Development/Plasticity/Repair

Schwann Cell-Specific Ablation of Laminin γ1 Causes Apoptosis and Prevents Proliferation

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To investigate the function of laminin in peripheral nerve development, we specifically disrupted the laminin γ1 gene in Schwann cells. Disruption of laminin γ1 gene expression resulted in depletion of all other laminin chains known to be expressed in Schwann cells. Schwann cells lacking laminin do not extend processes required for initiating axonal sorting and mediating axon–Schwann cell interaction. They fail to downregulate Oct-6 and arrest at the premyelinating stage. The impaired axon–Schwann cell interaction prevents phosphorylation of β-neuregulin-1 receptors and results in decreased cell proliferation. Postnatally, laminin-null Schwann cells exhibit reduced phosphatidylinositol 3 (PI3)-kinase activity and activation of caspase cascades, leading to apoptosis. Injection of a laminin peptide into mutant sciatic nerves partially restores PI3-kinase activity and reduces apoptotic signals. These results demonstrate the following: (1) that laminin initiates axonal sorting and mediates axon–Schwann cell interactions required for Schwann cell proliferation and differentiation, and (2) that laminin provides a PI3-kinase/Akt-mediated Schwann cell survival signal.

Key words: Schwann cell; laminin; proliferation; apoptosis; phosphatidylinositol 3-kinase; myelin

Introduction

Myelination in the peripheral nervous system (PNS) is accomplished by Schwann cells. Schwann cell precursors are derived from neural crest cells and differentiate to immature Schwann cells. Before birth, Schwann cells destined to myelinate differentiate to promyelinating Schwann cells and extend cytoplasmic processes into axonal bundles, progressively separating these bundles to form a 1:1 ratio with individual axons (radial sorting) and enwrapping them with a myelin sheath (Webster, 1993; Mirsky and Jessen, 1999).

There is substantial evidence that Schwann cells require the formation of a basal lamina to properly ensheath and myelinate axons (Bunge, 1993). Laminins are major components of the basal lamina that appear to be especially important. In vitro studies using Schwann cell/neuronal coculture have shown that laminin deposition is required for myelination (Fernandez-Valle et al., 1993; Fernandez-Valle et al., 1994; Podratz et al., 2001). Both dystrophic mice (Xu et al., 1994; Sunada et al., 1995) and human congenital muscular dystrophy patients (Helbling-Leclerc et al., 1995) have mutations in their laminin α2 chain genes, resulting in muscular dystrophy and a dysmyelinating peripheral neuropathy. Schwann cell-specific disruption of β1 integrin, a component of many laminin receptors, also causes a dysmyelinating peripheral neuropathy with impaired radial sorting of axons (Feltri et al., 2002). Recently, we deleted laminin γ1 in the nervous system using the calcium/calmodulin-dependent protein kinase II α promoter to drive Cre expression (CaMKII/Cre/λAMγ1 mice) (Chen and Strickland, 2003). These mice have laminin γ1 gene disruption in a subpopulation of Schwann cells and exhibit dysmyelinating phenotype. All of these results suggest that laminin is critical for PNS development, but the mechanistic details are unknown.

Laminins are heterotrimeric glycoproteins composed of an α-, β-, and γ-chain. Five α-chains, four β-chains, and three γ-chains have been identified, and 15 isoforms have been observed (Yin et al., 2003). Laminin γ1 is one of the most abundant chains and is present in 11 isoforms, including all known laminin isoforms expressed in the PNS (Patton et al., 1997; Previtali et al., 2003). To study the mechanism of laminin function in PNS development, we specifically disrupted the laminin γ1 gene in Schwann cells at their early development stages using the Mpz (myelin protein zero) promoter to drive Cre expression. Because laminin γ1 chain is a common component in all laminin isoforms expressed in Schwann cells, knocking out laminin γ1 depleted most if not all of the laminin isoforms in these cells. By doing so, this study revealed novel functions of laminin in the PNS and also uncovered the mechanism by which laminin regulates PNS development. Schwann cells that lack laminin have impaired axon interaction, which leads to impaired proliferation and differentiation. These cells also have reduced phosphatidylinositol 3 (PI3)-kinase signaling needed to maintain viability and therefore increased apoptosis. Our results, coupled with other studies (Feltri et al., 2002; Chen and Strickland, 2003), indicate a critical role for laminin in establishing a stable axon interaction...
that provides proliferation and differentiation signals and in maintaining Schwann cell viability.

