Development/Plasticity/Repair

Glypican-1 and α4(V) Collagen Are Required for Schwann Cell Myelination

Michael A. Chernousov, Katrina Rothblum, Richard C. Stahl, Ann Evans, Lisa Prentiss, and David J. Carey
Weis Center for Research, Geisinger Clinic, Danville, Pennsylvania 17822-2601

Schwann cell myelination requires interactions with the extracellular matrix (ECM) mediated by cell surface receptors. Previously, we identified a type V collagen family member, α4(V) collagen, which is expressed by Schwann cells during peripheral nerve differentiation. This collagen binds with high affinity to heparan sulfate through a unique binding motif in the noncollagenous N-terminal domain (NTD). The principal α4(V) collagen-binding protein on the Schwann cell surface is the heparan sulfate proteoglycan glypican-1. We investigated the role of α4(V) collagen and glypican-1 in Schwann cell terminal differentiation in cultures of Schwann cells and dorsal root ganglion neurons. Small interfering RNA-mediated suppression of glypican-1 expression decreased binding of α4(V)-NTD to Schwann cells, adhesion and spreading of Schwann cells on α4(V)-NTD, and incorporation of α4(V) collagen into Schwann cell ECM. In cocultures, α4(V) collagen coassembles with laminin on the surface of polarized Schwann cells to form tube-like ECM structures that are sites of myelination. Suppression of glypican-1 or α4(V) collagen expression significantly inhibited myelination. These results demonstrate an important role for these proteins in peripheral nerve terminal differentiation.

Key words: **Schwann cell; extracellular matrix; proteoglycan; myelin; collagen; dorsal root ganglion**

Introduction

A number of studies have demonstrated a requirement for interaction of Schwann cells with molecules in the basal lamina, which assembles on the surface of axon-Schwann cell units, to induce myelination. In cocultures of Schwann cells and sensory neurons, Schwann cells fail to myelinate axons in the absence of basal lamina (Moya et al., 1980). Myelination is restored by providing exogenous basal lamina (Carey et al., 1986) or ascorbic acid, which stimulates endogenous extracellular matrix (ECM) assembly (Carey and Todd, 1987; Eldridge et al., 1987). Laminin-2 is a major component of the Schwann cell basal lamina. *Dystrophic* (*dy*) mice, which lack laminin-2 because of a mutation in the laminin α2 subunit, display abnormal basal lamina assembly and myelination defects (Bradley and Jenkison, 1973;Yang et al., 2005). Disruption of the laminin α4 chain, which is expressed at low levels in Schwann cells and is a component of laminin-8, causes dysmyelination without affecting basal lamina ultrastructure (Wallquist et al., 2005; Yang et al., 2005). Deletion of the laminin γ1 subunit, which is a component of laminin-2 and laminin-8, also causes peripheral dysmyelination (Chen and Strickland, 2003; Yu et al., 2005).

The interaction of Schwann cells with basal lamina is mediated by ECM receptors on Schwann cell surface. The best-characterized receptors are the heterodimeric integrin receptors. Function-blocking anti-β1 integrin antibodies inhibit myelination in Schwann cell–nerve cell cocultures (Fernandez-Valle et al., 1994). Conditional knock-out of Schwann cell β1 integrin produces dysmyelinating neuropathy characterized by inability of Schwann cells to segregate axons (Feltri et al., 2002). Dystroglycan is a nonintegrin laminin receptor. Conditional knock-out of Schwann cell dystroglycan causes multiple abnormalities, including abnormal myelin sheath folding (Saito et al., 2003).

Cell surface heparan sulfate proteoglycans can also mediate Schwann cell–ECM interactions. Schwann cells express at least two cell surface proteoglycans, the lipid-anchored glypican-1 (Carey and Stahl, 1990; Carey et al., 1993) and the transmembrane syndecan-3 (Carey et al., 1992, 1997). Although Schwann cell heparan sulfate proteoglycans bind ECM proteins (Chernousov et al., 1996; Rothblum et al., 2004), including laminin (Carey and Stahl, 1990; Carey et al., 1990), the functional importance of these interactions remains obscure.

Developing Schwann cells synthesize collagen type V molecules that contain a unique α4(V) chain that binds with high affinity to heparan sulfate (Chernousov et al., 1996, 2000). This binding is mediated by a high-affinity site in the noncollagenous N-terminal domain (NTD) of the α4(V) chain (Erdman et al., 2002). α4(V)-NTD mediates heparan sulfate-dependent Schwann cell adhesion (Erdman et al., 2002; Rothblum et al., 2004). Glypican-1 is the major α4(V)-NTD binding protein of cultured Schwann cells (Rothblum et al., 2004).

We investigated the function of α4(V) collagen in myelinating cocultures of Schwann cells and sensory neurons. α4(V) collagen is found in tube-like ECM structures that surround axon-Schwann cell units before and during myelination. Suppression of expression of α4(V) collagen or its receptor, glypican-1, by small interfering RNA (siRNA) transfection inhibits myelination.

Received June 21, 2005; revised Oct. 19, 2005; accepted Nov. 3, 2005.

This work was supported by National Institutes of Health Grant NS21925. We thank Robert Erdman and Kim Derr for providing Schwann cell cultures and John Shaw for assistance with electron microscopy. Hynda Kleinman (National Institutes of Health, Bethesda, MD) generously provided anti-collagen type IV antibodies.

Correspondence should be addressed to David J. Carey, Weis Center for Research, Geisinger Clinic, 100 North Academy Avenue, Danville, PA 17822-2601. E-mail: djcarey@geisinger.edu.

DOI:10.1523/JNEUROSCI.2544-05.2006

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This suggests an important and previously unrecognized role for these proteins in Schwann cell terminal differentiation.

Materials and Methods

Cell cultures. Schwann cells were isolated from newborn rat sciatic nerves as described previously (Carey and Stahl, 1990; Erdman et al., 2002). The cells were cultured on poly-L-lysine-coated culture dishes in DMEM, 10% fetal bovine serum, and 2 μM forskolin. To stimulate ECM assembly, Schwann cells were treated with 50 μg/ml L-ascorbic acid (Sigma, St. Louis, MO), with addition of fresh ascorbic acid every 24 h (Chernousov et al., 1998).

Cultures of dissociated dorsal root ganglion (DRG) neurons were prepared as described previously (Carey et al., 1986; Carey and Todd, 1987). Briefly, DRGs were dissected from 18-d-gestation rat embryos, and connective tissue capsules were removed. After trypsinization in 0.05% trypsin in calcium and magnesium-free HBSS, the cells were mechanically dispersed, resuspended in FUdR medium [Eagle's MEM, 10% fetal bovine serum, 10 ng/ml nerve growth factor (NGF), 10^{-6} M 5-fluorodeoxyuridine] and plated onto 11 mm diameter Aclar discs that were coated with ammoniated rat collagen type I (approximately six ganglia per disc). The cultures were incubated in multiwell chambers in a humidified tissue culture incubator at 37°C. The medium was changed every other day, alternating between serum-free N2 medium, FUdR medium, and AraC medium (Eagle's MEM, 10% fetal bovine serum, 10 ng/ml NGF, 10^{-6} M cytosine arabinoside). After 3 weeks, the cultures were seeded with Schwann cells ($\sim 3.0 \times 10^5$ cells/Aclar disc) in Eagle's MEM, 10% fetal bovine serum, and 10 ng/ml NGF. After 3 d, cells were switched to N2 medium plus NGF and incubated for 1 week before transfection. ECM assembly and myelination were stimulated by addition of 50 μg/ml ascorbic acid. Animal use was approved by the Institutional Animal Care and Use Committee.

