Identification of novel cell-adhesion molecules in peripheral nerves using a signal-sequence trap

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The development and maintenance of myelinated nerves in the PNS requires constant and reciprocal communication between Schwann cells and their associated axons. However, little is known about the nature of the cell-surface molecules that mediate axon–glial interactions at the onset of myelination and during maintenance of the myelin sheath in the adult. Based on the rationale that such molecules contain a signal sequence in order to be presented on the cell surface, we have employed a eukaryotic-based, signal-sequence-trap approach to identify novel secreted and membrane-bound molecules that are expressed in myelinating and non-myelinating Schwann cells. Using cDNA libraries derived from dbcAMP-stimulated primary Schwann cells and 3-day-old rat sciatic nerve mRNAs, we generated an extensive list of novel molecules expressed in myelinating nerves in the PNS. Many of the identified proteins are cell-adhesion molecules (CAMs) and extracellular matrix (ECM) components, most of which have not been described previously in Schwann cells. In addition, we have identified several signaling receptors, growth and differentiation factors, ecto-enzymes and proteins that are associated with the endoplasmic reticulum and the Golgi network. We further examined the expression of several of the novel molecules in Schwann cells in culture and in rat sciatic nerve by primer-specific, real-time PCR and in situ hybridization. Our results indicate that myelinating Schwann cells express a battery of novel CAMs that might mediate their interactions with the underlying axons.

Keywords: Myelin, Schwann cells, axon–glial interaction, signal-sequence trap

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INTRODUCTION

Myelination by Schwann cells allows fast impulse propagation along axons in the PNS. During development, Schwann-cell precursors originating from the neural crest give rise to immature Schwann cells, which eventually differentiate into the two major glial-cell types that are associated with axons in the adult, ensheathing and myelinating cells (Jessen and Mirsky, 2005). Whereas ensheathing cells surround multiple unmyelinated axons and form Remak bundles, myelinating cells sort larger axons into a 1:1 relationship (i.e. radial sorting) and form a multilammelar myelin sheath around individual axons. In general, Schwann cells myelinate axons of >1 μm diameter, indicating that the signal for their differentiation into myelinating cells is provided by the axons that they contact (Peters *et al.*, 1991).

Recent studies demonstrate that myelination by Schwann is regulated by distinct growth factors, including neurotrophins (Chan *et al.*, 2004), GDNF (Hoke *et al.*, 2003) and neuregulins (Michailov *et al.*, 2004; Taveggia *et al.*, 2005). Neurotrophins are important regulators of myelination that affect myelinating glia either directly (Chan *et al.*, 2001; Cosgaya *et al.*, 2002) or indirectly, by regulating the required axonal signals

that control their development (Chan *et al.*, 2004). The choice whether a particular Schwann cell will differentiate into a myelinating or ensheathing cell depends on the amount of type III neuregulin-1 (NRG1 type III) that is present on the surface of its associated axon together with other axonal signals (Taveggia *et al.*, 2005); low levels are required for ensheathment, whereas high levels induce myelination. Furthermore, the amount of NRG1 type III also regulates the thickness of the myelin sheath (Michailov *et al.*, 2004; Taveggia *et al.*, 2005). Interestingly, NRG1 type III is active only when membrane-bound and not as a soluble form, indicating that Schwann cell–axon contacts mediated by cell-adhesion molecules (CAMs) might be a prerequisite for myelination.

CAMs are implicated in various developmental stages of myelinating Schwann cells, including cell attachment, process extension, axon ensheathment, spiral enwrapping, compaction and the formation of the nodes of Ranvier (Quarles, 2002; Spiegel and Peles, 2002; Bartsch, 2003; Poliak and Peles, 2003; Feltri and Wrabetz, 2005). Schwann cell CAMs can be divided into several groups based on their proposed function. One group mediates axon–glia interactions and might have a role in myelination, such as N-cadherin (Wanner and Wood, 2002). Blocking N-cadherin binding by either antibodies or

competing for its binding with synthetic peptides, leads to a significant reduction in axon-aligned process growth and cell–cell interactions in DRG/Schwann cell co-cultures. Other suggested candidates for mediating Schwann cell–axon attachments are L1 (Seilheimer *et al.*, 1989) and myelin-associated glycoprotein (MAG) (Owens *et al.*, 1990). However, evidence from gene-targeting studies indicates that neither L1 nor MAG are involved in initiating and maintaining the axon–Schwann cell association (Li *et al.*, 1994; Montag *et al.*, 1994; Haney *et al.*, 1999).

Another group of CAMs that play a role in myelination includes integrin β1 and dystroglycan, both of which mediate the interactions between Schwann cells and extracellular components of their basal lamina (Feltri and Wrabetz, 2005). The addition of anti-β1-Integrin-blocking antibodies into co-cultures of Schwann-cells and DRG-neurons prevents myelination (Fernandez-Valle *et al.*, 1994). Schwann cells lacking either integrin β1 or laminin γ1 are arrested at the radial sorting stage (Feltri *et al.*, 2002; Chen and Strickland, 2003; Yang *et al.*, 2005; Yu *et al.*, 2005), which further supports a role for integrins in myelination. In addition, Schwann cell-specific deletion of dystroglycan results in polyaxonal myelination and a profound defect in the formation of the microvilli that contact the nodes of Ranvier (Saito *et al.*, 2003). The third group of molecules includes the structural CAMs, such as P0 and PMP22, that are required for the generation of compact myelin (Suter and Scherer, 2003), E-cadherin (Fannon *et al.*, 1995; Young *et al.*, 2003) and claudin-19 (Miyamoto *et al.*, 2005), which are important for generating specialized, non-compact myelin structures.

The fourth group of CAMs present in Schwann cells includes neurofascin 155 (NF155) (Tait *et al.*, 2000), TAG-1 (Traka *et al.*, 2002) and gliomedin (Eshed *et al.*, 2005), all of which mediate the interactions between myelinating Schwann cells and axons, and are crucial for the local differentiation of the axonal membrane at and around the nodes of Ranvier (Poliak and Peles, 2003; Salzer, 2003). NF155 is present at the paranodal axoglial junctions that are formed between the axon and the glial paranodal loops at both sides of the node of Ranvier (Tait *et al.*, 2000); it is associated with an axonal complex of Caspr and contactin, both of which are essential for the formation of axoglial junctions (Bhat *et al.*, 2001; Boyle *et al.*, 2001; Charles *et al.*, 2002; Gollan *et al.*, 2003). TAG-1 is a homophilic GPI-linked CAM that is localized to the juxtaparanodal region, where it forms a complex with Caspr2 that is required for the clustering of potassium channels at this site (Poliak *et al.*, 2003; Traka *et al.*, 2003). At the nodes of Ranvier, axon–glia interaction is mediated by binding of gliomedin, an olfactomedin-domain CAM that is concentrated at the Schwann cell microvilli, to the two axonal immunoglobulin superfamily (IgSF) CAMs, neurofascin 186 and NrCAM (Eshed *et al.*, 2005). Furthermore, binding of gliomedin to these CAMs is required for clustering of sodium channels at the nodal axolemma.

