Non-redundant function of dystroglycan and \(\beta1\) integrins in radial sorting of axons

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SUMMARY
Radial sorting allows the segregation of axons by a single Schwann cell (SC) and is a prerequisite for myelination during peripheral nerve development. Radial sorting is impaired in models of human diseases, congenital muscular dystrophy (MDC) 1A, MDC1D and Fukuyama, owing to loss-of-function mutations in the genes coding for laminin \(\alpha2\), Large or fukutin glycosyltransferases, respectively. It is not clear which receptor(s) are activated by laminin 211, or glycosylated by Large and fukutin during sorting. Candidates are \(\alpha\beta1\) integrins, because their absence phenocopies laminin and glycosyltransferase deficiency, but the topography of the phenotypes is different and \(\beta1\) integrins are not substrates for Large and fukutin. By contrast, deletion of the Large and fukutin substrate dystroglycan does not result in radial sorting defects. Here, we show that absence of dystroglycan in a specific genetic background causes sorting defects with topography identical to that of laminin 211 mutants, and recapitulating the MDC1A, MDC1D and Fukuyama phenotypes. By epistasis studies in mice lacking one or both receptors in SCs, we show that only absence of \(\beta1\) integrins impairs proliferation and survival, and arrests radial sorting at early stages, that \(\beta1\) integrins and dystroglycan activate different pathways, and that the absence of both molecules is synergistic. Thus, the function of dystroglycan and \(\beta1\) integrins is not redundant, but is sequential. These data identify dystroglycan as a functional laminin 211 receptor during axonal sorting and the key substrate relevant to the pathogenesis of glycosyltransferase congenital muscular dystrophies.

KEY WORDS: Peripheral nerve development, Schwann cells, Axonal sorting, Congenital muscular dystrophy, Dystroglycan, Integrins, Mouse

INTRODUCTION
Radial sorting of axons by Schwann cells (SCs) is a multistep developmental process required for myelination. It involves segregation of large caliber axons, and depends on signals exchanged between axons and SCs. Axon signals include the amount of neuregulin type III on axons, extracellular matrix signals are initiated by laminin 211 (\(\alpha2\beta1\gamma1\)) and 411 (\(\alpha4\beta1\gamma1\)) via \(\beta1\) integrin receptors on SCs (Feltri and Wrabetz, 2005).

Laminin 211 is missing in D\(y\)D\(y\) mutants (a model of congenital-muscular dystrophy 1A) and radial sorting is severely impaired in the proximal peripheral nervous system (i.e. spinal roots) and is mildly impaired in distal nerves in these mutants (Bradley and Jenkinson, 1973; Bradley and Jenkinson, 1975; Stirling, 1975). By contrast, absence of laminin 411 in mice affects proximal and distal districts only mildly (Wallquist et al., 2005), whereas absence of both laminin 211 and 411 blocks radial sorting completely in all districts (Chen and Strickland, 2003; Yang et al., 2005) (Fig. 1). The reasons for this topographical difference are unknown, but might derive from activation of different laminin receptors by different laminins.

Deletion of receptors containing the \(\beta1\) integrin subunit causes arrest in radial sorting distally, but only mild defects proximally (Feltri et al., 2002), leaving the laminin receptor involved in radial sorting in spinal roots unidentified. Radial sorting defects are also observed in models of other human muscular dystrophies (MDC1D and Fukuyama) that are the result of mutations in fukutin or LARGE glycosyltransferases (Levedakou et al., 2005; Saito et al., 2007), which have \(\alpha\)-dystroglycan as substrate. These data suggest either that dystroglycan mediates sorting or that these glycosyltransferases act on other proteins, such as \(\beta1\) integrins. However, the absence of Large or fukutin does not impair glycosylation of \(\beta1\) integrin (Levedakou et al., 2005; Saito et al., 2007) and deletion of dystroglycan in SCs does not affect radial sorting, at least in distal nerves (Saito et al., 2003). Thus, the molecular mechanisms by which these mutations alter sorting are unknown.

Finally, the mechanisms by which different laminin-receptor pairs promote sorting are incompletely understood. Axonal sorting requires multiple events: the formation of ‘families’ of SCs with multiple axons contained in a common basal lamina (Webster et al., 1973), the matching of the number of axons and SCs, the insertion of SC processes around axons to recognize and segregate large ones, and the defasciculation of single axons with their own daughter SC and basal lamina (Martin and Webster, 1973; Webster et al., 1973). It is unknown which laminin-receptor pairs contribute to each step. For example, laminins promote the formation of SC processes and the interaction with axons (Yu et al., 2009) via activation of Rac1 by a \(\beta1\) integrin receptor (Benninger et al., 2007; Nodari et al., 2007), but laminins also promote SC proliferation and survival via activation of Pi3K, Fak and Cdc42.
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Materials and Methods

