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ApoER2 and Reelin are expressed in regenerating peripheral nerve and regulate Schwann cell migration by activating the Rac1 GEF protein, Tiam1

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ABSTRACT

ApoER2 and its ligand Reelin participate in neuronal migration during development. Upon receptor binding, Reelin induces the proteolytic processing of ApoER2 as well as the activation of signaling pathway, including small Rho GTPases. Besides its presence in the central nervous system (CNS), Reelin is also secreted by Schwann cells (SCs), the glial cells of the peripheral nervous system (PNS). Reelin deficient mice (reeler) show decreased axonal regeneration in the PNS; however neither the presence of ApoER2 nor the role of the Reelin signaling pathway in the PNS have been evaluated. Interestingly SC migration occurs during PNS development and during injury-induced regeneration and involves activation of small Rho GTPases. Thus, Reelin–ApoER2 might regulate SC migration during axon regeneration in the PNS. Here we demonstrate the presence of ApoER2 in PNS. After sciatic nerve injury Reelin was induced and its receptor ApoER2 was proteolytically processed. In vitro, SCs express both Reelin and ApoER2 and Reelin induces SC migration. To elucidate the molecular mechanism underlying Reelin-dependent SC migration, we examined the involvement of Rac1, a conspicuous small GTPase family member. FRET experiments revealed that Reelin activates Rac1 at the leading edge of SCs. In addition, Tiam1, a major Rac1-specific GEF was required for Reelin-induced SC migration. Moreover, Reelin-induced SC migration was decreased after suppression of the polarity protein PAR3, consistent with its association to Tiam1. Even more interesting, we demonstrated that PAR3 binds preferentially to the full-length cytoplasmic tail of ApoER2 corresponding to the splice-variant containing the exon 19 that encodes a proline-rich insert and that ApoER2 was required for SC migration. Our study reveals a novel function for Reelin/ApoER2 in PNS, inducing cell migration of SCs, a process relevant for PNS development and regeneration.

1. Introduction

Reelin, a large extracellular matrix protein, plays a relevant role in the central nervous system (CNS), where it guides migration of neurons during development and is essential for synaptic plasticity and corticogenesis (Hiesberger et al., 1999; Weeber et al., 2002). Reelin also has been found in the PNS (Panteri et al., 2006) and has been implicated in axonal regeneration after nerve damage (Lorenzetto et al., 2008).

Reelin binds to the endocytic receptors ApoER2 and VLDL-R, inducing 1) the proteolytic processing of the receptor favoring its downregulation (Hoe et al., 2006; Larios and Marzolo, 2012) and 2) the recruitment of the adaptor protein, Dab1 which is phosphorylated by the SRC-family kinase Fyn (Ballif et al., 2003; Hiesberger et al., 1999). Subsequently, a variety of proteins involved in different cellular processes are activated, including PI3K. Among the main downstream targets of PI3K are the small GTPases of the Rho family (Courtney et al., 2010). These proteins are pivotal regulators of the actin cytoskeleton since control key process required for cell migration. Rho GTPases are active in the GTP-bound form and inactive GDP bound form. The cycle between active and inactive status is regulated by other specific group of proteins, the GEFs, including GAPs and GDIs. GEFs control the release

Abbreviations: ApoER2, apolipoprotein E receptor 2; VLDL-R, very low density lipoprotein receptor; GEFs, guanine nucleotide exchange factors; GAPs, GTPase activating proteins; DAI, Days after Injury; Dock7, Dedicator of cytokinesis 4; PAR3, Partitioning defective 3 homolog; Tiam1, T-cell lymphoma invasion and metastasis 1; JNK, c-Jun. N-terminal kinase; MMP, metalloproteinase; NRG1, Neuregulin 1; PBS, phosphate buffered saline; PNS, peripheral nervous system; PAK-CRIB, p21 activated kinase 1 (PAK)-Cdc42/Rac interactive binding (CRIB); FRET, Förster resonance energy transfer; RAP, Receptor-associated protein; SC, Schwann cell.

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of GDP to GTP, while GAPs regulate the GTPases ability to hydrolyze GTP and GDIs act as inhibitors of GDP dissociation (Etienne-Manneville and Hall, 2002).

The migration of SCs has a fundamental role in the process of axonal regeneration in the PNS. It has been well documented that after a nerve injury, axons distal to the damage site degenerate through a process known as Wallerian degeneration (Gaudet et al., 2011). As a result, SCs lose contact with the axon and experience morphological and genetic changes through cell dedifferentiation, thus, acquire the capacity to proliferate, migrate and myelinate injured axons. Several lines of evidence have highlighted the importance of Rho GTPases in SC during development and regeneration. For example, the neurotrophin NT3 stimulates cell migration through TrkC and in a process dependent of Rho GTPases Rac1 and Cdc42 (Yamauchi et al., 2003). In addition, the specific GEF of Rac1 Tiam1 is activated by the small GTPase Ras (Yamauchi et al., 2005b). Additional evidence shows that Rac1 and Cdc42 together c-Jun. N-terminal kinase positively regulate migration through NRG1 and Erb2/3 receptors (Yamauchi et al., 2008).

Reelin also induces actin cytoskeleton rearrangements in neurons activating Cdc42. Through ApoER2, Reelin induces filopodia formation and increases growth cone motility (Leemhuis et al., 2010). Despite detailed information about the participation of Reelin in neuronal migration, it is unknown if Reelin could activate Rho GTPases in SCs promoting changes of cytoskeleton required for cell migration. In this work, we presented evidence that Reelin, through the GEF Tiam1, activates Rac1 and stimulates migration in SCs. In addition, we showed that migration induced by Reelin requires ApoER2. These effects could be important after PNS injury for efficient axonal regeneration and functional recovery and might represent novel targets for therapeutic applications.

2. Material and methods

2.1. IF for nerve explants

Adult (8 weeks) C57BL/6 mice were obtained from the Animal Facility of the Biological Sciences Faculty of the Pontifical Catholic University of Chile. The animals were kept under controlled temperature (22–24 °C) and light cycle. Water and pelleted food were supplied ad libitum. Animals were killed by cervical dislocation and sciatic nerve extracted and collected in cold PBS. Nerve explants were fixed by immersion in 4% paraformaldehyde in 0.1 M pH 7.4 PBS (PBS 1X) for 1 h. Then the samples were washed three times, 10 min each, in 1X PBS, immersed in sucrose gradient (5%, 10%, 20% in 1X PBS, 1 h each step), and then embedded in OCT (Sakura Finetek). Once in OCT, samples were cut transversely/longitudinally and mounted on Superfrost Plus slides (Thermo Fisher Scientific). Sections were washed twice, 10 min each, in PBS 1X and then blocked/permeabilized in 0.1% Triton X-100, 5% fish skin gelatin (Sigma-Aldrich) in PBS 1X for 1 h at RT. Then, sections were incubated at 4 °C with primary antibodies (rabbit polyclonal that recognizes the cytoplasmic region of all variants of ApoER2 (Fuentealba et al., 2007) (ApoER2-total), preimmune serum or anti-Neurofilament M in blocking/permeabilizing solution for 12 h, washed three times in PBS 1X, 10 min each, and incubated with secondary antibodies at room temperature for 2 h. Finally, samples were washed in PBS 1X three times, 10 min each, and mounted in Vectashield (Vector Laboratories) containing Hoescht.

2.2. Sciatic nerve injury

Adult mice (9 to 12 weeks) were anesthetized using intraperitoneal tribromoethanol. Using aseptic technique, the right sciatic nerve was exposed at the sciatic notch and crushed 3 times, 5 s each, with fine forceps. The left sciatic nerve was also exposed at the sciatic notch but used as sham control. The skin was closed with metal sutures and mice were resuscitated by warming and then returned to their cages. Mice were killed after 3 or 7 days and 3 equally long segments (60 mm aprox) were collected with respect to the injured zone: proximal, injury and distal segment. Contralateral nerve was collected as a control. Immunofluorescence was carried out as described above.