siRNA transfection. siRNAs were selected using the Whitehead Institute siRNA Selection Program (Yuan et al., 2004) plus additional criteria for rational siRNA selection (Reynolds et al., 2004). The following target sequences were used: glypican-1 (GenBank accession number NM_030828), 5′-CCTGCTGCACCAGTGAGATG; collagen α 4(V) (GenBank accession number AF272661), 5′-CAGCTCTTTCCAGATGGACAT. Synthetic double-stranded siRNAs were purchased from Ambion (Austin, TX). siNegative 1 (siNeg) from Ambion was used as a control. Schwann cells were transfected using I-fect reagent (Neuromics Antibodies, Minneapolis, MN). Schwann cells were transfected at a density of 50–70% confluence in 60 mm dishes. Cells were washed and placed in 2.4 ml of Optimem I (Invitrogen, San Diego, CA). Twenty-six microliters of I-fect reagent were diluted into 25 μl of Optimem I. In another tube, 2 μl of 20 μM siRNA was diluted into 15 μl of Optimem and 65 μl of RNA diluent. After a 5 min incubation at room temperature, the siRNA mixture was gently mixed with the diluted I-fect, incubated for 5 min at room temperature, and added to the culture medium. After 4 h, 2.5 ml of DMEM, 20% fetal bovine serum, 4 μM forskolin, and 2% penicillin/streptomycin was added. For coculture transfection, 12 μl of I-fect reagent and 4 μl of 20 μM siRNA were used. The siRNA–I-fect mixture was added to cocultures that contained 200 μl of Eagle's MEM, 10% fetal bovine serum, and 10 ng/ml NGF. After incubation for 4 h, 250 μl of medium plus penicillin/streptomycin was added. For myelination experiments, Schwann cell-DRG neuron cocultures were transfected on day 1 and retransfected on days 3 and 11. Ascorbic acid was added daily beginning on day 4. Cultures were fixed and stained on day 14. In one series of experiments, Schwann cells were transfected with siRNAs and the following day replated onto DRG neurons. Ascorbic acid was added the following day, and the cells were fixed and stained 7 d later. All experiments involving siRNA transfection were repeated at least three times, and the results of representative experiments are shown.

Real-time reverse transcription-PCR. Total RNA was isolated using the Total RNA Isolation Mini-Kit (Agilent Technologies, Wilmington, DE). Total RNA (2 μg) was used in a reverse transcription (RT) reaction with Superscript II (Invitrogen). cDNA was purified on a DyeEx 2.0 spin column (Qiagen, Hilden, Germany), diluted, and used as template in real-time PCR assays in a Lightcycler (Roche Diagnostics, Welwyn Garden City, UK) with QuantiTect SYBR Green PCR mix (Qiagen). PCR primers were as follows: glypican-1 forward, 5′-CGAAGTCCGCCAGATCTACG; glypican-1 reverse, 5′-ATCCTGCAGTGTACGCTCC; collagen α4(V) forward, 5′-CAAGTCCAGCCTTCTCAGC; collagen α4(V) reverse, 5′-AACAGCAGCTCCTGGATGTC; β-actin forward, 5′-CCC TCTGAACCCTAAGGCCAACCG; β-actin reverse, 5′-GTGGTGGTGAAGCTG-TAGCCACGC.

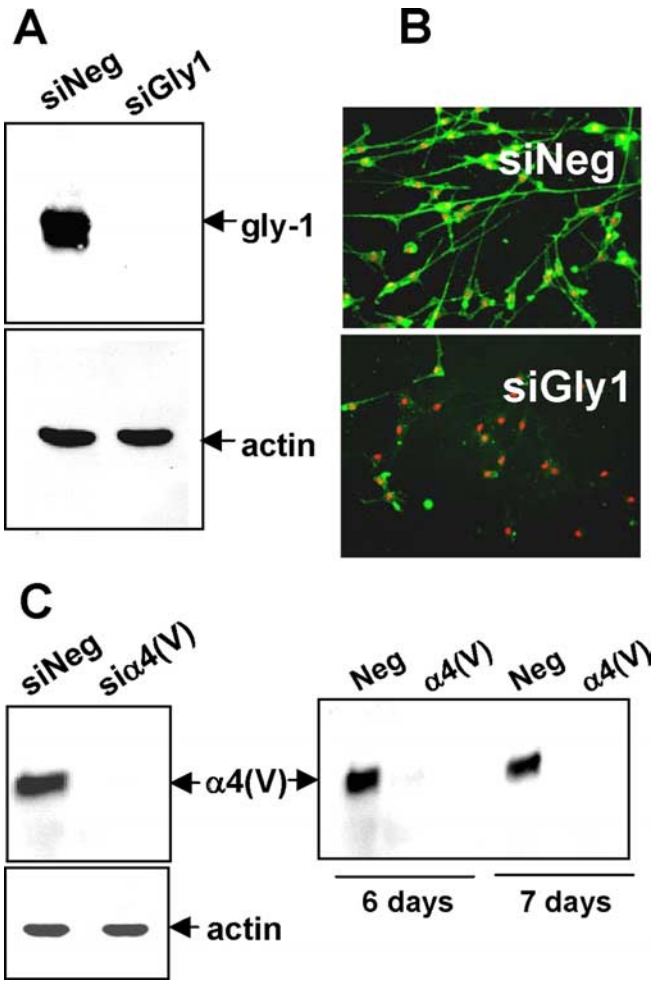

Figure 1. siRNA-mediated suppression of target gene expression in Schwann cells. ***A***, Detergent extracts of siNeg- or siGly1-transfected Schwann cells were digested with heparitinase and subjected to immunoblot analysis with anti-glypican-1 antibodies (top); aliquots of undigested extracts were immunoblotted with anti-actin antibodies (bottom) to verify equal sample loading. ***B***, Cell surface expression of glypican-1 was assessed by immunofluorescent staining of transfected cells 48 h after transfection using anti-glypican-1 antibodies (green) and DAPI (4′,6′-diamidino-2-phenylindole) to stain nuclei (red). ***C***, Schwann cells were transfected with siNeg or siα4(V), and conditioned media and cell lysates were harvested 48 h later (left) or at the indicated times after transfection (right); aliquots of medium (top) or cell lysates (bottom) were subjected to immunoblot analysis and stained with anti-α4(V) collagen (top) or anti-β-actin (bottom) antibodies.

Immunoblot analysis. To assess glypican-1 expression, Schwann cells were rinsed with PBS and scraped in 0.5% octyl β-D-glucopyranoside (Sigma) in PBS plus protease inhibitors. The cell extracts were vortexed for 15 min on ice followed by centrifugation for 15 min at 10,000 rpm at 4°C. $CaCl_2$ was added to a concentration of 10 μM, and the samples were digested with heparitinase (Seikagaku Kogyo, Tokyo, Japan) at 37°C for 18 h. Digestion was stopped by addition of electrophoresis sample buffer; the samples were resolved by electrophoresis on 7.5% polyacrylamide SDS gels.

To detect α4(V) collagen or laminin in culture medium, the medium was harvested and centrifuged at 1500 rpm for 10 min to remove cell debris. Aliquots were subjected to SDS gel electrophoresis on 6% polyacrylamide-SDS gels.

To detect α4(V) collagen or laminin in ECM, the cells were washed with ice-cold PBS and extracted with 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EGTA, and 0.25 mM DTT, pH 7.2, plus protease inhibitors. Detergent-insoluble material remaining on the plates was extracted with gel sample buffer containing 2% SDS and 5% 2-mercaptoethanol heated to 100°C. The proteins were resolved by electrophoresis in 7.5% polyacrylamide SDS gels.

Proteins were transferred to polyvinylidene difluoride membranes (Immobilon, Pall, Pensacola, FL) and detected, as described previously, using anti-glypican-1 antibodies (Carey and Stahl, 1990), anti-actin antibodies (Sigma), anti-α4(V) collagen antibodies (Chernousov et al., 1996), or anti-laminin antibodies (Proteus Biosciences, Ramona, CA). Immunoreactive bands were quantitated using Lumi-Imager (Roche Diagnostics).

α4(V)-NTD and laminin binding assays. α4(V)-NTD binding to Schwann cells and laminin binding and cluster formation were assayed as described previously (Tsiper and Yurchenco, 2002; Rothblum et al., 2004) with modifications. Briefly, cells grown in glass slide chambers were incubated for 20–30 min in serum-free medium with purified recombinant α4(V)-NTD (10 μg/ml) or for 18 h in growth medium supplemented with 25 μg/ml mouse laminin (Sigma). The cells were then rinsed with PBS and processed for immunofluorescent staining with anti-α4(V)-NTD antibodies or anti-laminin antibodies and rhodamine-phalloidin. Bound antibodies and phalloidin were visualized by fluorescent microscopy.