Generation of transgenic mice
β1 integrin floxed (β1F/F) (Graus-Porta et al., 2001), dystroglycan floxed mice (Dag1F/F) (Moore et al., 2002; Saito et al., 2003) and mPoTOCTCre transgenic mice (Feltri et al., 1999) have been described previously. Dag1F/F/Itgb1+/F/F mice were generated by crossing Itgb1+/+ and Dag1F/F mice. Dag1F/F/P0Cre and Itgb1F/F mice were crossed to obtain Itgb1F/F+/P0Cre mice. These were intercrossed to obtain Itgb1F/F+/P0Cre mice. Offspring were analyzed by PCR on tail genomic DNA as described (Feltri et al., 1999; Saito et al., 2003). Most progeny in this study resulted from Itgb1 floxed parents that were N15 congenic for C57BL/6, and from Dag1 parents that were N7-N9 congenic for C57BL/6.

TUNEL and proliferation assays

TUNEL assay and BrdU incorporation and staining were performed as described (Feltri et al., 2002). Proliferation assays were performed using rabbit α-PH3 antibodies (Upstate) as described (D’Antonio et al., 2006). Nuclei were counterstained with DAPI and counted with a Leica confocal microscope. For TUNEL and proliferation, three different mice per genotype and four different slides per animal were analyzed, for an approximate total number of nuclei of 85,000 for postnatal day (P) 3, 100,000 for P5, 120,000 for P15 and 140,000 for P28.

Immunohistochemistry

Sciatic nerves were processed and stained as described (Nodari et al., 2008) using rabbit antibodies against neurofilament (Sigma), goat antibody against tubulin and rabbit anti-calnexin (Sigma-Aldrich); mouse anti-α-tubulin and rabbit anti-calnexin (Sigma-Aldrich); mouse anti-glycosylated α-dystroglycan (IIH6) and goat anti-core α-dystroglycan (G20) (generous gifts from Kevin Campbell, University of Iowa, IA, USA).

Morphological analysis

Morphological analysis was performed as described (Wrabetz et al., 2000). Quantification of axonal sorting defects was performed on semi-thin and electron microscopy sections of roots or sciatic nerves from three animals per genotype.

Immu-electron microscopy

P3-P4 wild-type, β1 integrin-null and dystroglycan-null mice (negative controls) were perfused with 4% paraformaldehyde (PFA)/0.05% glutaraldehyde in 0.1 M sodium periodate, 0.1 M lysine, 3% sucrose in 0.1 M phosphate buffer pH 7.4. Sciatic nerves were dissected, fixed for 2 hours, and left overnight in 3.5% sucrose in 0.1 M phosphate buffer. Tissues were processed at 4°C as described (Scherer et al., 1995) with the following modifications: Tissue was stained with 0.25% tannic acid for 1 hour, quenched in 50 mM NH4Cl, washed in 4% sucrose in 0.1 M maleate buffer (pH 6.2), incubated for 1 hour with 1% uranyl acetate in maleate buffer, dehydrated to 90% ethanol (from 70% ethanol onward; all steps at –20°C) then dipped in absolute ethanol for 15 seconds and then infiltrated first with
1:1 ratio of L.R. Gold (Polysciences)/ethanol, then with a 7:3 ratio of L.R. Gold/ethanol followed by two changes of L.R. Gold (1 hour and then overnight). Next day, the tissues were infiltrated in L.R. Gold with 0.5% benzoin methyl ether for 1 hour and then overnight, embedded in gelatin and polymerized by UV irradiation (365 nm) for 48 hours at ~20°C. Sections were collected on nickel formvar/carbon grids, and stained according to the methods of Li et al. (Li et al., 2005a). After two washes in PBS and block with 0.25% fish skin gelatin and 0.1% Triton X-100 in PBS for 1 hour at room temperature, sections were incubated with hamster anti-β1 integrin (BD Biosciences) or mouse anti-β-dystroglycan (Novocastra) antibodies, rinsed with PBS, incubated with secondary antibody (custom-made anti-hamster, conjugated with 5 nm gold particles or anti-mouse, conjugated with 10 nm gold particles; British BioCell). In some experiments, the hamster anti-β1 integrin antibody (BD Biosciences) was biotinylated with an EZ-Link Sulfo-NHS-biotin (Thermo Scientific) in a molecular ratio of 1:20 for 2 hours on ice in the dark, dialyzed overnight against PBS and used for immunostaining at a 1:25 dilution. A goat anti-biotin antibody (British Biocell) conjugated with 5 nm gold particles was used as secondary antibody. After staining, grids were washed on drops of PBS and distilled water and stained with saturated uranyl acetate and lead citrate.