2.3. Cell culture

Primary SCs were obtained from sciatic nerves of rats at postnatal day 2. Briefly, sciatic nerves were dissected and cells dissociated using collagenase (Sigma type I) and trypsin 0.25% (Gibco) in PBS. After a centrifugation to remove the enzymes, cells were resuspended in low glucose DMEM medium supplemented with 10% heat-inactivated FBS and 100 U/ml of penicillin/streptomycin and the fibers were dissociated using a Pasteur pipette. Cells were plated in T25 bottle treated with laminin (400 μg/ml, Sigma) for 2 h and maintained in incubator (37 °C and 5% CO_2) with the medium describe above. After one day, the cells were treated with cytosine arabinoside (AraC, Sigma) for 48 h to suppress fibroblast growth. The cells were then cultured in low glucose DMEM as was described above supplemented with forskolin (2 μM) (SIGMA) and bovine pituitary extract (5 μg/ml) (GIBCO). After cells reached about 70% of confluence, the remaining fibroblasts were eliminated using complement-mediated lysis directed against the fibroblast antigen using Thy-1 antibody (clone T11D7e, Serotec, USA) and rabbit complement (SIGMA). Primary cells were used until passage 6. Our cultures consisted of 95% of Schwann cells, as determined by immunofluorescence for S100, a specific Schwann cell marker (DAKO, Denmark A/S). Immortalized Adult Schwann (IFRS1) cells were a gift of Dr. Kazuhiko Watabe (Tokyo Metropolitan Institute of Medical Science, Japan) and were used in some of the co-immunoprecipitation experiments. In some migration experiments, primary rat Schwann cells from ScienCell (#R1700, CARLSBAD, CA) were used, which were appropriately characterized for ApoER2/Reelin signaling pathway expression. These cells were cultured using the same conditions mentioned above and used until passage 4. Both, sciatic nerve injury and primary SC culture protocols were approved by the Bioethical Committee of our Faculty.

2.4. Production of Reelin

Recombinant mouse Reelin was obtained from HEK293 cells stably expressing the full-length protein according to Sotelo et al. (2014). Briefly, cells were cultured until 80% confluent in high glucose DMEM with 10% FBS containing penicillin and streptomycin and 0.5 mg/ml of G418 at 37 °C. After washing two times with PBS, the cells were cultured in DMEM without serum for 3 days. Every day the cell medium was collected and centrifuged at 1000 g for 5 min, and the supernatant was stored at 4 °C. The collected medium was concentrated using Amicon ultra-15 centrifugal filter units (filter membrane 100 kDa).

2.5. Cell migration assays

Cell migration was studied by using 12-well Boyden chambers (BD Biosciences). Briefly, polyethylene terephthalate (8 μm pore) filters were coated with laminin (400 ng/ml) for two hours and washed with sterile H_2O. Then, SCs (1×10^6) in 200 μl of serum free medium were loaded into the upper chambers, which were inserted into the tissue-culture wells. The wells were filled with 500 μl of media containing Reelin or control media. In some experiments we use recombinant mouse Reelin (R&D System (Minneapolis, MN) or vehicle. After incubation at 37 °C for 12 h, the filters were stained with 0.2% crystal violet/methanol (10%) for 15 min and then washed. The non-migrating cells were removed using cotton tipped applicator and the migrating cells at the bottom of the filters were counted under Olympus CKX41 microscope at 20× magnification. Migration values were determined by counting different fields (20×) per filter under microscope. Pictures

were taken using a MEM1300R USB digital microscope camera (Future-Optics, China).

2.6. Pull-down assay for Rac1

The activation of Rac1 by Reelin was monitored with a pull-down assay using PAK-CRIB fusion protein generously provided by Dr. Gregg Gundersen (Columbia University, New York, NY). GST fusion proteins (50 μg) and glutathione-agarose beads were incubated at 4 °C for 4 h. Cells were treated with Reelin o control media for 20 or 40 min washed with chilled PBS and the extracts prepared using lysis buffer (50 mM Tris/HCl, pH 7.5, 10 mM EGTA, 50 mM NaCl, Triton X-100, Tween-20 and protease and phosphatase inhibitors). Then, cell extracts were incubated with the beads at 4 °C for 4 h. The beads were washed five times in pull-down buffer, boiled in 2X sample buffer, separated by SDS-PAGE and immunoblotted with anti-Rac1 antibody (Cytoskeleton, Inc.) to monitor total Rac1 and GTP-bound (activated) Rac1.

2.7. GST-pull down assay

GST-fusion proteins (GST, GST-ApoER2T-Full-length, GST-ApoER2T-deltaPro) were prepared as described (Cuitino et al., 2005; Sotelo et al., 2014). Fusion proteins were expressed in *Escherichia coli* (BL21) and lysed with PBS, 1% Triton X-100, 10 mM EDTA and 1X Complete protease inhibitor mixture. All the purified proteins were sequentially dialyzed against PBS, 250 mM NaCl 50 mm Tris–HCl pH 8.0 and 5 0 mM Tris–HCl pH 8.0. HEK293 cells were transfected with the plasmids for PAR3 or Dab2-GFP, using calcium phosphate method; cell extracts were prepared lysing the cells with HUNT buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1X Complete protease inhibitor mixture); the cell extracts were incubated with GST fusion proteins and bound to glutathione agarose beads for 2 h at 4 °C. Beads were washed three times in HUNT buffer, boiled in 2X sample buffer and separated by SDS-PAGE. The presence of PAR3 or Dab2 associated to the GST proteins was determined by Western blot, using the anti-PAR3 or the anti-GFP polyclonal antibodies as required.

2.8. FRET analyses

Raichu-Rac1 biosensor was used for FRET experiments (Itoh et al., 2002). The Rac1 activation induces a conformational change of the probe resulting in energy transfer from CFP emission energy to YFP excitation energy, which is measured as FRET efficiency (Itoh et al., 2002). Cells (2×10^6) were transfected with the biosensor plasmid (4 μg) by electroporation (Amaxa) and plated on laminin coverslips. After 24 h of expression, recombinant Reelin (1 μg/ml) o vehicle was added for 10 and 20 min. The reaction was stopped washing the cells with chilled PBS and fixed with 4% paraformaldehyde/Sucrose for 10 min. After washing coverslips were mounted on glass slides with Fluoromount™.

(Sigma-Aldrich). FRET images were acquired in a DSU microscope (Olympus). For emission ratio imaging, the following filter set were used: CFP: 490/500 HQ, DM505, 515/560HQ; FRET: 490/500 HQ, DM505, 527/565HQ. FRET map images were calculated by Ratio imaging using ImageJ software using the following formula: (CFP emission/YFP emission, both exited at the same excitation wavelength (CFP). Before FRET map calculation, images were background subtracted and corrected for alignment channel. An average of 30 cells per condition was considered for FRET analyses.

2.9. Co-immunoprecipitacion assay

IFRS1 cells (2×10^6) were transfected by electroporation with DNA constructs encoding Tiam1-CA1199-HA (Meseke et al., 2013b) and PAR3 or ApoER2-Full-length-FLAG, Rap and PAR3 (10 μg in total). After 48 h of expression, cells were washed twice in ice-cold PBS and lysed in ice-cold immunoprecipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1.0% Triton X-100, 10 mM EDTA and protease inhibitors). Specific antibodies (Rabbit-PAR3 (Millipore 07-330) or mouse-HA) (Fuentealba et al., 2007) and Protein A-Sepharose beads were incubated for 4 h at 4 °C. After washing, the beads-antibody complex was incubated with the lysates for 2 h. Finally, the samples were suspended in sample buffer, resolved by SDS-PAGE, and transferred to Immobilon PVDF membrane (Millipore). The co-precipitating levels of proteins were visualized by Western blotting using a mouse anti-FLAG (Clontech) or Anti-PAR3 antibodies.