Materials and Methods

Mice, genotyping, and analysis of Cre-mediated laminin γ1 gene recombination. Mice in which the exon 2 of the laminin γ1 gene was flanked by two loxP sites ([lam1γ1] and CaMKII/Cre/lam1γ1 mice were generated as described previously (Chen and Strickland, 2003). mP_TOT(Cre) (P_cre) transgenic mice have been described previously (Feltli et al., 1999). To obtain P_cre/lam1γ1 mice, mice homozygous for the flam1γ1 allele were crossed with mice heterozygous for the lam1γ1 allele and hemizygous for the P_cre transgene. lam1γ1, P_cre, Z/Eg mice were generated by crossing lam1γ1+/, P_cre mice with a double reporter mouse line (Z/Eg) from The Jackson Laboratory (Bar Harbor, ME) (Novak et al., 2000). This reporter mouse line expresses lacZ throughout embryonic development and adult stages. During Cre expression, the lacZ gene is removed, which then activates expression of the second reporter gene, enhanced green florescent protein (EGFP). Genotypes of the resulting offspring were identified by PCR analyses of tail DNA were prepared from various tissues of male mice homozygous for the CaMKII/Cre:laminin γ1 allele and hemizygous for the P_cre transgene as described previously (Chen and Strickland, 2003). Nerves were fixed in 2.5% paraformaldehyde and semithin sections and electron microscopic analyses of sciatic nerves were performed as described previously (Feltri et al., 1999, 2000). Lamins were stained with anti-neurofilament antibodies to identify nerves. P15 sciatic nerve sections were stained with anti-S100, anti-laminin γ1, or anti-phosphoAkt antibodies, and nuclei were counterstained with DAPI. Double-labeled nuclei were determined. At each time point, six control and six mutant animals were analyzed. The differences in percentage of TUNEL-positive nuclei in control and mutant sciatic nerves were analyzed by two-tailed Student’s t test.

Western blot analysis. Control and mutant mice at different ages were anesthetized. The sciatic nerves were exposed, immersed in PBS containing the phosphatase inhibitor Cocktail I and II (Sigma), and dissected. The nerves were homogenized in 50 mM Tris, pH 7.4, containing 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, 1 mM EDTA, 1 mM EGTA, 10% glycerol, phosphatase inhibitor Cocktail I and II, and protease inhibitor cocktail (Sigma). Protein concentrations were determined by the method of Lowry (Bio-Rad, Hercules, CA). For immunoblotting of caspase-9, total protein of sciatic nerves was extracted from 3–5 chondromodopropyl(dimethylammonio)-1-propanesulfonate cell extract buffer (Cell Signaling Technology) containing protease inhibitors. Proteins (15–20 μg) were fractionated on 4–15% SDS-PAGE, blotted onto polyvinylidene difluoride membrane (Millipore, Billerica, MA), and probed with primary antibodies. Antibodies against phosphorylated (p)-ErbB2, ErbB2, p-ErbB3, p-Akt, Akt, p-glycogen synthase kinase 3β (GSK-3β), GSK, and mouse caspase-9 were purchased from Cell Signaling Technology and used at 1:1000 dilution. Other antibodies used were anti-ErbB3 (1:200; Abcam), anti-β-actin (1:8000; Sigma), and rabbit anti-Oct-6 (1:400; Abcam) and was injected with control peptides. Injections were performed on postnatal day 1 and was injected intraperitoneally with 100 μg of BrdU per gram of body weight. One hour later, the embryos or the sciatic nerves of P5 mice were dissected and frozen in dry ice, and the upper bodies or tails were used for genotyping. Whole embryos (embryonic day 13 (E13), E15.5, or E17.5) or sciatic nerve tangential cryosections (E17.5, E19.5, or P5) were prepared, fixed in cold methanol, denatured with 2N HCl for 20 min at 37°C, and neutralized in 0.1 M sodium borate, pH 8.5, for 10 min. Sections were coincubated with rat anti-BrdU (Abcam) and rabbit anti-neurofilament H (Chemicon). After staining with the appropriate secondary antibodies, the nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Only culture-shaped nuclei inside the nerve tissues were counted, and double-labeled nuclei (both BrdU and DAPI) were determined. At each time point, six control and six mutant mice were analyzed. The differences in percentage of BrdU-incorporated nuclei between control and mutant nerves were analyzed by two-tailed Student’s t test.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling assay. Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) was performed using the In Situ Cell Death Detection kit (Roche Applied Science, Indianapolis, IN) according to the instructions of the manufacturer. Whole embryo (E15.5) or sciatic nerve tangential cryosections (P0 to adult) were prepared, fixed in 4% PFA in PBS, pH 7.4, for 20 min, and permeabilized in 0.1% Triton X-100/0.1% sodium citrate on ice for 2 min. E15.5 embryo sections were stained with anti-neurofilament antibodies to identify nerves. P15 sciatic nerve sections were stained with anti-S100, anti-laminin γ1, or anti-phosphoAkt antibodies, and nuclei were counterstained with DAPI. Double-labeled nuclei were determined. At each time point, six control and six mutant animals were analyzed. The differences in percentage of TUNEL-positive nuclei in control and mutant sciatic nerves were analyzed by two-tailed Student’s t test.