**Rac1-Pak1 binding domain localization**

Organotypic neuron/SC cultures, cold jet, mouse SC cultures and immunocytochemistry for Rac1-Pak1 binding domain localization were performed as described (Nodari et al., 2007). The following antibodies were used: goat anti-GST (GE Healthcare), mouse anti-βDG (Novocastra), rabbit anti-S100 (Dako) antibody to discriminate SC from fibroblasts (not shown).

**Rat SC cultures**

Rat SCs were produced and expanded as described previously (Feltri et al., 1992). For β1 silencing, rat SCs were plated on poly-l-lysine and transfected the next day with 66 nM ITG1 and control siRNA (ON-TARGET plus, Dharmacon) using Lipofectamine 2000 (Invitrogen). Next day, cells were starved for 24 hours in DMEM containing 2 μM forskolin, stimulated for 30 seconds with 1 mM of soluble NRG1-β1 (R&D Systems) or dorsal root ganglia membranes, which were prepared as described (Taveggia et al., 2005). Proteins were extracted on ice with 25 mM Tris pH 7.4, 95 mM NaCl, 10 mM EDTA, 1% SDS, 1 mM NaF, 1 mM NaVO3.

**RESULTS**

**Ablation of dystroglycan alone is sufficient to cause arrest in radial sorting of axons**

Mutations in the genes encoding the α-dystroglycan glycosyltransferases Large and fukutin cause sorting defects, suggesting that dystroglycan might be involved (Levedakou et al., 2002; Saito et al., 2003). However, mice lacking SC dystroglycan have no sorting defects (Saito et al., 2003). These mice were generated by crossing mice carrying the Dag1 gene flanked by LoxP sites (Moore et al., 2002) with Mpc-Cre transgenic mice (mpC_TOTCre) that express Cre in SCs from embryonic day (E) 13.5 (Feltri et al., 2002; Saito et al., 2003). Only the distal district (sciatic nerves) of resulting mice in a mixed background (129/Sv and C57BL/6) was analyzed (Saito et al., 2003). To determine whether dystroglycan was involved in radial sorting, we first bred dystroglycan mutants on a congenic C57BL/6 background, and analyzed proximal and distal peripheral nervous system districts. Notably, in this background the absence of SC dystroglycan caused a radial sorting defect that was mild in sciatic nerve and severe in ventral roots (Fig. 1B,F). Further breeding of dystroglycan mutants in the C57BL/6 background (N10-N13) greatly diminished the phenotype (not shown), indicating that we identified a genetic window (N5-N9) in which the role of dystroglycan in radial sorting could be unraveled. Based on the distribution of the radial sorting defect, these mice closely resemble dystrophic dy/dy or dy(2)/dy(2) mice, lacking laminin 211.

**The function of SC dystroglycan and β1 integrin in axonal sorting is not redundant**

To determine whether there is redundancy between β1 integrin and dystroglycan in sorting, we generated mice lacking both receptors in SCs. As described for Dag1 floxed mice, Itgb1 floxed mice were bred into a congenic C57BL/6 background. Immunofluorescence confirmed ablation of both proteins in SCs (see Fig. S1 in the supplementary material).

Similarly to the dystroglycan mutant, mice lacking SC β1 integrin in a pure C57BL/6 background have a more severe phenotype than those previously characterized in a mixed 129/sv-C57BL/6 background (Feltri et al., 2002). C57BL/6 β1 integrin mutants showed an almost completely arrested phenotype in sciatic nerves, which contained only few myelinated fibers and larger bundles of unsorted axons (Fig. 1C). Importantly, radial sorting defects were now apparent in spinal roots (Fig. 1G).

Double mutants were viable but, by P15, developed tremor with impaired gait, which evolved to hindlimb paralysis and severe atrophy by P60. Onset of symptoms was earlier than in single mutants, with more severe tremor and a wide-based gait. Ventral roots and sciatic nerves were completely occupied by unsorted bundles of axons (Fig. 1D,H). Dorsal roots presented defects in radial sorting, whereas small, unsorted bundles were rarely seen in single mutants (not shown). Occasional myelinated fibers were found in double-null mice (Fig. 1). The severity of double mutants is comparable to that of mice lacking both 211 and 411 laminins (Chen and Strickland, 2003; Yang et al., 2005), and suggests that dystroglycan and β1 integrins represent all the laminin receptors involved in radial sorting.

Although loss of β1 integrin causes a more prominent defect than loss of dystroglycan in distal nerves (compare Fig. 1B with 1C), the extent of the defect was similar between the two single mutants in ventral roots, where the area occupied by unsorted bundles was 30% in each single mutant and 93% in double mutants (Table 1).