Adhesion assays. Twenty-four-well culture plates were coated with 20 μg/ml α4(V)-NTD, collagen type IV, or laminin. Schwann cell adhesion was assayed as described previously (Chernousov et al., 2001; Erdman et al., 2002). Adhesion and spreading of Schwann cells on DRG neurons was assayed as described previously (Wanner and Wood, 2002), with modifications. Briefly, Schwann cells were labeled with 0.77 μM CellTracker Green (5-chloromethylfluorescein diacetate; Invitrogen) in N2 medium according to the recommendation of the manufacturer. The cells were harvested, resuspended in N2 medium supplemented with NGF and heregulin, and added to DRG neuron cultures. The cultures were incubated for 18–20 h, fixed and permeabilized with cold methanol, and stained with monoclonal anti-neurofilament antibody (Sigma). Schwann cells and axons were visualized by fluorescence microscopy. To analyze adhesion and neurite extension of DRG neurons on Schwann cells, a suspension of dissociated DRG neurons in N2 plus NGF and heregulin were seeded on monolayers of transfected Schwann cells and incubated for 18–20 h or 5 d. The cells were then fixed, permeabilized, and stained with anti-neurofilament antibody. Nerve cells were visualized by fluorescence microscopy. All adhesion experiments and binding assays (see above) were repeated at least three times.

Immunocytochemistry. To analyze cell surface or ECM proteins live, unfixed cells were incubated with primary antibodies, rinsed with PBS, and fixed with 3% formaldehyde. To stain intracellular proteins, the formaldehyde-fixed cells were permeabilized with 0.05% Triton X-100 in PBS for 5 min. The cells were incubated with a mixture of Alexa-conjugated secondary antibodies (Invitrogen) and, in some cases, rhodamine-phalloidin (Invitrogen) for 1.5 h and processed for fluorescence microscopy. To stain myelin segments, formaldehyde-fixed cells were permeabilized with methanol at −20°C for 10 min, rinsed, and stained with monoclonal anti-myelin basic protein (MBP) antibody (SMI 94; Sternberger Monoclonals, Exeter, UK). The following antibodies were used: polyclonal anti-α4(V) collagen antibodies, polyclonal anti-α1(V) collagen antibodies (Chernousov et al., 2000), polyclonal anti-collagen type IV antibodies (a gift from Hynda Kleinman, National Institutes of Health, Bethesda, MD), polyclonal anti-glypican-1 antibodies (Carey and Stahl, 1990), and polyclonal (Carey and Stahl, 1990) and monoclonal (anti-laminin B2, clone D18; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) anti-laminin antibodies.

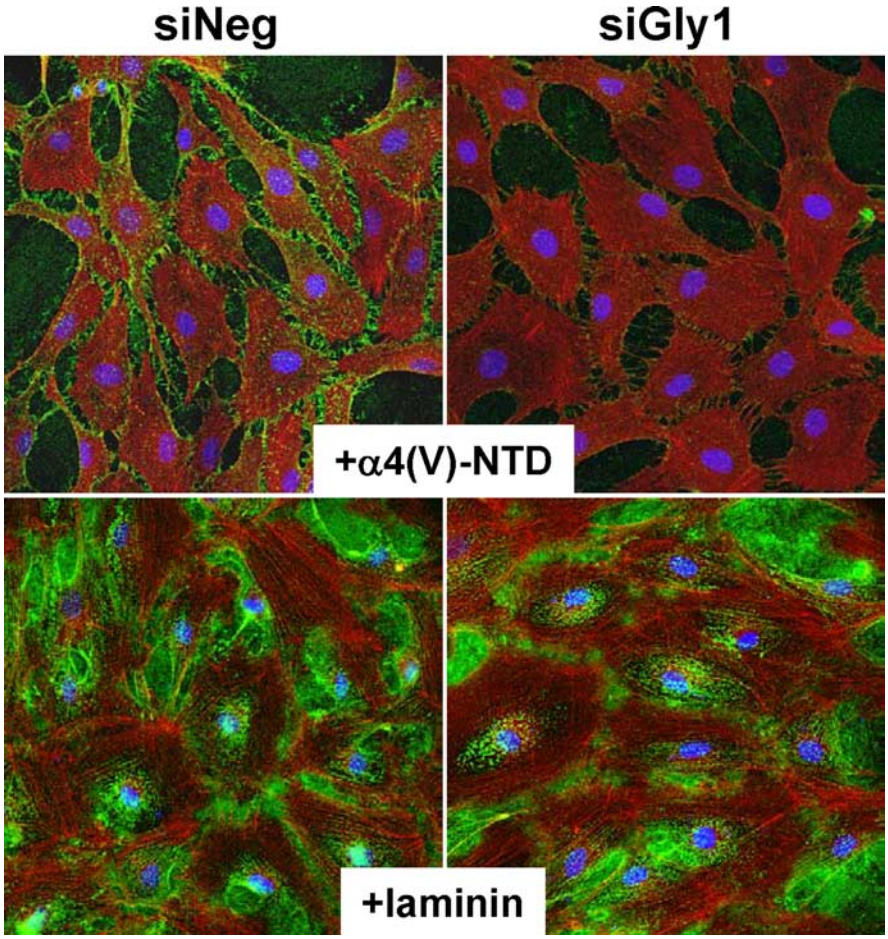

Figure 2. Binding of exogenous ECM proteins to Schwann cells. siNeg- or siGly1-transfected Schwann cells were incubated in medium with 10 μg/ml recombinant α(4)-NTD (top) or 25 μg/ml laminin (bottom) as described in Materials and Methods. The cells were dual stained with anti-α(4)-NTD antibodies (top) or anti-laminin antibodies (bottom) and rhodamine-phalloidin. Cell nuclei were stained with DraQ5. Images were obtained by confocal microscopy.

For conventional fluorescence microscopy, images were collected using a Nikon (Tokyo, Japan) Eclipse E800 microscope equipped with a Photometric (Huntington Beach, CA) Cool Snap ES digital camera. Image acquisition and processing were performed with MetVu software (version 6.2r2; Universal Imaging Corporation, West Chester, PA). For confocal microscopy, images were collected using a Leica (Nussloch, Germany) DM IRE2 laser scanning microscope equipped with a Leica TCS SP2 scanner. A 63× HCS PL APO [numerical aperture (NA), 1.40] and 100× NCS PL APO (NA, 1.40) were used. The acquisition software was Leica Confocal Software (version 2.5). Image analysis was performed using MetaMorph (version 6.1r0; Universal Imaging Corporation).

Electron microscopy. Cultures were fixed by perfusion with 2% glutaraldehyde, 100 mM sucrose, and 50 mM phosphate buffer, pH 7.4. The cultures were rinsed in 150 mM phosphate buffer, pH 7.4, and postfixed in 2% osmium tetroxide in 100 mM phosphate buffer. The cells were dehydrated in ethanol and embedded in Epox 812 (Fullam, Latham, NY). Ultrathin sections were placed on copper grids, stained with lead citrate and uranyl magnesium acetate, and examined in a JEOL (Peabody, MA) JEM-1200EX transmission/scanning electron microscope.

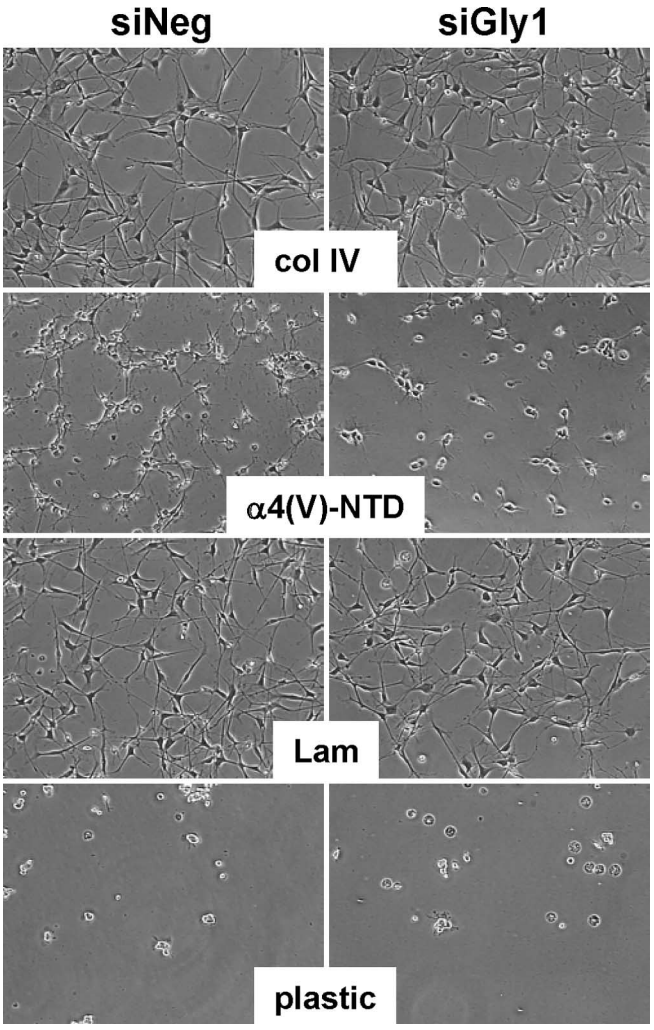

Figure 3. Schwann cell adhesion and spreading on ECM proteins. siNeg (left)- or siGly1 (right)-transfected Schwann cells were plated on wells coated with ECM proteins or on uncoated plastic as indicated in serum-free medium. Three hours after plating, cells were examined by phase-contrast microscopy, and photographs were taken. Col IV, Collagen type IV; Lam, laminin.

