Because L-periaxin interacts with DRP2, we then attempted to isolate the L-periaxin complex from peripheral nerve lysates by immunoaffinity chromatography using an affinity-purified antibody to L-periaxin (Figure 4B). As expected, DRP2 copurified with L-periaxin, but, importantly, the complex also contained α- and β-dystroglycan which indicated that L-periaxin and DRP2 were part of a DGC (Figure 4B). Since the utrophin and dystrophin complexes have recently been shown to interact in Schwann cells (Imamura et al., 2000), it was of interest to determine if utrophin and Dp116 were also associated with the DRP2 complex. Both were present, which probably accounts for the presence of actin in the complex because utrophin has an actin binding domain (Figure 4B). The low signal for β-dystroglycan in the Western blots is consistent with the proposal that the interaction of DRP2 with β-dystroglycan is relatively weak, as has been found for the association of β-dystroglycan with both utrophin and Dp116 in the Schwann cell plasma membrane (Imamura et al., 2000; Saito et al., 1999). In addition to dystroglycan, myelinating Schwann cells express a second laminin receptor, α6β4-integrin (Einheber et al., 1993). However this was not present in the L-periaxin-DRP2 complex as shown by the absence of the β4 polypeptide (Figure 4B).
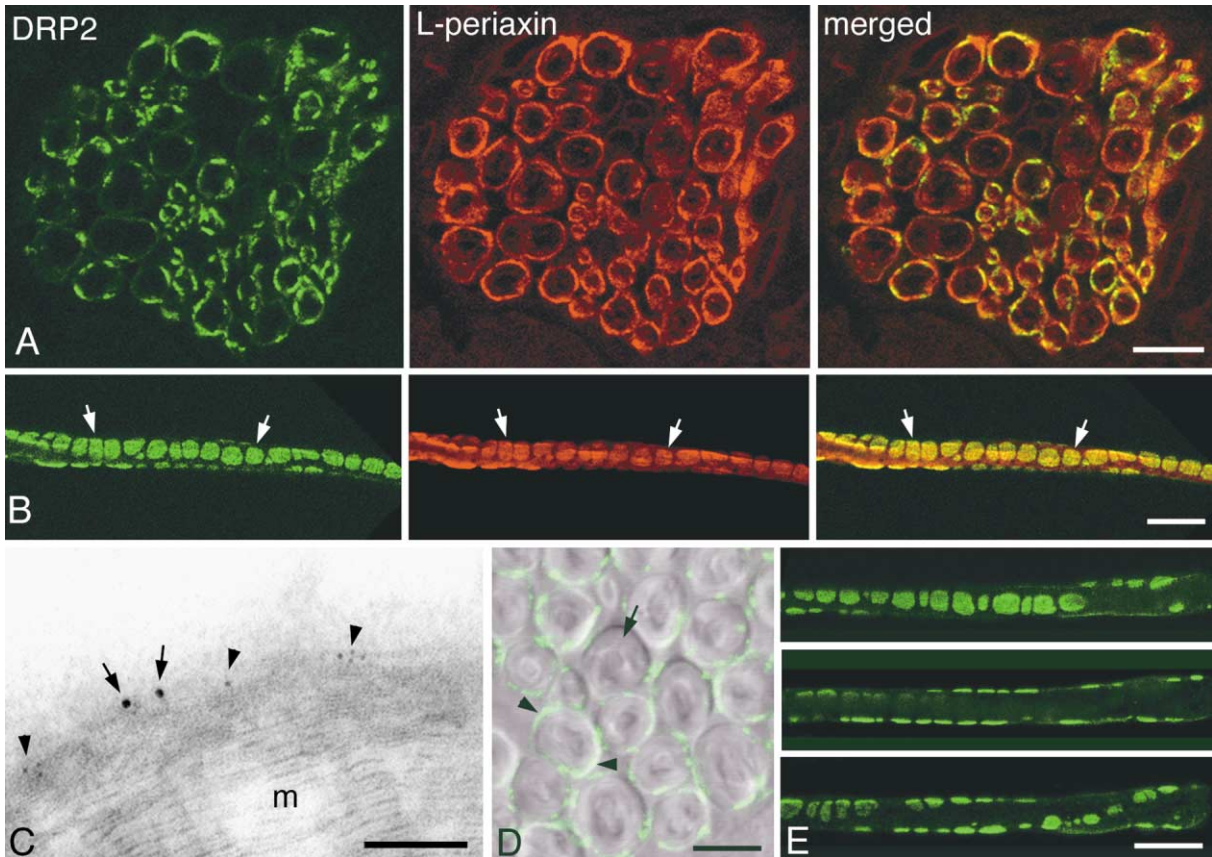
Figure 2. Localization of DRP2 and L-Periaxin

(A) Confocal immunofluorescence analysis of transverse sections (P21) of mouse sciatic nerve double-labeled with a rabbit antibody against DRP2 and a mouse antibody against L-periaxin. DRP2 and L-periaxin colocalize. Scale bar, 10 μm.