**Dystroglycan is required at a later step than β1 integrins during sorting**

Unsorted bundles in the absence of dystroglycan were smaller than in the absence of β1 integrin. Sciatic nerves bundles in dystroglycan mutants contained fewer axons of smaller caliber (Fig. 2A and Table 2). By contrast, most bundles in β1 integrin and

<table>
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<tr>
<th>Genotype</th>
<th>β1 integrin null (n=3)*</th>
<th>Dystroglycan null (n=3)*</th>
<th>Dystroglycan/β1 integrin null (n=3)</th>
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<tbody>
<tr>
<td>Bundle area/total area</td>
<td>0.30 μm²</td>
<td>0.30 μm²</td>
<td>0.93 μm²</td>
</tr>
<tr>
<td>Average axonal diameter in bundles</td>
<td>5.0 μm</td>
<td>4.3 μm</td>
<td>n.d.</td>
</tr>
<tr>
<td>Number of axons per bundle</td>
<td>72±24</td>
<td>44±2</td>
<td>n.d.</td>
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*41 bundles for β1 integrin and 43 bundles for dystroglycan mutants were analyzed. n.d., not done.
Fig. 2. Radial sorting and myelin defects in dystroglycan and double mutants. Electron microscopy of transverse sections of dystroglycan, β1 integrin and double mutant sciatic nerves at P28. (A-D) Nerves of all the mutants contain bundles of naked axons (A-D), but the size of those axons is smaller in dystroglycan (A) compared with β1 integrin (B) and double mutants (C). (See also Table 2.) Occasionally, large axons are found in bundles of dystroglycan mutants (D). (E-G) Detached and redundant basal lamina (arrowheads) in β1 integrin (E), dystroglycan (F) and double (G) mutants. (H-L) Myelination defects in dystroglycan mutants: disorganization of inner and outer wraps (arrowheads in H,I) and outer mesaxons (arrow in I), non-compaction of outer myelin lamellae (K) or throughout the whole myelin thickness (L). (M-O) Hypomyelination in double mutants (M). A myelin-like process was observed separating two axon bundles (arrowheads and inset in O). Wild type (wt) is shown in N. Scale bar: in C, 5 μm for A-C, 3.2 μm for D,O, 1.6 μm for E-G, 1.25 μm for M,N, 1 μm for J-L.

double mutants were large, occupied the majority of the endoneurium and contained mixed caliber axons (Fig. 2B,C and Table 2). Axons in both mutants were naked, although in certain bundles dystroglycan mutants SCs had unsheathed axons. Basal lamina was discontinuous, detached or redundant in both mutants, especially around bundles of unsorted axons (Fig. 2E-G, arrowheads). In double mutants, basal lamina defects were more severe and were present in the majority of fibers.

To determine at what stage radial sorting was arrested, we performed electron microscopy at P3 and P5, when radial sorting is active in sciatic nerves (Webster et al., 1973). At these ages, wild-type (wt) nerves contained small bundles with few axons (Fig. 3A), with large axons that were already segregated at the periphery (~45%), pro-myelinating and myelinating fibers (Fig. 3A) (Webster et al., 1973). By contrast, most axons in β1 integrin nerves were trapped in large bundles (Fig. 3C-E), including large axons that were completely unsegregated (~80% at P3 and ~60% at P5). Less than ~5% of large axons were segregated at the periphery (Fig. 3C, asterisks and 3D). Overall, nerves appeared more immature than previously described in mixed C57BL6/129 nerves (Feltri et al., 2002). P3 and P5 dystroglycan mutant nerves appeared delayed compared with wt, but were further along in morphogenesis than β1 mutants, as bundles contained fewer axons (Fig. 3E), and ~40% of large axons were segregated at the periphery of the bundles (Fig. 3B, asterisks and 3D). Thus, in the absence of dystroglycan, SCs can engage axons and segregate them, but they are then arrested before defasciculating the axon into a 1:1 relationship. By immunoelectron microscopy of P3-P4 wt nerves we found more gold grains on SC processes that contacted small axons or were not engaged (stage 0) when using the anti-β1 integrin antibody than when using the dystroglycan antibody (Fig. 4C and Table 3). The number of gold grains marking β1 integrin did not increase between SC processes at stage 0 and SCs segregating or defasciculating large axons (stage 4-6). By contrast, the number of gold grains marking dystroglycan increased strikingly between the two stages (Fig. 4A,C and Table 3). Finally, α-dystroglycan glycosylation, which increases as nerves mature (Court et al., 2011), was significantly reduced in β1 integrin-null nerves. Collectively, these data suggest that dystroglycan acts after αβ1 integrin during axonal sorting.

