Roles of Meltrin-β/ADAM19 in Progression of Schwann Cell Differentiation and Myelination during Sciatic Nerve Regeneration

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Remyelination is an important aspect of nerve regeneration after nerve injury, but the underlying mechanisms are not fully understood. Here, we show that meltrin-β (ADAM19), a member of the ADAM (a disintegrin and metalloprotease) family, plays crucial roles in nerve regeneration after a crush injury to the sciatic nerves. The expression of meltrin-β was up-regulated in neurons after the crush injury. Morphometrical analysis revealed a delay in remyelination in meltrin-β-deficient nerves, whereas no significant defects were observed in their axon elongation. The activation of Krox-20, an indispensable transcription factor for myelination, was delayed in meltrin-β-deficient nerves and was accompanied by the retarded expression of myelin-related proteins. Expression of Krox-20 in Schwann cells was mediated by Akt. Phosphorylation of Akt but not that of Erk was reduced in regenerating nerves of meltrin-β-deficient mice. The cell membrane fraction prepared from meltrin-β-deficient nerves showed a defective activation of Akt in the membrane-loaded Schwann cells. Meltrin-β-deficient mice exhibited delayed sciatric functional recovery after the nerve crush. Altogether, these results reveal a role of meltrin-β in Schwann cell differentiation and re-myelination in nerve regeneration. Moreover, this study suggests that meltrin-β functions as a modulator of juxtacrine signaling from axons that activate the Akt pathway and the Krox-20 expression, which is the prerequisite for Schwann cell differentiation.

The peripheral nervous system (PNS) is mainly composed of neurons and glial cells. Differentiation of Schwann cells, a group of glial cells that sheathe the axons of the peripheral nerves, including sensory and motoneurons, is a multistep process. Immature Schwann cells migrate along axons and then differentiate to promyelin-stage Schwann cells, which sheathe the single axons, before differentiation into myelinating Schwann cells. Differentiation from immature to promyelin and from promyelin to myelinating Schwann cells requires the POU domain transcription factor Oct-6 (also called suppressed cAMP-inducible protein, SCIP) and the zinc-finger transcription factor Krox-20 (Egr-2), respectively (2–4).

Differentiation of Schwann cells is regulated by various growth factors, such as neuregulin-1 (NRG1), the major ErbB ligand acting as a glial growth factor (5). Receptor-tyrosine kinases, including ErbBs, transmit intracellular signals mainly through extracellular signal-regulated kinases (Erks) and phosphatidylinositol 3-kinase (PI3K)-Akt pathways (6), of which the latter pathway is crucial for initiation of myelination (7, 8). NRG1-ErbB signaling in promyelin and myelinating Schwann cells activates Erks and PI3K-Akt pathways, respectively. These studies reveal the importance of identifying modifiers that alter growth factor signaling, including NRG1, from the Erks- to PI3K-Akt-dependent pathway for the initiation of myelination.

Here, we show that meltrin-β (ADAM19) plays roles in Schwann cell differentiation during regeneration of the sciatic nerves after a crush injury. Meltrin-β is a member of the ADAM (a disintegrin and metalloprotease) family of proteins that contains the active metalloproteases domain. Evidence suggests modulatory roles of ADAM proteases in the ectodomain shedding of various membrane proteins. Kuzbanian/ADAM10 is involved in the ectodomain shedding of multiple substrates, including Notch ligands, ephrins, and cadherins (9–11). Tumor necrosis factor-α-converting enzyme/ADAM17 plays essential roles in the phorbol ester-stimulated ectodomain shedding of various membrane-anchored growth factors, receptors, or adhesion molecules (12). We and Inoue et al. identified mouse meltrin-β cDNA previously (13–15). Meltrin-β is highly expressed in the developing PNS and enhances the generation of soluble ligands from membrane-anchored type I NRG1 in cultured neurons (5, 14, 16–18). Although physiological substrates of meltrin-β remain elusive, the protease domain of meltrin-β could, thus, regulate the growth factor signaling required for PNS development. From the structural point of view, meltrin-β and several other ADAM proteins can be classified as subfamily proteins; these proteins contain well conserved dis-
Delayed Myelination in the Absence of an ADAM

integrin and other cysteine-rich domains, conserved domains originally found in viper venoms, whereas these domains are less conserved in tumor necrosis factor-α-converting enzyme/ADAM17 and Kuzbanian/ADAM10 (13, 19, 20). The functions of disintegrin and cysteine-rich domains of meltrin-β have not been elucidated.