(B) Confocal immunofluorescence analysis of a teased fiber (P26) of mouse sciatic nerve double-labeled as in (A). DRP2 and L-periaxin colocalize in clusters at the upper surface of a teased fiber in confocal optical sections. Scale bar, 10 μm.

(C) DRP2 is localized with L-periaxin at the Schwann cell plasma membrane. In transverse section, DRP2 (10 nm gold particles, arrows) and L-periaxin (5 nm gold particles, arrowheads) are primarily localized to the abaxonal Schwann cell plasma membrane in P28 rat trigeminal nerve by immunoelectron microscopy. Myelin (m) is unlabeled. Scale bar, 100 nm.

(D) DRP2 clusters at the abaxonal Schwann cell surface. Transmitted light images of trochlear nerve sections showing the myelin sheath (arrow) were overlayed with the immunofluorescence labeling pattern of DRP2 (arrowheads) Scale bar, 5 μm.

(E) Spheroidal clusters of DRP2 at the surface of the Schwann cell. Three longitudinal confocal-optical sections at the top, middle, and bottom of a teased sciatic nerve fiber immunostained for DRP2 demonstrate that the surface of the Schwann cell is extensively decorated with spheroidal clusters of DRP2. Scale bar, 10 μm.

These experiments established that L-periaxin and DRP2 are constituents of a transmembrane complex of the DGC type.

L-Periaxin Is Required to Cluster and Stabilize the DRP2-Dystroglycan Complex

We have previously shown that the peripheral nerves of periaxin-deficient mice display a late-onset, demyelinating neuropathy (Gillespie et al., 2000). However, it was unclear why loss of the periaxins should destabilize axon-Schwann cell interaction after myelination was essentially complete. Hence, we wished to determine if the loss of L-periaxin might influence the organization of the DRP2-dystroglycan complex. Immunofluorescence analysis of $Prx^{-/-}$ mice revealed an apparent loss of DRP2 in the Schwann cell plasma membrane by comparison with wild-type nerves, and the little DRP2 that remained was no longer clustered in patches but diffusely distributed (Figure 5A). In contrast, there was no obvious disruption to the localization of β-dystroglycan (Figure 5A). The specificity of the dramatic loss of DRP2 in periaxin-deficient peripheral nerves was confirmed by Western blot which also showed that α-dystroglycan, β-dystroglycan, Dp116, and utrophin were unaffected (Figure 5B).

Clustering of DRP2 appeared to require L-periaxin, which suggested that the latter might provide the scaffold on which the DRP2 DGC assembles. An analogous function has been proposed for a variety of other PDZ-domain containing proteins in multiprotein, cortical signaling complexes. In order to identify other proteins with which the PDZ domain in the N terminus of L-periaxin might interact, we repeated the yeast two-hybrid screen of a rat peripheral nerve cDNA library using the N terminus of the protein (aa 2–164) as bait. This truncated version of L-periaxin contained the PDZ domain and part of the basic domain, although it would have been insufficiently large to interact with DRP2. Ten overlap-
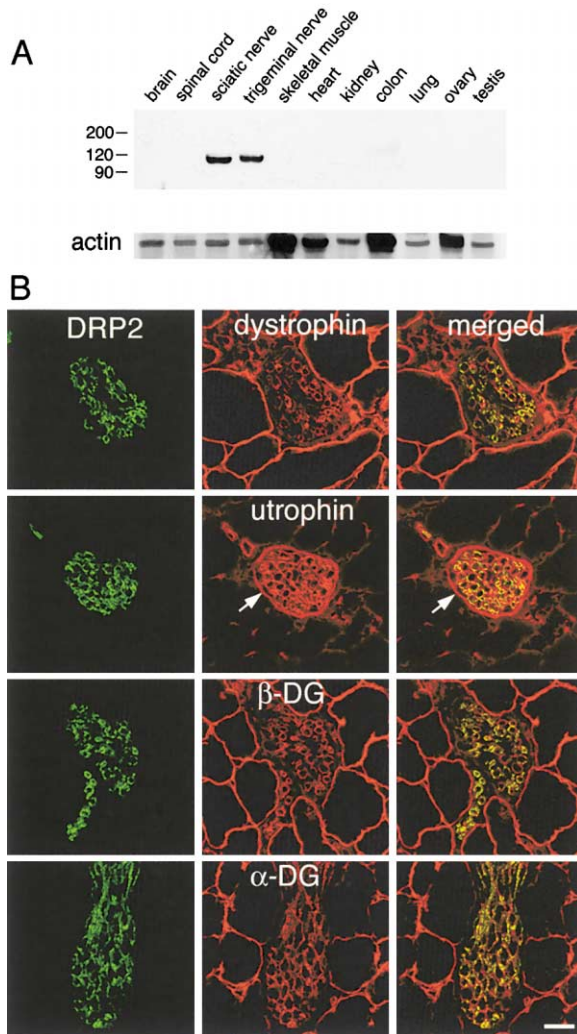
Figure 3. DRP2 Is Primarily a PNS Protein

(A) Homogenates (15 μg protein) of mouse tissues were separated by SDS-PAGE and DRP2 was detected by Western blotting using a DRP2 antibody. The blots were stripped and reprobed with an antibody against actin as a loading control for PNS and CNS tissue. DRP2 protein was only detected in the PNS.