Imaging analysis. Images of immunostained sections were acquired by using an AxioVision System (Zeiss, Oberkochen, Germany) and processed with Adobe Photoshop (Adobe Systems, San Jose, CA). The electron micrograph and Western blot film were digitized using a scanner (Microtek, Carson, CA). The signal intensity of the Western blot film was quantified by NIH Image.

Results

Generation of mice lacking laminin γ1 in Schwann cells

To specifically disrupt the laminin γ1 gene in Schwann cells, we created mice homozygous for a floxed laminin γ1 allele ([lam1γ1] (Chen and Strickland, 2003) and carrying a P_cre transgene, mP_TOT(Cre), which activates Cre-mediated recombination specifically in Schwann cells between E13.5 and E14.5 (Feltli et al., 1999, 2002). P_cre/lam1γ1 mice (referred to mutant mice hereafter) were born normally in accordance with the predicted Mendelian
including Schwann cells. Adjacent transverse sections of control and mutant sciatic nerves at P0 were stained for different laminin subunits, P0 \( / \text{Cre}:fLAM \) depletion of other laminin chains. Note that the laminin bundles of unsorted axons with some Schwann cells located outside (SC). cytoplasmic processes and leave axons unsorted.

Perineurium (arrows).

Nerves show that mutant nerves have large unsorted axonal bundles and few Schwann cells with myelin sheaths near the remanent in the perineurium (arrowheads), and MBP expression was not detected. Scale bar: 25 \( \mu \text{m} \).

Disruption of laminin \( \gamma 1 \) gene expression resulted in concurrent depletion of all other laminin chains known to be expressed in Schwann cells. Adjacent transverse sections of control and mutant sciatic nerves at P0 were stained for different laminin subunits, including \( \alpha 1 \), \( \alpha 2 \), \( \alpha 4 \), \( \beta 1 \), and \( \gamma 1 \). In the mutant nerves, disruption of laminin \( \gamma 1 \) gene expression resulted in concurrent depletion of other laminin chains. Note that the laminin \( \alpha 1 \) chain, which is expressed in the perineurium in mature nerves, and the laminin \( \alpha 4 \) chain, which is expressed at low level in adult nerves, are both nearly undetectable at the P0 stage. Scale bar, 25 \( \mu \text{m} \).

Figure 1. Schwann cells lacking laminin \( \gamma 1 \) expression are severe hypomyelination and fail to extend processes to initiate axonal sorting. A, PCR analysis of genomic DNA from various tissues of wild-type, homozygous fLAM-\( \gamma 1 \) mice (f/f; control) and \( P0_/ \text{Cre}:fLAM-\( \gamma 1 \) mice (f/f, \( P0_/ \text{Cre}; \) mutant). The primers used amplified the wild-type (1.3 kb), unrecombined (3.2 kb), and recombined (2.3 kb) fLAM-\( \gamma 1 \) alleles. B, Transverse sections of control and mutant sciatic nerves at P0 were double stained for laminin \( \gamma 1 \) (red) and MBP (green). In the mutant nerve, laminin \( \gamma 1 \) expression was absent in the endoneurium (asterisks) and only remained in the perineurium (arrowheads), and MBP expression was not detected. C, Transverse semithin sections from P28 sciatic nerves show that mutant nerves have large unsorted axonal bundles and few Schwann cells with myelin sheaths near the perineurium (arrows). D, Electron micrographs of P1 sciatic nerves show that mutant Schwann cells (arrows) do not extend cytoplasmic processes and leave axons unsorted. E, Electron micrograph of P28 sciatic nerves show that mutant nerves have large bundles of unsorted axons with some Schwann cells located outside (SC). F, Higher magnification of the boxed region in E shows that the mutant Schwann cell closely associated with unsorted axonal bundles lacks a continuous basal lamina (compare fuzzy materials indicated by arrows and denuded areas indicated by arrowheads) and does not extend processes between axons. Scale bar: B, 76 \( \mu \text{m} \); C, 18.5 \( \mu \text{m} \); D, 5.6 \( \mu \text{m} \); E, 1.7 \( \mu \text{m} \); F, 0.3 \( \mu \text{m} \). wt, Wild type; ct, control; mt, mutant; Ln \( \gamma 1 \), laminin \( \gamma 1 \).