Results

We reported previously that glypican-1 is a major cell surface binding protein for α(V) collagen on cultured Schwann cells (Rothblum et al., 2004). Their interaction is based on heparan sulfate-mediated binding of glypican-1 to a high-affinity heparin-binding site in the α4(V)-NTD (Erdman et al., 2002). The purpose of this study was to investigate the role of glypican-1 and α4(V) collagen in Schwann cell terminal differentiation.

Transfection with glypican-1-specific siRNA (siGly1) was used to selectively suppress glypican-1 expression in Schwann cells. A double-stranded RNA of the same length and similar base composition that does not match any sequences in the rat genome (siNeg) was used as a control for siRNA transfection experiments. Transfection of neonatal rat Schwann cells with siGly1 caused an 85–95% reduction in glypican-1 mRNA levels, as determined by quantitative RT-PCR analysis (data not shown). Steady-state levels of glypican-1 proteoglycan, determined by immunoblot analysis of heparitinase-digested Schwann cell lysates with anti-glypican-1 antibodies, were decreased by a similar amount (Fig. 1*A*). Time course studies showed that the steady-state level of glypican-1 protein was reduced ≥85% by 24 h after transfection and remained at this low level for at least 7 d after siGly1 transfection (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material). This is consistent with the relatively rapid rate of membrane proteoglycan turnover in

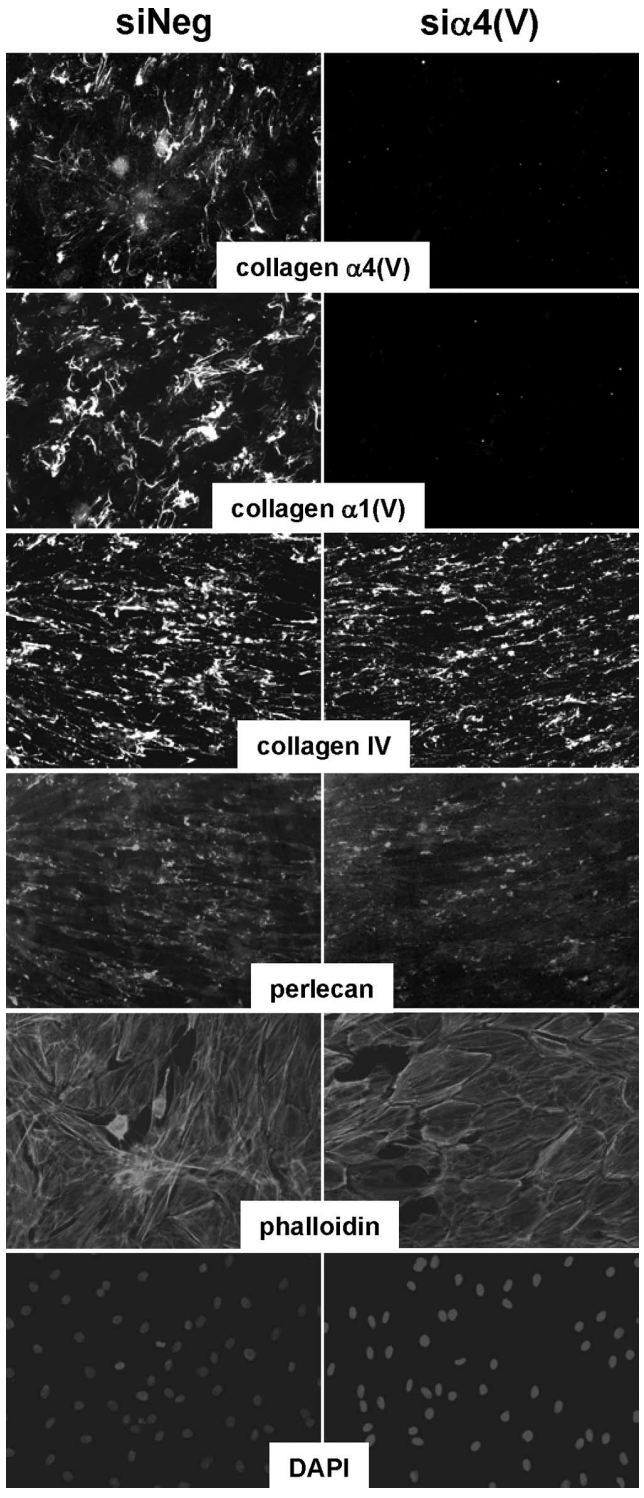

Figure 4. Suppression of α4(V) collagen expression leads to loss of incorporation of α1(V) and α4(V) collagen subunits into Schwann cell ECM. siNeg (left)- or siα4(V) (right)-transfected Schwann cells were treated with ascorbic acid as described in Materials and Methods and stained with anti-α(4) collagen antibodies, anti-α(1) collagen antibodies, anti-collagen type IV antibodies, or anti-perlecan antibodies. One set of cultures was also stained with rhodamine-phalloidin to visualize actin filaments; nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole).

Schwann cells (Carey and Evans, 1989). Immunofluorescent staining of transfected Schwann cells revealed a near total loss of glypican-1 immunoreactivity on >90% of the cells (Fig. 1*B*). These results demonstrate that siRNA transfection effectively suppresses Schwann cell glypican-1 expression.

The effect of loss of glypican-1 expression on α4(V) collagen binding was investigated. Binding of α4(V)-NTD to plasma membranes of siNeg-transfected Schwann cells was readily observed by immunofluorescent microscopy. Bound α4(V)-NTD was especially prominent on thin filopodial processes that projected from the cell surface (Fig. 2). Binding of α4(V)-NTD to siGly1-transfected cells was almost completely absent. These results are consistent with our previous findings (Rothblum et al., 2004) and demonstrate that glypican-1 is the major α4(V)-NTD binding protein on the Schwann cell surface.

Schwann cells bind exogenous laminin on their cell surface and form laminin clusters (Tsiper and Yurchenco, 2002). Because glypican-1 has been found to bind laminin (Carey and Stahl, 1990; Carey et al., 1990), we investigated the effect of suppression of glypican-1 expression on cell surface laminin binding. Binding of exogenous laminin to siNeg-transfected Schwann cells was observed by fluorescence microscopy. There was no effect of siGly-1 transfection on the amount or distribution of laminin bound to Schwann cells (Fig. 2). These results demonstrate that laminin binding and cluster formation are not dependent on cell surface glypican-1.

Previously, we reported that α4(V) collagen mediates Schwann cell adhesion and spreading via a heparan sulfate-dependent mechanism that utilizes the high-affinity heparin binding in the α4(V)-NTD (Erdman et al., 2002). We investigated the effect of siGly1 transfection on Schwann cell adhesion and spreading on ECM-coated dishes. Transfection with siGly1 produced a modest (~20%) but reproducible decrease in Schwann cell adhesion to α4(V)-NTD (data not shown). However, there was a nearly complete loss of Schwann cell spreading and process extension (Fig. 3). In contrast, transfection with siGly1 did not decrease Schwann cell adhesion or spreading on dishes coated with collagen type IV or laminin.

siRNA transfection was used to selectively suppress α4(V) collagen expression by Schwann cells. Transfection of Schwann cells with α4(V) collagen-specific siRNA [siα4(V)] caused a 75–85% reduction in α4(V) collagen mRNA levels, as determined by quantitative RT-PCR analysis (data not shown), and protein levels, as determined by immunoblot analysis of Schwann cell conditioned medium with anti-α4(V) collagen antibodies (Fig. 1*C*).