(B) Specificity of DRP2 expression in the PNS. Cross sections of mouse quadriceps muscle and a myelinated nerve fiber at P21 were double-labeled with a rabbit antibody against DRP2 and mouse monoclonal antibodies against dystrophin, utrophin, β-dystroglycan, or α-dystroglycan. Only Schwann cells in the nerve contain DRP2, whereas the other dystroglycan-complex proteins are expressed in both Schwann cells and skeletal muscle. Utrophin is also found in the perineurium (arrow). Scale bar, 20 μm.

ping L-periaxin clones were recovered from a screen of one million clones, which strongly suggested that L-periaxin could homodimerize. Interestingly, truncation of the N-terminal fragment of L-periaxin revealed that the minimal PDZ domain (aa 2–103) formed homodimers less effectively (Figure 6A). In order to determine if homodimerization involved the peptide binding regions analogous to those of other PDZ domains, we mutated the adjacent G and F amino acids in the carboxylate binding loop (Dytrych et al., 1998). Homodimerization was attenuated even further in the mutated-core PDZ domain

(Δ2–103) (Figure 6A) suggesting that the canonical peptide binding groove of periaxin's PDZ domain participated in homodimerization.

Biochemical evidence for the ability of L-periaxin to dimerize was obtained by means of a "pull-down" assay. A GST-fusion protein that included the same region of L-periaxin as was used in the two-hybrid screen was able to bind intact L-periaxin in a sciatic nerve lysate (Figure 6B). In support of the two-hybrid data, truncation of the N terminus of L-periaxin to the core PDZ domain attenuated homodimerization with the full-length protein to a point where it was no longer detectable by affinity chromatography (Figure 6B). To confirm that full-length L-periaxin could homodimerize in a cellular environment, we showed that myc-tagged periaxin could be coimmuneprecipitated with FLAG-tagged periaxin when both were expressed in COS cells (Figure 6C). These results point to the ability of L-periaxin to cross-link DRP2-dystroglycan complexes in the submembranous cortex of the Schwann cell by virtue of its ability to homodimerize and account for the loss of DRP2 clustering in the peripheral nerves of $Prx^{-/-}$ mice. A model for the DRP2 complex is presented in Figure 7.

Discussion

We have identified a peripheral demyelinating neuropathy in which a specific dystrophin-related protein complex is disrupted by the loss of L-periaxin. A central feature of this macromolecular structure is the importance of L-periaxin to the stability of the complex through its interaction with the dystrophin-related protein, DRP2. This interaction is specific in that L-periaxin does not associate directly with either of the other two members of the dystrophin family found in Schwann cells, namely dystrophin (Dp116) and utrophin, which suggests that these proteins are members of distinct dystroglycan complexes. This conclusion is supported by the fact that neither utrophin nor DRP2 can functionally substitute for Dp116 in the peripheral nerves of patients with a severe form of muscular dystrophy (Comi et al., 1995).

Although Prx null mice myelinate in an ostensibly normal fashion, they develop a severe, late-onset demyelinating neuropathy in which the myelin sheath becomes unstable (Gillespie et al., 2000). In the absence of L-periaxin, the earliest evidence for morphological disruption of the axon-Schwann cell unit is the focal thickening of the sheath. The ensuing overproduction of myelin is followed by demyelination and remyelination. The results reported here implicate the L-periaxin-DRP2-dystroglycan complex in the regulation of the terminal stages of myelination. This view is consistent with the fact that the Prx gene is regulated by axonal contact, and that the thickness of the sheath is believed to be determined by the axon (Lemke and Chao, 1988; Scherer et al., 1995). It is also consistent with the fact that the translocation of L-periaxin to a predominantly abaxonal location at the Schwann cell plasma membrane is a relatively late event during myelination (Dytrych et al., 1998; Gillespie et al., 1994; Scherer et al., 1995). Therefore, association of L-periaxin with the cortical DRP2 complex appears to be a feature of the maturation of the sheath, in concordance with the late-onset phenotype of the periaxin-deficient mouse.
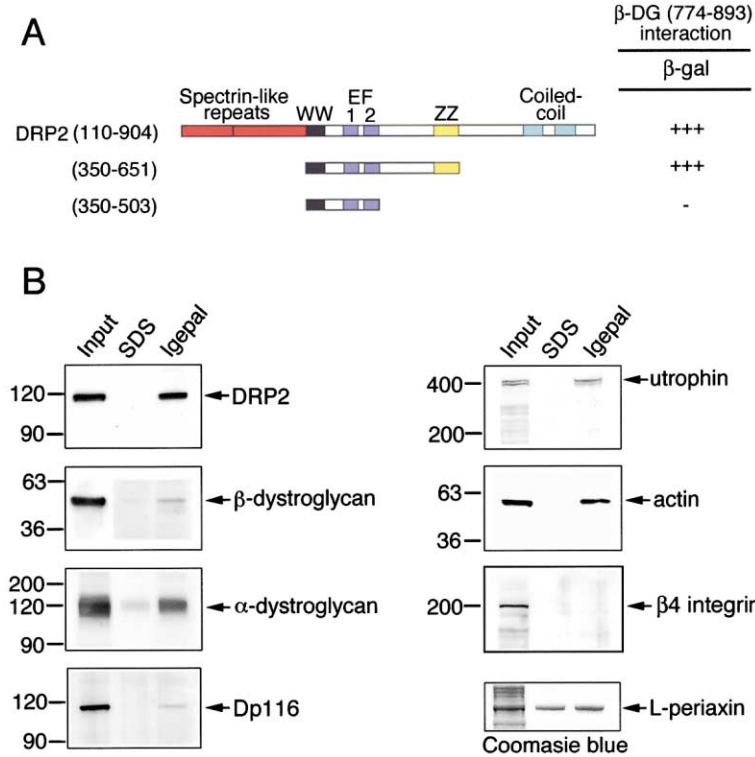
The production and deposition of basement mem-