Figure 2. Disruption of laminin \( \gamma 1 \) gene expression resulted in depletion of all other laminin chains known to be expressed in Schwann cells. Adjacent transverse sections of control and mutant sciatic nerves at P0 were stained for different laminin subunits, including \( \alpha 1 \), \( \alpha 2 \), \( \alpha 4 \), \( \beta 1 \), and \( \gamma 1 \). In the mutant nerves, disruption of laminin \( \gamma 1 \) gene expression resulted in concurrent depletion of other laminin chains. Note that the laminin \( \alpha 1 \) chain, which is expressed in the perineurium in mature nerves, and the laminin \( \alpha 4 \) chain, which is expressed at low level in adult nerves, are both nearly undetectable at the P0 stage. Scale bar, 25 \( \mu \text{m} \).

Mutant Schwann cells are severely hypomyelinated and fail to extend processes to initiate radial sorting of axons and mediate axon–Schwann cell interaction

We compared the onset of myelination in mutant and control sciatic nerves at P0 by staining transverse sections for MBP, a myelin marker. In control nerves, MBP was expressed normally, whereas at this time, no MBP expression could be detected in mutant nerves (Fig. 1B). At late postnatal stages (P28), myelination was complete in control nerves. In contrast, mutant sciatic nerves showed large unsorted axonal bundles and a few Schwann cells with normal myelin sheaths (Fig. 1C, arrows). In mice lacking \( \beta 1 \) integrin in Schwann cells, perineurial cells are abnormally located along microfasciculations in the center of the nerve (Feltri et al., 2002). In agreement with this observation, laminin \( \gamma 1 \) expression was not detectable in the endoneurium outside mutant Schwann cells before P5 (Figs. 1B, 2) but appeared at later stages in the en-
doneurium of some centrally located Schwann cells in mutant sciatic nerves (P28) (supplemental Fig. 1A, available at www.jneurosci.org as supplemental material). Therefore, these centrally located Schwann cells with normal myelin sheaths in mutant nerves might obtain laminin from nearby perineurial cells or escape recombination of the laminin γ1 gene and thus undergo normal differentiation. To address this question, we crossed the mutant mice with an EGFP reporter mouse line Z/EG (lacZ/EGFP) (Novak et al., 2000) and obtained mice that are homozygous for the floxed laminin γ1 allele and are also hemizygous for both P0-Cre transgene and the Z/EG reporter transgene. In mice carrying the reporter gene, during Cre-mediated recombination, EGFP will be expressed. We analyzed the sciatic nerves from these mice at P28 and found that the few Schwann cells that have laminin γ1 surrounding them expressed the reporter gene EGFP. This result indicated that these Schwann cells have undergone Cre-mediated laminin γ1 gene recombination since the reporter gene was expressed, but they have obtained laminin γ1 from other cells (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

However, it is also possible that some Schwann cells might escape Cre-mediated laminin γ1 gene recombination or undergo incomplete (monoallelic) recombination.

We next compared the morphology of control and mutant Schwann cells at P1 and P28 by electron microscopy. In control sciatic nerves at P1, most Schwann cells had extended processes to segregate axons, and some axons had formed a 1:1 relationship with individual Schwann cells (Fig. 1D). In contrast, mutant Schwann cells at P1 did not extend processes (Fig. 1D, arrows) and left axons unsorted. Ultrastructural analysis of mutant sciatic nerves at late postnatal stages showed that Schwann cells closely associated with unsorted axonal bundles (Fig. 1E, SC) lacked a continuous basal lamina (Fig. 1F, compare fuzzy materials indicated by arrows and denuded areas indicated by arrow-heads) and did not extend their cytoplasmic processes between axons, thus failing to segregate, interact, and myelinate axons.

**Mutant Schwann cells fail to downregulate Oct-6 and arrest in premyelinating stages**

During the deposition of the basal lamina, the essential step for Schwann cell differentiation is the upregulation of the transcription factor Oct-6 (Bermingham et al., 1996; Jägle et al., 1996). Oct-6 expression in Schwann cells is transient and peaks in the promyelinating stage and progressively downregulated during postnatal development. Oct-6 function is required in promyelinating Schwann cells for timely differentiation into myelinating Schwann cells (Jägle et al., 1996). We examined Oct-6 at different developmental stages to detect whether their expression is...
Oct-6 is required for the peak of myelination and for the activation of the major myelin genes, including P0 and MBP. Therefore, we examined Krox-20 expression in mutant peripheral nerves. At approximately E15, Krox-20 was detected at very low levels in both control and mutant nerves (data not shown). At E19.5, normal Krox-20 expression was observed in the absence of laminin γ1. In contrast, during postnatal development, when control Schwann cells progressively upregulated Krox-20, only a few Schwann cells in mutant nerves expressed high levels of Krox-20 (Fig. 3C), indicating that upregulation of Krox-20 is impeded in mutant Schwann cells.

Together, these results suggest that laminins are not required for the induction of Oct-6 and Krox-20 but are critical for the maintenance of Krox-20 expression and downregulation of Oct-6 and are therefore necessary for differentiation of myelinating Schwann cells.