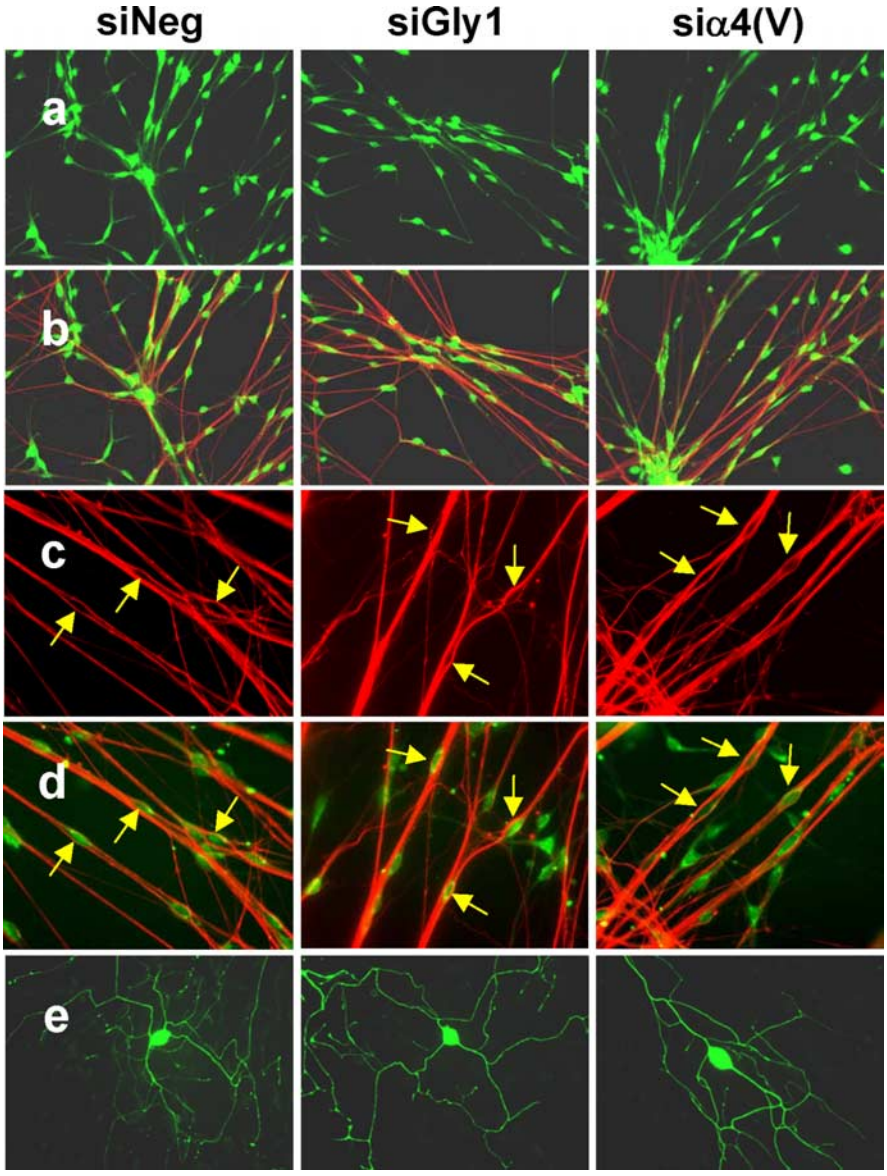

Figure 5. Suppression of glypican-1 or α4(V) collagen expression does not influence association of Schwann cells with DRG neurons. ***a–d***, Schwann cells transfected with siNeg, siGly1, or siα4(V) were labeled with Cell Tracker (green) and seeded on DRG neuron cultures. After 18 h (***a***, ***b***) or 5 d (***c***, ***d***), cocultures were stained with anti-neurofilament antibodies (red) to visualize DRG axons. ***a*** and ***b*** show Schwann cells only (***a***) and merged images (***b***) of the same fields. ***c*** and ***d*** show stained neurons only (***c***) and merged images (***d***) of the same fields; arrows mark Schwann cell intercalation into axon bundles. ***e***, DRG neurons were plated on monolayers of Schwann cells transfected with the indicated siRNAs. Eighteen to 20 h after plating, the neurons were stained with anti-neurofilament antibodies (green).

The effect of siRNA transfection on α4(V) collagen expression was maximal by 48 h after transfection and persisted for at least 7 d (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material).

We reported previously that in Schwann cell cultures treated with ascorbic acid, α4(V) collagen is assembled into collagen heterotrimers that also contain α1(V) collagen subunits (Chernousov et al., 2000). These type V collagen heterotrimers are incorporated into a subset of ECM fibrils that also contains collagen type IV (Chernousov et al., 1998). In siα4(V)-transfected Schwann cell cultures, incorporation of both α4(V) and α1(V) collagen subunits into the ECM was almost completely absent. In contrast, incorporation of collagen type IV or the basal lamina heparan sulfate proteoglycan perlecan was unaffected (Fig. 4). Together, these results demonstrate that siRNA

transfection effectively suppresses α4(V) collagen expression in Schwann cells.

The effect of suppression of glypican-1 and α4(V) collagen expression on Schwann cell terminal differentiation was investigated in cocultures of Schwann cell and DRG neurons. To determine the effects on Schwann cell adhesion to axons, transfected Schwann cells were fluorescently labeled and seeded onto cultures of DRG neurons. The cocultures were incubated for 18–20 h or 5 d and fixed and stained with anti-neurofilament antibody. Labeled Schwann cells and axons were visualized by fluorescence microscopy. Numerous Schwann cells adhered to axons and extended processes along them (Fig. 5*a*,*b*). By 5 d, Schwann cells had intercalated into axon bundles (Fig. 5*c*,*d*). There was no apparent effect of transfection with siGly1 or siα4(V) on the number of axon-adherent Schwann cells, Schwann cell extension on axons, or intercalation of Schwann cells into axon bundles.

In complementary experiments, we examined neurite extension by DRG neurons plated on transfected Schwann cells. The neurons attached avidly to the Schwann cells and elaborated extensive axonal outgrowths (Fig. 5*e*). The adhesive interactions required to support this outgrowth were dependent on cell–cell interactions between the Schwann cells and neurons, because the process was observed in the absence of ascorbic acid treatment (i.e., under conditions of absence of matrix) and was not affected by ascorbic acid treatment of the Schwann cells (data not shown). Transfection of the Schwann cells with siGly1 or siα4(V) had no effect on nerve cell adhesion or axonal extension (Fig. 5*e*).

We also investigated the effects of siRNA transfection on ECM assembly in cocultures of Schwann cells and DRG neurons. In cocultures treated with ascorbic acid, Schwann cells assemble a complex ECM that contains fibrillar matrix structures plus basal lamina matrix that contains laminin (Carey and Todd, 1987; Eldridge et al., 1987). Transfection with siα4(V) caused a significant reduction in the secretion of α4(V) collagen into the medium and incorporation of this collagen into the ECM but had no effect on secretion or matrix-deposition of laminin (Fig. 6). Transfection with siGly1 did not inhibit α4(V) collagen secretion but markedly inhibited α4(V) collagen deposition into the ECM in Schwann cell-DRG neuron cocultures. Secretion and matrix incorporation of laminin were not affected (Fig. 6).