Figure 4. The L-Periaxin-DRP2-Dystroglycan Complex

(A) DRP2 interacts with β-dystroglycan. The strength of the interaction of the cytoplasmic carboxy-terminal domain of β-dystroglycan (β-DG) with truncated versions of DRP2 was assessed by a semi-quantitative yeast two-hybrid assay.

(B) Immunoaffinity-purification of the L-periaxin-DRP2-dystroglycan complex. Detergent extracts of mouse sciatic nerve in the non-ionic detergent Igepal were incubated with beads to which affinity-purified sheep anti-L-periaxin antibodies had been covalently coupled. After extensive washing, bound proteins were eluted and analyzed by Western blotting after SDS-PAGE. The SDS control lane contains proteins that bound to the beads after first solubilizing the nerves in SDS followed by dilution of the SDS with Triton X-100. The presence of the proteins in the Igepal lysate is shown in the input lane. Immunaffinity purification of L-periaxin from both SDS- and Igepal-solubilized lysates is shown in the Coomassie-blue-stained gel.

brane by the Schwann cell around the axon-Schwann cell unit is also under axonal control (Bunge and Bunge, 1978; Doyu et al., 1993). The basal lamina has an essential role in promoting the development of the myelinating Schwann cell and in stabilizing Schwann cell-axon interaction (Carey et al., 1986; Eldridge et al., 1989). The

autocrine nature of this relationship is illustrated by the defects in myelination observed in laminin-2 deficiency, as occur in congenital muscular dystrophy (CMD) and in the *dy/dy* mouse (Arahata et al., 1993; Matsumura et al., 1997b; Sunada et al., 1994; Uziyel et al., 2000; Xu et al., 1994). Derangements in the structure or signaling
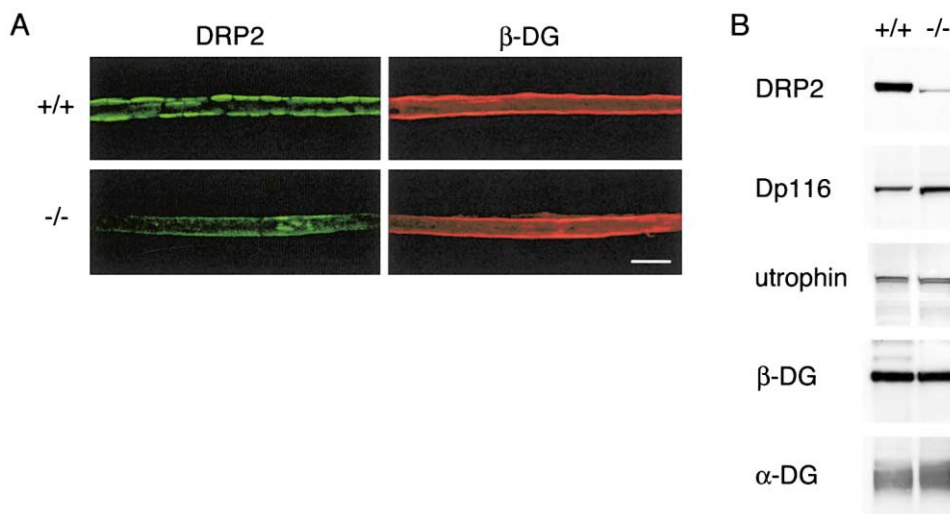

Figure 5. DRP2 Is Specifically Mislocalized and Depleted in Periaxin Null Mice

(A) Immunofluorescence localization of DRP2 and β-dystroglycan (β-DG) in teased sciatic nerve fibers of wild-type mice (+/+) and periaxin null homozygotes (−/−). Confocal optical sections through the center of the $Prx^{+/+}$ fiber reveal the clustered localization of DRP2, whereas β-dystroglycan has a more uniform distribution at the Schwann cell plasma membrane which is not changed in the $Prx^{-/-}$ nerve. In contrast, DRP2 is no longer clustered in $Prx^{-/-}$ sciatic nerves, it also appears to be reduced in quantity.

