INTRODUCTION

Neuregulin-1 (NRG1) is an essential molecule in the development of peripheral nerves and neuromuscular system of the vertebrates. NRG1 in the axonal membrane regulates Schwann cell (SC) myelination during postnatal development [1-3]. In addition, the nrg1 gene encodes ARIA, Acetylcholine Receptor (AChR)-Inducing Activity, that plays a role in the development and maturation of the neuromuscular junction (NMJ) [4-6]. Signals downstream of membrane bound (NRG1-type III) or soluble NRG1 products are mediated by the receptor tyrosine kinase ErbB2. NRG1 binding to the heterodimeric ErbB2/ErbB3 triggers recruitment of several adaptors to transmit signaling cascades [7]. We have recently shown that Grb2-associated binder 1 (Gab1), a scaffolding protein, is required for NRG1-type III-induced peripheral nerve myelination [8].

The vertebrate neuromuscular junction (NMJ) is considered as a "tripartite synapse" consisting of a motor axon terminal, a muscle endplate, and terminal Schwann cells that envelope the motor axon terminal. The neuregulin 1 (NRG1)-ErbB2 signaling pathway plays an important role in the development of the NMJ. We previously showed that Grb2-associated binder 1 (Gab1), a scaffolding mediator of receptor tyrosine kinase signaling, is required for NRG1-induced peripheral nerve myelination. Here, we determined the role of Gab1 in the development of the NMJ using muscle-specific conditional Gab1 knockout mice. The mutant mice showed delayed postnatal maturation of the NMJ. Furthermore, the selective loss of the gab1 gene in terminal Schwann cells produced delayed synaptic elimination with abnormal morphology of the motor endplate, suggesting that Gab1 in both muscles and terminal Schwann cells is required for proper NMJ development. Gab1 in terminal Schwann cells appeared to regulate the number and process elongation of terminal Schwann cells during synaptic elimination. However, Gab2 knockout mice did not show any defects in the development of the NMJ. Considering the role of Gab1 in postnatal peripheral nerve myelination, our findings suggest that Gab1 is a pleiotropic and important component of NRG1 signals during postnatal development of the peripheral neuromuscular system.
a docking site for SH2-domain-containing signaling proteins such as SH2-containing protein-tyrosine phosphatase (Shp2) and phosphatidylinositol-3 kinase regulatory subunit p85 [9], thereby activating many signal transduction pathways in SCs for myelination. Previous studies employing knockout out (KO) mice indicate that Gab1 also appears to mediate NRG1-ErbB2 signaling in cardiovascular development [10]. In contrast to these developmental defects observed in Gab1-null mice, the mice null for Gab2, an isoform of Gab1, do not show any defects in organ development but exhibit limited abnormalities in mast cell degranulation and osteoclast activity [11-12], potentially suggesting that functions of Gab1 and 2 may be distinct in most cells.

The NMJ in vertebrates is a “tripartite synapse” consisting of a motor axon terminal, a muscle endplate, and terminal Schwann cells (tSCs) that envelope the motor axon terminal (Fig. 1A) [13]. NRG1 expressed in neurons and tSCs appears to contribute to NMJ development and maturation directly via NRG1/ErbB2 signaling in muscles [14] and indirectly via tSC [13, 15-16]. In par-

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**Fig. 1.** Abnormal postnatal development of NMJ in Gab1-MSKO mice. (A) Schematic diagram showing the tripartite neuromuscular synapse: tSC; terminal Schwann cell, AChR; acetylcholine receptor; (B) Western blot analysis shows the expression of Gab1 and Gab2 in the brain and skeletal muscles. (C) The endplate morphology of adult Gab1^+/+ and Gab1-MSKO mice (8–10 weeks old). Z-stack images of NMJ stained with α-bungarotoxin (α-BT) (green) show an AChR cluster that normally consists of 2–4 AChR islands (arrowheads). Arrows indicate fragmented AChR islands in Gab1-MSKO mice. NF; neurofilament stain. Scale bar=20 µm. (D, E) Quantitative results showing the number of AChR islands in a NMJ (D) and mean area of a NMJ (E, AChR area) in Gab1^+/+ and Gab1-MSKO mice at adulthood. (F) The endplate morphology of adult Gab2 HT and Gab2 KO mice at adulthood. Scale bar=20 µm. (G, H) Quantitative results showing the numbers of AChR islands (H) and areas (I) of NMJs in Gab2 HT and KO mice. Data are mean±SEM. *p<0.05, **p<0.01. NS, non-significant.
ticular, it was recently shown that axonal NRG1-type III signaling regulates synaptic elimination in NMJ development by modulating tSC activity [16, 17]. However, it is still unclear whether the signaling mechanism of NRG1-type III in tSCs for synaptic elimination functions through a pathway similar to that employed in SC myelination.