2.10. Western blotting

Cells lysates were prepared using lysis buffer (50 mM Tris, pH 7.4, 2 mM EDTA, 1% PMSF, 1% Triton X-100 and protease inhibitors). The proteins in the cell lysates were denatured and then separated on SDS-PAGE gels. The electrophoretically separated proteins were transferred to nitrocellulose membranes, blocked with 5% no-fat milk, and immunoblotted with the respective antibodies. The bound antibodies were detected by using the enhanced chemiluminescence (ECL) system (Amersham Biosciences). To determine activation of Reelin signaling in Schwann cells the antibodies were mouse monoclonal anti-actin (MAB1501R, Chemicon), rabbit polyclonal anti-AKT antibody (#9272, Cell Signaling) and rabbit monoclonal anti-phosphorylated AKT antibody (#4060, Cell Signaling).

2.11. cDNA synthesis and analysis of ApoER2 expression by conventional RT-PCR

Total RNA was extracted from cells using RNA-SOLV reactive (OMEGA, Biotech). The extracted RNA was quantified by spectrophotometry at 260-nm optical density in a NanoDrop (ND-1000) Spectrophotometer (NanoDrop Technologies, Rockland, DE). Total RNA was isolated using RNA-Solv and following the manufacturer instructions. RNA (1 μg) was first digested by DNAse I and then subjected to reverse transcription in a 20 μl reaction mixture using random primers and RevertAidTM MMuLV Reverse Transcriptase in the presence of RNAseOUT. The resulting cDNA was used for qPCR. Primers for ApoER2, Reelin and Dab1 were designed for optimal performance using the Net primer free software from PREMIER Biosoft International (Table S1, supplementary information).

2.12. Small interfering RNA (siRNA) and constructs transfection

Rat Tiam1 specific siRNA (ON-TARGETplus SMARTpool) and Rat ApoER2 specific siRNA (siGENOME SMART Pool) were purchased from Dharmacon (Thermo Scientific). Rat PAR3 specific siRNA was a gift from Dr. Lissette Leyton (Universidad de Chile). Briefly, Schwann cells (R1700) (2×10^6) were transfected with Tiam1-siRNA (50 nM), ApoER2 (50 nM) or PAR3 (500 nM) siRNA or control-siRNA by electroporation using the Primary Mammalian Neurons kit (Amaxa) with the pulse code CA138. After electroporation cells were plated on culture dishes treated with polylisine (10 μg/ml). For Boyden chamber migration and respective WB experiments, cells were used after 24 h of expression (Tiam1 or ApoER2) and 48 h (PAR3) and plated on laminin treated filters.

3. Results

3.1. Reelin and ApoER2 are expressed in mouse sciatic nerve

Mice devoid of Reelin (reeler mice) present defects in PNS regeneration (Lorenzetto et al., 2008). Therefore, we decided to study the expression of the Reelin receptor ApoER2 in rodent PNS. By Western blot we detected a predominant band corresponding to the full-length version of ApoER2 (Fig. 1A). By immunofluorescence, performed on

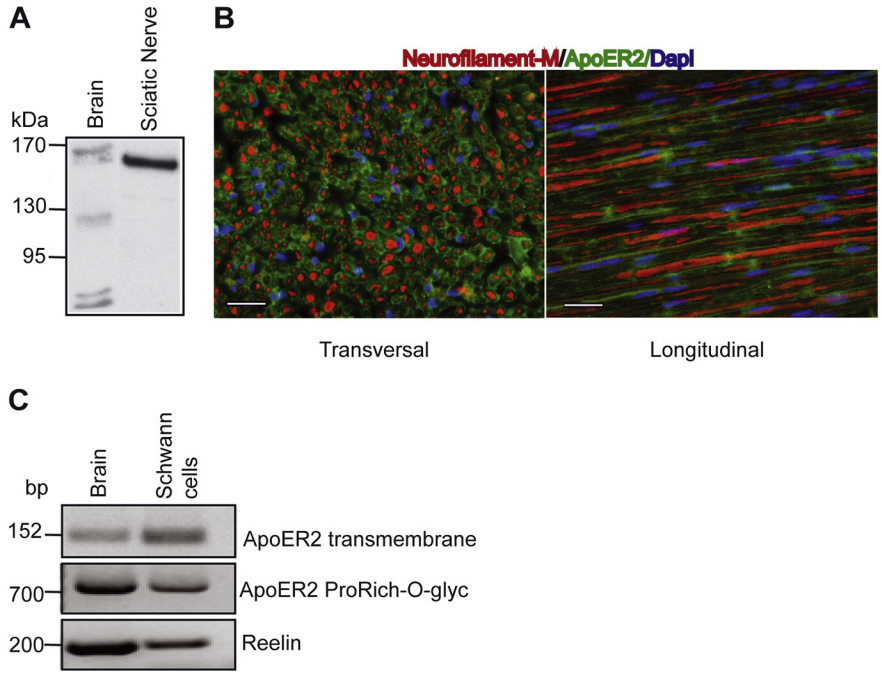

Fig. 1. Expression of ApoER2 in peripheral nervous system. (A) The expression of ApoER2 was determined in mouse sciatic nerve (60 μg of total tissue extract) and compared to a positive control (20 μg of brain homogenate). (B). Immunofluorescence on cross (left) and longitudinal (right) sections of mouse sciatic nerve using anti-ApoER2-total antibody (green). Neurofilament staining was used as neuronal marker (red). Nuclei were counterstained with Hoechst. Scale bars, 25 μm). (C) Detection by PCR. The transmembrane domain of ApoER2 common to all isoforms was detected with specific primers. To detect the expression of variant containing a proline-rich insert and O-glycosylation domain, primers for both variants were combined. Reelin was detected with specific primers. Sizes are indicated on the left. Mouse brain was used as positive control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

longitudinal and transverse sections of adult mouse sciatic nerve, the presence of ApoER2 was also detected (Fig. 1B). Importantly, the immunoreactivity for the receptor was not associated to axons, labeled with neurofilament antibody, suggesting that ApoER2 could be preferentially expressed by SCs, the glial cell associated to peripheral axons. The expression of ApoER2 in primary cultured SCs was investigated by RT-PCR, detecting mRNA expression corresponding to the full-length splice variant of ApoER2 and also variants containing the O-glycosylation domain (encoded by exon 16) and cytoplasmic proline-rich insert (encoded by exon 19) (Fig. 1C). In addition, we detected the expression of Reelin mRNA (Fig. 1C).

3.2. ApoER2 proteolytic processing is upregulated following crush injury of mouse sciatic nerves

The next step was to study the expression of ApoER2, and its ligand Reelin, after an injury to the sciatic nerve of adult mice. After performing crush injuries and contralateral sham lesion in sciatic nerves we compared ApoER2 expression through immunofluorescence in different regions of injured nerves. ApoER2 immunostaining was increased in the distal portion of the crushed sciatic nerve as soon as 3 days after injury (dai) (Fig. 2A). ApoER2 up regulation continued increasing in the crushed nerve at 7 dai. At this stage, strong immunoreactivity was detected on the injured and distal portions of the nerves (Fig. 2A). In both cases we confirmed that the immunoreactivity was not associated to axons. Interestingly, when the expression of the receptor was assessed by Western blot (using two different antibodies recognizing the cytoplasmic domain of the receptor), we found that while the full-length protein has already decreased significantly at 3 dai predominantly at the level of the injury and in the distal stump (Fig. 2B,C), there was a significant accumulation of low molecular weight receptor species recognized by the same antibody; therefore the increased immunostaining observed after injury was a result of the detection of the accumulated fragments. Furthermore, at 7 dpi we detected the expression of Reelin, determined by Western blot in the distal portion of the crushed nerve (Fig. 2D). This last observation is in agreement with previous results (Panteri et al., 2006) showing upregulation of Reelin following crush injury in adult mouse. Taken together, our results show for the first time the expression of ApoER2 on PNS and its proteolysis after injury.

