

# Caveolin-1 Expression in Schwann Cells

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**ABSTRACT** Caveolae are non-clathrin-coated invaginations of the plasma membrane, which are present in most cell types. An integral component of caveolae is the caveolin family of related proteins, which not only forms the structural framework of caveolae, but also likely subserves its functional roles, including regulation of signal transduction and cellular transport, in particular, cholesterol trafficking. Although caveolae have been identified ultrastructurally in the peripheral nervous system (PNS), caveolin expression has not previously been studied. To date, three caveolin genes have been reported. Here, we show for the first time that caveolin-1 is expressed by Schwann cells (SC) as well as several SC-derived cell lines. Caveolin-1 is enriched in the buoyant, detergent-insoluble membranes of rat sciatic nerve (SN) and SC, a hallmark of the caveolar compartment. Caveolin-1 exists as both soluble and insoluble forms in rat SN and SC, and localizes to SC cytoplasm and abaxonal myelin. SC caveolin-1 decreases after axotomy, when SC revert to a premyelinating phenotype. We speculate that caveolin-1 may regulate signal transduction and/or cholesterol transport in myelinating SC. *GLIA* 27:39–52, 1999. © 1999 Wiley-Liss, Inc.

## INTRODUCTION

Caveolae are plasma membrane microdomains that can invaginate to form 50–100 nm vesicles, and are enriched in cholesterol, glycosphingolipids, a variety of signaling molecules, and the caveolins, a family of 18–24 kD proteins. There are four known structurally homologous caveolin isoforms transcribed from three separate genes (Glenney and Soppet, 1992; Scherer P et al., 1996; Tang et al., 1996). Caveolin-1 consists of 21 kD and 24 kD isoforms derived from alternate translational start sites, and it was the first characterized member of the caveolin family (Rothberg et al., 1992). It is present in most cell types, but is especially abundant in epithelial cells, fibroblasts, type I pneumocytes, adipocytes, and smooth muscle cells (Lisanti et al., 1994a, 1995a; Severs, 1988). Caveolin-1 is a membrane protein that forms part of the cytoplasmic coat of caveolae (Lisanti et al., 1993), interacting with itself to form homo-oligomers (Fra et al., 1995b). Caveolin-2 (20 kD) has not been extensively studied, but has a similar distribution to caveolin-1 (Scherer P et al., 1996), with which it can form hetero-oligomeric complexes (Scheiffele et al., 1998), whereas caveolin-3 (18 kD) is considered muscle-specific (Tang et al., 1996).

A number of functions are proposed for caveolae: (1) the uptake of small molecules such as folate into cells, a process known as potocytosis (Anderson et al., 1992); (2) the transcytosis of molecules such as low density lipoprotein across cells (Simionescu, 1983); (3) polarized trafficking of proteins, especially in epithelial cells (Simons and Wandinger-Ness, 1990); (4) cholesterol transport (Murata et al., 1995); and (5) regulation of signal transduction (Anderson, 1993; Lisanti et al., 1994b, 1995c; Wu et al., 1997). A “caveolae signaling hypothesis” proposes that compartmentalization of signaling molecules within caveolae allows for efficient coupling of activated receptors to a number of different effector systems (Lisanti et al., 1994a; Okamoto et al., 1998; Sargiacomo et al., 1993).

By virtue of its ability to self-oligomerize, caveolin may serve a scaffolding role (Sargiacomo et al., 1995).