In this study, we examined the roles of Gab1 and Gab2 in the development of the NMJ using muscle-specific Gab1 KO and conventional Gab2 KO mice. Muscle-specific Gab1 null mice exhibited abnormal development of the NMJ. We also identified an important role for Gab1 in tSCs in synaptic elimination during postnatal development of the NMJ using SC-specific Gab1 conditional KO mice. However, the NMJ developed normally in Gab2 knockout mice. Thus, our data show that Gab1 plays a pleiotropic and non-redundant function in the NRG1-mediated development of the peripheral neuromuscular system.

MATERIALS AND METHODS

Materials

Antibodies against p75 neurotrophic receptor (Cat No: sc-6188) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to Gab1 (Cat No: 3232S), horseradish peroxidase (HRP)-linked anti-rabbit IgG (Cat No: 7074), HRP-linked anti-mouse IgG (Cat No: 7076S) were obtained from Cell Signaling Technology (Boston, MA, USA). Antibody against S100 (Cat No: Z0311) was obtained from Dako (Carpinteria, CA, USA). Anti-neurofilament (NF) antibody (Cat No: N4142) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorolink Cy3-labelled goat anti-rabbit (Cat No: PA43004) and anti-mouse IgG (Cat No: PA43002) were purchased from Amersham Biosciences/GE Healthcare (Piscataway, NJ, USA). Alexa Fluor 488-labeled donkey anti-rat (Cat No: A21208) and anti-rabbit IgG (Cat No: A21206), and Alexa Fluor 488 conjugated alpha-bungarotoxin (Cat No: B13422, α-BT) were purchased from Molecular Probes (Carlsbad, CA, USA). All other undesignated reagents were purchased from Sigma-Aldrich.

Animals

Genotyping for the Gab1 locus, myelin protein zero (MPZ)-Cre transgene, and Gab2 knockout mice were performed by PCR analysis of tail genomic DNA as we described previously [8]. Myogenic factor 5-Cre (Myf5-Cre) transgenic mice (B6.129S4-MyfStm3(cre) Sor/J; Stock No: 007893B6) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All surgical procedures were performed according to protocols approved by Dong-A University Committee on animal research, which follows the guidelines for animal experiments that were established by the Korean Academy of Medical Sciences.

Western blot analysis

For Western blot analysis, muscles were harvested and transferred to tubes containing lysing matrix beads and a lysis buffer [8]. Tubes were placed on TissueLyser LT (Qiagen) and then subjected to homogenization for 2–3 min. The lysates were centrifuged at 8,000 g for 10 min at 4°C, and the supernatant was collected. Protein (25–35 μg) was separated by SDS-PAGE, and then transferred onto a nitrocellulose membrane (Amersham Biosciences). After blocking with 0.1% Tween-20 and 5% nonfat dry milk in Tris buffered saline (pH 7.2, TBS) at room temperature for 1 hr, the membranes were incubated with primary antibodies (1:500–1,000) in TBST containing 2% nonfat dry milk at 4°C overnight. After three 15 min washes with TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:3,000) for 1 hr at room temperature. The signals were detected using the ECL system (ECL Advance kit, Amersham Biosciences).