3.3. Reelin induces Schwann cell migration

As part of the Wallerian degeneration process after injury, SCs dedifferentiate, proliferate and migrate from the proximal and distal stumps of the nerves forming cellular guides for axon regeneration also known as Bands of Bungner (Gaudet et al., 2011). In order to examine the biological relevance of the presence of ApoER2 and its ligand Reelin after injury, we studied the effect of the latter on the migration of SCs in primary culture. Migration assays were performed using Boyden chambers. Interestingly, we observed that recombinant Reelin greatly increased SC migration compared to vehicle treated cells (Fig. 3A–B). Similar results were obtained using Reelin-conditioned media compared to mock-conditioned media (not shown). SCs also respond to Reelin activating its downstream cascade. At the molecular level, Reelin induces tyrosine phosphorylation of the adaptor protein Dab1 (Disabled-1) by Src family kinases, promoting the activation of PI3K and PKB/AKT (Ballif et al., 2003). Akt is activated by phosphorylation at Ser473. Scs exposed to Reelin increased the levels of phosphorylated AKT determined by Western blot (Fig. 3C) reaching a peak at 40 min of incubation, compared with mock media. In addition, by PCR we detected the presence of the adaptor protein Dab1 in total sciatic nerve and in primary SCs (Fig. 3D).

3.4. Reelin activates Rac1 GTPase

Among some key downstream effectors of the Reelin signaling pathway are the small Rho GTPases, which regulate cytoskeletal dynamics required for cell migration in several cell types (Etienne-Manneville and Hall, 2002). In SCs it has been demonstrated that Rac1 is an essential

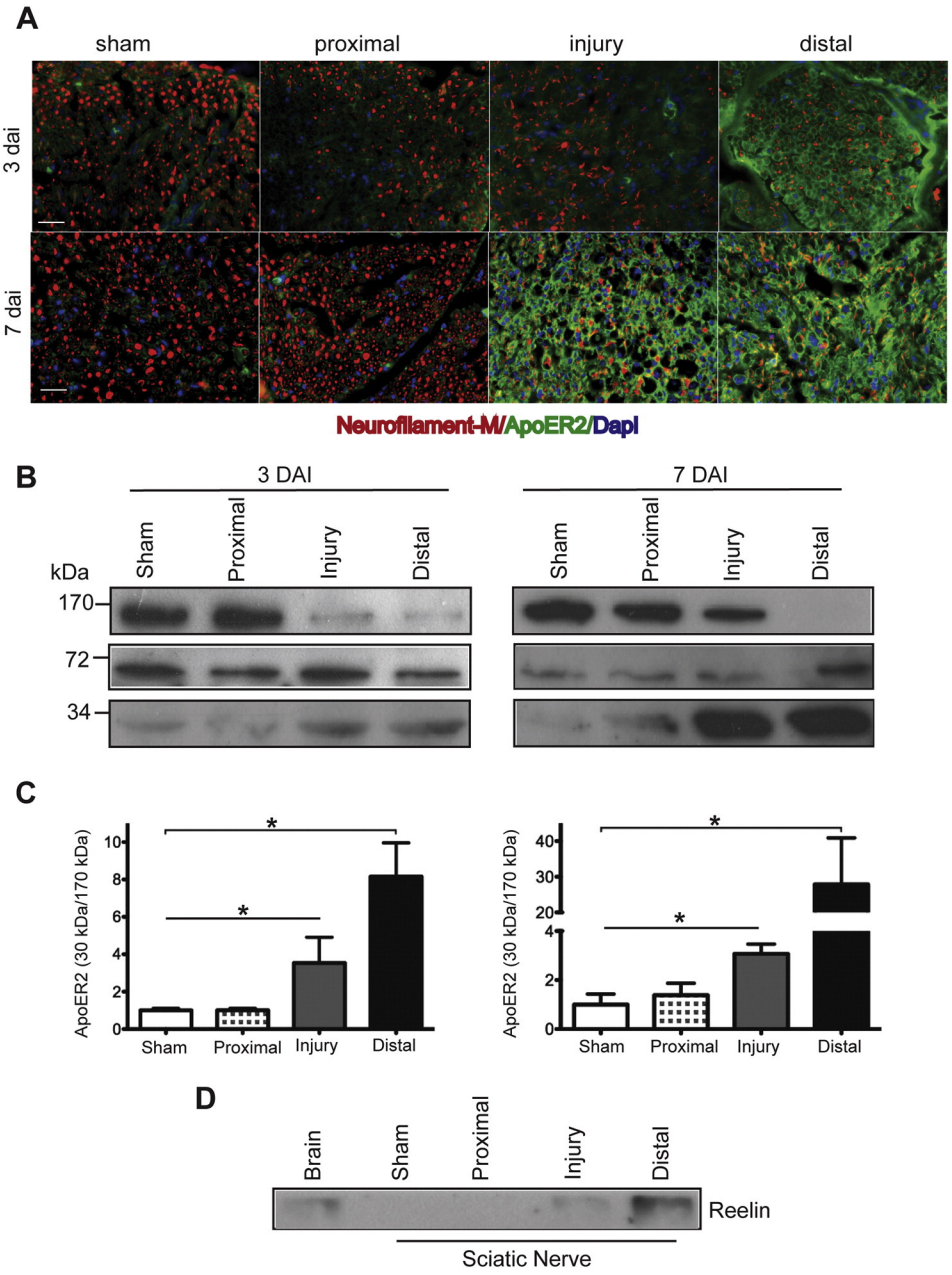

Fig. 2. ApoER2 and Reelin expression after sciatic nerve injury. (A) The immunohistochemistry analysis of ApoER2 expression after injury was performed in transverse sections of sham and crushed sciatic nerve (proximal, injury and distal) at 3 and 7 days after injury (dai) with anti-ApoER2-total antibody. Neurofilament staining was used as neuronal marker (red). ApoER2 is shown in green. Nuclei are counterstained with Hoechst. Scale bars, 25 μm. (B–C) Sciatic nerve extracts from sham, proximal, injured and distal portions were used to detect the expression of ApoER2. The full-length receptor was detected in all cases but experienced a notorious decrease from proximal to distal areas relative to the lesion. In contrast, the detection of ApoER2 fragments was increased. To detect the receptor, an antibody that recognizes the cytoplasmic tail of the protein was used. The change was significant at 3 and 7 dai. The results correspond to $n = 3$ animals per group for two independent experiments, mean ± SE (* $p < 0.05$ one way ANOVA). (D) Protein extracts were prepared and the expression of Reelin was evaluated with a specific antibody on different segments of sciatic nerve at 7 dai. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mediator of cell migration (Yamauchi et al., 2003). Therefore to elucidate the molecular mechanisms by which Reelin induces migration, we examined whether Reelin could activate Rac1. First, we studied the levels of Rac1-GTP in response to Reelin by pull-down assay using a PAK-CRIB-GST fusion protein. The results indicated that SCs treated with Reelin for 20 min have higher levels of Rac1-GTP than non-treated cells (Fig. 4A–B). Next, we studied the spatiotemporal activation of Rac1 in Schwann cells in response to Reelin by FRET with the Raichu-Rac1 biosensor (Nakai et al., 2006). Raichu-Rac1 is a fusion protein that includes the Cdc42- and Rac-interactive binding motif (CRIB) of PAK fused to CFP and YFP on each of its terminus (Itoh et al., 2002). We monitored the activation of the Raichu-Rac1 biosensor in SCs treated with recombinant Reelin for 10 and 20 min. In Reelin-treated cells, the Rac-GTP activity was significantly higher than in control cells (Fig. 4C–D). SCs showed high Rac activity in the peripheral lamellipodial veils of the cells suggesting local activation of the GTPase.