Most meltrin-β-deficient mice died soon after birth, probably due to developmental defects in the heart (19, 21, 22). However, some of meltrin-β-deficient mice carrying a mixed genetic background of 129/Sv and C57BL/6, but not those carrying a single C57BL/6 background, survived to adulthood despite these defects (22). These survivors enabled us to investigate whether meltrin-β participates in the development of the PNS after birth. In this study we found meltrin-β-deficient mice exhibited a delay in remyelination and a prolonged period of hind limb dysfunction after injury. Meltrin-β-deficient nerves showed delayed up-regulation of Krox-20, leading to retarded activation of the genes for myelin-related proteins. Moreover, Akt activation, an essential process for expression of Krox-20 in Schwann cells, is less efficient in lesioned meltrin-β-deficient nerves. These results revealed that meltrin-β functioned as one of the modulators of Schwann cell differentiation from promyelins to myelinating stages during regeneration through the activation of Akt signaling pathway.

EXPERIMENTAL PROCEDURES

Animals—Meltrin-β-deficient mice were generated as previously described (22). Heterozygous mice of the mixed background (129Sv × C57BL6) were crossed, and the offspring were genotyped by PCR. No gross abnormalities were observed in wild type and heterozygous littermates on overall development, growth characteristics, and histology (22); therefore, littermates with wild type genotypes were used as controls throughout this study. All animals were maintained in accordance with the guidelines of Kyoto University. The technical protocols for animal experiments in this study were approved by a review committee of the Institute for Frontier Medical Sciences, Kyoto University.

Antibodies—The antibodies used and their sources were as follows: rabbit polyclonal anti-C terminus of meltrin-β antibody (16), rabbit polyclonal anti-Akt and anti-phospho-Akt antibodies (9272, 9271, Cell Signaling Technology), mouse monoclonal anti-Erk1 and anti-Erk2 antibodies (601030, 610103, BD Biosciences), rabbit polyclonal anti-phospho-Erk1/2 antibody (9101S, Cell Signaling), rabbit polyclonal anti-Krox-20 antibody (PRB-236P, Covance), goat polyclonal anti-Oct-6 antibody (sc-11661, Santa Cruz), rat monoclonal anti-F4/80 antibody (clone Ab-3, Serotec), rabbit polyclonal anti-myelin basic protein antibody (A0623, Dako), rabbit polyclonal anti-GAP-43 antibody (AB5220, Chemicon), mouse monoclonal anti-neurofilament 160 antibody (clone NN18, Sigma), and horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories).

Immunoblotting and Histology—Several days after surgery, distal portions of the sciatic nerve from the crush-lesioned or unlesioned side were homogenized in radiolmmune precipitation assay buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5). Twenty micrograms of sciatic nerve lysates were analyzed by SDS-PAGE followed by immunoblotting. Immunoblots were developed with a horseradish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence. The intensity was determined by laser densitometry of immunoblots using a densitometer and ImageQuant software (GE Healthcare). Scans at multiple exposures were obtained to ensure that the results fell within the linear range of the instrument.

For immunohistochemical analyses, sciatic nerves and lumbar DRG were dissected at various days after the nerve crush, flash-frozen in optimal cutting temperature compound, and cryosectioned at 8 μm. After preparation, sections were fixed with ice-cold acetone and immunostained using antibodies against NF160, GAP-43, and meltrin-β. For the staining with antibodies against Krox-20 and Oct-6, sections were fixed with 4% paraformaldehyde in PBS. For toluidine blue staining, sciatic nerves were postfixed overnight in PBS containing 2% glutaraldehyde and then dehydrated and embedded in plastic for microtome sectioning. For quantification of immunostained nerves, longitudinal cyrosections (10 μm) of uninjured or injured nerves at 7, 12, and 21 days after crush were prepared from wild type and mutant mice. Each section was immunostained with antibodies against transcription factors Oct-6 and Krox-20 and counterstained with 4′,6-diamidino-2-phenylindole. Photographs were taken from three wild type and three mutant nerves, and immunostained nuclei were counted with reference to the 4′,6-diamidino-2-phenylindole-stained signals.