Whole mount immunohistochemistry

Whole mount staining for NMJ analysis was performed according to the previous article with minor modifications [18]. Briefly, dissected hind-limbs (neonates) or extensor digitorum longus (EDL) muscles were fixed with 4% PFA, and EDL muscles were teased apart into four pieces under a stereomicroscope. Teased EDL muscle fibers were blocked with blocking buffer (5% BSA, 0.5% Triton X-100/PBS) and were then incubated with primary antibodies (S100 or NF) for 1–3 days at 4°C. EDL muscles were rinsed three times (2 hr per wash) with 0.5% Triton X-100 in PBS and were incubated with Alexa Fluor 488-conjugated α-BT (1:1,500), together with Cy3-labeled secondary antibodies (1:4,000 dilution), for 24 hr at 4°C. Samples were washed and whole-mounted on glass slides with mounting medium. Z serial sections were collected with a Zeiss confocal laser-scanning microscope (LSM700, Carl Zeiss) and projected into a single image. AChR staining with α-BT reveals the typical pretzel-like fluorescent cluster which normally consists of two or four AChR islands or strips. The area of each AChR cluster (an endplate) was determined by measuring the total areas of AChR islands in a cluster using Image J software. At least 200 stacked NMJ images were captured from 3–6 animals for analyzing AChR morphology.

Polyinnervation is observed in the immature state, wherein NMJs are innervated by more than one motor axon [19]. To count the polyinnervated endplates, at least 100 stacked NMJ images were captured from 3–6 animals at P10 and in adulthood. To count tSCs at NMJ, Hoechst staining was used in combination with...
S100 labeling to mark tSCs at NMJs in EDL muscles at P10 and approximately 100 stacked images were used (n=3).

**Primary Schwann cell culture**

Primary SCs were isolated from the sciatic nerves of 4–5 day old Sprague-Dawley pups as we previously reported [8]. When the cell density reached 70% confluency, lentiviral particles expressing Gab1-shRNAi or scrambled shRNAi were administrated at various concentrations for 18–24 hr. Then, the medium was replaced with fresh DMEM containing 10% FBS and cultured for another 24 hr. The medium was then replaced with the growing medium (DMEM containing 1% FBS, NRG1 (30 ng/ml), forskolin (5 µM)) for one day and the morphology of SCs was analyzed following immunostaining against SC markers such as S100 or p75 neurotrophin receptor (p75). The length of SCs was measured from captured image using Image J software. Approximately 200 SCs from each of shRNAi-infected SCs (n=3) were analyzed.

To analyze the role of Gab1 in SC proliferation, BrdU incorporation assay were performed. Scrambled or Gab1-shRNAi lentivirus-infected SCs were plated on PDL-coated 96 well plates at a density of 8×10⁴ cells/well, and cells were cultured overnight in DMEM containing 1% FBS and 50 ng/ml NRG1. For the BrdU assay, infected SCs were plated on a coverslip coated with PDL at a density of 1×10⁵ cells/well and cultured in DMEM containing 0.5% FBS and 200 ng/ml NRG overnight. Cells were incubated with 20 µM BrdU for 6 hr and then fixed with 4% PFA for 10 min. BrdU immunostaining was performed as we described previously [20], and the number of BrdU-positive cells per coverslip was counted and three independent experiments were performed for quantitative analysis.

**Statistical analysis**

Statistical analysis was performed by using GraphPad Prism software (GraphPad, San Diego, CA). p-values were from Student’s two-tailed test, and results were expressed as mean and SEM.

**RESULTS**

**Abnormal endplate morphology in Gab1-MSKO mice**

In Gab1(-/-) embryos, the migration of muscle precursor cells into limbs is hampered, and this genotype is embryonic lethal [21], precluding the determination of the role of Gab1 in the postnatal development of the NMJ. To characterize this process, skeletal muscle-specific Gab1 conditional knockout (Gab1-MSKO) mice were established by removing floxed Gab1 (Gab1f/f) with Cre recombinase under the myogenic factor 5 (Myf5) promoter, which begins to be active at approximately E10 [22]. Gab2 KO mice, that is viable [23], were also used to examine the role of Gab2 in postnatal NMJ development. Gab1-MSKO mice showed smaller body size compared to the control mice (Gab1f/f) at birth and it was maintained throughout postnatal life as we recently reported [24]. Western blot analysis showed that the expression levels of Gab1 in skeletal muscles, but not in the brain, were significantly reduced in adult Gab1-MSKO mice compared to their control mice and that the expression of Gab2 both in the brain and skeletal muscles was not detected in Gab2 KO mice (Fig. 1B). The low levels of Gab1 expression in the skeletal muscles of Gab1-MSKO mice might represent the expression of Gab1 in non-muscle tissues such as connective tissue coverings and vessels within muscles.