3.5. The Rac1-specific GEF Tiam1 mediates Schwann cell migration induced by Reelin

The next step was to determine if Tiam1, the major Rac1-specific GEF in SCs, is involved in the increased migration induced by Reelin. To this end, cells in which Tiam1 expression was downregulated by a Tiam1-specific siRNA (Fig. 5A) were used for migration assays in

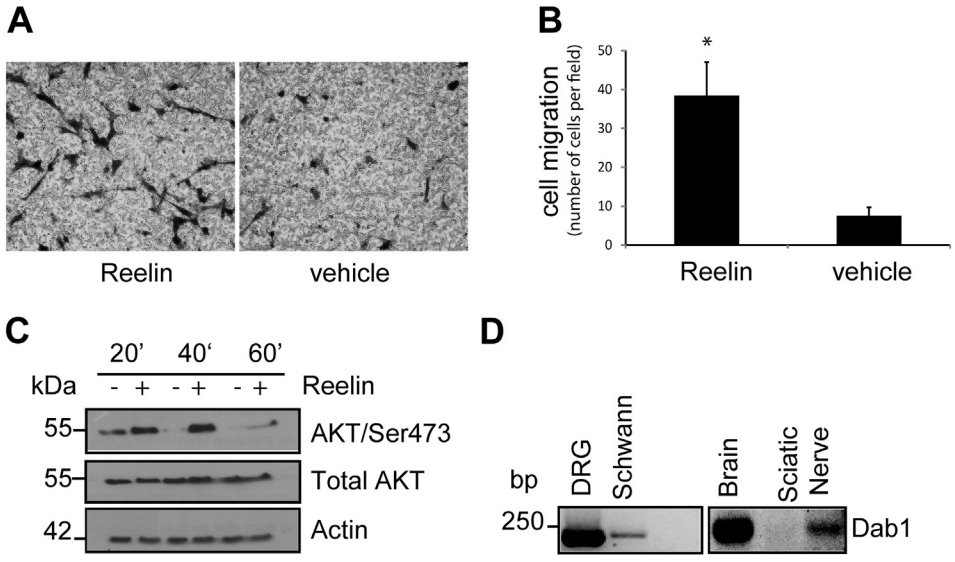

Fig. 3. Reelin mediates migration of primary Schwann cells. Schwann cells were serum-deprived by 4 h and plated on the upper insert of a Boyden chamber in a serum free medium. Then, cells were allowed to migrate out onto the lower well, which was filled with containing recombinant Reelin or vehicle, for 16 h. (A) Representative pictures (40×) are shown. (B) The number of migrating cells was counted (6 independent fields) under microscope. Data are expressed as mean ± SE ($p < 0.05$ *t*-test). *Significant change compared with vehicle. (C) Reelin induces Akt phosphorylation. Schwann cells were serum starved for 10 h and treated for 20, 40 and 60 min with conditioned media containing Reelin (R) or control media (mock) and subjected to Western blot using an antibody for Akt phosphorylated at Ser473 residue. Total AKT was not changed and actin loading indicates equal amounts of protein. (D) Detection by PCR of Dab1 in sciatic nerve, DRG and isolated Schwann cells. Rat brain was used as positive control.

Boyden chambers. After reelin treatment, SCs transfected with Tiam1 siRNA migrate significantly less than the equivalent cells expressing a control scramble siRNA (Fig. 5B). These results identify Tiam1 as the Rac1-specific guanine-nucleotide exchange factor involved in Reelin-induced migration of SCs.

3.6. PAR3 interacts with Tiam1 and ApoER2 and is required for migration

PAR3 and Tiam1 association is functionally important for the polarization and hence for cell migration (Goldstein and Macara, 2007). To explore a possible role of PAR3 in the Reelin-induced SC migration, we first determined if the known interaction of Tiam1 with PAR3 (Nishimura et al., 2005; Wang et al., 2012) could take place in these cells. For such a purpose, SCs were transiently transfected with an activated form of Tiam1 (Tiam1-CA1199-HA) that has deletion in the N-terminus and a full length version of PAR3 and the interaction was effectively detected by co-immunoprecipitation (Fig. 5C). Next, we studied if Reelin induced cell migration is affected by siRNA knock-down of PAR3 (Fig. 5D). The results showed that in PAR3-suppressed cells Reelin-induced SC migration was significantly decreased (Fig. 5E), suggesting that PAR3 and Tiam1 are key regulators of the Reelin induced cell migration of SCs.

On the other hand, ApoER2 splice variants including exon 19, which encodes a proline-rich insert in the cytoplasmic domain, interact with proteins harboring PDZ domains, such as PSD95 and X11a (Minami et al., 2010). Therefore the receptor potentially could interact with PAR3. To test this hypothesis cells were transfected either with a full-length form of ApoER2 or with a construct that lacks of this insert (ApoER2delta-Pro) together with the full-length PAR3. By co-immunoprecipitation it was demonstrated that PAR3 binds the full-length isoform of ApoER2 and this interaction was weaker when the form ApoER2delta-Pro was expressed (Fig. 6A). This result was corroborated by GST-pull down assays (Fig. 6B), in which GST-fusion proteins containing the full-length and the deltaPro receptor tails were used to detect interaction with PAR3. Again, the interaction was more evident with the full-length tail, suggesting that PAR3 interacts with the proline-rich insert. In contrast, and as a positive control for the interaction, both receptor tails interacted similarly with the endocytic adaptor protein Dab2 that recognizes the NPxY motif of the tail (Cuitino et al., 2005). To directly evaluate the role of ApoER2 in the Reelin effects over SC migration, the expression of the receptor was down regulated in using a specific siRNA for ApoER2 (Fig. 6C). Our results demonstrated that SCs with reduced expression of the receptor showed a significantly decreased Reelin-stimulated migration (Fig. 6D).

4. Discussion

Reelin corresponds to an extracellular matrix glycoprotein of about 400 KDa expressed in various body tissues, particularly in nervous tissue (Forster et al., 2010). Reelin signaling is transmitted through two receptors, ApoER2 or VLDL/R and regulates various biological processes associated predominantly to neuronal migration, synaptic plasticity, neurogenesis and neurodegeneration (Larios and Marzolo, 2012). Here, we show that ApoER2, one of the Reelin receptors, is expressed in adult sciatic nerve of mouse and its protein levels are modified after peripheral crush injury. Our immunofluorescence results showed that ApoER2 is not associated with neurofilament, a major component of the neuronal cytoskeleton; therefore we suggest that the cell type expressing this protein is the SC, which has an essential role in PNS regeneration. Furthermore, analysis of mRNA expression of primary SCs isolated from sciatic nerves confirmed the expression of different isoforms of ApoER2, the ligand Reelin and of the signaling adaptor protein Dab1. One of the most striking characteristics of these cells is their plasticity. It has been described that peripheral nerve injury is followed by an increase in SC proliferation in the distal nerve stumps, which begins 3 to 4 days after injury (Gaudet et al., 2011). SCs secrete soluble factors that facilitate their migration and also nerve regeneration (Gaudet et al., 2011). Consistent with these evidences, it has been demonstrated that SCs secrete Reelin during development and after sciatic nerve injury (Panteri et al., 2006). Moreover, Lorenzetto et al. (Lorenzetto et al., 2008) demonstrated that mice deficient in Reelin (reeler mice) have impaired axonal regeneration after nerve injury. Thus, these findings suggested that this extracellular matrix protein could induce a cellular response that allows successful axon regeneration. It is widely known that Reelin plays a role in neuronal migration and in the positioning of cells in the brain during development, acting as a stop signal inhibiting migration of cortical neurons