Membrane Preparation and Analysis of Akt or Erks Activation in Schwann Cells—DRG neurons from 35-mm dish cultures were washed twice with ice-cold PBS and collected with fine forceps and homogenized with 20 strokes of a 0.2-ml micro tissue grinder (Wheaton, Millville, NJ) in 150 μl of ice-cold PBS. The volume was brought up to 1.5 ml with ice-cold PBS, and the homogenate was centrifuged (100 × g, 20 min, 4 °C) to remove debris. The supernatant, diluted up to 3 ml, was then centrifuged at 35,000 × g for 1 h at 4 °C. The supernatant was discarded, and the pellet was resuspended in culture media by vortexing. To analyze the activation levels of either Akt or Erks in Schwann cells, the membrane fraction prepared from either wild type or meltrin-β-deficient neurons was loaded onto Schwann cell cultures, and after 20 min cell lysates were prepared, blotted, and probed for phospho-Akt, total Akt, phospho-Erk, and total Erks as described above. Conditioned media were prepared as previously described (23). Briefly, conditioned media from ~12 cultures each of wild type and meltrin-β-deficient DRG were collected and concentrated 100-fold. Concentrated media were diluted 10-fold with Dulbecco’s modified Eagle’s medium and exposed to the Schwann cell for 20 min. Schwann cell lysates were prepared, blotted, and analyzed described above.

Quantitative Reverse Transcription (RT)-PCR—Total RNA was extracted from distal parts of sciatic nerves at various days after crush injury or cultured Schwann cells using the RNaseasy MiniKit (Qiagen, Basel, Switzerland). RNA samples were further purified by DNase digestion and extraction with phenol and chloroform and subjected to RT-PCR. Briefly, 0.5 μg of
total RNA was converted to cDNA using Superscript II (Invitrogen) in the presence of random hexamer primers. The mRNAs for Krox-20, P0, and myelin basic protein were measured by real-time quantitative RT-PCR using the Applied Biosystems prism model 7700 sequence detection instrument. The mRNA levels were corrected for glyceraldehyde-3-phosphate dehydrogenase.

Transfection of siRNA—For transfection of siRNA, control or Akt targeting siRNA were electroporated into primary Schwann cells using the nucleofector mouse embryo fibroblast mixture 1 (program T20; Amaza Biosystems) and then plated onto 6-well culture plates (100,000 cells per well). The siRNA effect was analyzed by immunoblotting 48 h after transfection. All the siRNAs used in this study were purchased from Qiagen (negative control siRNA, catalog no. 1033076; Akt targeting siRNAs, catalog no. 1022427 and SIO2652419). RNA samples were prepared from Schwann cells and analyzed as described above.

Sciatic Nerve Crush—Mice were anesthetized by intraperitoneal injection of 2.5% avertin. The sciatic nerve was exposed at the mid-thigh region and crushed for 30 s using Dumont no. 5 forceps. This was repeated at the same site to completely sever the axons.

Quantitation of Degenerating and Myelinated Axons—For the assessment of the extent of axonal degeneration and regeneration, we measured numbers of degenerating and myelinated axons. Toluidine blue-stained sections prepared from at least five control mice and five mutant mice were photographed and 10,000 μm² areas were chosen at random for analysis. Irregular and darkly stained myelin profiles were classified as degenerating myelin sheaths. Myelin sheaths that wrap axons to a greater or lesser extent were counted as myelinated.

Tracing of DRG Neurons and Motoneurons—Crystals of Fluorogold (Molecular Probes) were applied onto the cut nerve 7 mm distal to the crush lesion. After 48 h mice were deeply anesthetized with pentobarbital and perfused with 4% paraformaldehyde. Spinal cords and DRG were removed and processed for cryosectioning. For counting DRG neurons, all experiments were evaluated using cresyl violet staining for serial sections and Fluorogold-labeled large cells with abundant cytoplasm and prominent nucleolus in each section were counted. Thus, the number of neurons can be counted based on the number of nucleolus in Fluorogold-labeled cells. Numbers of motoneurons were counted similarly using these criteria.

Behavior Analysis—At various day points after unilateral crush injury, mice were tested for recovery of hind limb function on the crushed side by measuring toe spread and the ability to grip an inverted wire screen (24, 25). For the outer toe spread assay, the hind feet of the mice were painted with black pigment ink; the mice were then allowed to walk freely on a blank sheet of paper. The distance between the first and fifth digits (outer toe spread) was measured. For the grip assay mice were placed on a wire screen mesh, which was then turned over to test the ability of the mice to grip the screen correctly with their hind paws. The test was repeated 10 times for each mouse, and the number of failed trials was recorded.

Statistical Analysis and Ethical Considerations—Results were expressed as the mean ± S.E. Differences between groups were examined for statistical significance using Fischer’s protected least significant difference (PLSD) test. p < 0.05 denoted the presence of a statistically significant difference.