OBJECTIVES

Given the importance of Schwann cell–axon interactions and the relatively small known repertoire of cell surface molecules that mediate them, we set out to identify novel secreted and transmembrane proteins expressed in peripheral myelinated nerves using a unique signal-sequence trap approach. Screening cDNA expression-libraries prepared from either primary rat Schwann cells that were stimulated with dbcAMP to induce their differentiation, or 3-day-old rat sciatic nerves, identified many CAMs, some of which are likely to mediate Schwann cell–axon communication.

MATERIALS AND METHODS

REX-SST library construction

Poly-A$^+$-RNA was isolated from dbcAMP-stimulated rat Schwann cells or 3-day-old rat sciatic nerves using FastTrack 2.0 kit (Invitrogen) according to the manufacturer's instructions. The original pMX-SST-vector (a generous gift from Y. Kitamura, University of Tokyo, Japan) was modified slightly by introducing EcoRI and XhoI sites to the multiple cloning site to allow directional cloning of cDNAs. cDNA was synthesized with the cDNA synthesis kit (Stratagene) using custom-made random-primers containing an XhoI-site. The cDNAs were size-selected on ChromaSpin TE-400 columns (Clontech), ligated into the EcoRI–XhoI-digested REX-SST-vector and electroporated into ElectroMax DH10B-cells (Invitrogen). The primary libraries (5×10^5 *cfu* for the iSC-library and 1.7×10^6 *cfu* for the 3drSN-library) were titrated and amplified by growing 2.5×10^4 clones on 15-cm agar LB-Amp plates overnight at 37°C. Plasmid DNA was prepared and used for transfection of Phoenix-Eco packaging cells to prepare viral stocks.

Screening and isolation of cDNA-inserts

Screening of the REX-SST libraries was done as previously described (Kojima and Kitamura, 1999). Ba/F3-cells ($2–6 \times 10^7$) were infected with the iSC- and the 3drSN-retroviral libraries and grown in the presence of interleukin 3 (IL-3). After 24 hours, the infected Ba/F3-cells were washed three times in RPMI 1640 medium without IL-3, seeded in 96-well plates at a density of 3.3×10^3 cells well^{-1} and grown for 10 days in selection-medium without IL-3. Surviving clones were transferred to new 96-well plates, and confluent wells were passaged three further times. Cells were then lysed in lysis-buffer (10 mM Tris-Hcl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1.7 μM SDS, 0.5 mg ml^{-1} Proteinase K) at 56°C in a humid chamber, followed by heat-inactivation at 85°C for 20 minutes. Lysed cells (3 μl) were used for PCR (5'-primer, GAAGGCTGCCGACCCCG; 3'-primer, GGCGCGCAGCTGTAAACG) to isolate the cDNA-inserts, and the resulting products were separated on agarose gels. When two or more PCR products were detected, additional PCR was performed on the respective bands using the same primers. Pre-screening for highly abundant genes was done by spotting the PCR-products onto Hybond$^+$ nylon membranes and hybridizing them to a mix of P^{32}-labeled probes derived from clones representing four genes: osteonectin (bp 11–356; D28875), collagen 1α1 (bp 1–405; Z78279), collagen 18α1 (bp 15–586; AK031798) and tyrosinase-related protein 1 (bp 330–867; XM_238398). Hybridization-negative PCR-products were purified and sequenced using the original 5' primer of the PCR.

Tissue culture methods

Phoenix-Eco cells were grown in DMEM medium containing 10% FCS. Ba/F3-cells were grown in RPMI 1640 medium supplemented with 10% FCS and either with or without 0.5% IL-3 conditioned medium. Retroviral infections of Ba/F3-cells were made overnight by adding viral supernatant to Ba/F3-cells (3×10^5 cells ml^{-1}) in the presence of 4 μg ml^{-1} Polybrene (Sigma). Induced primary rat Schwann cell cultures – Schwann cells isolated from postnatal day 4 rat sciatic nerve and brachial plexus – were plated on PLL/laminin-coated dishes in DMEM/10% FCS and next day treated with cytosine arabinoside (10^{-5} M) for 3 days. The cells were then re-plated and grown in 10% FCS/7.5 ng ml^{-1} βNRG (Amgen Inc. or R&D Systems) and 10^{-4} M dbcAMP until confluent. After two passages, the medium was changed to DMEM/5% FCS, 7.5 ng ml^{-1} βNRG, 10^{-5} M dbcAMP for 2 days, then to DMEM/0.5–1% FCS/βNRG without cAMP for 2 days, and finally to DMEM/0.5–1% FCS/βNRG and 10^{-3} M dbcAMP for 2 days.

Real-time, quantitative PCR

Total RNA isolation was performed using TRI-reagent (Sigma) and random-primed cDNA synthesis was done using 2 μg total RNA and 50 U of SuperScript-II Reverse Transcriptase (Invitrogen) according to the manufacturers' instructions. Specific PCR primers were designed using Roche's Applied Science Universal Probe Library Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) and mRNA sequences from the Genebank database; the sequences of the primers are available on demand. Quantification of cDNA targets was performed on ABI Prism® 7000 Sequence Detection System (Applied Biosystems), utilizing SDS 1.1 Software. Optimal reaction conditions for amplification of the target genes were performed according to manufacturer's (Applied Biosystems) recommendation. All reactions were run in duplicate and transcripts were detected using SYBR Green I. Results were recorded as mean threshold cycle (C_T), and relative expression was determined using the comparative C_T method (Livak and Schmittgen, 2001). The ΔC_T was calculated as the difference between the average C_T value of the endogenous control, ribosomal Protein S9 RNA (Accession No. X66370), from the average C_T value of test gene and was used to calculate a relative quantity of gene expression. Relative expression of the target gene was calculated by the formula, $2^{-\Delta\Delta C_T}$, which is the amount of gene product, normalized to the endogenous control and relative to the calibrator sample.