We next examined the role of muscular Gab1 and Gab2 in NMJ development by analyzing the morphological characteristics of AChR clustering using fluorescein-conjugated α-BT. EDL muscles from adult Gab1f/f and Gab1-MSKO mice (8–10 weeks old) were whole-mount stained with α-BT and an anti-neurofilament antibody to label motor nerve terminals. As shown in Fig. 1C, normal AChR staining in Gab1f/f mice revealed the typical pretzel-like endplate structure (a NMJ, an AChR cluster) that contains two to four AChR islands [25], whereas AChR clustering in Gab1-MSKO mice exhibited abnormally fragmented endplate morphology with a higher number of α-BT-labeled AChR islands in a cluster (2.7±0.06 vs. 6.6±0.19, p<0.01) (Fig. 1C, D). We also measured the area of α-BT-labeled clusters, and found that the mean area of AChR clusters in Gab1-MSKO mice was smaller than that of Gab1f/f mice (284.4±9.6 µm² vs. 352.1±5.7 µm², p<0.01) (Fig. 1E). In contrast, the number of islands and the mean area of AChR clusters in Gab2 KO mice were not different from those of Gab2 (+/-) mice (Fig. 1G–I).

**A delay in postnatal developmental maturation of the endplate in Gab1-MSKO mice**

We investigated the developmental maturation of AChR clusters in Gab1f/f and Gab1-MSKO mice by classifying the maturation of endplate morphology at P1 and P14. Based on previous reports [19, 26], the maturation of endplate morphology was classified into ovoid plaque, perforated and branched morphologies in the order of maturation and the branched form shows AChR islands in a cluster (Fig. 2A). At P1, approximately 75% and 25% of endplates were ovoid plaque and perforated types, respectively, in control mice, whereas in Gab1-MSKO mice, only 10% of endplates showed perforated morphology (Fig. 2B, C). At P14, most endplates (~60%) belonged to perforated type morphology in control mice. However, in Gab1-MSKO mice, there was a marked delay in the maturation of endplate morphology, with the immature ovoid plaque type being the most frequent type of endplate morphology.
even at P14 (Fig. 2B, D).

**Gab1 in terminal Schwann cells is required for synaptic elimination in neuromuscular junction development**

An immature NMJ is innervated by more than one motor axon (polyneuronal innervation), and synaptic elimination during the second postnatal maturation period results in mononeuronal innervation in adulthood [13]. It has been believed that tSCs play an essential role in synaptic elimination [13], possibly via receiving axonal NRG1-type III signals [16]. To determine the role of Gab1 in tSCs in synaptic elimination, we thus examined the polyinnervation states of motor endplates in mice in which the gab1 gene was excised in tSCs using MPZ-cre transgenic (MPZ is consistently expressed in tSCs [27]) (Gab1-SCKO) during postnatal development. We immunostained using an anti-neurofilament (NF) antibody and α-BT in the EDL muscle to count NMJs containing more than two axons. At P10, 8.25% and 17.54% of motor endplates were polyneuronally innervated in control and Gab1-SCKO mice, respectively (p<0.05, n=3) (Fig. 3A, C). In adults, synaptic elimination was completed in control mice (fewer than 1% were polyneuronally innervated) whereas 8.07% of endplates in Gab1-SCKO mice still received polyneuronal innervation (p<0.01, n=3) (Fig. 3B, C).