**Laminin mediated axon–Schwann cell interactions are critical for Schwann cell proliferation**

By gross observation, mutant sciatic nerves were thinner than controls (Fig. 1C), and this could be attributable to reduced total cell number inside mutant nerves. We stained transverse sections from similar distal parts of mutant and control nerves at various ages with DAPI to measure the total number of nuclei. The cell number in mutant sciatic nerves compared with controls was diminished to 43% at P0, 28% at P5, and 33% at P28 (Fig. 4A).

During late embryonic and perinatal stages, Schwann cells proliferate vigorously to rearrange, sort, and ensheath axons (Stewart et al., 1993). Reduced Schwann cell proliferation could cause the decreased total cell number. To determine whether Schwann cells proliferate normally, we compared BrdU-incorporating nuclei in control and mutant nerves at various ages. During the peak of Schwann cell proliferation (E17.5–E19.5) (Stewart et al., 1993), control nerves showed extensive cell proliferation, whereas mutant nerves had few proliferating cells (Fig. 4B). Statistical analyses showed that the percentage of BrdU-incorporating nuclei was similar before laminin γ1 gene disruption (E13) but significantly decreased in mutant nerves compared with controls at E15.5 (20.8 vs 30.6%), E17.5 (12.7 vs 29.2%), E19.5/P0 (11.8 vs 29.6%), and P5 (3.8 vs 5.6%) (Fig. 4C), indicating that mutant Schwann cell proliferation is reduced.

Because axons are a major source of Schwann cell mitogens (Wood and Bunge, 1975; Morrisey et al., 1995), impaired axon–Schwann cell interaction could result in reduced Schwann cell proliferation. β-Neuregulin-1 (NRG-1) is a major axon-derived actor in Schwann cell proliferation at various ages. The response of Schwann cells to NRG-1 at P0 and P2 was assessed on immunoblots with antibodies recognizing p-ErbB2 and p-ErbB3. β-Actin is the loading control. Mutant Schwann cells show severe reduction in response to axonal mitogens.

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Schwann cell mitogen and can interact with and stimulate the phosphorylation of receptor tyrosine kinases ErbB2 and ErbB3 on Schwann cells (Morrissey et al., 1995). We measured the phosphorylation level of ErbB2 and ErbB3 in sciatic nerve extracts during early postnatal stages (Fig. 4 D). We found that, although ErbB2 and ErbB3 receptor levels were not significantly changed, the phosphorylation of both proteins was greatly reduced, suggesting that the inability of mutant Schwann cells to be exposed to axonal mitogens is a major cause of decreased proliferation. This result indicates that laminin-mediated axon–Schwann cell interactions are essential for proliferation.

A previous study using a CaMKII-Cre transgene to direct conditional laminin γ1 gene knock-out (Chen and Strickland, 2003) showed a similar but much less severe peripheral nerve phenotype compared with this mouse line (P0/Cre:fLAMγ1 mice). In the CaMKII/Cre:fLAMγ1 mice (mice that are homozygous for a floxed laminin γ1 gene and also hemizygous for CaMKII-Cre transgene), Schwann cell proliferation was not affected during embryonic stages, in contrast to the P0/Cre:fLAMγ1 mice. We compared the patterns of laminin γ1 disruption in these two mouse lines during embryonic stages. We found that, in the endoneurium of P0/Cre:fLAMγ1 mice, laminin γ1 immunoreactivity was completely absent from E15.5 to E19.5/P0, and cell proliferation was dramatically reduced [Fig. 5 (quantitative result by Chen and Strickland, 2003)]. However, in the CaMKII/Cre:fLAMγ1 mice, laminin γ1 immunoreactivity was absent in some small patches in the endoneurium and Schwann cell proliferation was not significantly reduced [Fig. 5 (quantitative result by Chen and Strickland, 2003)]. This dramatic difference in the patterns of laminin γ1 gene disruption in these two mouse lines may explain the difference in Schwann cell proliferation.

Together, our results show that complete ablation of laminin expression during embryonic stages dramatically reduces Schwann cell proliferation.

Schwann cells lacking laminin γ1 expression undergo apoptosis at postnatal stages

Extracellular matrix molecules such as laminin are often essential for maintenance of cell viability (Meredith et al., 1993; Chen and Strickland, 1997). Disruption of laminin γ1 may affect Schwann cell survival. We performed TUNEL to determine the extent of Schwann cell apoptosis at various ages (Fig. 6 A,B). There was little apoptosis at E15.5 and E17.5 (data not shown), and the percentages of apoptotic cells were similar in control and mutant nerves. However, in early postnatal stages, the percentage of dying cells in mutant nerves progressively increased, peaked at P15, and gradually declined as the nerves matured. In contrast, we seldom found apoptotic cells inside control nerves during these stages. TUNEL-positive cells in mutant nerves were significantly higher than in controls at P0 (1.8 vs 0.5%), P5 (3.3 vs 0.1%), P15 (7.1 vs 0.04%), and P28 (3.0 vs 0.2%) but not at E15.5 (1.1 vs 0.6%) and adult (0.5 vs 0.2%).