In situ hybridization

Synthesis of riboprobes and *in situ* hybridizations were performed as previously described (Eshed *et al.*, 2005). Templates for riboprobes were cloned by RT-PCR on total RNA isolated from brains of 3-day-old rat pups or adult mice; the resulting PCR-fragments were purified, cloned into pGEM-T easy (Promega) and verified by sequencing. A list of the primers used for RT-PCR cloning, and the position of the riboprobes is available upon request. Riboprobes for β-tubulin and MBP were described previously (Schaeren-Wiemers and Gerfin-Moser, 1993). Rat pups of the indicated age were dissected, frozen in OCT (Tissue-Tek) and stored at –70°C until use; cryosections of 14 μm were mounted on SuperFrost slides (Menzel-Glaeser) and processed as described. All hybridizations were done at 72°C.

RESULTS

Constructing viral cDNA libraries from Schwann cells and rat sciatic nerve

Membrane targeting of secreted and cell-surface proteins requires the presence of a short amino-terminal hydrophobic peptide, termed a signal peptide (von Heijne, 1985). Here we report the use of a signal-sequence trap (SST) to isolate sequences that encode signal peptides from a large pool of cDNAs to identify new potential CAMs and signaling molecules from Schwann cells. The method used detects signal sequences in cDNA fragments based on their ability to redirect a constitutively active mutant of the thrombopoietin receptor MPL (MPL^M) to the cell surface, thereby permitting IL-3-independent growth of Ba/F3 cells, which, otherwise, require IL-3 for survival (Kojima and Kitamura, 1999). The retroviral SST-vector (pMX-SST, also known as REX-SST for 'retrovirally expressed SST') contains a truncated MPL^M variant that lacks most of the extracellular domain, including the signal sequence. Cloning a cDNA that contains a signal sequence in frame to the MPL^M sequence will direct the expression of this fusion protein to the plasma membrane and result in IL-3-independent growth of Ba/F3-cells.

We have constructed two cDNA-libraries in the REX-SST vector. The first library was made using PolyA$^+$ RNA extracted from rat primary Schwann cells that were stimulated with the cAMP analogue dibutyryl cAMP (dbcAMP) to induce their differentiation. For the second library, sciatic nerves of 3-day-old rats were used as the source of RNA. We chose these two sources because both are expected to be enriched in mRNAs that might be important for myelination. Treating Schwann cells with dbcAMP mimics axonal contact and induces the genetic myelination program (Morgan *et al.*, 1991). Accordingly, stimulating primary Schwann cells with dbcAMP should generate a highly enriched source of mRNAs expressed in Schwann cells once they have contacted their axons. The 3-day-old sciatic nerve was selected because it represents a stage when most Schwann cells have already contacted the axons and begun to myelinate (Friede and Samorajski, 1968), thereby allowing the identification of proteins that are expressed during the early, active period of myelination.

Selecting cDNAs that encode for signal-sequence-containing proteins

An outline of the strategy used is depicted in Fig. 1. Random-primed plasmid cDNA libraries of the above sources (5×10^5 and 1.7×10^6 independent clones for the Schwann cell and the sciatic nerve libraries, respectively) were converted into retroviral libraries as described in the Materials and Methods. The resulting retroviral stock was used to infect the IL-3-dependent Ba/F3 cells that were grown further in 96-well dishes. After selecting Ba/F3 clones that grow in the absence of IL-3, genomic DNA was extracted and the integrated, virally delivered inserts were rescued by PCR using primers flanking the cloning site. The reaction products were analyzed by gel electrophoresis and clones containing a single band were used directly for further processing. Clones containing multiple
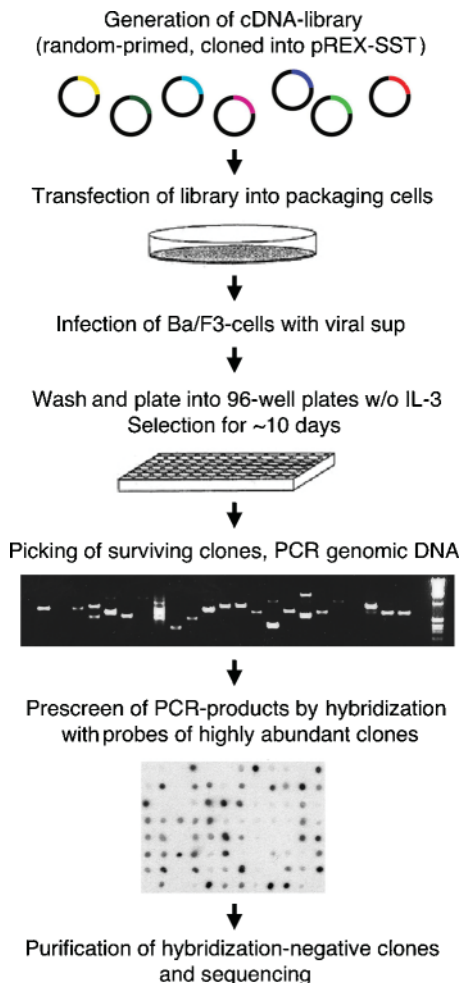
Fig. 1. The screening strategy. Rat sciatic nerve and dbcAMP-stimulated Schwann cells cDNAs were cloned into a vector containing the retrovirally expressed SST (REX-SST). Plasmid DNA of the two libraries was then transiently transfected into packaging cells and Ba/F3-cells infected with the resulting retroviral stock in the presence of IL-3. After recovery, IL-3 was removed, and the cells plated into 96-well plates and grown in the absence of IL-3. Surviving clones were picked and cDNA-inserts rescued by PCR using primers flanking the insert. To avoid repetitive isolation of prevalent clones, the PCR-products were spotted onto nylon membranes and hybridized with probes of these few, highly abundant genes. Hybridization-negative clones were sequenced and the identity of the respective genes determined.

bands (~10%) were separated further from the gels into single bands by an additional PCR reaction. The size of the PCR products ranged from 0.2–3 kb with the majority 0.5–1 kb. Initial round of sequencing of 96 clones revealed that a few genes account for the majority of the clones isolated (data not shown). These genes include collagen $1\alpha1$ and osteonectin, which are present in both libraries, and collagen $18\alpha1$ and tyrosinase-related protein TRP, which occur only in the Schwann-cell library. We reasoned that the high prevalence of these genes among the clones isolated results from their ability to efficiently bring the MPL^M-fusion protein to the cell surface rather than from their high abundance in either Schwann cells or sciatic nerves. To avoid re-isolating these few cDNAs, we prescreened all PCR products by dot-blot hybridization with ^{32}P-labeled probes prepared from the genes identified repeatedly. PCR products with no hybridization signal (443

from Schwann cells and 420 from sciatic nerve) were purified and sequenced. Of all the sequences analyzed, 95% encoded for genes (known and unknown) that contain a signal sequence.