(B) DRP2 is specifically depleted in the absence of L-periaxin. SDS-solubilized sciatic nerves (10 μg protein) from wild-type mice (+/+) and periaxin null homozygotes (−/−) were analyzed by Western blot after SDS-PAGE. DRP2 is the only major constituent of the DGC to be depleted.

Figure 7. Model of the L-Periaxin-DRP2 Complex at the Schwann Cell Plasma Membrane

The ability of the PDZ domain of L-periaxin to homodimerize may account for the clustering of DRP2. The mechanism of linkage of the utrophin and Dp116 complexes to the L-periaxin-DRP2-dystroglycan complex is not yet clear. SG, sarcoglycans; α, β, α- and β-dystroglycan.
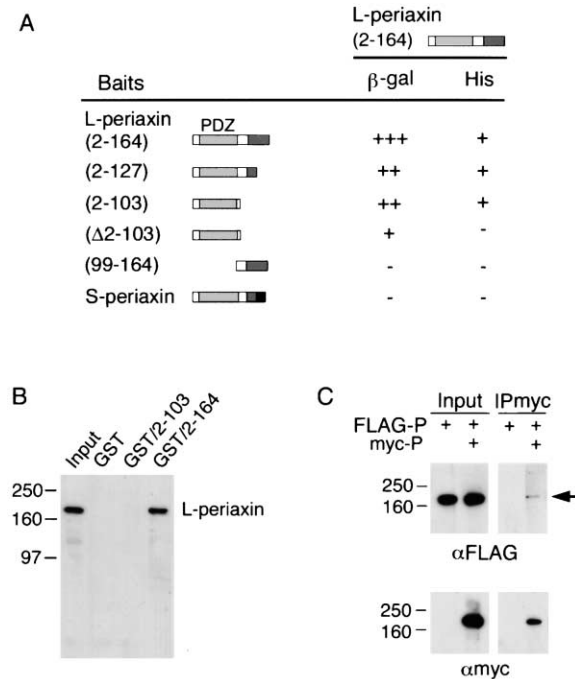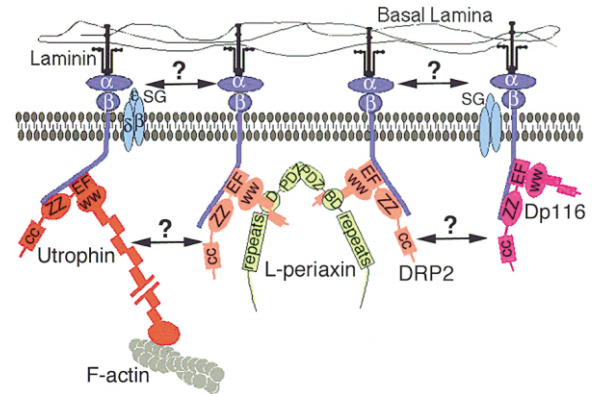
Figure 6. L-Periaxin Homodimerizes

(A) Yeast two-hybrid analysis of the region required for L-periaxin homodimerization. The ability of truncated forms of L-periaxin to homodimerize with the N terminus of the protein was analyzed semi-quantitatively by determining the time taken for the development of β-galactosidase activity and the ability of cotransfected yeast colonies to grow in the absence of histidine. Amino acids are in brackets. The construct $\Delta 2$–103 was mutated by the substitution of the adjacent G and F amino acids in the carboxylate binding loop by alanines. S-periaxin represents the full-length isoform of the alternative product of the *Prx* gene.

(B) The N terminus of L-periaxin interacts with full-length L-periaxin in vitro. Sciatic nerve lysates in 0.5% CHAPS were incubated with GST or GST-L-periaxin fusion proteins (GST/2–103, or 2–164) bound to agarose. Bound proteins were eluted and analyzed by SDS-PAGE and Western blotting with a rabbit anti-L-periaxin antiserum. The input lane represents a sample of lysate.

(C) Full-length L-periaxin homodimerizes in transfected COS7 cells. COS7 cells transiently expressing FLAG-tagged L-periaxin (FLAG-P) alone or with myc-tagged L-periaxin (myc-P) were lysed in 0.2% CHAPS and myc-P was immunoprecipitated using a myc antibody. Immunoprecipitates were analyzed by Western blotting with FLAG (αFLAG) or myc antibodies (αmyc). The input lanes contain samples of cell lysate.

properties of receptor complexes for basement membrane proteins, such as laminin-2, are also likely to affect myelination, as has been found when Dp116 is absent from the PNS (Comi et al., 1995).

PDZ domains normally interact with a group of conserved peptide sequences found at the C termini of certain transmembrane proteins (Kornau et al., 1997). The carboxylate binding loop has been shown to be a key structural element of the peptide binding groove in the PDZ domain and the GLGF motif is a highly conserved feature of this structure (Doyle et al., 1996). In periaxin, the equivalent sequence is GVSGF (Dytrych et al., 1998). It is now clear that PDZ domains can interact with internal peptide sequences and that they may also homodimerize and heterodimerize (Cuppen et al., 1998; Garner et al., 2000; Xu et al., 1998). The dimer formed

between the PDZ domains of neuronal nitric oxide synthase (nNOS) and syntrophin has been shown to be a consequence of the interaction of the canonical peptide binding groove of syntrophin with a β-hairpin "finger" within the nNOS PDZ domain in a head-to-tail conformation (Hillier et al., 1999). Because mutation of two of the amino acids in the GLGF motif in the putative carboxylate binding loop of the periaxin PDZ domain from GF to AA attenuated homodimerization, it seems likely that self-association of periaxin proceeds by a parallel mechanism.