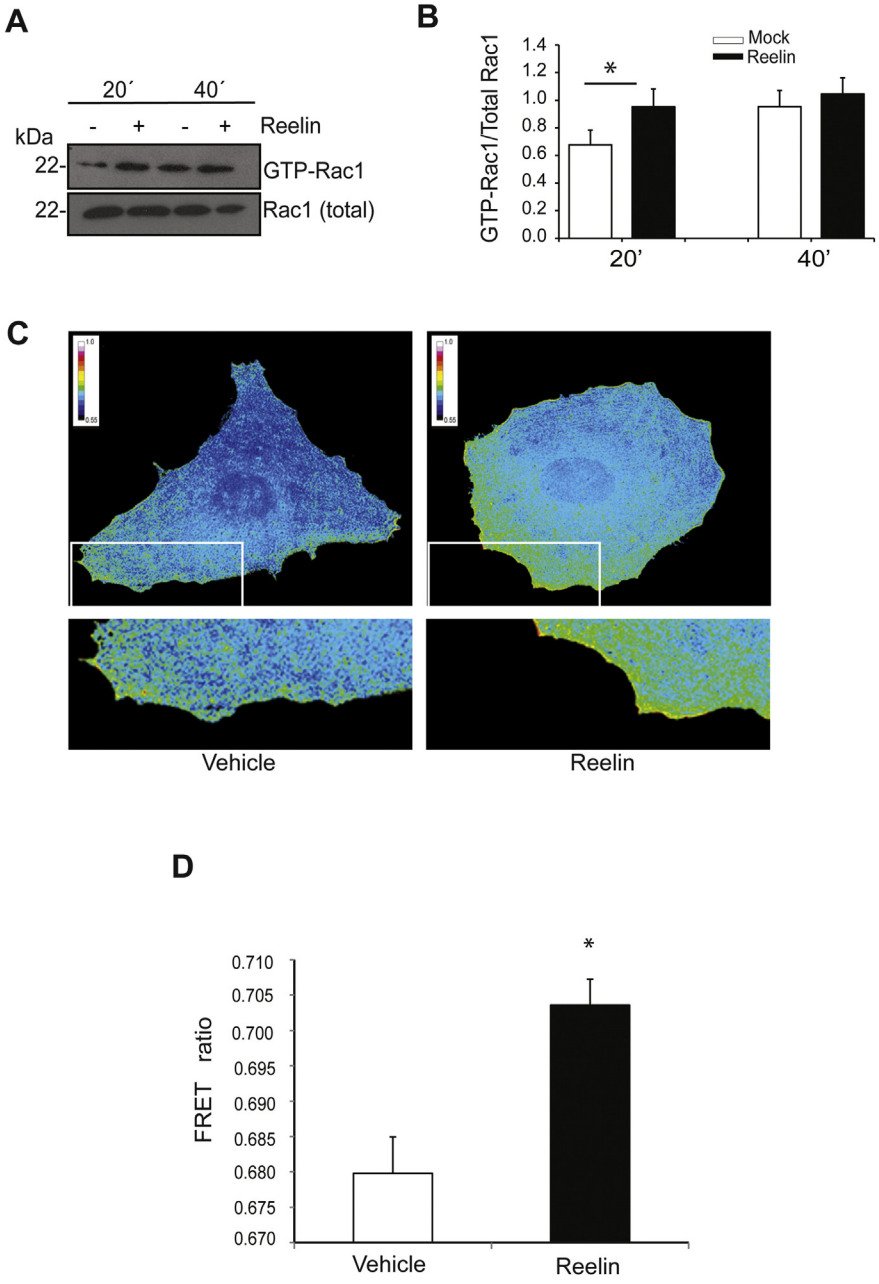

Fig. 4. Reelin enhanced GTP binding of Rac1 in a time-dependent. (A) Primary Schwann cells were serum starved for 10 h and treated for 20 and 40 min with conditioned media containing Reelin or control media (mock). Subsequently, cells were lysed and GTP-Rac1 was precipitated using 50 μg of GST-PAK-CRIB fusion protein for 2 h. Finally, the complexes were analyzed by Western blot with and antibody anti-Rac1. (B) Quantification of GTP-Rac1 normalized by total Rac1 showed higher Rac1 activity in treated Schwann cells compared with untreated Schwann cells. *Significant change compared with control media. Values represent the mean ± SE of three separate experiments ($p < 0.05$; *t*-test). (C) Distribution of Rac1 activity in Schwann cells. Schwann cells were electroporated with a plasmid for the Raichu-Rac1 biosensor and plated on laminin coated coverslip. After 24 h cells were serum starved for 10 h and treated with recombinant Reelin or vehicle for 20 min. After fixing, YFP/CFP ratio images of cells expressing Raichu-Rac1 ($n = 30$ per condition) were acquired at the 20 min time point. (D) The graphs represent the quantification of FRET efficiency in treated and untreated cells. *Significant difference compared with control ($p < 0.05$ *t*-test). Scale bar 20 μm.

(Dulabon et al., 2000) or as a chemoattractant for migrating neurons (Gilmore and Herrup, 2000). Thus, we decided to study whether Reelin could be associated to SC migration in the PNS. Our migration experiments indeed demonstrated that Reelin is involved in the migration of SCs.

At the molecular level, the signaling pathway ApoER2/Reelin induces phosphorylation of Dab1, a cytoplasmic adapter protein that activates tyrosine kinase by NPxY cytoplasmic motif (Ballif et al., 2003; Hiesberger et al., 1999). Dab1 is phosphorylated by SRC family kinases, such as Fyn and Src, which regulate survival, proliferation, differentiation and mobility in different cell types (Brown and Cooper, 1996). In turn, phosphorylated Dab1 activates the PI3K and downstream PI3K, the Rho families of GTPases. These proteins are key players in cell migration, since they regulate the cytoskeletal rearrangements required for cell migration (Hall, 1998). Therefore, to clarify the mechanisms by which Reelin influences cell migration we studied the activation of the GTPase Rac1 demonstrating that its activation is regulated by Reelin. It is worth mentioning that in cortical neurons, by GST-pull-down assays it was demonstrated that Reelin induced a robust Cdc42 activation and in a minor magnitude localized Rac activation (Leemhuis et al., 2010). Reelin, through Dab1 and PI3K, also activates the small GTPase Rap1 via its specific GEF C3G (Ballif et al., 2004). Our observations were further confirmed with FRET analysis. We detected polarized Rac1 activation