cDNAs identified encode for distinct functional groups of proteins

Sequence analysis of the 863 isolated clones resulted in the identification of 158 cDNAs (Table 1 and Table 2). Of these 158 cDNAs, 57 (36%) were identified only from Schwann cells, 73 (46%) were isolated only from the sciatic nerve library, and 28 (18%) were found in both sources. Although most genes were identified in only one of the two sources of cDNA, the combined list of genes identified included those that have been described previously in PNS myelination, further validating our approach. Notably, many of the genes identified have not been described previously in myelinating Schwann cells: these novel genes include some with known or suggested functions in other cell types and completely new sequences without a known function. Our SST approach isolated a wide range of structurally different proteins, including secreted factors and transmembrane proteins with varying topology that contain one-, four- and seven-transmembrane domains. Based on structural features and information from the literature, we have divided the identified encoded proteins into several functional groups (Table 1). These groups contain 35 extracellular matrix (ECM) components, with many collagens; 19 different receptors and signaling molecules, including receptor tyrosine and serine kinases, members of the TNF-R family, cytokine receptors and G-protein-coupled receptors; eight growth and differentiation factors, and two growth-factor-associated proteins; 18 ecto-enzymes, including proteases and enzymes that regulate lipid metabolism; 16 genes that encode proteins that are associated with protein processing and trafficking in the endoplasmic reticulum and Golgi network. The last group we identified contained 51 cell-adhesion and recognition molecules, 30 of which were identified from the sciatic nerve library, 14 from cultured Schwann cells and seven from both sources. As depicted in Table 2, these proteins are grouped based on their domain organization and include proteins of the immunoglobulin superfamily, tetraspanins, GPI-linked proteins, EGF-domain containing proteins, integrins, cadherins and proteins that contain other domains that occur in proteins that are involved in cell–cell and cell–ECM interactions. In this group we also include proteins that mediate intercellular communication such as Slit, Notch and Delta-like, as well as membrane-bound semaphorins and their neuropilin and plexin receptors. Interestingly, only a few of these proteins have been reported previously in myelinating Schwann cells (MAG, P0, CD44, neurofascin, PMP22, claudin-19, integrin $\alpha7$ and syndecans), whereas the function of most of the genes identified in myelinating glial cells is unknown. Comprehensive descriptions of the genes we identified and their possible involvement in the development of myelinated fibers is provided below.

Relative expression of the CAMs identified in Schwann cells and sciatic nerve

To further verify the expression of the identified CAMs and of other genes of interest in myelinating and non-myelinating

Table 1. Genes identified by SST

The names and Genebank accession numbers of the genes isolated, and their division into functional groups. Red indicates cDNAs isolated from the Schwann cell library, blue indicates those isolated from the sciatic nerve library, and black indicates those isolated from both sources.

Protein	Accession
ECM	
Biglycan	U17834
Collagen 1a1	Z78279
Collagen 1a2	AF121217
Collagen 3a1	BC087039
Collagen 5a1	AJ005394
Collagen 5a3	AF272661
Collagen 6a1	XM215375
Collagen 6a2	XM342115
Collagen 7a1	XM238554
Collagen 9a1	XM223124
Collagen 9a3	XM342599
Collagen 15a1	XM216399
Collagen 18a1	XM241632
Collagen 22a1	NM152888
Dystroglycan 1	AF357215
Endoglin	AY562420
Fibronectin 1	X15906
Fibulin 2	XM232197
Integrin-binding sialoprotein	AB001383
Laminin alpha-4	XM228209
Matrix Gla protein	J03026
Microfibrillar-associated p2	XM233602
Microfibrillar associated p5	XM342750
Nidogen (Entactin)	XM213954
Nidogen 2	XM573694
Osteonectin	D28875
Perlecan	XM233606
Procollagen proteinase enhancer	U94710
Reelin	AB049473
SPARC-like 1	U27562
Tenascin C	D90343
Thrombospondin 1	AF309630
Thrombospondin 4	X89963
von Willebrand factor A-related	BC081983
WD repeat domain 34	BC082103

Protein	Accession
Receptors	
Activin IIA	XM342431
Complement related	NM1005330
Endothelin B	NM017333
FGF	NM024146
Frizzled homolog 8	XM344616
G protein-coupled 108	BC061996
G protein-coupled 37-like	XM573457
G protein-coupled 56	AF529886
GDNF-R (alpha 3)	BC079378
GM-CSF-R (alpha)	AJ628424
IL6-R	M92340
Interferon gamma-R	AF201901
LRC-8	XM341785
p75NTR	X05137
TrkB	M55291
PDGF beta-R	AY090783
SDF-R	NM019380
TROY	XM214214
VEGF-R	U93306

Protein	Accession
Growth and Differentiation	
Betacellulin	AB028862
Dickkopf homolog 3	AF245040
Follistatin-like 1	BC087014
Granulin	M97750
Gremlin 1 homolog	Y10019
IGFBP-5	L08275
IGFBP-6	M69055
MCP-1	AF058786
Nephroblastoma gene	AF171936
Prosaposin	M19936
ER/Golgi	
S-100 calcium binding protein	J03628
Calnexin	L18889
Calumenin	AJ001929
Evectin 1	AF081582
Golgi apparatus protein gp25L	BC088422
Intracellular chloride channel	AF104119
Nuclear pore glycoprotein 62	M62992
Peripheral myelin protein 2	S39508
Phosphatidylinositol glycan T	XM_215919
Prolyl 4-hydroxylase, beta	X02918
Reticulocalbin 3	BC083719
Scotin	BC083710
Signal sequence receptor, alpha	AY321345
Signal sequence receptor, beta	XM215619
Trans-golgi network protein 1	BC098512
Transmembrane trafficking pr21	AJ004912
Ectoenzyme	
AE binding protein 1	XM223583
Aminopeptidase A	AF146044
Carboxypeptidase	XM215840
Cathepsin B	X82396
Cathepsin C	D90404
Cathepsin H	Y00708
delta-6 Fatty acid desaturase	NM031344
Matrix metalloproteinase 2	U65656
Presenilin-like protein 1	BC087132
Protease inhibitor 16	XM215351
Serpin-g1	BC061860
Serpin-h1	M69246
Similar to 1110065L07 Riken	XM225044
similar to HSPC288	XM216756
Sphingomyelin phosphodiesterase	BC083780
Stearoyl-Coenzyme A destaruase2	NM031841
Tyrosinase-related protein 1	XM238398
beta 1,4-galactosyltransferase	AF048687
Unknown function	
Castration induced protein-1	AJ010750
Growth arrest specific 7	AJ131902
LOC296115 (similar to KIAA0256)	XM215843
Pleckstrin domain containing	BC061738
Protein kinase C substrate 80	XM_238534
Similar to adipocyte protein 4	BC085824
Similar to MGC8721	BC078979
Similar to RIKEN 1810007P19	BC098048
Similar to RIKEN D130038B21	BC079082