RESULTS

Expression of Meltrin-β (ADAM19) in Peripheral Neurons after Crush Injury—To investigate whether meltrin-β participates in the development of the PNS after birth, heterozygous mice of the mixed background of 129/Sv and C57BL/6 were crossed, and the meltrin-β-deficient mice were generated. Approximately 10–15% of the meltrin-β-deficient mice survived to adulthood despite the heart defects (22). These survivors enabled us to investigate roles of meltrin-β in the development of the PNS after birth. Although the numbers of axons in the meltrin-β-deficient mice were slightly lower than those in the wild type mice (about 77% at L5 level), both myelinating and non-myelinating axons were normal in appearance. The g-ratio of myelinating axons in the meltrin-β-deficient mice was similar to that in the wild type mice. IB4-positive, calcitonin gene related peptide-positive, and parvalbumin-positive neurons of meltrin-β-deficient mice were more or less equally reduced in number compared with those of wild type mice, suggesting that the smaller number of axons in the sciatic nerves is not due to defects in the formation of certain types of neurons. Meltrin-β-deficient mice showed no abnormality in walking or in sensing heat or touch (data not shown).

We adopted another approach to explore the roles of meltrin-β in PNS, hypothesizing that the roles of meltrin-β might be elucidated by examining the regeneration processes after the sciatic nerve crush, which is a well established paradigm for nerve regeneration. We examined the expression of meltrin-β before and after the nerve crush by Western blotting and immunohistochemical analyses (Fig. 1). Meltrin-β protein was scarcely detectable in uninjured DRG neurons. Several days after the sciatic nerve crush at the mid-femoral level, meltrin-β expression increased strongly in L3-L5 DRG neurons (Fig. 1, C and G) and motor neurons (data not shown). GAP-43, which is a highly specific marker for regenerating peripheral neurons including DRG neurons (Fig. 1, E and I) (26), was induced similarly after the sciatic nerve crush. Thus, expression of meltrin-β is up-regulated after the sciatic nerve crush at the early phase of regeneration and persists at least for 2 weeks.

Decreased Myelination in the Early Stage of Regeneration after Sciatic Nerve Crush in Meltrin-β-deficient Mice—To understand the roles of meltrin-β in nerve regeneration processes, we first examined whether the absence of meltrin-β affected nerve regeneration at the morphological level. The sciatic nerves of wild type and meltrin-β-deficient animals were crush-lesioned at the mid-femoral level, and the extent of morphological degeneration and regeneration was assessed by serial sectioning and microscopic analyses of the lesioned nerves. After the nerve damage, self-destruction of axons or Wallerian degeneration (27) was observed in the nerve distal to the lesion, resulting in loss of axon-Schwann cell contact. Five days after the nerve injury, demyelinating features were prominent in the part distal to the lesion in both wild type and mutant mice nerves (Fig. 2A). There was no significant differ-
Delayed Myelination in the Absence of an ADAM

FIGURE 1. Induction of meltrin-β in DRG by sciatic nerve crush. A, an immunoblot for meltrin-β protein that indicates its up-regulation in adult L3-L5 DRG at 7 days after nerve crush. Open and filled arrowheads indicate ~120-kDa prodomain-bearing and ~100-kDa prodomain-lacking, active forms of meltrin-β, respectively. The active form of meltrin-β is scarcely detectable in uninjured samples. Molecular weight markers are shown at the left (upper panel). The expression of β-tubulin protein was used as a control for protein loading (lower panel). B–I, immunohistochemical analysis that demonstrates activation of meltrin-β in DRG neurons after sciatic nerve crush. DRG sections before (B–E) and 7 days after injury (F–I) were double-stained with anti-neurofilament 160 (green) and anti-meltrin-β (red) antibodies (B, C, F, and G) or with anti-neurofilament 160 (green) and anti-GAP-43 (red) antibodies (D, E, H, and I). Meltrin-β is activated similarly to GAP-43, a specific marker for regenerating peripheral neurons. Scale bar, 50 μm.