Myelination defects in dystroglycan and double mutant mice

Both mutants showed myelin defects. In β1 integrin mutants, the few myelin sheaths were too thin for the caliber of the axon (data not shown). For dystroglycan mutants, in addition to an earlier onset of the redundant myelin loops previously described at 6 months of age (Saito et al., 2003) (data not shown), we observed disorganization and redundancy of inner and outer mesaxons (Fig. 2H,I, arrows), lack of compaction of myelin throughout the whole thickness of the myelin sheath (Fig. 2L) or in outer lamella (Fig. 2K) and inappropriate myelin wraps in the cytoplasm (Fig. 2J). The few myelinated fibers of double mutants were severely hypomyelinated (Fig. 2M). Myelin periodicity appeared normal, although non-compaction of the outer and/or innermost lamellae was observed (Fig. 2M, inset).

β1 integrin receptors, but not dystroglycan, transmit survival and proliferation laminin signals

Laminins promote SC proliferation (Yang et al., 2005; Yu et al., 2005) and survival (Yu et al., 2005) and dystroglycan is required in vitro for laminin-mediated protection of SC from apoptosis (Li et al., 2005b). To determine which laminin receptor promotes

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<th>Genotype</th>
<th>β1 integrin null (n=3)*</th>
<th>Dystroglycan null (n=3)*</th>
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<tr>
<td>Average axonal diameter in bundles</td>
<td>1.47±0.10 μm (range 0.12-5.91)</td>
<td>0.64±0.01 μm (range 0.08-3.92)</td>
</tr>
<tr>
<td>Number of axons per bundle</td>
<td>124±8</td>
<td>73±12</td>
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*44 and 36 bundles of unsorted axons in dystroglycan and β1 integrin-null sciatic nerves were analyzed, respectively. Bundles in dystroglycan mutants contain significantly fewer axons (P=0.0009) with smaller diameters (P<0.0001) than in β1 integrin mutants. Student’s t-test, errors indicate s.e.m.
proliferation and survival in vivo, we measured the rate of apoptotic and proliferating cells in mutant nerves by TUNEL and anti-histone H3 staining (D’Antonio et al., 2006). Proliferation was significantly decreased in β1 integrins and double mutants at P3 and P5 (Fig. 5). The decreased proliferating rate at P3 was confirmed using BrdU incorporation (see Fig. S2 in the supplementary material). By contrast, dystroglycan mutants showed a transient increase in proliferation at P15. Apoptosis was increased in β1 integrin and double mutants, but not dystroglycan mutants at P3 and P5 (Fig. 6). Staining with the SC marker periaxin (Gillespie et al., 1994) showed that 80% of the TUNEL positive cells were SCs (see Fig. S3 in the supplementary material). Thus, only β1 integrin receptor(s) mediate the pro-survival and proliferation signal from laminins during axonal sorting.

The decreased proliferation and survival in β1 integrin mutants was surprising, as they displayed normal proliferation and survival when in a mixed 129/C57BL6 background (Feltri et al., 2002). The increased severity of the sorting defects in congenic mice could be due to a reduction in SC number in addition to the described inability to make lamellipodia (Nodari et al., 2007). Another surprise was that double mutant nerves are as severe as nerves lacking all laminins, yet the decrease in SC survival in double receptor mutants is much smaller (<1%) than that reported in mice lacking all laminins (Yu et
To clarify these points, we confirmed our ability to detect apoptosis in nerves, and then compared β1 integrin mutants in congenic versus mixed background and compared β1 integrin mutants with laminin γ1 mutants. We performed TUNEL on P1 rat nerves after axonal transection, which is known to cause extensive SC apoptosis (Grinspan et al., 1996), and we detected a rate of apoptosis (7%), similar to that reported by Grinspan et al. (Fig. 7).

When we compared the fraction of TUNEL positive SCs in P3 nerves lacking laminin γ1 with controls, we found a significant increase in apoptosis, but at a low level of <1%, similar to what we found in β1 integrin mutants (Figs 6 and 7). Finally, we re-analyzed β1 integrin mutant nerves in a mixed genetic background at P5, and we confirmed that in this situation no increase of SC apoptosis is detectable (Fig. 7), as reported (Feltri et al., 2002). We conclude that lack of β1 integrin causes a significant, but modest, decrease in SC proliferation and survival. The decrease in SC survival is similar to

### Table 3. Subcellular quantification of β1 integrin and dystroglycan on SC processes at different stages of radial sorting at P3 and P5

<table>
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<tr>
<th></th>
<th>Total number of gold grains*</th>
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<tr>
<td></td>
<td>On SC processes around</td>
</tr>
<tr>
<td></td>
<td>small axons or near untouched axons (stage 0)</td>
</tr>
<tr>
<td>Anti-β1 integrin antibody</td>
<td>54</td>
</tr>
<tr>
<td>Anti-β1 dystroglycan antibody</td>
<td>32</td>
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</table>

*Total number of gold grains found on wt SC processes classified based on their relationship with axons as depicted in Fig. 10.
that observed in the absence of laminins, indicating that receptor(s) containing the β1 integrin subunit transmit a laminin survival signal in developing nerves.