The mislocalization and concomitant loss of DRP2 in periaxin-deficient mice suggests not only that the ability of L-periaxin to dimerize helps to cluster the complex but that the protein also selectively stabilizes the DRP2 DGC. In similar fashion, a reduction in membrane-associated dystrophin in the *Mdx* mouse leads to the loss of other components of the DGC, and mice deficient in the dystrophin Dp71 have significantly less dystroglycan in the brain (Ervasti et al., 1990; Greenberg et al., 1996). Hence, it appears that assembly of the DGC stabilizes its component proteins. PDZ domain proteins have been shown to perform similar functions in other types of signaling complex. In *Drosophila*, INAD, a homodimeric protein of the rhabdomere with five PDZ domains, stabilizes the TRP channel protein, and the product of the *Dlg* gene selectively prevents the destruction of the Fas II and Shaker channel proteins (Chevesich et al., 1997; Li and Montell, 2000; Zito et al., 1997).

Schwann cells are polarized cells with a membrane in contact with basal lamina that is analogous to the basolateral surface of an epithelial cell. DGCs have been proposed to play a critical role in the differentiation of epithelial cells by helping to define the basolateral membrane and by acting as foci for the assembly of cytoskeleton-associated complexes (Drubin and Nelson, 1996; Durbeej et al., 1995; Kachinsky et al., 1999). In this context, and in contrast to Dp116, utrophin or, indeed, dystrophin, the localized, focal nature of DRP2 in the Schwann cell plasma membrane is of particular interest; it suggests that the clustering of the DRP2 DGC might influence the way the complex responds to extracellular

signals from basement membrane proteins such as laminin. These DGC clusters may also reflect the necessity of bringing other signaling molecules that associate with L-periaxin into close proximity. The other components of these clustered complexes remain to be determined. However, it appears that they do include both utrophin and dystrophin, proteins known to associate in peripheral nerve with a sarcoglycan complex comprising β-, δ- and ε-sarcoglycan (Imamura et al., 2000). The fragile nature of the interaction between β-dystroglycan and both utrophin and Dp116 in the Schwann cell plasma membrane has been attributed to the absence of γ-sarcoglycan (Imamura et al., 2000; Saito et al., 1999). This might also explain the relatively low amounts of β-dystroglycan in the L-periaxin-DRP2 complex.

The importance of L-periaxin to the function of the human PNS has been established by the recent discovery of autosomal-recessive forms of hereditary demyelinating sensorimotor neuropathy of the CMT type caused by a variety of mutations in the human *PRX* gene (Boerkoel et al., 2001; Guilbot et al., 2001). The similarities of the human disease, both clinically and histopathologically, to the phenotype of the periaxin-deficient mouse are striking (Boerkoel et al., 2001; Gillespie et al., 2000; Guilbot et al., 2001). The current work shows that such animal models can provide a powerful means to elucidate the molecular pathophysiology of disease (Dyck et al., 1993; Muller et al., 1997; Warner et al., 1999). Perhaps, even more importantly, the characterization of the rest of the periaxin complex is likely to identify new candidate genes for other autosomal recessive forms of CMT.

Experimental Procedures

Yeast Two-Hybrid Screening and Interactions

Two-hybrid screening was performed in the yeast strain Y190 (Clontech). The N terminus of rat L-periaxin (aa 2–459) was the bait, and was obtained by PCR amplification and subcloned in frame into the EcoRI and BamHI sites of pAS2-1 (Clontech) to generate a GAL4 binding domain fusion protein. A random-primed rat sciatic nerve library was generated in λACTII. The entire library was subjected to Cre-lox excision and purified plasmid cDNAs were obtained using a Qiagen Maxiprep kit. After cotransformation of the bait with 1.6×10^6 library cDNA clones (Gietz and Schiestl., 1995) in the yeast strain Y190 (Clontech) harboring *HIS3* and *lacZ* as reporter genes, positive clones were selected on plates lacking leucine, tryptophan, and histidine containing 30 mM 3-aminotriazole. Positive clones were tested for β-galactosidase activity by filter-lift assay. Interacting clones were rescued and interactions were confirmed by yeast mating according to the manufacturers instructions (Clontech), and sequenced. For yeast mating, the bait in yeast strain Y187 (Clontech) was mated with the prey in strain Y190. The full-length DRP2 cDNA was obtained by screening a random-primed P10 rat sciatic nerve library generated in λgt11 using a 217 bp PCR fragment corresponding to amino acids 734–805 and by 5′ RACE using the GeneRacer Kit (Invitrogen). Deletion constructs of L-periaxin, S-periaxin, DRP2 and the C terminus of β-dystroglycan were generated by PCR and subcloned into either pAS2-1 to produce GAL4 binding domain fusions or pACT2 to produce GAL4 activation domain fusions. PCR mutagenesis was performed using a Quikchange site-directed mutagenesis kit (Stratagene). The mutations were subsequently confirmed by sequencing. The PDZ domain mutation was generated by replacing glycine and phenylalanine at aa 31 and 32 of L-periaxin with alanines. pACT-Dp116 (aa 86–379) and pACT-utrophin (aa 2552–2848) were obtained by reverse transcription (RT) PCR using P10 and P12 mouse sciatic nerve RNA as template. The interactions were performed by cotransformation in Y190 or by yeast mating and assayed for reporter gene expression. β-galactosidase activity by filter lift assay was scored semiquantitatively by the time taken