Schwann cells, we performed real-time PCR analysis using specific primer sets for their corresponding genes on total RNA isolated from cultured Schwann cells and 1-week-old rat sciatic nerves. This analysis revealed that the genes identified were expressed at different levels in the sciatic nerve (genes are sorted by their expression levels in rat sciatic nerve from high to low in Table 3). Several genes (i.e. MUC-18, CD81 and CD63) that were isolated from Schwann cells but not from the

Table 2. CAMs and related molecules identified in the screen

The names and Genebank accession numbers of genes isolated that encode for proteins that mediate cell-adhesion and intercellular communication. Membrane topology and organization of the extracellular domain of each group of proteins is presented schematically. Genes were isolated from Schwann cells (red), sciatic nerve (blue) and both libraries (black).

Scheme	Protein	Accsesion	Comments
	CD24	NM012752	GPI-linked
	CD34	XM223083	1x TM Sialomucin
	CD164, Endolyn	AJ238574	1x TM Sialomucin
	Endoglin	AY562420	1x TM
	Similar to BC013529	XM341726	1x TM
	HTGN29	BC079420	1x TM Syndecan-like
	Syndecan 2	BC070890	1x TM, HSPG
	Syndecan 3	U73184	1x TM, HSPG
	Syndecan 4	S61868	1x TM, HSPG
	CD63	NM_017125	TM4SF
	CD81	NM_013087	TM4SF
	CD82, Kangail	AF049882	TM4SF
	Claudin 19	BC079172	TM4SF
	EMP-1	NM012843	TM4SF
	PMP 22	X62431	TM4SF
	CD44	U96138	1x TM 1x LINK (hyaluronan-binding)
	CD97	NM1012164	7x TM EGF domain
	Osteoactivin	AF184983	1x TM 1x PKD
	Similar to AU040320 (PKD1-like)	XM216537	1x TM 5x PKD
	Myelin protein zero (P0)	NM017027	1x TM 1x Ig
	Similar to B7-like protein GL50-B	XM574731	2x Ig
	Basigin	X54640	1x TM 2x Ig
	ZIg 1	XM217106	1x TM 2x Ig
	Neurotrimin	U16845	1x TM 3xIg
	IL1-R associated	U48592	1x TM; 3x Ig 1x TIR
	Necl 1	XM_341157	1x TM; 3x Ig
	Necl 2	BC078966	1x TM; 3x Ig
	Necl 4	XM344870	1x TM; 3x Ig
	Myelin-associated glycoprotein (MAG)	M14871	1x TM 4x Ig
	Activated leukocyte cell adhesion molecule (AlCAM)	AB008538	1x TM 5x Ig
	MUC-18	AB035507	1x TM; 5x Ig
	NrCAM	AY574277	1x TM 6x Ig, 4x FN3
	Neurofascin	AY061639	1x TM 6x Ig, 4x FN3

Scheme	Protein	Accsesion	Comments
	DiGeorge syndrome gene critical region 2	XM573327	1x TM 1x LDLa 1x CLECT 1x VWC
	Dysadherin (FXYD domain-containing ion transport regulator 5)	NM021909	1x TM
	Integrin alpha 7	BC088846	1x TM 3x Integrin alpha (beta-propellor repeats)
	Integrin beta 8	XM343126	1x TM 1x integrin beta-repeat 1x vWF-repeat
	Low density lipoprotein-related protein 12	XM235261	1x TM 2x CUB 5x LDLa
	Milk fat globule-EGF factor 8 protein	D84068	1x TM 2x Calcium-binding EGF 2x Discoidin
	N-Cadherin	AB017695	1x TM 5x Cadherin-repeats
	Neuropilin-2	AF016297	1x TM 2x CUB 2x Discoidin 1x MAM
	Notch gene homolog 2	M93661	1x TM 34x EGF 3x NL 2x NOD
	Olfactomedin-like 2B	XM222868	1x TM 1X Olfactomedin
	Semaphorin 3B	XM343479	1x TM 1x Semaphorin 1x PSI 1x Ig
	Semaphorin 6D	XM230583	1x TM 1x Semaphorin 1x PSI
	Similar to HGFL protein	BC092208	1x TM 1x Kringle
	Pituitary tumor-transforming 1 interacting prot	BC061813	1x TM 1x PSI
	Plexin domain-containing 2	XM341567	1x TM 1x PSI
	Amyloid beta (A4) precursor protein	NM019288	1x TM 1x Amyloid A4 1x Kunitz 1x Beta-APP,
	Slit-like 2	BC050274	1x TM 1x LRR 1x EGF 1x FNIII
	Delta-like 1 homolog (Dlk1)	D84336	1x TM 6x EGF

Legend: Ig-loop, FN3, PKD, EGF-domain, β-Propellor repeat, Ca²⁺-binding EGF-domain, Discoidin-domain, LDLα-domain, CUB-domain, Semaphorin-domain, PSI-domain, Olfactomedin-domain, Kringle-domain, Syndecan-domain, Hyaluronan-binding, C-type lectin domain, von Willebrand factor type C domain, MAM domain, Integrin-domain, Leucine-rich repeat, Cadherin domain.

sciatic nerve library, have relatively high mRNA expression in sciatic nerve. Furthermore, the genes analyzed can be classified further into two main groups according to their relative expression in rat sciatic nerve and primary cultured Schwann cells. Genes that have higher expression in sciatic nerve than in isolated Schwann cells (brown and red in Table 3) might have a role during myelination, whereas genes that have higher levels in Schwann cells in culture compared with sciatic nerve (blue in Table 3) might be important in early stages of axonal contact.