Previous studies have shown that basal lamina assembly induces myelin formation by Schwann cells in cocultures with DRG neurons (Carey and Todd, 1987; Eldridge et al., 1987). We investigated the relationship between α4(V) collagen-containing matrix and myelin assembly in Schwann cell-DRG neuron cocultures. Cocultures incubated in serum-free medium without ascorbic acid lack any fibrillar or basal lamina matrix structures that can be detected by immunofluorescent staining with specific antibodies (Fig. 7) or by electron microscopy (Moya et al., 1980). One day after addition of ascorbic acid to initiate ECM assembly deposition of α(V) collagen into matrix is discernible by confocal microscopy (Fig. 7). α4(V) collagen is incorporated into a network of ECM fibrils as well as distinct tube-like matrix structures. α4(V) collagen-containing matrix tubes are fully formed by day 3. The α4(V) collagen-containing matrix tubes also contain laminin, suggesting they are nascent basal laminas (Fig. 7). The tube-like basal lamina structures form on the surfaces of a subpopulation of Schwann cells in the cocultures. The α4(V) collagen-containing tubes spanned the lengths of several Schwann cells. Myelin segments, visualized by staining with anti-MBP antibodies, were visible in the cocultures beginning on day 5 after ascorbic acid addition. Myelin segments increased in number and in length over the ensuing days. Myelin segments were formed only

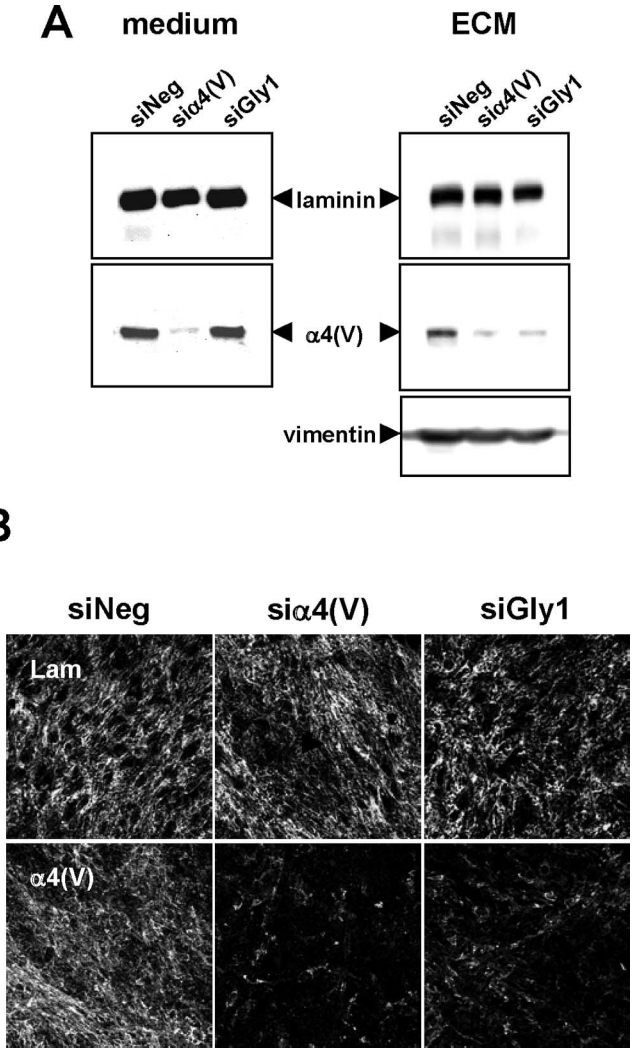

Figure 6. Suppression of α4(V) collagen or glypican-1 expression inhibits incorporation of α4(V) collagen, but not laminin, into ECM of Schwann cell–DRG neuron cocultures. ***A***, Aliquots of conditioned media and detergent-insoluble fractions (ECM) of cocultures transfected with siNeg, siGly1, or siα4(V) were subjected to immunoblot analysis with anti-laminin, anti-α4(V) collagen, or anti-vimentin antibodies. The cultures were incubated in medium with ascorbic acid for 3 d before harvesting. ***B***, Immunofluorescent staining of siRNA-transfected cocultures with anti-laminin and anti-α4(V) collagen antibodies; the cultures were incubated in medium with ascorbic acid for 48 h before staining. Lam, Laminin.

in association with α4(V) collagen-containing matrix tubes (Fig. 7). In some cases, adjacent myelin internodes separated by nodes of Ranvier were observed.

To determine whether α4(V) collagen is required for Schwann cell myelination, cocultures were transfected with siα4(V). The cultures were stained with anti-MBP antibodies 10 d after ascorbic acid addition to induce ECM assembly and were visualized by confocal microscopy. Transfection with siα4(V) produced an ~50% reduction in the number of myelin segments that were formed ($p < 0.01$) (Fig. 8*A*,*B*). A similar reduction in MBP steady-state levels was observed by immunoblot analysis of lysates of transfected cocultures (Fig. 8*C*).

We also investigated the effect of siGly1 transfection on myelination. SiGly1 transfection produced an ~75% reduction in the number of myelin segments ($p < 0.001$) and MBP steady-state level (Fig. 8). Essentially identical results were obtained when Schwann cells were first transfected with siGly1 and then

replated onto DRG neurons and incubated in medium with ascorbic acid. (data not shown). These results demonstrate that α4(V) collagen and glypican-1 play important roles in ECM-induced Schwann cell myelination.

Schwann cell and DRG neuron cocultures were also examined by transmission electron microscopy. During peripheral nerve development, myelin assembly is preceded by the sorting of axon bundles by processes of associated Schwann cells (Webster, 1971; Webster et al., 1973). Segregation of individual axons was observed in siNeg-transfected cultures 10 d after addition of ascorbic acid to initiate terminal differentiation (Fig. 9). There was no apparent effect of transfection with siGly1 or siα4(V) on the ability of Schwann cells to associate with or segregate axons. In addition, there was no apparent effect of transfection with siGly1 or siα4(V) on the ability of Schwann cells to assemble morphologically identifiable basal lamina matrix on their surfaces (Fig. 9). This result is consistent with the biochemical and light microscopic data demonstrating that siRNA transfection did not reduce incorporation of laminin into the ECM (Fig. 6).

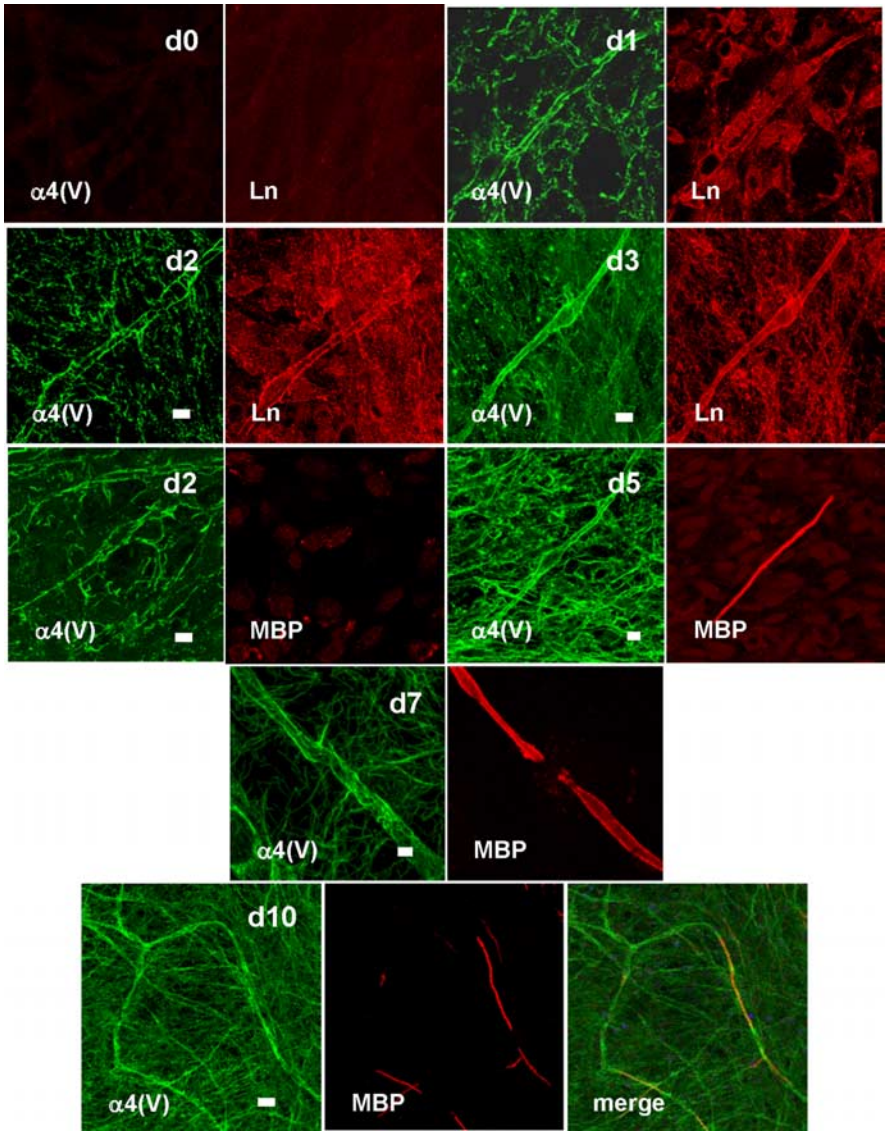

Figure 7. α4(V) collagen forms tube-like ECM structures that colocalize with laminin and are sites of myelin assembly in Schwann cell–DRG neuron cocultures. Cocultures were treated with ascorbic acid for the indicated days and dual stained with anti-α4(V) collagen and anti-laminin (Ln) antibodies or anti-α4(V) collagen and anti-MBP antibodies. Scale bars represent 10 μm, except for the d7 (5 μm) and d10 (50 μm) micrographs.