Mesenchymal cells are recruited to peripheral nerves to generate the perineurium at E15–E17 (Parmantier et al., 1999). To determine whether the apoptotic cells in the sciatic nerve were Schwann cells and lack laminin γ1 expression, we stained mutant nerve sections at P15 for laminin γ1, TUNEL, and a Schwann cell marker, S100. Cytoplasmic S100 staining showed that the apoptotic cells in mutant nerves were Schwann cells (Fig. 6 C) that had lost laminin γ1 expression (Fig. 6 D).

Because axonal survival signals are only important in early (before P6) but not for later postnatal developmental stages (Grinspan et al., 1996), it is unlikely that late postnatal apoptosis (P15–P28) (Fig. 6 B) is attributable to a lack of proper Schwann cell/axon relationship. This delayed apoptosis may indicate that laminin is required for long-term survival of Schwann cells, which has been suggested previously (Meier et al., 1999). Consistent with this observation, the CaMKII/Cre:fLAMγ1 mice, in which laminin disruption in Schwann cells happens later and is incomplete (at approximately E17.5–E19.5; data not shown) (Fig. 5) compared with that in P0/Cre:fLAMγ1 mice (Cre expression at approximately E13.5–E14.5) (Felti et al., 1999) showed a later onset of Schwann cell apoptosis (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Schwann cell apoptosis continued through adulthood in CaMKII/Cre:fLAMγ1 mice (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) but not in P0/Cre:fLAMγ1 mice (Fig. 6 B), probably because most Schwann cells die before P0/Cre:fLAMγ1 mice reach adulthood, and fewer Schwann cells remain within the sciatic nerve. Therefore, significant increases in apoptosis cannot be detected.

Together, our results coupled with previous studies indicate that loss of laminin could be a direct cause of apoptosis. However, we cannot rule out the possibility that impaired ErbB signaling in early postnatal development (Fig. 4 D) may contribute to part of increased apoptosis because the axon-derived neuregulin signal still regulates Schwann cell survival to some extent in early postnatal stages (Grinspan et al., 1996).

Laminin γ1-null Schwann cells show reduced PI3-kinase activities and elevated apoptosis/caspase signaling

The PI3-kinase/Akt pathway plays a critical role in controlling the balance between cell survival and apoptosis (Burgering and Cof-

Figure 5. Schwann cell proliferation is not impaired in CaMKII/Cre:fLAMγ1 mutant mice. Longitudinal sections of control (fLAMγ1), CaMKII/Cre:fLAMγ1, and P0/Cre:fLAMγ1 mutant sciatic nerves at E19.5 were triple stained for BrdU (red), laminin γ1 (green), and DAPI (blue) after a 1-h pulse of BrdU, and the images of BrdU/laminin γ1 and BrdU/DAPI were merged. Laminin γ1 expression was disrupted in some small patches in the sciatic nerves of CaMKII/Cre:fLAMγ1 mutant mice (stars), and Schwann cell proliferation was not impaired (quantitative analysis was shown previously (Chen and Strickland, 2003)). However, laminin γ1 expression was almost completely disrupted in the Schwann cells in P0/Cre:fLAMγ1 mutant sciatic nerves (stars), and cell proliferation was dramatically reduced (quantitative analysis shown in Fig. 4). Laminin γ1 expression in the epineurium of P0/Cre:fLAMγ1 mutant sciatic nerves was normal (arrows).
Several in vitro studies have shown that this pathway is important for Schwann cell viability (Cheng et al., 2000; Maurel and Salzer, 2000). To investigate whether the laminin γ1-null Schwann cells have reduced PI3-kinase/Akt signaling, we assessed PI3-kinase activity by measuring the phosphorylation level of Akt/PKB protein in sciatic nerve extracts at various ages (Fig. 7A). Akt phosphorylation progressively increased in control nerves during postnatal development. However, in mutant sciatic nerves, Akt phosphorylation decreased from P0 to P28 compared with control, with a maximum decrease at approximately P15. We stained nerve sections for both S100 and p-Akt and compared with PI3-kinase/Akt signaling, thus leading to apoptosis. To test this possibility, we performed a rescue experiment by injecting a laminin peptide into mouse sciatic nerves. It has been shown that a short peptide derived from laminin containing the sequence EIKLLIS, which may represent the integrin-activating site of laminin, can activate PI3-kinase/Akt-mediated survival signaling in neuronal cell culture (Tashiro et al., 1999). During activation, Akt phosphorylates and inactivates several downstream targets, including GSK-3 and caspase-2, to execute its anti-apoptotic effect. GSK-3 promotes apoptosis, and its activity can be inhibited by Akt-mediated phosphorylation at Ser21 of GSK-3α and Ser9 of GSK-3β (Cross et al., 1995). To determine the Akt-mediated anti-apoptotic effect, we examined the endogenous level of p-GSK-3β in sciatic nerve extracts at P5 and P15, the peak of apoptosis in mutant nerves. In mutant sciatic nerves, GSK-3β protein was comparable with controls, but its phosphorylation level was reduced at both ages (Fig. 7B).