Some cDNAs are identified genes in both Schwann cells and DRG neurons

Next, we examined the expression of three CAMs (Muc18, PDK1-like and Dgcr2), and two growth factors (betacellulin

Table 3. Expression of selected genes in sciatic nerve and Schwann cells
The expression of the indicated genes in P7 rat sciatic nerves and primary Schwann cells in culture determined by quantitive RT-PCR. The genes are sorted according to their abundance in the sciatic nerve, with the most abundant at the top. The relative expression of the indicated genes in sciatic nerve compared with primary rat Schwann cells is described as a fold-change (color-coded on the right). Letters to the right of the gene name indicate whether it was isolated from Schwann cells (S), sciatic nerve (N) or both (B) libraries.

Gene	Library	Fold-change
P0	B	↑
HTGN29	B	NS
CD164, Endolyn	N	↑
CD34	N	↑↑↑↑
MUC-18	S	↓↓↓
Plexin domain containing 2	N	↑↑↑↑
Necl 4	B	↓
CD81	S	↓↓↓
CD63	S	↓↓↓
Integrin beta 8	N	↑↑
ZIg 1	B	↓↓
Semaphorin 6D	N	↓↓
Semaphorin 3B	N	↓
Endoglin	N	↑↑↑↑
DiGeorge syndrome gene critical region 2	S	↓
Similar to HGFL protein	N	NS
Neuropilin-2	S	↑↑
Pituitary tumor-transforming 1 interacting	N	↓↓↓
Necl 2	N	↑
Dysadherin	S	↓↓↓
Osteoactivin	S	↑↑
Delta-like 1 homolog (Dlk1)	N	↓
Similar to B7-like protein GL50-B	N	↑↑
Milk fat globule-EGF factor 8 protein	N	↑↑
Basigin	N	↓↓
CD24	S	↑↑↑↑
Granulin	B	NS
PKD1-like	N	↓↓↓
Olfactomedin-like 2B	B	↓↓↓
Notch gene homolog 2	N	↓↓
Interleukin 1 receptor accessory	N	↓↓↓↓
Betacellulin	S	↓↓↓
Integrin alpha 7	N	↓↓↓↓
Slit-like 2	N	↓↓
CD97	S	↓
CD82, Kangai1	N	↓↓↓↓

Color key:
- UP >20
- Up 2.5 to 7
- Up 1.5 to 2.5
- Non Significant
- DOWN 1.5 to 2
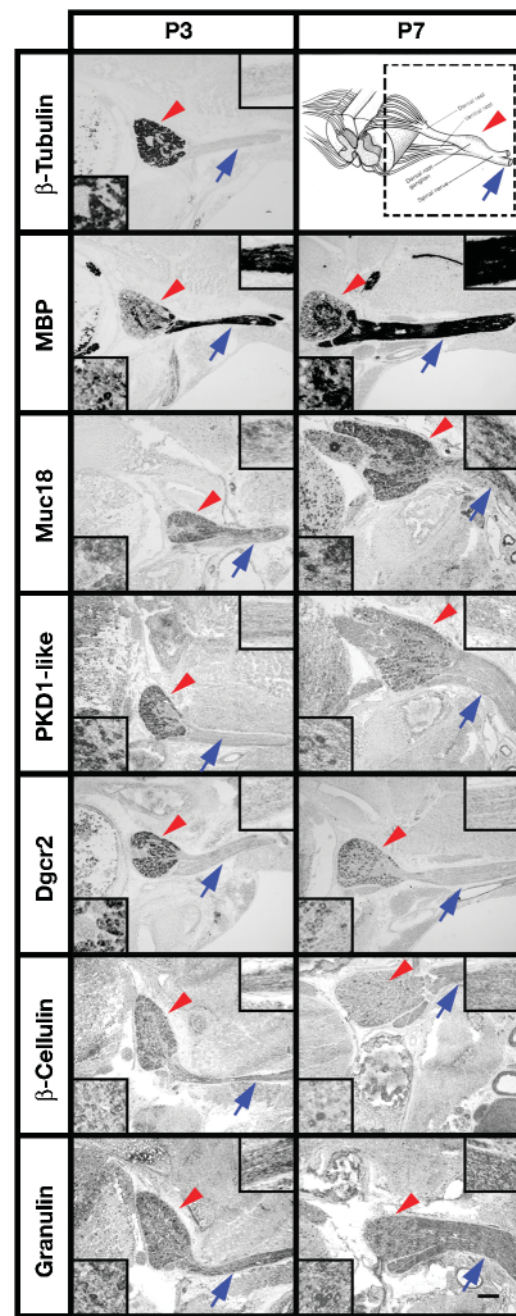- DOWN 3 to 5
- DOWN 6 to 10
- DOWN >20

Fig. 2. *In situ* hybridization analysis of selected genes. Expression of the genes indicated in rat PNS was examined by *in situ* hybridization. Cross-sections containing dorsal root ganglia (red arrowheads) and spinal nerve (blue arrows) of either P3 or P7 rats were hybridized with the respective antisense probe. Control hybridizations with the sense probes did not yielded signals (data not shown). A scheme depicting the location of the tissue examined is shown in the upper right panel. β-Tubulin and MBP were used as markers for neurons and myelinating glia, respectively. Higher magnifications of the signals in the spinal nerve (upper right corner of each panel) and the DRG (lower left corner) are in the insets. Scale bar, 200 μm.

and granulin) in the PNS by *in situ* hybridizations on transverse sections of 3-day-old (P3) and P7 rats, containing the dorsal root ganglion (DRG) and spinal nerve (Fig. 2). The resulting pattern of expression of each gene was compared with that of β-tubulin (as a neuronal marker) and MBP (as a marker for myelinating Schwann cells). Strong expression of Muc18 and granulin was detected in Schwann cells along

the nerve, and in DRG neurons in both P3 and P7 (Fig. 2). Although betacellulin was also detected in both DRG neurons and Schwann cells, its expression was lower than that of Muc18 and granulin, particularly in Schwann cells where its expression decreased further during development. Dgcr2 was detected weakly in Schwann cells and strongly in DRG neurons, with mRNA-levels reducing with age. By contrast,

PDK1-like was detected only in DRG neurons. This is surprising because this gene was clearly detected by real-time PCR in sciatic nerves (Table 3). One possibility is that expression in myelinating Schwann cells is below the detection level of our *in situ* hybridization. In this regard, it is relevant that the identification of Dgcr2 (which had either a weak or undetectable signal in *in situ* hybridization) by Northern-blot analysis required a very long exposure (data not shown). Interestingly, PDK1-like and a few other genes that showed similar neuronal expression patterns (data not shown), were identified only in the sciatic nerve library, which indicates that this library might also contain neuronal mRNAs that were present along the axons (Piper and Holt, 2004). Nevertheless, our analysis demonstrates that all of the genes examined are found in Schwann cells, in neurons, or in both cell types.