for the development of blue colonies: +++, <30 min; ++, 30–60 min; +, 60–180 min. All constructs generated by PCR were confirmed by DNA sequencing.

Primary Antibodies

All peptides were coupled to Keyhole Limpet hemocyanin and affinity purified as described (Dytrych et al., 1998). DRP2 antibodies 2164 and 2155 were raised in New Zealand white rabbits against synthetic peptides CSDVTANTLLAS (aa 947–957) and CEKLRHAFPSVRSSD (aa 935–948), respectively. A C-terminal L-periaxin antibody (Peri-C2) was raised in sheep (Diagnostics Scotland, Carluke, UK) and directed against the synthetic peptide SGSKDREEGG-FRVRLPSVGFSETAC (Research Genetics) corresponding to amino acids 1345–1368 of rat L-periaxin. The L-periaxin antibody, 170pep1, was previously characterized (Gillespie et al.,1994). A mouse antibody raised against the same peptide 170pep1M was also used for immunofluorescence. Monoclonal antibodies to β-dystroglycan (MANDAG 2), and utrophin, (MANCHO 3), were generous gifts from Dr. G.E. Morris (Morris et al., 1998) and an anti-β4-integrin (clone 346–11A) was from Dr. S.J. Kennel (Oak Ridge). Anti-α-dystroglycan antibody and a monoclonal antibody against the C terminus of dystrophin, DYS2, were from Upstate Biotechnology and Novacastro, Newcastle, UK respectively.

Western Blotting

For tissue Western analysis, tissue homogenates were separated by electrophoresis on 10% SDS-PAGE gels and subsequently transferred electrophoretically to nitrocellulose (Schleicher & Schuell) as previously described (Gillespie et al., 1994). Membranes were blocked with PBS, 0.1% Tween 20, and 5% nonfat milk. Primary antibody dilutions for Western blots were DRP2 (2155) 1:2000, dystrophin (DYS2), 1:40, utrophin (MANCHO 3), 1:500, β-dystroglycan (MANDAG 2), 1:500, α-dystroglycan 1:2000, actin (Sigma) 1:1000, β4-integrin 1:2000.

GST Fusion Protein Production

Rat L-periaxin (aa 2–103, aa 2–164) and DRP2 (aa 110–384) were amplified by PCR and subcloned in frame into pGEXKG for GST fusion protein production. Recombinant proteins were overexpressed by induction of transformed *E. coli* BL21 CodonPlus-RIL(DE3) (Stratagene) at log phase with 1 mM isopropyl-1-thio-D-galactopyranoside (IPTG) for 3 hr. The bacterial pellet was lysed by sonication and the supernatant from a 15,000 rpm centrifugation (SS34 rotor, Sorvall) was purified on a Glutathione-Sepharose 4B (Amersham Pharmacia Biotech) column. Recombinant proteins were eluted from the column with excess reduced glutathione (10 mM).

In Vitro Transcription and Translation
and Pulldown Experiments

A truncated L-periaxin (aa 2–379) was generated and labeled by in vitro transcription followed by translation using ^{35}S-methionine in a reticulocyte lysate kit (Promega). Purified GST fusion proteins (GST and GST-DRP2, 6 μg) were coupled to Glutathione-Sepharose beads (25 μl, Amersham Pharmacia Biotech) by incubation for 40 min in binding buffer (50 mM Tris HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 1 mM DTT, 1 mM PMSF, 5 μg/ml leupeptin, 2 μg/ml pepstatin, 0.5 mM TLCK). The washed beads were incubated with the ^{35}S-labeled translation product (1×10^6 cpm) in binding buffer for 1.5 hr at 4°C. The beads were washed extensively in binding buffer before solubilization in SDS-PAGE sample buffer. Proteins were resolved in a 12% SDS-PAGE gel, Coomasie stained to confirm equivalent fusion protein loading and processed for fluorography using Amplify (Amersham Pharmacia Biotech). For sciatic nerve pull-down assays, the perineurial sheaths of P16 or P21 rat sciatic nerves were removed, following which they were homogenized on ice in 25 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.5% CHAPS, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml antipain, 0.5 mM TLCK, 0.7 μg/ml chymostatin, 10 μg/ml benzamidine, 1 mM PMSF. The homogenate was centrifuged for 20 min at 13,000 g and the lysates were precleared by incubation for 45 min at 4°C with 25 μl Glutathione-Sepharose beads. The cleared lysate was then incubated for 45 min at room temperature with GST

fusion protein bound to beads. The beads were washed five times with lysis buffer, proteins were eluted with SDS-PAGE sample buffer, resolved on 8% SDS polyacrylamide gels, transferred to nitrocellulose, and analyzed by Western blotting using anti-170pep1 (1:20,000).