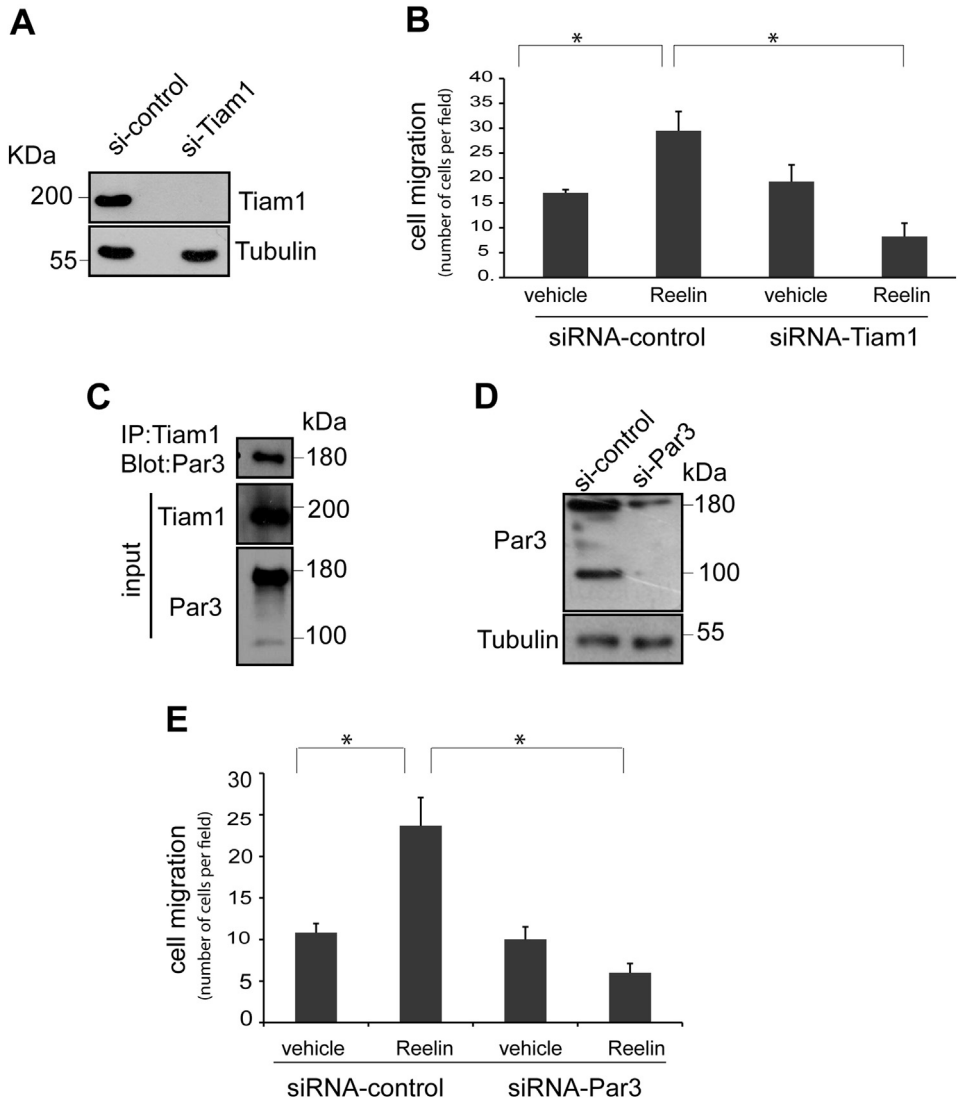

Fig. 5. Tiam1, a Rac1-GEF, and the scaffold protein PAR3 mediate Reelin-induced cell migration in Schwann cells. (A) Cells were transfected with control siRNA o Tiam1-specific siRNA (siTiam) and Tiam1 was analyzed by immunoblot. Tubulin was measured as a loading control. (B) Quantification of cell migration of Schwann cells transfected with siRNA control or siRNA for rat-Tiam1 in Boyden-chambers. The number of migrating cells was counted (6 independent fields) under microscope. (C) Immortalized Schwann cells were transiently transfected Tiam C1199-HA and full length PAR3. Cell lysates were immunoprecipitated with anti-HA and probed with anti-PAR3. The bottom panel shows total levels of proteins in lysates. (D) Immunoblot of siPAR3 transfected cells. Tubulin was measured as a loading control. (C) Quantification of cell migration of Schwann cells transfected with siRNA control or siRNA for rat-PAR3. The number of migrating cells was counted (6 independent fields) under microscope. Data are expressed as mean ± SE and evaluated by using *t*-test (*p < 0.05 *t*-test).

close to the plasma membrane. This distribution could be associated to migration. However our FRET experiments were done in non-migrating cells and we do not know if Rac1 activation is persistent over time during migration or if Reelin induces changes on the Schwann cell morphology essential for migration. The Rho GTPases are involved in many aspects of cell motility through their ability to promote reorganization of the cytoskeleton. Rho protein is essential for cell adhesion, Rac1 is required for lamellipodia and ruffles formation and Cdc42 mediates formation of filopodia (Hall, 1998). In SCs LPA activates Rac1 inducing cytoskeletal changes (Barber et al., 2004).

We identify Tiam1 as the Rac1-specific GEF responsible for Reelin-induced Schwann cell migration. A similar finding was observed in Schwann cell migration induced by TrkC (Yamauchi et al., 2005a). Tiam1 is a modular protein harboring a variety of interaction domains, including a PDZ domain. Previous evidences have determined that Tiam1 participates in polarized cell migration interacting with the PAR complex of PAR3 (Wang et al., 2012). Moreover, both PAR3 and Tiam1 play critical roles in cell polarization (Mertens et al., 2006). The Tiam1 PHnCCEx domains bind directly to the PAR3 c-terminal which contains PDZ domains (Chen and Macara, 2005). In neurons, this association participates in axon specification and dendritic spine maturation (Zhang and Macara, 2006) and in epithelial cells controls the formation of tight junctions (Chen and Macara, 2005; Mertens et al., 2005). Here we showed evidence for a complex formation between PAR3 and Tiam1 and more importantly, we demonstrated by knocking down this protein, that PAR3 is also involved in the migration of SCs induced by Reelin. Besides Reelin-induced activation of Rac1, other trophic factors such as NT3 promote migration mediated by Rac1/Cdc42 and c-Jun N-terminal kinase (Yamauchi et al., 2003). As in our system, NT3 also involves Tiam1 activation, mediated by Ras (Yamauchi et al., 2005a). During development, SC precursors proliferate and migrate along growing axons to trigger the onset of myelination. BDNF takes a role during this process by recruiting PAR3 and activating Rac1 (Tep et al., 2012). Thus, Rac1 appears to be the main mediator of migration in SCs.

The effects of Reelin are mediated by ApoER2 because it did not induce migration in ApoER2 silenced cells, indicating that the effect of Reelin on SC migration is not mediated by VLDLR, the other Reelin receptor. Our results are consistent with a previous study demonstrating that in epithelial cells (MCF10A) ApoER2 through its interaction