CONCLUSIONS

- Screening expression cDNA libraries prepared from primary Schwann cells and sciatic nerves from rats has identified a large number of secreted and membrane-bound molecules that are present in these sources in different amounts.
- The proteins identified include multiple cell-adhesion molecules and other proteins that are involved in cell–cell communication in other tissues, growth and differentiation factors and their receptors, extracellular components, and proteins that are associated with functions of the endoplasmic reticulum and Golgi.
- This approach provides us with a wealth of novel molecules among which several are reasonable candidates to be mediators of axon–glial communication.

DISCUSSION

The main goal of the work presented here has been to identify molecules that are expressed in the PNS at the onset of myelination and that might be involved in Schwann cell–axon communication. By screening two different sources of mRNA for genes that encode signal peptides, we have identified a wide range of structurally and functionally diverse molecules, many of which have been implicated previously in cell adhesion and cell–cell interactions. In the following section we describe the different functional groups of proteins identified in our screen and their possible relevance to myelinating Schwann cell biology.

Signaling systems

Several of the proteins identified in our screen regulate early development of the Schwann-cell lineage such as endothelin, insulin and BMP (Jessen and Mirsky, 2005), and neurotrophins and their receptors, which are important for myelination (Cosgaya *et al.*, 2002; Chan *et al.*, 2004), betacellulin, which signals through receptor tyrosine kinases of the ErbB-family (Pinkas-Kramarski *et al.*, 1998), and granulin, a potent growth factor with diverse actions (Ong and Bateman, 2003). Given the importance of the neuregulins and their receptors in Schwann-cell development and myelination (Michailov *et al.*, 2004; Taveggia *et al.*, 2005), it is reasonable to suggest that

autocrine stimulation of Schwann cells by betacellulin might allow their axon-independent survival. Another putative novel factor that might affect Schwann-cell physiology is granulin (also known as epithelin and acrogranin), which we identified in both Schwann cell and sciatic nerve libraries. Granulin is a secreted mitogen that is implicated in several biological processes including embryogenesis (blastocyst formation), wound healing and tumorigenesis (Ong and Bateman, 2003). Like betacellulin, granulin induces phosphorylation of key signal-transducing molecules (Shc in the ERK and PI 3-kinase) that are known to regulate Schwann-cell differentiation (Jessen and Mirsky, 2005; Taveggia *et al.*, 2005).

Another potential signaling system identified in our screen consists of Notch and Delta-like molecules. These molecules mediate important developmental processes between neighboring cells (Kanwar and Fortini, 2004; Yoon and Gaiano, 2005). It is, therefore, not surprising that this system is implicated also in glial biology, namely in the differentiation of myelinating cells in the CNS (Wang *et al.*, 1998; Hu *et al.*, 2003), and early in the development of Schwann cells from the neural crest (Morrison *et al.*, 2000). However, the involvement of Notch and its ligands in PNS myelination is still elusive. The Notch-ligand Delta-like 1 (Costaglioli *et al.*, 2001), which we also isolated in our screen, is expressed by Schwann cells and is downregulated during myelination, which indicates that it might be involved in negatively regulating the process.

ECM components and their receptors

Myelinating Schwann cells are surrounded by a well-developed basal lamina composed of a variety of collagens, laminin, fibronectin, entactin and heparan sulfate proteoglycan (Bunge, 1993), most of which we identified in our screen. Many of these components are also produced by isolated Schwann cells in cultures and are present in depositions that surround the cultured cells. The importance of the basal lamina has long been known and the interaction of laminin with its integrin receptors for myelination has been demonstrated recently (Colognato *et al.*, 2005). In addition to the highly abundant components of the basal lamina mentioned above, we isolated various ECM molecules that have been described previously in Schwann cell, such as tenascin C (Fruttiger *et al.*, 1995) and tissue plasminogen activator (Akassoglou *et al.*, 2002), thrombospondin 1 (Burstyn-Cohen *et al.*, 1998), and novel molecules that are yet to be characterized in the PNS (biglycan and the WD repeat domain protein 34). Another ECM component we isolated repeatedly is osteonectin/SPARC, a secreted Schwann cell protein that has been suggested to mediate axon–glial communication (Chlenski *et al.*, 2002; Bampton *et al.*, 2005). No defects in myelination are reported in mice that lack this gene (Gilmour *et al.*, 1998), but given the wide range of phenotypes that are linked to disarrangement of the ECM, careful analysis of the peripheral nerves of these animals by electron microscopy, and determining the molecular composition of the nodes and adjacent domains might be worthwhile. We have also isolated two laminin receptors, integrin α7, which is expressed in both axons and Schwann cells but does not seem to have a role in myelination (Previtali *et al.*, 2003), and integrin β8, which is novel and previously undescribed in the PNS.

Finally, we isolated three of the four known syndecans and a novel molecule (HTGN29) that shares important structural features with these. Syndecans are a family of transmembrane

proteoglycans that interact with numerous extracellular ligands through specific sequences in their heparan sulfate chains, and are considered to be co-receptors for ECM molecules and growth factors. In addition to their roles as co-receptors, many recent studies indicate that signaling through the core protein of syndecans can regulate cytoskeletal organization (Yoneda and Couchman, 2003). Syndecan 3 and syndecan 4 were shown recently to localize on the microvvili that contact the node of Ranvier (Goutebroze et al., 2003). However, targeted disruption of syndecan 3 did not result in an overt nodal phenotype (Melendez-Vasquez et al., 2005), which indicates that other syndecans present in myelinating Schwann cells might compensate for its loss.