FIGURE 2. Decreased myelination in the early stage of regeneration after sciatic nerve crush in meltrin-β-deficient mice. A, semi-thin toluidine-blue-stained sections of sciatic nerves 3 mm proximal or distal to the lesion at 3, 10, and 21 DAC. Degenerating profiles from wild type (WT) and meltrin-β-deficient (KO) mice on panels of five DACs are indicated with arrowheads. The number of myelinating nerves in meltrin-β KO mice was less than that in wild type mice at 10 DAC, whereas similar numbers of myelinated nerves were found in wild type and meltrin-β KO mice at 21 DAC. Scale bar, 25 μm. B, the densities of myelinated fibers and degenerating axon profiles were measured in wild type (open bars) and meltrin-β-deficient nerves (filled bars) 3 mm proximal or distal to crushed sites at 5 days after the injury. Degeneration occurred similarly in the nerves of both genotypes. The mean ± S.E. obtained with five wild type and five mutant mice are represented. C, immunostaining of invading macrophages using an antibody against F4/80 in the lesioned nerves. Macrophages invaded into injured nerves similarly in wild type and mutant-deficient mice. Scale bar, 100 μm. D, the densities of myelinated axon profiles were measured in wild type and mutant-deficient nerves 3 mm distal to crushed sites at 5, 10, and 21 days after injury. Scale bar, 25 μm.
meltrin-β-deficient mice, although the progression of the myelination recovered in the late stage of regeneration in these mice.

The delay in the early stage of regeneration in meltrin-β-deficient mice could be because of the decreased rate of axon elongation after the lesion. To evaluate the axonal regeneration rate, spinal motoneurons and DRG neurons were labeled in retrograde with Fluorogold 5, 10, and 21 days after the crush at a site 7 mm distal to the lesion (Fig. 3). Fluorogold was used as a marker because it is a long-lasting and non-diffusible tracer that undergoes rapid retrograde axonal transport (26, 28). Meltrin-β-deficient mice had similar numbers of labeled spinal motoneurons as wild type mice at any day point after the crush, suggesting that the axons of meltrin-β-deficient mice elongated over the lesion and reached the injection site as efficiently as those of the wild type mice (Fig. 3F). Similar results were obtained in the DRG neurons (data not shown). These results exclude primary defects of meltrin-β-deficient mice in the axonal elongation during regeneration.

Delay in Progression of Schwann Cell Differentiation in Meltrin-β-deficient Nerves during Regeneration—The delayed regeneration of meltrin-β-deficient nerves could be caused by a defect in development of the Schwann cell, including its proliferation, viability, and differentiation. For this reason, we compared Schwann cell viability and proliferation in wild type and meltrin-β-deficient sciatic nerves during regeneration. There was little apoptosis in the lesioned nerves of meltrin-β-deficient mice, as evidenced by similar numbers of cleaved caspase-3-positive apoptotic cells that were observed in the part distal to the lesion of wild type and meltrin-β-deficient nerves 4 days after nerve crush (data not shown). Bromodeoxyuridine incorporation into glial fibrillary acidic protein-positive immature Schwann cells was similar in the nerves from both genotypes at 7 days after nerve crush (bromodeoxyuridine incorporation in each microscopic field of 100 × 100 μm²: wild type, 13.7 ± 1.0; mutant, 13.0 ± 2; n = 4). Similar numbers of GFAP-positive cells were found in wild type and meltrin-β-deficient nerves (data not shown). Thus, the viability and
Schwann cells in addition to Oct-6+/Krox-20− cells, whereas the majority of Schwann cells in mutant nerves were Oct-6+/Krox-20− (Fig. 4, B, E, and G, and supplemental data). The result indicated that activation of Krox-20 initiates in promyelin Schwann cells in regenerating nerves of wild type mice but not in those of the mutant mice at 7 DAC. Twelve days after the crush, a prominent increase in the number of Oct-6−/Krox-20−-myelinating cells was observed in wild type nerves, whereas most Schwann cells in mutant nerves were Oct-6+/Krox-20− and Oct-6−/Krox-20+. Still devoid of Oct-6−/Krox-20+-myelinating cells (Fig. 4, C, F, and G, and supplemental data; percentage of Oct-6−/Krox-20+ cells in each microscopic: wild type, 52.8 ± 6.1; mutant, 11.0 ± 1.6; n = 3). Subsequently, a prominent activation of Krox-20 also occurred in the mutant nerves between 2 and 3 weeks after the injury that resulted in increased Oct-6−/Krox-20−-myelinating cells in these mice (Fig. 4G). These results showed that the progression of Schwann cell differentiation from Oct-6−/Krox-20− to Oct-6+/Krox-20+ and then to Oct-6−/Krox-20−-myelinating cells is delayed in the meltrin-β-deficient nerves.