**Akt signaling is impaired in SCs lacking β1 integrins**

PI-3K and MAPK-Erk kinase are survival, proliferative and pro-myelinating signals in SCs (Goebbels et al., 2010; Maurel and Salzer, 2000; Newbern et al., 2011; Ogata et al., 2004). Absence of laminins causes defective activation of Akt which might explain the decreased survival and hypomyelination (Yu et al., 2005). We thus measured the levels of phospho-Akt473 and phospho-p42/44 (Erk1/2), as effectors of PI-3 and MAP kinases, in mutant nerves during sorting (P3). Similarly to nerves lacking laminins, p-Akt473 is significantly reduced in the absence of β1 integrins in SCs, whereas phospho-p42/44 (p-p42/44) is normal (Fig. 8A,B). Akt in SCs is phosphorylated in response to growth factors, such as neuregulin-type III, on axons (Taveggia et al., 2005). We therefore investigated whether the defective Akt473 phosphorylation in β1 mutants was an indirect consequence of the lack of axonal contact, or whether it represented a direct requirement of laminin/β1 integrin signaling to activate Akt. We exposed β1-deficient SCs in culture to neuregulins and examined whether they could phosphorylate Akt473. Rat SCs were transfected with three β1 integrin silencing oligonucleotides, which efficiently reduced expression (Fig. 8D), and were stimulated with either the soluble EGF domain common to all neuregulins or neuronal membranes containing neuregulin-type III (Taveggia et al., 2005). Reduction of β1 integrin did not preclude Akt phosphorylation in response to either soluble or axonal-bound neuregulins. Thus, β1 deficient SCs are competent to activate Akt in response to neuregulin provided both as a paracrine or a juxtacrine signal, suggesting that the reduced Akt473 phosphorylation observed in vivo is a consequence of the failure of mutant SCs to contact axons.
Src activation is decreased in SCs missing β1 integrins

Binding of laminins to cultured SCs recruits dystroglycan and utrophin, and causes a dystroglycan and sulfatide-dependent phosphorylation of Src at position p-416 (Li et al., 2005b). In turn, Src phosphorylation is required for laminin-dependent protection of SCs from anoykisis (Li et al., 2005b). Src is also implicated downstream of neuregulins and the phosphatase Shp2 in SCs (Grossmann et al., 2009). To test whether the absence of dystroglycan or β1 integrin impaired Src activation during sorting, we measured Src(p416) phosphorylation in P3 mutant nerves. This antibody crossreacts also with activated Fyn and Lyn (Li et al., 2005b). Interestingly, β1 integrin deletion alone was sufficient to impair Src activation. These data are consistent with the fact that only β1 integrin-null SCs displayed decreased survival in vivo. To test whether dystroglycan was required for signaling after P3, we measured p-Src(p416), p-Akt(Thr473), p-p42/44(Tyr202/Tyr204) and p-p38(Thr180/Tyr182) at P15 and P28, but found no significant differences between dystroglycan-null and wt nerves (see Fig. S4 in the supplementary material).

Laminin signaling to p38 MAPK requires both β1 integrins and dystroglycan

Other MAP kinases in SCs are Jnk/Sap and p38 (Cavaletti et al., 2007). Jnk/Sap is induced by NT3-TrkC in SCs (Yamauchi et al., 2003) and inhibits SC differentiation through c-Jun (Parkinson et al., 2008; Parkinson et al., 2004). By contrast, p38 is induced by laminins, and is required for myelination in neuron-SC co-cultures (Fragoso et al., 2007; Fragoso et al., 2003). To determine whether the activation of p38 MAPK by laminins requires β1-class integrins or dystroglycan, we measured phospho (p)-p38(Thr180/Tyr182) in P3 SCs. Dystroglycan-null SCs spreading on laminin, and visualized its localization using anti-GST antibodies. Dystroglycan-null SCs were able to produce lamellipodia with PBD enrichment, suggesting that active Rac1 is properly recruited at the leading edge of these cells. We found that the levels of RhoE were significantly reduced in nerves from dystroglycan-null mice compared to controls, indicating that RhoGTPases are active in dystroglycan-null SCs. Indeed, RhoGTPases are GTP-bound, integrins promote their targeting to the plasma membrane to interact with effectors (del Pozo et al., 2000). Dystroglycan-null SCs are unable to translocate GTP-bound Rac1 to the membrane and to produce radial lamellipodia (Nodari et al., 2007). To test whether dystroglycan has a similar role, we added purified GST-Pak-binding domain (GST-PBD) to wt or dystroglycan-null SCs spreading on laminin, and visualized its localization using anti-GST antibodies. Dystroglycan-null SCs were able to produce lamellipodia with PBD enrichment, suggesting that active Rac1 is properly recruited at the leading edge of SCs in the absence of dystroglycan (Fig. 9).