Because synaptic elimination affects the maturation of endplate morphology [13], we analyzed the morphology of endplates in adult Gab1-SCKO mice. We found that postsynaptic AChR aggregates were fragmented into small islands, a pattern similar to that found in adult Gab1-MSKO mice (Fig. 3D). However, the mean area of AChR clusters was not significantly different between control and Gab1-SCKO mice (Fig. 3E). Finally, we investigated the developmental maturation of AChR clusters in Gab1^f/f and Gab1-SCKO mice at P14 as we done in Gab1-MSKO

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**Fig. 2.** Delayed neuromuscular synapse maturation in Gab1-MSKO mice. (A) Schematic drawing showing the postnatal maturation of the endplate morphology from ovoid to branched type. (B) Representative confocal micrographs showing the endplate morphology of Gab1^f/f and Gab1-MSKO mice at P1 and P14. Single arrows indicate ovoid morphology, whereas double arrows indicate perforated or branched morphology. (C, D) Quantitative analysis of endplate maturation in Gab1^f/f and Gab1-MSKO mice at P1 (C) and P14 (D) (n=3). Scale bar=20 µm. Data are mean±SEM. *p<0.05, **p<0.01. NS, non-significant.
analysis. Approximately 20% of endplates belonged to immature oval type morphology in control mice whereas ~50% of endplates remained as oval type in Gab1-SCKO mice (Fig. 3F). Thus, Gab1 deficiency in tSCs resulted in abnormal development not only in motor innervation but also in endplate maturation.

**Gab1 regulates proliferation and process formation in Schwann cells**

It was previously shown that synaptic elimination by tSCs requires an adequate number of tSCs and tSC process elongation [13, 16, 26]. Furthermore, it has been reported that the number of tSCs during postnatal development would be regulated by axonal NRG1-type III [16]. We thus counted the number of tSCs in end-
plates of Gab1-SCKO mice at P10 after triple fluorescent staining with an anti-S100 antibody, α-BX and Hoechst staining. The mean number of tSCs in endplates of EDL muscle was 3.1 at P10 in control mice, whereas it decreased to 2.54 (p<0.01) in Gab1-SCKO mice (Fig. 4A, B), suggesting that Gab1 is required for the generation of adequate number of tSCs during postnatal development.

To examine whether Gab1 is required for the proliferation of NRG1-induced SCs, we cultured primary rat SCs and investigated the effect of Gab1 silencing in NRG1-induced SCs proliferation using BrdU incorporation analysis. One day after lentiviral Gab1 shRNAi or scrambled shRNAi infection, cells were cultured in the presence of recombinant NRG1 (200 ng/ml) for one day and then proliferating cells were determined using BrdU incorporation assay. We found that the number of BrdU-positive cells was significantly reduced in Gab1-silenced primary SCs, compared to scrambled shRNAi-infected SCs (Fig. 4C, D), suggesting the implication of Gab1 in NRG1-induced SC proliferation.

We next investigated whether Gab1 mediates the process-forming activity of NRG1 in cultured SCs. To address this question, primary SCs, that were infected with Gab1 or scrambled shRNAi lentivirus, were grown in the presence of NRG1 for one day, and the length of SCs was measured after immunostaining against a

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**Fig. 4.** Gab1 is required for SC proliferation and process elongation. (A) Representative confocal micrographs showing tSC nuclei in NMJs of EDL muscle of Gab1<sup>f/f</sup> and Gab1-SCKO mice at P10. S100 was labeled for tSC, and note the decreased number of tSC nuclei in Gab1-SCKO mice compared to the control. Scale bar=20 µm. (B) Quantitative result showing the mean number of tSCs/endplate at P10. (C~F) Primary rat SCs were infected with lentiviral Gab1 or scrambled (Scr) shRNAi, and silencing of Gab1 was confirmed by Western blot analysis (C). (D) Quantitative result of BrdU incorporation assay following Gab1 silencing (n=3). Lentivirus-infected SCs were cultured for one day in the presence of NRG1 (200 ng/ml) and then BrdU incorporation assay was performed. (E) Representative confocal micrographs showing the morphology of cultured primary SCs after Gab1 lentiviral shRNAi or scrambled shRNAi infection. SCs were cultured for one day in the presence of NRG1 (200 ng/ml) immunostained for p75 neurotrophin receptor (p75) which is a marker for cultured SCs. (F) Quantitative results showing the length of cultured primary SCs which was measured after anti-p75 immunostaining. Scale bar=20 µm. Data are shown as the mean±SEM. *p<0.05, **p<0.01. NS, non-significant.
SC marker protein, p75. Gab1-silenced SCs exhibited shorter processes compared to control cells which were infected with scrambled shRNAi (Fig. 4E, F). Together, our results indicate that proper development of tSCs via Gab1 may regulates the rate of redundant motor axon pruning at developing NMJ.