To quantify the levels of Oct-6 and Krox-20 during the nerve regeneration, we collected sciatic nerves of wild type and mutant mice at various days after crush injury and examined the expression of both of these transcription factors by immunoblot analysis (Fig. 5A). We found that the transition of Schwann cells from Oct-6-dominant to Krox-20-dominant stages in meltrin-β-deficient mice lagged about 1 week behind that in wild type mice (Fig. 5B). Analyses with quantitative RT-PCR revealed that the delayed activation of Krox-20 resulted in retarded transcriptional activation of the P0 gene, one of the major myelin components of the PNS, in regenerating nerves of meltrin-β-deficient mice (Fig. 5C). Together with morphological analyses, these results indicate that meltrin-β participates in the progression of Schwann cell differentiation, mainly in the transition from Oct-6-dominant promyelin to Krox-20-dominant myelinating stages.

**Decreased Activation of PI3K—Akt signals in regenerating meltrin-β-deficient nerves.** Schwann cell development, including proliferation and differentiation, is regulated by various growth factors, such as NRG and insulin-like growth factor I (IGF-I). Receptors for these growth factors are classified as receptor-tyrosine kinases, which transmit intracellular signals mainly through the Erk and PI3K pathways (6). To compare

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**FIGURE 5.** Delayed activation of Krox-20 during regeneration in meltrin-β-deficient nerves. A, Western blots of cell lysates prepared from the lesioned nerves at the indicated days after nerve crush using antibodies against Oct-6 (upper panel) and Krox-20 (middle panel). Twenty micrograms of proteins were contained in each lane. A representative experiment is shown here. The expression of β-tubulin protein was used as a control for protein loading (bottom panel). B, expression of Krox-20 (red) and that of Oct-6 (green) in the lesioned nerves of wild type (solid lines) and mutant (dotted lines) mice after nerve crush were quantified on the basis of the amount of β-tubulin. Note that lags of several days occurred in the activation of Krox-20 and down-regulation of Oct-6 in mutant nerves. AU, arbitrary units; u.c., uncrushed. C, levels of Krox-20 and P0 transcripts in the lesioned nerves specified days after the crush. Quantitative RT-PCR products of Krox-20 and P0 mRNA were normalized with those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The mean ± S.E. from three wild type mice and four mutant mice are represented (*, p < 0.01; **, p < 0.005, Fischer’s PLSD test.).
Akt was significantly reduced in meltrin-β-deficient nerves, a similar level of phosphorylation of intracellular signals mediated by Erk and PI3K-Akt pathways in wild type and meltrin-β-deficient mice, sciatric nerves were isolated at 7 days after the crush injury, and the phosphorylation of Erk and Akt was examined by immunoblotting and densitometric analysis (Fig. 6, A and B). In wild type and meltrin-β-deficient regenerating nerves, a similar level of phosphorylation of Erks was observed. In contrast, the level of phosphorylation of Akt in the wild type or mutant nerves 7 days after the crush injury. The bar graph indicates the ratio of phospho-Akt to total Akt. The means ± S.E. from six wild type and five mutant mice are represented (*, p < 0.05; Fischer's PLSD test). C, membranes or conditioned media (CM) prepared from wild type and mutant neuron cultures were plated onto Schwann cell cultures, and after 20 min lysates were prepared, blotted, and probed for phospho-Akt, total Akt, phospho-Erk, and total Erks as shown.

Recent studies indicate that signals mediated by PI3K-Akt are crucial for initiation of myelination and that the effects of growth factors on the progression of Schwann cell differentiation are primarily dependent on the balance between the Erk and PI3K-Akt activation (7, 8). We examined whether the pharmacological inhibition or the targeted knockdown of Akt with siRNA against it in Schwann cells affects activation of Krox-20 and P0. As shown in Fig. 7, activation of Krox-20 and P0 transcripts by the treatment of IGF-I were prominently reduced by a pharmacological inhibition of Akt activation or a targeted knockdown of Akt expression but not by an inhibition of Erk activation, suggesting that Akt and its signaling pathway mediates Krox-20 activation and the myelination program that requires Krox-20.

Contact-dependent, juxtacrine signaling from axons is required for the activation of PI3K-Akt pathway in Schwann cells that initiate myelination (31, 32). To explore whether the impaired contact-dependent signaling in meltrin-β-deficient nerves causes the decreased phosphorylation of Akt in Schwann cells, we plated membrane fractions prepared from wild type and meltrin-β-deficient neuron cultures onto Schwann cells for 20 min and then measured the levels of phospho-Akt and phospho-Erk (Fig. 6C). As a result, the activation of PI3K-Akt pathway was consistently reduced with the meltrin-β-deficient membrane fraction, whereas it activated Erk similarly to the wild type membrane fraction. No significant difference was found between the effects of conditioned media prepared from wild type and meltrin-β-deficient neuron culture. The effects of conditioned medium and membrane preparation of primary nerve culture should represent the involvement of paracrine and juxtacrine signaling mechanisms, respectively. These results suggest that meltrin-β-deficient nerves are defective in presenting contact-dependent signaling from axons to Schwann cells that is required for the activation of PI3K-Akt pathway, the up-regulation of Krox-20, and the onset of myelination during regeneration.