Discussion

The importance of Schwann cell interaction with ECM as a prerequisite for myelination is well established (Bunge et al., 1986). Several matrix protein-receptor interactions that are important for Schwann cell myelination have been described. These include the adhesive protein laminin in the basal lamina (Chen and Strickland, 2003; Wallquist et al., 2005; Yang et al., 2005; Yu et al., 2005) and laminin-binding integrins (Feltri et al., 2002; Previtali et al., 2003b) and α/β-dystroglycan (Saito et al., 2003) on the Schwann cell membrane. The findings presented here strongly suggest another interaction that is important for myelination, between α4(V) collagen in the matrix and glypican-1. We have shown previously that glypican-1 is the primary receptor for α4(V) collagen on the surface of cultured Schwann cells (Rothblum et al., 2004). We now demonstrate that specific suppression of expression of α4(V) collagen or glypican-1 by siRNA transfection significantly reduces the amount of myelin that is formed in Schwann cell-DRG neuron cocultures. Additional evidence implicating α4(V) collagen in Schwann cell myelination is its presence in distinct tube-like matrix structures that are the sites of myelin assembly. These results suggest that Schwann cell-ECM interaction mediated by these proteins plays an important role in matrix-stimulated myelination by Schwann cells.

The available evidence suggests that the effect of suppression of glypican-1 expression on myelin formation is manifested because of perturbation of Schwann cell interactions with α4(V) collagen. We have shown previously that glypican-1 is a major cell surface binding protein for α4(V) collagen on cultured Schwann cells, through a binding interaction between the α4(V)-NTD and glypican-1 heparan sulfate chains. As shown here, glypican-1 mediates α4(V) collagen NTD binding to Schwann cells and Schwann cell adhesion and spreading on surfaces containing the α4(V)-NTD. Suppression of glypican-1 expression also significantly reduces incorporation of α4(V) collagen into the ECM. This suggests that α4(V) collagen binding to glypican-1 plays a dual role, modulating both matrix assembly and cell-matrix adhesion. This is reminiscent of fibronectin matrix assembly, which is dependent on fibronectin binding to cell surface integrin receptors that also mediate cell adhesion to fibronectin (Fogerty et al., 1990; Wu et al., 1993). Despite the fact that the matrix adhesion protein laminin binds heparan sulfate (Skubitz et al., 1988; Kouzi-Koliakos et al., 1989), suppression of glypican-1 expression had no observable effects on laminin binding to the Schwann cell surface, Schwann cell adhesion to or spreading on laminin-containing surfaces, or laminin incorporation into the ECM. These results strongly suggest that the effects

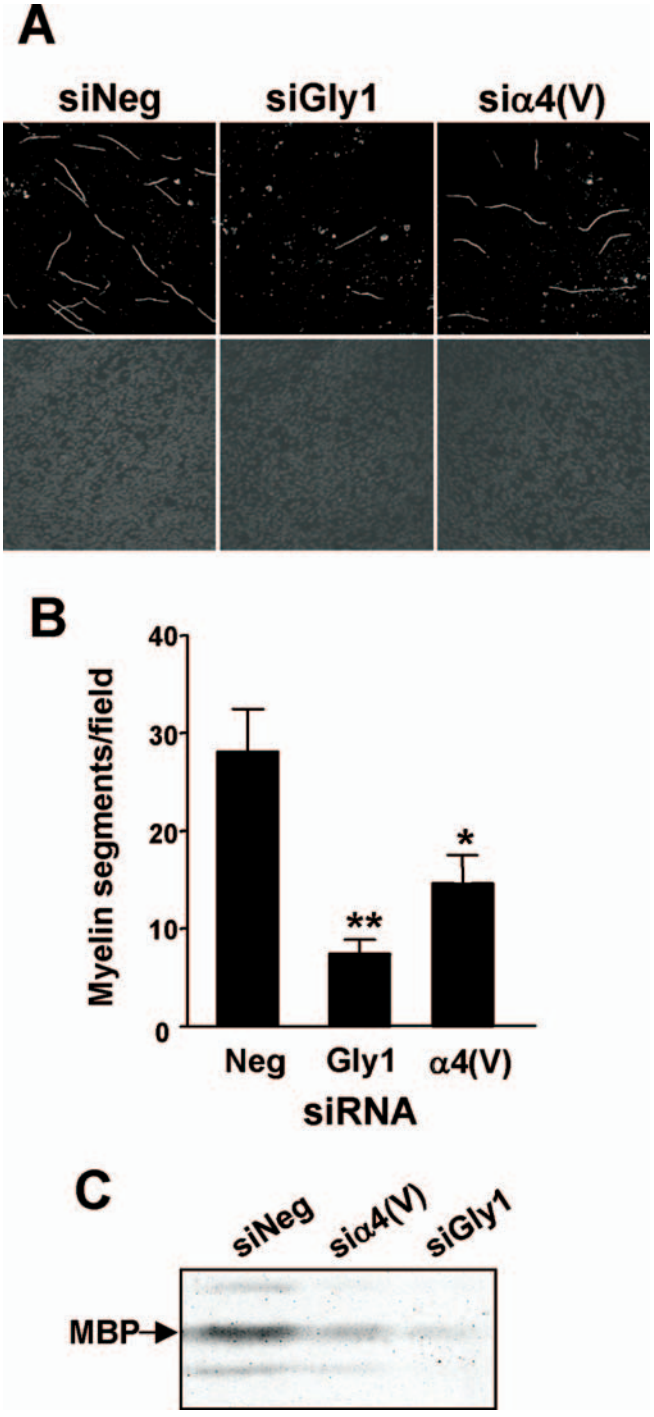

Figure 8. Suppression of α4(V) collagen or glypican-1 expression inhibits myelination in cocultures. ***A***, siRNA-transfected cocultures were incubated in ascorbic acid medium for 10 d to induce myelination; the cultures were fixed and stained with anti-MBP antibodies (top) and with DraQ5 (bottom) to visualize Schwann cell nuclei. ***B***, Quantification of myelin segments produced in siRNA-transfected cocultures. Data represent mean $\pm$ SD of myelin segments in 32 fields in four independent cultures for each condition. ***C***, Immunoblot analysis of MBP levels in cocultures transfected with the indicated siRNAs. *$p < 0.01$ versus siNeg; **$p < 0.001$ versus siNeg.

of suppression of glypican-1 expression on myelination are independent of Schwann cell-laminin interaction. Additional data and similar arguments appear to rule out a direct role of collagen type IV, an important and ubiquitous component of basal lamina, in glypican-1 function.

The precise role of glypican-1-α4(V) collagen binding in myelin assembly is not known. This binding presumably promotes Schwann cell adhesion to and spreading on basal lamina matrix that contains the collagen. Such effects can be demonstrated directly in cultures of Schwann cells plated on surfaces coated with α4(V) collagen. Under these conditions, the adhesive interaction is mediated by binding between a high-affinity heparan sulfate binding motif in the α4(V) collagen NTD and heparan sulfate chains on glypican-1 (Erdman et al., 2002). Glypican-1 is attached to the Schwann cell plasma membrane by a glycosylphosphatidylinositol anchor and therefore lacks a cytoplasmic domain that can interact directly with cytosolic structural or signaling proteins to activate downstream pathways important for myelin assembly. Whether glypican-1 is part of an adhesive/signaling complex that contains other functional components remains to be investigated.

Binding of Schwann cells to axons and spreading and alignment along the axons are early and necessary steps that are observed before the onset of myelination (Webster, 1971; Webster et al., 1973). Specific Schwann cell surface proteins, such as N-cadherin and L1, have been shown to be important for these processes (Wanner and Wood, 2002). Our findings demonstrated that glypican-1 and α4(V) collagen are not involved in these processes.