DISCUSSION

Although it has long been suggested that trans-synaptic signaling by NRG1 regulates AChR expression and early NMJ development in skeletal muscle [28–29], recent studies have revealed that the initial development of the AChR clusters in endplates was not significantly affected by direct NRG1 signaling to muscle [15, 30]. However, it appears that the trans-synaptic NRG1 signaling is required for the synaptic maturation and maintenance. First, it was reported that the amplitude of synaptic miniature endplate potentials and AChR density were mildly reduced in muscle-specific ErbB2/ErbB4-null mice [15]. In addition, Schmidt et al. [14], suggest that trans-synaptic NRG1 signaling is important in stabilizing AChR during postnatal maturation. They showed that NRG1 maintains synaptic AChR clusters by promoting the anchoring of AChR to the synaptic membrane via muscular ErbB2-dependent tyrosine phosphorylation of alpha-dystrobrevin, a component of the dystrophin complex. We found that muscle-specific knockout of the gab1 gene resulted in fragmented and small AChR clusters in adulthood as well as a delay in the maturation of the endplate. Although the mechanism by which Gab1 regulates endplate maturation is unknown at present, it may be possible that NRG1-dependent Gab1 phosphorylation in muscles may regulate AChR stability during postnatal development. Further studies on the possible role of Gab1 as a scaffold protein in AChR recycling and alpha-dystrobrevin signaling induced by NRG1 may provide a mechanistic insight into the role of Gab1 in endplate maturation.

On the other hand, recent studies have demonstrated a critical role for tSCs in synaptic elimination in polyinnervated motor synapses during postnatal development. During early postnatal stages, tSCs extend their processes between the nerve terminals and developing AChR clusters, rendering some postsynaptic AChRs inactive due to the loss of contact with the nerve terminal [13, 26]. These changes result in the pruning of the inactive motor terminal, i.e., synaptic elimination, as well as changing the AChR clustering pattern from plaque to pretzel-like. It has recently been shown that manipulations of axonal NRG1-type III signaling alter the rate of synapse elimination at developing NMJs through tSC [16]. For example, transgenic mice over-expressing NRG1-type III or NRG1(+/-) mice exhibited enhanced and delayed synaptic elimination, respectively. This is consistent with findings that forced ErbB2 activation in tSCs or the exposure of tSCs to high levels of NRG1-type III both produced increases in synapse-modifying activities such as extending processes [16, 17, 26]. Thus, it is believed that tSCs are primary targets of axonal NRG1-type III for synaptic elimination at NMJs during postnatal development. Our findings provide important in vivo evidence not only for the involvement of Gab1 in tSC-mediated synaptic elimination but also to support the role of NRG1-type III in postnatal synaptic remodeling through tSCs. In particular, we show here that Gab1 in SCs regulated proliferation and process formation, two putative mechanisms of synaptic pruning by tSCs [13, 16, 26]. Therefore, identifying the downstream signaling of Gab1 in tSCs will be the next step in understanding the molecular mechanism of synaptic elimination in the NMJ.

Considering the role of NRG1 in peripheral axons in postnatal myelination through Gab1 [8], the present study reveal that NRG1-type III-Gab1 signaling has at least two independent functions, myelination and synaptic elimination through SCs during postnatal development. Further studies on the mechanistic aspects underlying Gab1 function will provide important insights into the understanding of postnatal development of peripheral nerves and NMJ.

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Gab1 in Neuromuscular Junction

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www.enjournal.org 149
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