DISCUSSION

Role of dystroglycan in peripheral nerve disease

We show that dystroglycan has a previously unrecognized role in the radial sorting of axons during peripheral nervous system development. Of note, the topography of the sorting defects in the absence of dystroglycan is severe in spinal roots and mild in distal
nerves, and recapitulates precisely the defect observed in the absence of laminin 211 (Bradley and Jenkison, 1973; Stirling, 1975; Yang et al., 2005). Our data nicely complement the recent finding of incomplete rescue of $D_{3k}^{3k}/D_{3k}^{3k}$ mice using a transgenic laminin 111 lacking the Lg4-5 domain. This domain binds dystroglycan specifically, and its absence could not rescue the radial sorting defects present in roots (Gawlik et al., 2010). This, together with older data indicating that dystroglycan binds laminin 211 in SCs (Yamada et al., 1996), indicates that dystroglycan is a functional laminin 211 receptor during radial sorting. Multiple muscular dystrophies carry mutations in genes coding for both extracellular (laminin 211) and intracellular (sarcoglycans, dystrophin)

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**Fig. 8.** Impaired Akt, Src and p38MAPK activation during sorting. (A-C) Western blots of P3 sciatic nerves pooled from three to six animals from the indicated genotypes. Graphs indicate mean of at least three experiments using different pools of animals. Error bars indicate s.e.m. *$P \leq 0.05$, **$P \leq 0.005$, ***$P \leq 0.0005$, n.s., non significant, by Student's t-test. (B) Western blot using anti-p-Akt$^{Ser473}$ antibodies and normalized for total Akt levels show a significant decrease in p-Akt$^{Ser473}$ only in $\beta_1$ mutants. (A) p-P44/42 levels are similar in mutant and wt. (C) p-Src$^{ Tyr416 }$ is decreased in $\beta_1$ integrin mutants whereas p-p38$^{ Thr180/Tyr182 }$ (E) is decreased only when both $\beta_1$ integrins and dystroglycan are deleted. (E) Western blot shows the efficient silencing of $\beta_1$ integrin, and the appropriate phosphorylation of Akt on Ser$^{473}$ in $\beta_1$ integrin-silenced SCs. (D) Rat SCs transfected with either $\beta_1$ integrin silencing or control siRNA, starved and stimulated with either the soluble EGF domain or dorsal root ganglia membranes containing neuregulin-type III (Taveggia et al., 2005).
components of the dystroglycan complex, as well as in the genes for at least six enzymes that mediate dystroglycan post-translational modifications, including acetylglucosaminyltransferase-like protein (Large) and fukutin. Thus, the genetic defects of multiple myopathies ultimately converge on the dystroglycan complex (reviewed by Barresi and Campbell, 2006; Michele et al., 2002; Muntoni et al., 2004). Dystroglycanopathies are often syndromic, reflecting the role of dystroglycan in many tissues. In the central nervous system, for instance, deletion of dystroglycan in mice recapitulates neuronal migration defects and other cortical abnormalities found in MDCs (Moore et al., 2002). Similarly, we show here that dystroglycan deficiency recapitulates the peripheral neural defects seen in MDC1A, MDC1C and MDC1D. These data strongly suggest that dystroglycan is the substrate of Large and fukutin during axonal sorting, explaining the puzzling observation of defective radial sorting in animal models of MDC1C and MDC1D.

Dystroglycan and αβ1 integrins cooperation in SCs

We have shown that αβ1 integrins and dystroglycan cooperate in the radial sorting of axons. Sorting is completely arrested in the absence of both αβ1-integrins and dystroglycan, indicating that they are the only laminin receptors required for this process, and, similar to laminins themselves, the receptors are also not redundant. Cooperation between these receptors can occur in various, non-mutually exclusive ways. αβ1 integrins and dystroglycan might have identical roles, interacting with the same laminins and transmitting the same signals, but still be required together to achieve full activation. Our data argue against this possibility: αβ1 and dystroglycan mediate different morphogenetic steps and activate different signals during radial sorting. Alternatively, it is possible that αβ1 integrins and dystroglycan interact with different laminins, or that they interact with the same laminins but the consequences of the ligation are different. Our in vivo analysis indicates that dystroglycan acts as a laminin 211 receptor during axonal sorting. By contrast, published data in vitro suggest that αβ1 integrins are required for SC adhesion to both laminin 211 and 411 (Wallquist et al., 2005; Yang et al., 2005). Whether this means that one or more αβ1 integrin receptor in SCs binds to different laminins is not clear at the moment. Independently of how many αβ1 integrins are required, our data so far suggest that at least one αβ1 integrin is required before dystroglycan, because the β1 subunit is present in immature SCs contacting many axons, and its absence arrests radial sorting at an early stage. By contrast, dystroglycan acts at subsequent steps, as it is mostly detectable in SCs contacting many axons, and during radial sorting of SCs before defasciculation of the axon. The idea that αβ1 integrins and dystroglycan act in sequence is supported by our epistatic genetic experiments in single and double-null mice and by the immature glycosylation of α-dystroglycan in β1 integrin-null nerves.