Delayed Functional Recovery after Nerve Crush in Meltrin-β-Deficient Mice—Finally, we examined whether the retardation of remyelination after nerve crush affected functional recovery of the injured nerves in meltrin-β-deficient mice. Two types of behavior tests, toe-spreading and grip strength tests (25, 26, 33), were performed (Fig. 8). Rodents spread the toes of their hind feet on contact with a solid surface, which is a reflex that requires sensory innervation (25, 26, 33). The ability to spread the toes, therefore, is diminished after the crush injury and returns to normal as sensory axon reinnervation recovers.
Delayed Myelination in the Absence of an ADAM

FIGURE 8. Sciatic functional recovery after crush injury is delayed in meltrin-β-deficient mice. A, toe-spreading test of mice after sciatic nerve injury (see "Materials and Methods"). B, footprints of wild type (WT) and meltrin-β-deficient (KO) mice at the specified days after sciatic nerve injury. In the first column the toe-spread distance is indicated. C, recovery of toe spread after sciatic nerve crush. Retarded recovery is found in mutant mice. D, grip failures after sciatic nerve crush. All data are represented as means ± S.E. from five wild type mice and seven mutant mice. *, p < 0.05; **, p < 0.005 compared with wild type, Fischer’s PLSD test.

the toe-spreading measurement, wild type mice exhibited significant declines in the index of toe spreading immediately after sciatic injury and then recovered progressively from 7 to 21 days post-injury. In meltrin-β-deficient mice, the index began to recover at day 7 similarly to that in wild type mice. However, the recovery rate of the index was significantly slower, with a lag period of several days behind that of wild type mice. On the other hand, when injured in the hind limb, mice frequently fail to grip gridd wire with the injured leg until grip strength recovers. In this grip strength test, the number of failed trials until each mouse succeeds in gripping the grids with its injured leg is measured (25). The grip strength recovery of meltrin-β-deficient mice lagged significantly behind that of wild type mice 1 to 2 weeks after injury but by 3 weeks after injury had attained a level comparable with that of wild type mice. These results indicated that the sciatic functional recovery is delayed in meltrin-β-deficient mice.

DISCUSSION

Using the sciatic nerve crush as a well established paradigm of regenerating lesions, we showed here that meltrin-β (ADAM19) participates in efficient progression of nerve regeneration. Tissue regeneration recapitulates developmental programs in general, and PNS regeneration is preceded by Wallerian degeneration (27). This process involves degradation of injured axons and myelin sheath and removal of phagocytic debris by macrophages. Several proteases are involved in these early events. Membrane type-5 matrix metalloproteinase, for example, degrades the core proteins of chondroitin sulfate proteoglycans, which are abundant in neuronal tissues and inhibitory to axonal outgrowth (34, 35). In contrast, the early events after the crush injury including Wallerian degeneration and axon elongation were not affected in meltrin-β-deficient mice (Figs. 2 and 3). Instead, meltrin-β played roles in Schwann cell differentiation. Immature Schwann cells differentiate to myelinating cells via a promyelinating stage during which Schwann cells establish a one-to-one relationship with axons. The Schwann cells are arrested at the promyelinating stage in Krox-20 hypomorphic mice (Egr2<sub>Lo/Lo</sub>) and in mice deficient in Nab proteins that associate with Krox-20 (36, 37); expression of Oct-6 remains high in both of these mice. This indicates that Schwann cells pass through an Oct-6<sup>+</sup> transitional stage before entering the Krox-20<sup>+</sup> myelinating stage and that functional Krox-20 is required for myelination (38, 39). Analyses of meltrin-β-deficient nerves revealed that meltrin-β is involved in the efficient progression from the Oct-6<sup>+</sup> to the Krox-20<sup>+</sup> stage. Thus, delayed transition of Schwann cells from the Oct-6<sup>+</sup> promyelinating stage to the Krox-20<sup>+</sup>-myelinating stage in meltrin-β-deficient nerves could be caused by inefficient activation of Krox-20 in nerve regeneration.