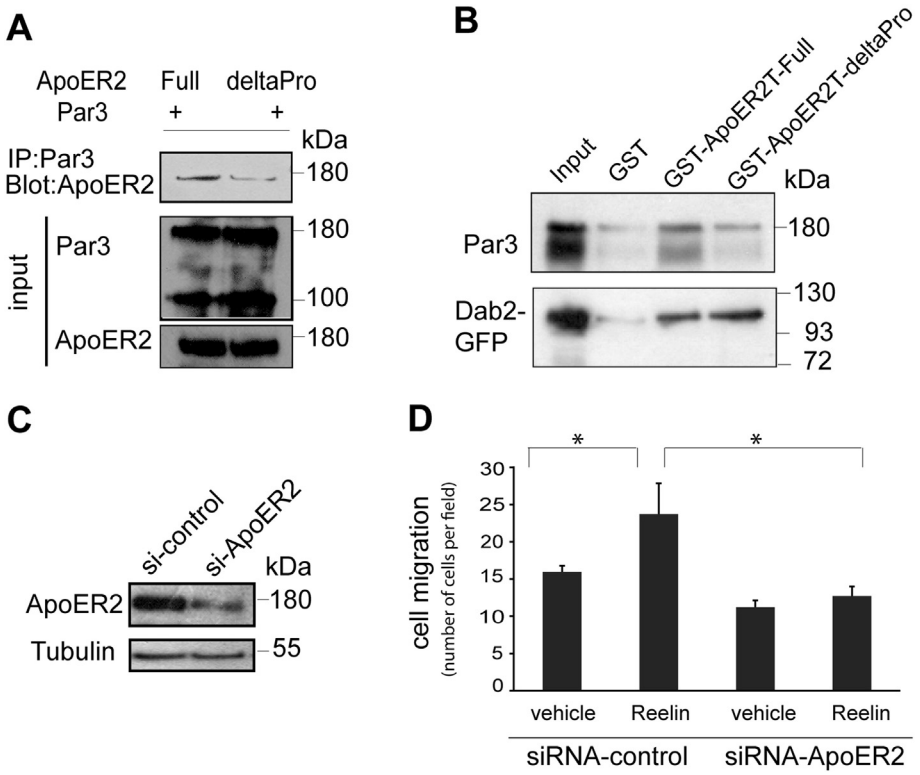

Fig. 6. ApoER2 interacts with PAR3 and mediates migration of primary Schwann cells induced by Reelin. (A) Immortalized Schwann cells (IFRS1) were transiently transfected with plasmids encoding either the full-length ApoER2-Flag or the DeltaPro ApoER2 (a construct lacking of proline-rich insert), the endoplasmic reticulum chaperone RAP and PAR3. Cell lysates were immunoprecipitated with anti-PAR3 and probed with anti-Flag to detect the transfected receptor. The bottom panel shows total levels of protein in lysates (input). (B) HEK293 cells were transfected with full-length PAR3 or with Dab2-GFP. After 48 h of expression, cell lysates were obtained and incubated with 50 μg of the fusion protein, GST, GST-ApoER2-full length, or GST-ApoER2 DeltaPro for 2 h. Finally, the complexes were analyzed by Western blot with antibodies for PAR3 and anti-GFP (to detect the interaction with Dab2). (C) ApoER2 expression levels were determined by Western blot in primary Schwann cells transfected with ApoER2-siRNA or siRNA Control. Tubulin was measured as a loading control. (D) Quantification of cell migration of Schwann cells transfected with siRNA control or siRNA for rat-ApoER2. The number of migrating cells was counted (12 independent fields) under microscope. Data are expressed as mean ± SE and evaluated by using *t*-test (*$p < 0.05$ *t*-test).

with the adapter protein X11α increase cell motility in a wound healing assay and this effect is further enhanced with the addition of Reelin (Minami et al., 2010). The alternatively spliced isoform of ApoER2 which contain a proline-rich 59 amino acid domain allows its interaction with different adaptor proteins, such us the c-jun N-terminal kinase (JNK) interacting proteins, JIP1 and JIP2, and the synaptic protein, PSD-95 (Herz and Chen, 2006; Larios and Marzolo, 2012; Stockinger et al., 2000). An important and new finding of our study was the identification of an unexpected interaction between ApoER2 and PAR3. We observed that the interaction between these two proteins is decreased if the receptor expressed has a deletion in the proline-rich domain, which corresponds to an ApoER2 natural splice variant. We suggest that ApoER2 could recruit the scaffold protein PAR3 and participates in cell migration induced by Reelin. PAR3 contains PDZ domains in its C-terminal end through which interacts with a large number of proteins (Gao et al., 2002; Gundersen and Worman, 2013). Considering this ability it is conceivable that PAR3 could interact with ApoER2 specifically binding to the internal PDZ motif present in the proline-rich insert. However, it remains unknown if ApoER2-PAR3-Tiam forms a tripartite complex induced or increased by Reelin. Recently, LRP1, another receptor belonging to the family of LDLR-family receptors, was involved in SC migration. LRP-1 is expressed in adult sciatic nerve and is upregulated after injury. LRP-1 participates in SC survival after injury (Campana et al., 2006; Mantuano et al., 2010). Therefore, through the interaction with different ligands, the receptors of the LDL-R family expressed in SCs accomplish relevant roles during PNS regeneration.

Cell migration also involves the participation on microtubules (Gundersen and Bulinski, 1988; Gundersen et al., 2005; Wen et al., 2004; Wojnacki et al., 2014). In this regard, it has been found that the microtubule-associated protein MAP1B is involved in LPA-induced migration of SCs in vitro, coordinating actin filament and microtubule remodeling (Bouquet et al., 2007). In neurons, Map1B is phosphorylated in response to Reelin and this has an impact in neuronal migration during development (Gonzalez-Billault et al., 2005). In addition, Reelin promotes microtubule dynamics specifically inducing the formation of dendritic comets with microtubule plus end binding protein 3 (EB3) (Meseke et al., 2013a) and induces the translocation of the Golgi apparatus into developing dendrites (Meseke et al., 2013b). Work in progress from our laboratory supports the idea that Reelin could regulate the formation of stable detyrosinated microtubules (Glu microtubules) and reorientation of microtubule-organizing center (MTOC) toward the leading edge, both relevant processes associated to migration (Gundersen and Bulinski, 1988). Our results indicating the participation of PAR3 in the Reelin-induced SC migration could be also related to the MTOC orientation toward the leading edge and stabilization of microtubules as has been described in other systems (Schmoranzer et al., 2009). Other effects that are possibly induced by Reelin in the migration of SCs should include the promotion of membrane trafficking and vesicle formation, as has been found in neurons (Leemhuis et al., 2010).

Besides the expression of ApoER2 and Reelin in SCs and their role in migration, we found that the receptor experienced a massive down-regulation of its full-length form and the concomitant accumulation of receptor fragments after nerve injury. These fragments are, with high probability, produced by a massive proteolytic processing of the receptor since we did not detect changes in the receptor mRNA expression at 3 or 7 days after the lesion (Fig. S1). SCs could respond to secreted Reelin, which is known to trigger ApoER2 proteolysis (Duit et al., 2010; Hoe et al., 2006). However, the receptor fragments were clearly detected three days after injury, in contrast to Reelin expression that was more

evident at 7 dai. It is known that within the first 24 h after injury, there is a massive increase in the SC expression of metalloproteinase-9 (MMP-9), that is essential for the activation of trophic signaling pathways controlling proliferation and avoiding apoptotic cell death (Chattopadhyay and Shubayev, 2009). In addition, in vivo studies indicate that the inhibition of proteolysis using the broad-spectrum MMP inhibitor Ilomastat (GM6001) immediately after injury, promotes axonal regeneration by favoring SC proliferation and dedifferentiation (Liu et al., 2010). ApoER2 proteolysis is also inhibited by the broad MMP inhibitor GM6001 (Larios et al., 2014; Larios and Marzolo, 2012). Therefore it is possible that the receptor is exposed to different agents promoting its proteolysis and accordingly, the inhibition of this process could be associated to a better regeneration outcome.

Given the importance of SC migration during PNS injury, the regulation of migration by Reelin is likely to be important for regeneration. Our findings could serve as basis for developing new therapeutic strategies in the treatment of diseases or injuries associated to the PNS. Actually, thousands of people are afflicted by lesions in the PNS. Therefore, studying new cellular and molecular mechanisms favoring regenerative conditions, including those inducing proper SC survival and migration, could favor the speedy recovery of affected patients. By other hand, since cell migration is a key event for carcinogenesis, the ability of Reelin to promote cell migration may be important not only for regeneration but also for cancer. Reelin induces migration of neuroblastoma cells (Becker et al., 2012) and Reelin expression is altered in breast cancer (Stein et al., 2010). Thus, Reelin signaling emerges as a relevant target in pathologies or physiologic conditions associated with cell migration.

5. Conclusions

This work demonstrates that ApoER2 and Reelin expression are modified after PNS injury. Moreover, we have demonstrated a novel function for Reelin/ApoER2 in PNS, inducing cell migration of SCs, a process relevant for PNS development and regeneration. As a working model we propose that the binding of Reelin to the receptor could mediate the recruitment of PAR3 to the cytoplasmic tail of ApoER2. This activates Rac1, through Tiam1 recruitment, at the leading edge of the plasma membrane allowing cell migration.

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Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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