Assembly of morphologically intact basal lamina matrix by Schwann cells was previously thought to be required for Schwann cell myelin assembly. More recent data, however, suggest that specific interactions between particular basal lamina proteins and their receptors are required, rather than basal lamina assembly per se. Some perturbations that eliminate the synthesis of specific basal lamina components negatively affect myelination without causing morphologically visible defects in basal lamina assembly. For instance, mice engineered to lack laminin α4, a relatively minor component of peripheral nerve basal lamina, develop a dysmyelinating neuropathy despite the presence of ultrastructurally intact basal lamina (Wallquist et al., 2005; Yang et al., 2005). Peripheral nerves of mice with Schwann cell-specific disruption of dystroglycan display significant myelin abnormalities but contain structurally intact basal lamina around myelinating Schwann cells (Saito et al., 2003). Spinal roots of double mutant laminin dy2J/α4 null mice are sorted and myelinated in the absence of morphologically recognizable basal lamina (Yang et al., 2005). This is also consistent with other data showing that in Schwann cell-DRG neuron cocultures maintained in defined medium, some myelination can be observed in the absence of basal lamina formation (Podratz et al., 1998, 2001). Interestingly, however, low levels of laminin are detected around myelinated fibers in these cultures, as well as surrounding myelinated fibers of dy2J/α4 null mice. These results suggest that it is the interaction between laminin and appropriate laminin receptors that is critical for myelination, not the assembly of basal lamina. The findings presented here demonstrate that a defect in α4(V) collagen incorporation into the ECM, caused by suppression of expression of the collagen or its receptor, glypican-1, significantly inhibits myelin assembly but with no apparent effects on laminin deposition into matrix or basal lamina assembly. Together, these data demonstrate the need for specific interactions between particular basal lamina components and their Schwann cell receptors to successfully execute the terminal differentiation program that leads to myelin assembly. The apparent requirement for multiple matrix-receptor interactions to achieve normal myelination is not surprising, given the complexity of the process. A challenge for future studies will be to elucidate the specific molecular path-

ways and functional consequences regulated by these diverse matrix-receptor interactions.

This concept that different ECM receptors perform distinct roles during myelination is supported by a detailed study of the expression of the laminin receptors dystroglycan and integrins. In late embryogenesis, before the onset of active myelination, Schwann cells express only $\alpha6\beta1$ integrin (Previtali et al., 2003b). Dystroglycan expression begins immediately after birth, when myelination is initiated. $\alpha6\beta4$ integrin also appears in Schwann cells after birth but becomes polarized only after the onset of myelination (Previtali et al., 2003b), and $\alpha7$ integrin expression is detected only after myelination begins (Previtali et al., 2003a). This expression pattern correlates with *in vitro* and *in vivo* data on the functions of these receptors as gleaned from studies in which they are inactivated. Thus, genetic inactivation of Schwann cell $\beta1$ integrin inhibits axonal sorting (Feltri et al., 2002), which is a prerequisite for myelination. Dystroglycan null Schwann cells progress through the promyelination stage but exhibit abnormal myelin assembly (Saito et al., 2003). Genetic inactivation of $\alpha7$ (Previtali et al., 2003a) or $\beta4$ integrins (Frei et al., 1999) does not affect myelination. The data presented here demonstrating accumulation of $\alpha4$(V) collagen in matrix tubes before myelination is observed and inhibition of myelination by suppression of $\alpha4$(V) collagen expression are consistent with our previously published data on $\alpha4$(V) collagen expression in rodents *in vivo*. $\alpha4$(V) collagen is expressed by Schwann cells in the late embryonic stage of nerve development, continues to be expressed at high levels through the first few weeks of postnatal development when myelination is most active, and then declines to low but detectable levels in adult nerves (Chernousov et al., 1996, 1999). Similarly, both premyelinating and myelinating Schwann cells abundantly express glypican-1 (Carey and Stahl, 1990) (R. Stahl and D. Carey, unpublished observations), consistent with an important role for this protein in myelination.

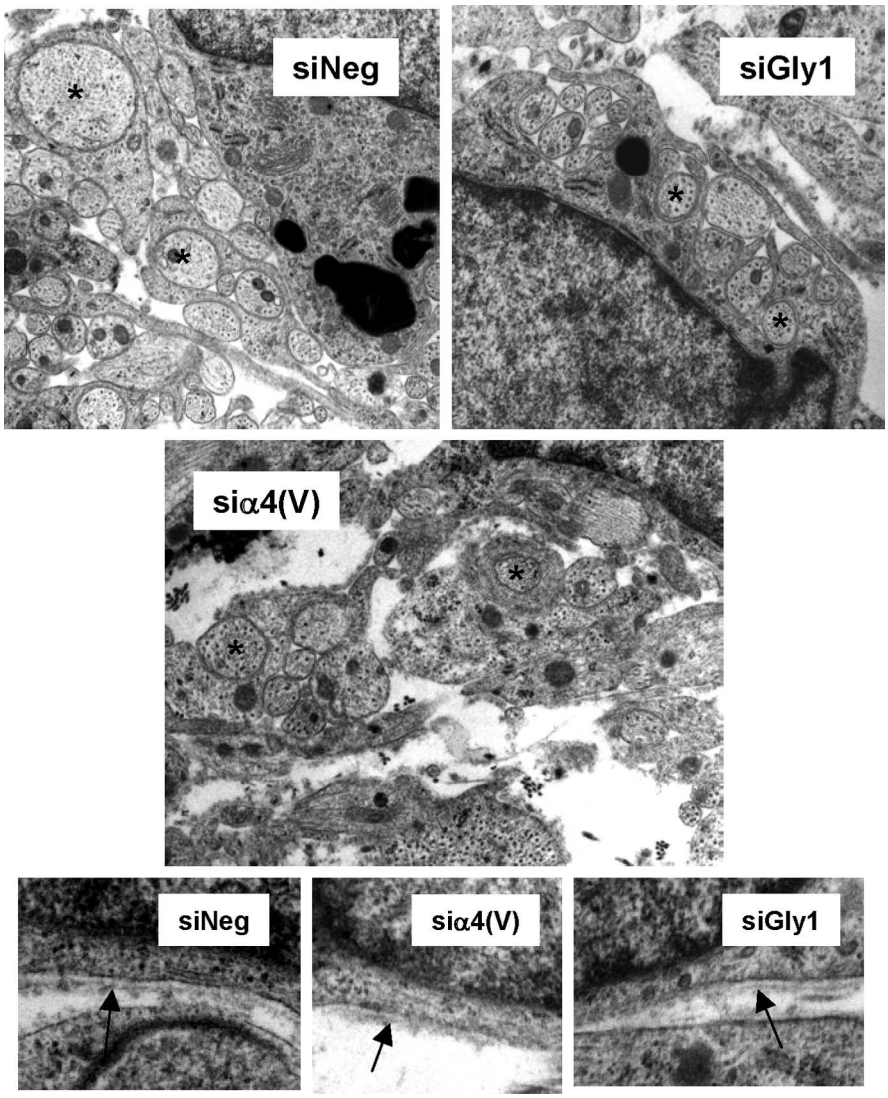

Figure 9. Axonal sorting and basal lamina assembly in siRNA-transfected cocultures. siRNA-transfected cocultures were incubated in ascorbic acid medium for 10 d to induce myelination; the cultures were fixed and processed for transmission electron microscopy. Asterisks indicate individual axons sorted by processes of neighboring Schwann cells. Arrows indicate basal lamina sheets assembled on the surfaces of Schwann cells.

These findings also demonstrate the utility of combining the primary cell coculture model, in which various aspects of peripheral nerve terminal differentiation can be readily observed, with the use of siRNA transfection to selectively block the expression of target proteins of interest. Although this approach will not replace genetically engineered animals as experimental models, there are certain advantages of the approach. Functions of target proteins can be investigated in a physiologically relevant context without the financial cost and time required to generate and characterize genetically engineered animal lines. Problems associated with embryonic lethality, which preclude studying effects on a late developmental event such as peripheral nerve terminal differentiation, and would require use of conditional knock-outs or other approaches, are avoided. Because the cell culture model contains only two cell types, the likelihood of compensatory alterations that mask potential phenotypes is reduced. Finally, the cell culture model provides ready access to and visualization of the cells of interest for detailed observation.

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