Previous studies showed that the PI3K-Akt pathway is crucial for initiation of myelination, whereas those of Erks have an opposing effect on Schwann cells; that is, cell proliferation and/or the inhibition of myelination (7, 8). Moreover, contact-dependent, juxtacrine signaling from axons mediates the activation of PI3K-Akt pathway, but not the activation of Erk pathway, in Schwann cells that initiate myelination (31, 32). In this study we showed that activation of Akt is required for transcriptional activation of Krox-20 in Schwann cells. Thus, activation of Krox-20 is dependent on activation of Akt, and juxtacrine mechanisms mediate the activation of Akt pathway in Schwann cells during myelination. We, therefore, asked whether the inability of meltrin-β-deficient nerves to activate Krox-20 in Schwann cells could be due to an impaired activation of Akt and deficient juxtacrine signaling critical for the activation of Akt in the nerves. As a result, a significant decrease in Akt activation was observed in meltrin-β-deficient nerves during regeneration. Moreover, activation of Akt in Schwann cells with the membrane fraction prepared from meltrin-β-deficient nerves was less efficient than that with the membrane fraction prepared from wild type nerves (Fig. 6C). The effect of membrane preparation of primary nerve culture should represent the involvement of juxtacrine signaling mechanisms. These data suggest that meltrin-β is involved in the activation of juxtacrine signaling from the nerves that stimulates the Akt pathway in Schwann cells.
Genetic evidence suggests that the axon-derived juxtaocular signal that activates a PI3K-Akt pathway for myelination is dependent on NRG1 type III, an isoform of NRG1 containing two membrane-spanning regions (31, 32), and BACE1, a β-secretase that is involved in the production of β-amyloid (40). BACE1 is responsible for the ectodomain shedding of NRG1 type III and activates the Akt pathway for the axon myelination (41). The mechanism of how NRG1 type III activates Akt has not been elucidated.

In contrast to BACE1, meltrin-β does not enhance the cleavage of NRG1 type III but enhances the cleavage of NRG1 type I (16, 17). Although the cleavage of a single site in the ectodomain of NRG1 type III results in the generation of a growth factor as a form of type I membrane protein (42), the ectodomain shedding of NRG1 type I, an isoform sharing an ErbB ligand domain with NRG1 type III but containing a single membrane-spanning region, generates soluble ligands for ErbB that activates Erk. The soluble NRG1 not only plays a role in differentiation of immature Schwann cell precursors to the promyelins stage but also transforms myelinated Schwann cells into promyelins cells through the reverse activation of Oct-6 in the myelinated cells (28, 43). Thus, the progression of Schwann cell differentiation from promyelins to myelinating stages accompanies the transition of ErbB signaling from NRG1 type I to type III-dependent or from paracrine to juxtaocular mode. In contrast to tumor necrosis factor-α-converting enzyme, which is essential for phorbol ester-enhanced ectodomain shedding of NRG1 type I, meltrin-β is involved in its constitutive ectodomain shedding, leading to the depletion of soluble NRG1 to be secreted (18). Based on these genetic and biochemical analyses, we hypothesize that depletion of NRG1 type I with meltrin-β is required for the activation of NRG1 type III-dependent signaling, which proceeds Schwann cell differentiation from Erk/Oct-6 promyelins to Akt/Krox20-dependent myelination stage. Not only delayed activation of Krox-20 but also prolonged activation of Oct-6 in meltrin-β-deficient nerves during regeneration (Fig. 5B) support this hypothesis. Alternatively, meltrin-β may be involved in peri-membranous events that cooperate with NRG1 type III signaling during myelination. Domains of meltrin-β other than the protease domain may also play some roles in the myelination pathway. Further studies will elucidate the regulatory mechanisms by which meltrin-β activates Akt signaling required for myelination.

In summary, this study showed that meltrin-β is involved in Schwann cell differentiation and myelination of regenerating axons after crush injury. The impairment of remyelination in meltrin-β-deficient nerves was accomplished at a later time point after the crush. Roles of meltrin-β in nerve regeneration may be compensated by other proteases such as tumor necrosis factor-α-converting enzyme. Two types of behavior tests were used to confirm delayed functional recovery of PNS after the nerve crush in meltrin-β-deficient mice. These tests evaluate crucial aspects of locomotion involving recovery of hindlimb sensory and motor function (25, 26, 33). The functions of ADAM proteins including meltrin-β as modulators of growth factor signaling and cell-cell interactions in neural development and regeneration provide novel avenues for understanding development and maintenance of neural tissues.

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Delayed Myelination in the Absence of an ADAM

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