Immunoaffinity Chromatography

Immunoaffinity beads were prepared by coupling affinity-purified anti-Peri-C2 antibody to Affi-Gel 10 (5 mg antibody per ml resin, Bio-Rad) in 100 mM HEPES (pH 7.5), for 48 hr at 4°C. The beads were washed in the same buffer followed by 100 mM HEPES (pH 7.5), 1M NaCl. Residual reactive groups were blocked by overnight incubation with 100 mM ethanolamine (pH 7.5), followed by successive washes with 100 mM HEPES, 100 mM HEPES-500 mM NaCl, and 100 mM HEPES, all at pH 7.5. Sciatic nerves were removed from mice aged 3 to 7 weeks old and homogenized in 200 μl ice-cold lysis buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 10 μg/ml leupeptin, 10 μg/ml antipain, 1 mM benzamidine, 0.5 mM TLCK, 0.7 μg/ml chymostatin, and 1 mM PMSF with 0.5% Igepal CA630, Sigma). The homogenate was solubilized for 10 min on ice. After centrifugation at 4° for 20 min at 13,000g, anti-Peri-C2-Affigel 10 resin was added to the cleared supernatant and tumbled for 3 hr. As a control, sciatic nerves were boiled in 2% SDS and the supernatant was diluted with lysis buffer containing 2.5% Triton X-100 in place of Igepal. The beads were washed five times with lysis buffer and once with lysis buffer without detergent. Proteins were solubilized, resolved on 4%–12% Nupage gels (Invitrogen), transferred to nitrocellulose, followed by Western analysis.

Transfection and Coimmunepecipitation

To generate L-periaxin with either a C-terminal myc-epitope tag or a C-terminal FLAG tag, full length L-periaxin cDNA was cloned into the BglII and XbaI sites of the mammalian expression vector pCB6 (gift of D. Russell, University of Texas) in which a C-terminal triple myc-epitope tag was inserted, it was also cloned into the SalI and BamHI sites of the expression vector pFLAG-CMV5a (Sigma). COS7 cells were grown on 35 mm petri dishes and transiently transfected by incubation with 1 ml OPTIMEM (Life Technologies) containing 1.7 μg DNA and 6.7 μl Lipofectamine (Life Technologies) for 4 hr. 48 hr following transfection, cells were washed in HBSS, harvested by scraping in HBSS containing 1 mM PMSF, pelleted by centrifugation, and lysed in buffer containing 25 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.2% CHAPS, 10 μg/ml leupeptin, 10 μg/ml antipain, 0.5 mM TLCK, 0.7 μg/ml chymostatin, 10 μg/ml benzamidine, 1 mM PMSF. Following centrifugation at 13,000 rpm the lysate was precleared with protein G agarose prior to incubation with 6 μg/ml anti-c-myc monoclonal antibody (9E10, Santa Cruz Biotechnology). After 3 hr incubation at 4°C, 30 μl of protein G agarose was added to precipitate antibodies. The agarose was washed five times with IP buffer and the immunoprecipitated proteins were denatured with SDS sample buffer and resolved by SDS-PAGE on an 8% gel. Western blots were probed with anti-FLAG monoclonal antibody (Sigma), 1:6000 and anti-c-myc, 1:200.

Immunofluorescence and Immunoelectron Microscopy

For teased fiber preparation, mice were perfused intravascularly with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3). Nerves were removed, fixed for 1 hr in the same fixative, washed in several changes of phosphate buffer and teased onto 3-aminopropyltriethoxysilane (TESPA)-coated glass slides followed by air-drying. Immunofluorescence of cryosections was as previously described (Gillespie et al., 2000). Primary antibodies were used at the following dilutions; affinity-purified anti-DRP2 (2164), 1:400, anti-Peri-C2, 1:5000, mouse anti-periaxin, 1:200, β-dystroglycan (MANDAG 2), 1:500, α-dystroglycan, 1:200, utrophin (MANCHO 3), 1:40, dystrophin (DYS2), 1:80. Secondary antibodies were FITC-conjugated anti-rabbit IgG (Cappel), TRITC-conjugated anti-mouse IgG$_1$, (Southern Biotechnology), and TRITC-conjugated anti-sheep IgG (Jackson Laboratories). All secondary antibodies were used at a dilution of 1:200. Trigeminal nerves from P28 rat trigeminal nerves were fixed by immersion in 4% formaldehyde (freshly prepared from paraformaldehyde) in a 0.01 M periodate, 0.075M lysine, 0.1M phosphate buffer containing 3% sucrose (pH 7.4) and processed for immunoelectron microscopy as described (Gillespie et al., 1994).

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