Caspase-9 is inhibited by Akt-mediated phosphorylation (Cardone et al., 1998) and is the key initiator of the intrinsic apoptotic pathway (Budihardjo et al., 1999). During apoptotic stimulation, procaspase-9 (49 kDa in mice) is processed into a large active subunit (37 or 39 kDa) by self-cleavage. Cleaved caspase-9 activates other effector caspases, including caspase-3 and caspase-7, and initiates a caspase cascade, leading to programmed cell death. We examined P5 and P15 sciatic nerves for the large active fragment of caspase-9 by Western blotting (Fig. 7C) and the activation of downstream effector caspase-3 and caspase-7 (Fig. 7D) by immunostaining. At these ages, activated caspase-9, caspase-3, and caspase-7 could be detected in mutant nerves but not in controls (Fig. 7C,D). These results further suggest that PI3-kinase/Akt-mediated anti-apoptotic effects are impaired in Schwann cells lacking laminin γ1, resulting in elevated GSK-3 activity and the initiation of a caspase cascade to promote apoptosis.

Laminin-induced PI3-kinase/Akt activation is required for Schwann cell survival

The increased apoptosis and decreased PI3-kinase activities in mutant sciatic nerves are temporally correlated (Fig. 8A). This suggests that the disruption of laminin γ1 in Schwann cells may cause impaired PI3-kinase/Akt signaling, thus leading to apoptosis. To test this possibility, we performed a rescue experiment by injecting a laminin peptide into mouse sciatic nerves. It has been shown that a short peptide derived from laminin containing the sequence EIKLLIS, which may represent the integrin-activating site of laminin, can activate PI3-kinase/Akt-mediated survival signaling in neuronal cell culture (Tashiro et al., 1999; Gary and Mattson, 2001).

Laminin peptide was injected into one sciatic nerve of mutant mice, whereas the contralateral nerve was injected with a scrambled control peptide ILEKSLI (Tashiro et al., 1999). Injection of laminin peptide but not the control peptide along the mutant sciatic nerves partially restored Akt phosphorylation (increase 75% compared with contralateral side; normalized with total Akt) and also partially suppressed the activation of caspase-9 (decrease 50% compared with contralateral side; normalized with β-actin) (Fig. 8B,C). The control peptide was not toxic because injection into control nerves did not induce apoptosis. Together, these data indicate that the disruption of laminin γ1 in Schwann cells, at least in part, accounts for the impaired PI3-kinase/Akt signaling and increased Schwann cell apoptosis.
Discussion

Using the Cre/LoxP system, we specifically disrupted the laminin γ1 gene in Schwann cells during their early developmental stages. Disruption of laminin γ1 gene resulted in complete depletion of all laminins in Schwann cells. These mice have revealed novel mechanistic insights into laminin function in Schwann cell development. First, laminin is essential for differentiation of Schwann cells; mutant Schwann cells fail to downregulate Oct-6 expression; this is essential for axon–Schwann cell interaction, axonal sorting, and myelination. Impaired axon–Schwann cell interaction prevents exposure of the cells to axon-derived mitogens and causes severe reduction of proliferation. Third, laminin provides a PI3-kinase-mediated signal to maintain Schwann cell survival. Disruption of laminin results in reduced PI3-kinase activity, leading to apoptosis. Injection of a laminin peptide into mutant peripheral nerves partially restores this signaling pathway and suppresses caspase-mediated death signal.