Multiple laminin receptors function in the multistep process of axonal sorting

Previous experiments in mice identified roles for Cdc42, Rac1, Fak and Ilk in radial sorting (Benninger et al., 2007; Grove et al., 2007; Nodari et al., 2007; Pereira et al., 2009). Having identified the two
of axons (Figs 4 and 9). By contrast, laminin 211 and 411, with a common basal lamina, insertion of processes within axons, (3) contact and recognition of large axons, and (4) segregation of large axons at the periphery, (5) matching of axons and SC number and (6) defasciculation of the axon with formation of an independent basal lamina. From our data it appears that the laminin 211-dystroglycan pair is implicated in the final steps of radial sorting, defasciculation of the axon with formation of an independent basal lamina (steps 4-6). Subcellular analysis of the localization of these molecules and the timing of their activation, together with comparisons of their null phenotypes and new experimental paradigms to model each of these steps in vitro will be required to further combine molecules into pathways at the appropriate step.

Control of cell number during radial sorting
The absence of laminins 211 and 411, B1 integrin (this paper), Fak, and Cdc42 in SCs causes an arrest in axonal sorting accompanied by a decrease in proliferation. In the absence of Cdc42 and y1 laminins, cell death is also increased, in the latter case at high levels, in contrast with similar studies conducted in the absence of laminins o2 and o4 (Benninger et al., 2007; Grove et al., 2007; Yang et al., 2005; Yu et al., 2005). Thus, whether laminins promote SC survival in vivo remains controversial. In our hands, y1 laminin and B1 integrin mutants showed a modest increase in apoptosis. We found that the genetic background of the mutants influences SC survival (Fig. 7), possibly explaining the conflicting findings. Still, having compared all the mutants with the same method we conclude that in laminin and integrin mutants apoptosis rates are only modestly increased (below 1%). Although minimal, this increase in apoptosis with concomitant decrease in SC proliferation could contribute to diminishing the number of SCs available to ensheath axons during radial sorting. Owing to the altered shape and ensheath axons during radial sorting. Owing to the altered shape of developmentally arrested SCs in these mutants, unbiased stereology with cell-specific marking will be required to determine whether this is the case. At least for B1 integrin, the decreased proliferation and survival is, at least in part, an indirect consequence of the failed access of mutant SCs to neuregulins on axons, as providing neuregulins directly to B1 integrin-null SCs in culture rescues phosphorylation of Akt1-3.

Surprisingly, dystroglycan mutants showed instead a transient increased in proliferation at P15. Because dystroglycan-null SCs form short internodes evident after P10 (Court et al., 2009), it is possible that mutant SCs require a burst of proliferation at this time to cover up all the axolemma. Alternatively, the incomplete radial sorting could cause the SCs around bundles to proliferate because they are not proceeding to myelinate. This transient proliferation was not due to decreased expression of p21Kip1 and p27Cip1 (cyclin-dependent kinase inhibitors p21 and p27) (data not shown).

Laminin receptors and myelination
It is unclear whether laminins and receptors are required for myelination after radial sorting is complete. Direct in vivo evidence is lacking, owing to the redundancy and compensation of the
laminin isoforms when only one is missing (Patton et al., 1997; Previtali et al., 2003) and the arrest before myelination when all are lacking (Yu et al., 2005). Our data show that the few SCs that escape arrest in sorting in the absence of β1 integrins or β1 integrins and dystroglycan form thin myelin sheaths (Figs 1, 2), suggesting a role for laminin receptors after radial sorting. In support of this, lamins are strictly required for myelination in vitro (Podratz et al., 2001). It is thus possible that laminins and laminin receptors would modulate signaling pathways required for myelination. A possible effector is p38MAPK, which is activated by laminins and is required for myelination in vitro by both SCs and oligodendrocytes (Bhat et al., 2007; Chew et al., 2010; Fragoso et al., 2007; Fragoso et al., 2003). Interestingly, we show that the absence of either β1 integrins or dystroglycan is insufficient to impair the activation of p38MAPK, but when both are missing phosphorylation of p38MAPK is reduced. Thus, p38 MAPK might act downstream of laminins to promote myelination in vivo.

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Dystroglycan in axonal sorting