Cell-adhesion and cell-recognition molecules

Tetraspanin proteins
Several members of this family were identified in myelinated fibers and their function in the system is emerging slowly (Poliak et al., 2002; Spiegel and Peles, 2002). Although these proteins lack a typical signal sequence, they were detected in our screen because of the close proximity of the first hydrophobic transmembrane sequence to the initiation methionine. We isolated cDNAs that encode six such proteins, including the most prominent peripheral myelin proteins, PMP22 (Suter et al., 1992) and its close homologue EMP1, and the tight-junction protein claudin-19, which is required for the formation of autotypic tight junctions in myelinating Schwann cells (Miyamoto et al., 2005). CD9, CD63, CD81 and CD82 belong to the tetraspanin-family. These proteins form a 'tetraspanin-web' by interacting laterally in the plasma membrane with each other, additional tetraspanins and various other proteins (Levy and Shoham, 2005).

This web locally and transiently clusters transmembrane and intracellular components, thereby facilitating specific, highly regulated responses to diverse extracellular signals, which affect a wide range of cellular processes including cell aggregation, cell motility and cell adhesion. Among the transmembrane proteins that interact with the tetraspanin-web are integrins, G-protein-coupled receptors and members of the immunoglobulin superfamily (Levy and Shoham, 2005). Of the classic tetraspanin-molecules, only CD9 has been described previously in myelinated fibers. This protein is concentrated at the paranodal loops of both myelinating Schwann cells and oligodendrocytes and its ablation in mice by homologous recombination leads to paranodal abnormality and minor myelin defects (Ishibashi et al., 2004). One of the questions that arises from this paper is whether CD9 is the only tetraspanin found in the paranodes or whether, for example, one of the molecules identified in our screen might be part of such a paranodal 'tetraspanin-web'. Indeed, CD81 has been described as a myelin protein that is associated with CD9 (Terada et al., 2002). However no myelin defects were reported in CD81-null mice (Geisert et al., 2002). So far, CD63 and CD82 have not been reported in myelinating cells, but they are good candidates to be *cis*-partners of CD9 in myelinating Schwann cells.

Immunoglobulin superfamily (IgSF) members
This is largest subgroup of CAMs isolated in our screen. In this group we include molecules with polycystic kidney disease (PKD)-domains, which exhibit an Ig-fold. This group contains several molecules with important roles in myelinated nerves, including P0, MAG and neurofascin. Surprisingly, we isolated NrCAM, a protein that is localized at the nodal axolemma (Lambert et al., 1997), from both the Schwann cell and sciatic nerve libraries, which indicates that NrCAM might also be expressed in myelinating Schwann cells. Several additional IgSFs isolated in our screen (basigin, neurotrimin, IL1-R associated protein, AlCAM and Muc18) have been implicated previously in nervous system development. Basigin is a widely expressed glycoprotein that regulates matrix metallo-proteases and is involved in several cellular processes (Gabison et al., 2005). Neurotrimin inhibits the adhesion and growth of neurons but not of Schwann cells (Clarke and Moss, 1997), and Muc18 and AlCAM, which are structurally similar IgSF-members, are reported to regulate the extension of neuritis from motor and sensory neurons in the PNS (Shih et al., 1998; Burns et al., 1991; Taira et al., 2004). Another subfamily of the IgSF identified in our screen are the nectin-like molecules. Two members of this subgroup have been described previously in the nervous system: Necl-1 at non-junctional contact sites between neuron and glia in the CNS and PNS (Kakunaga et al., 2005); and Necl-2 (also termed SynCAM1), which induces synaptogenesis in the CNS (Biederer et al., 2002). However, no information is available on the third member of this group (Necl-4), which we also isolated from both the Schwann cell and sciatic nerve libraries. Together with the known ability of IgSFs to interact homophilically and with other members of this family, our results indicate a possible role for neurotrimin, Muc18, AlCAM, and the Necls in Schwann cell–axon interactions. Finally, we identified several other IgSFs that are proposed to function as CAMs outside the nervous system, including ZIg-1/HepaCAM in the liver (Chung Moh et al., 2005; Moh et al., 2005), the rat ortholog of GL50 in the immune system (Ling et al., 2000) and osteoactivin in skeletal muscle (Ogawa et al., 2005). The potential importance of all of these proteins in myelinating Schwann cells requires more extensive, detailed analysis of their expression and localization during PNS development.

Other novel molecules of interest

In addition to the groups of molecules listed above, we identified several other novel molecules that might mediate cell–cell contacts between axons and glia. Among these is a gene mapped to the DiGeorge syndrom gene critical region (called Dgcr2) on chromosome 22q11.2 (Kajiwara et al., 1996; Taylor et al., 1997; Iida et al., 2001). This chromosomal microdeletion underlies the velocardiofacial syndrome, a syndrome that primarily affects cells that originate from the neural crest, including Schwann cells. Structurally, Dgcr2 contains a LDLa and a c-lectin domain, and a chordin-like, cysteine-rich repeat (also referred to as von Willebrand factor type C module), all of which mediate cell adhesion, which indicates that Dgcr2 might mediate interactions between Schwann cells and surrounding cells in the PNS. In keeping with such a role, *in situ* hybridization (Fig. 2) and Northern blots (data not shown) show that Dgcr2 is expressed in myelinating Schwann cells. Another novel, potentially relevant molecule is dysadherin; this cancer-associated glycoprotein downregulates E-cadherin, a molecule that is found in the paranodal loops and the Schmidt-Lanterman incisures of myelinating Schwann cells (Fannon et al., 1995). Dysadherin is also reported to regulate

Na, K-ATPase (Lubarski *et al.*, 2005). This is particularly interesting because Na, K-ATPase is required for the formation of septate junctions in *Drosophila* (Genova and Fehon, 2003), which bear structural and molecular similarities with paranodal axoglial junctions in myelinated fibers (Poliak and Peles, 2003). Whether dysadherin is involved in axon–glia contact at the paranodal junction is of interest for future studies.

Concluding remarks

The goal of the present work was to identify novel molecules that might mediate axon–glial interactions at the onset of myelination in the PNS. For this purpose, we used a eukaryotic SST system to screen cDNA expression libraries made from dbcAMP-stimulated primary rat Schwann cells and 3-day-old rat sciatic nerves. We identified many structurally and functionally diverse molecules. We further verified and compared the expression of many of the novel cell-adhesion and signaling molecules in primary cultures of rat Schwann cells and in sciatic nerves, which enabled us to estimate the abundance of the newly identified genes in the respective source. Finally, we examined the expression of selected genes in myelinating nerves by *in situ* hybridization. By applying this rational approach, we have identified and verified a large number of novel molecules that are expressed in the PNS during myelination. These molecules might potentially communicate important axon–glial signals that are necessary for proper myelination by Schwann cells. Therefore, future studies should evaluate the function of these novel molecules in axon–glial interactions.

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