Laminin γ1 and β1 integrin in Schwann cell development

Many phenotypes in mice lacking laminin γ1 in Schwann cells (Fig. 1C,E) are similar to those observed in mice lacking β1 integrin in Schwann cells (Feltri et al., 2002), indicating that β1 integrin plays a major role in mediating laminin signaling. However, proliferation is reduced in Schwann cells lacking laminin γ1 but is not significantly affected in β1 integrin-null Schwann cells. There are three likely possibilities for why laminin γ1 affects proliferation: (1) laminin might act as a direct Schwann cell mitogen because this effect has been observed in vitro (McGarvey et al., 1984; Baron-Van Evercooren et al., 1986); in this case, laminin γ1 would use receptors other than β1 integrin; (2) the laminin basal lamina could act as a scaffold to attract and bind growth factors and influence Schwann cell proliferation; and (3) the proliferation effect might be secondary to axonal sorting and provision of axon-derived growth factors (Fig. 4D). We think the third possibility is most likely based on our results (Fig. 4D) and the following observations. In early postnatal stages (P1), Schwann cells lacking β1 integrin send abnormally shaped, thick cytoplasmic processes to ensheathe groups of axons. The formation of un-
Schwann cells express several potential laminin receptors, including α6β1 and α6β4 integrins and dystroglycan (Previtali et al., 2003), and α6β1 is thought to be the major laminin receptor in Schwann cells. The β1 integrin-null Schwann cells can ensheathe axons prenatally and myelinate axons after birth with some delay (Feltri et al., 2002) in contrast to laminin γ1-null Schwann cells that do not exhibit these processes. The postnatal myelination difference could be attributable to compensation of β1 integrin by another laminin receptor, for example α6β4 or dystroglycan, both of which are expressed in postnatal Schwann cells (Previtali et al., 2003). The prenatal ensheathe ment difference is harder to explain, because only α6β1 integrin is observed before birth (Previtali et al., 2003). This suggests that an unidentified laminin receptor expressed in embryonic Schwann cells is involved during the ensheathe ment of axons.

The defects in the PNS observed in mice lacking laminin γ1 (this report) are more severe than those found in dystrophic mice (dy2J/dy2J or dy3K/dy3K), which have a mutation or complete deficiency in their laminin α2 gene, resulting in a lack of laminin-2 (α2 β1 γ1) (Xu et al., 1994; Nakagawa et al., 2001). However, in the laminin α2 mutant mice, laminin-1 and laminin-8 are upregulated, which can partially compensate for the loss of laminin-2 (Patton et al., 1997; Previtali et al., 2003; Yang et al., 2005). Because laminin-8, laminin-1, and laminin-2 all contain the γ1 chain, compensation in the laminin γ1-depleted mice is not possible and results in a more severe phenotype. Consistent with this observation, combined deficiency of laminin 2/8 (dy2J/α4null mice) caused more severe defects than those in dystrophic mice (Yang et al., 2005), and the severity is similar to our mutant mice (this report). Yang et al. also provided evidence that laminins are important for Schwann cell proliferation.

Neuregulin-ErbB and PI3-kinase pathway in Schwann cell survival
At E12–E13 in mice, the survival of Schwann cell precursors depends on axon-derived β-neuregulin 1/Erb B pathway (Dong et al., 1995; Riethmacher et al., 1997). After this stage, Schwann cells establish an autocrine loop but also require laminin for long-term survival (Meier et al., 1999). Because P0/Cre-mediated laminin γ1 disruption occurs between E13.5 and E14.5 and because there was no significant cell death at approximately this stage (Fig. 6B), it is unlikely that the neuregulin 1/Erb B signaling pathway is affected in mutant nerves.

The PI3-kinase activity in mutant Schwann cells was severely reduced (Fig. 7A). This reduced PI3-kinase activity may be a cause or a consequence of the impaired differentiation of mutant Schwann cells because the PI3-kinase pathway is important for Schwann cell differentiation and myelination (Maurel and Salzer, 2000; Ogata et al., 2004). However, disruption of laminin γ1 may also contribute to the reduction of this survival signaling pathway and result in apoptosis based on the following observations. At P0/P1, both control and mutant Schwann cells are at similar differentiating stages (premyelinating stage), but the mutant Schwann cells had reduced PI3-kinase activity and increased apoptosis. Additionally, mutant Schwann cells infused with laminin peptides showed partial restoration of PI3-kinase activity and reduced apoptosis.

Krox-20 and Oct-6 in laminin γ1-mediated effects
We observed reduced Krox-20 expression and increased Schwann cell death in mutant sciatic nerves (Figs. 3, 6). Because Krox-20 can suppress c-Jun-mediated TGFβ-induced Schwann cell apoptosis (Parkinson et al., 2004), increased Schwann cell apoptosis could result from the failure of Krox-20 to inhibit c-Jun activation. However, the phosphorylation of c-Jun at postnatal stages between control and mutant sciatic nerves was similar (data not shown), suggesting that the TGFβ pathway did not play a major role in the increased apoptosis of mutant Schwann cells.

The observation of laminin-independent initiation of Oct-6 and Krox-20 (Fig. 3) raises interesting questions: can laminin directly induce myelinating signals, or is the main function of laminin in early Schwann cell development to initiate radial sorting and ensheathment of axons? Radial sorting and ensheathment of axons are prerequisites for myelination. Whether laminin produces a signal to induce myelin gene expression and what this signal is are future questions of interest.

References