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Caveolin-1 directly binds cholesterol (Murata et al., 1995) and possibly glycosphingolipids (Fra et al., 1995a; Parton, 1994), both of which are abundant in caveolae (Simionescu et al., 1983). The unique lipid microenvironment of caveolae accounts for the insolubility of caveolae-associated molecules in most nonionic detergents and the low density of caveolae relative to other cellular membranes, properties used to isolate caveolin-enriched membranes (CEMs) from a variety of cells and tissues (Lisanti et al., 1995b; Song et al., 1996a). CEMs are thought to be the biochemical counterparts of caveolae and are thus enriched in a variety of signaling molecules. For example, all of the following are concentrated within caveolae-like membranes: the receptors for insulin (Mastick and Saltiel, 1997), platelet-derived growth factor (Liu et al., 1996), the nerve growth factors p75<sup>NTR</sup> (Bilderback et al., 1997) and trkB (Wu et al., 1997), basic fibroblast growth factor (Gleizes et al., 1996), epidermal growth factor (Smart et al., 1995), bradykinin (B<sub>2</sub>) (Haasemann et al., 1998), and atrial natriuretic peptide (type B) (Doyle et al., 1997). In addition, caveolin-1 binds directly to the epidermal growth factor receptor (Couet et al., 1997; Smart et al., 1995), p75<sup>NTR</sup> (Bilderback et al., 1997), and a variety of lipid-modified signaling molecules, such as Ras (Song et al., 1996a), Src-like kinases (Li et al., 1996a), endothelial nitric-oxide synthase (Shaul et al., 1996) and G-proteins (Li et al., 1995).

Plasma membrane invaginations with the morphologic appearance of caveolae are present in blood vessels (Olsson and Reese, 1971), cells forming the perineurial sheaths of the PNS (Akert et al., 1976; Hall and Williams, 1971; Olsson and Reese, 1971; Reale et al., 1975; Sandri et al., 1977; Waggenger et al., 1965), and SC (Mugnaini et al., 1977). However, caveolin expression within SC has not been explored. We have previously reported caveolin immunoreactivity in detergent-insoluble fractions of rat SN (Mikol and Feldman, 1997). Here, we demonstrate that caveolin-1 is expressed by SC and is enriched in a low-density membrane fraction using a detergent-free method to enrich for CEMs. Caveolin-1 from SN or SC is present in both soluble and membrane-associated forms and localizes to SC cytoplasm and abaxonal myelin. Caveolin-1 immunoreactivity is significantly decreased after axotomy, suggesting that its expression is greatest in differentiated SC.

## MATERIALS AND METHODS

### Materials

Rat SN and lung were purchased from Pel-Freez (Rogers, AR) for immunoblotting. Adult rat SN was frozen in OCT compound (Ted Pella, Inc., Redding, CA) and sectioned for immunohistochemistry. Adult rat teased SN fibers and transected SN (distal stumps collected 12 days after axotomy) (Scherer S et al., 1995), fixed in 4% paraformaldehyde, were generously pro-

vided by Dr. S. Scherer (University of Pennsylvania, Philadelphia, PA). The mouse monoclonal antibody (mAb) (C37120), the affinity-purified rabbit polyclonal antibody (pAb) against caveolin-1, and anti-Fyn rabbit pAb were purchased from Transduction Laboratories (Lexington, KY). The anti-S100 mAb (S-2532) was purchased from Sigma (St. Louis, MO), while the anti-myelin basic protein (MBP) mAb was obtained from Boehringer Mannheim (Indianapolis, IN). Dr. J. Archelos (Max Planck Institute for Psychiatry, München, Germany) kindly provided the anti-P<sub>0</sub> mAb. Anti-PMP-22 and anti-myelin associated glycoprotein (MAG) rabbit pAbs were generous gifts from Dr. E. Shooter (Stanford University, Stanford, CA) and Dr. J. Salzer (New York University, New York, NY), respectively.

### Cell Culture

In general, all cells were grown at 37°C in a humidified atmosphere with 10% CO<sub>2</sub>. For analysis by SDS-polyacrylamide gel electrophoresis (PAGE), cells were grown to ~80% confluency and collected for protein quantification. SH-SY5Y and SHEP human neuroblastoma cells and NIE-115 rat neuroblastoma cells were kindly provided by Drs. S. Fisher, V. Castle, and S. O'Shea, respectively, all at the University of Michigan. PC12 cells (rat pheochromocytoma origin) and N2a mouse neuroblastoma cells were purchased from American Type Culture Collections (Manassas, VA). PC12 cells were grown in RPMI 1640 medium with 5% fetal bovine serum (FBS) and 10% horse serum, while N2a cells were grown in minimal essential media plus 10% FBS and supplemented with 1% nonessential amino acids (Gibco BRL Products, Gaithersburg, MD). SH-SY5Y, SHEP and NIE-115 cells were cultured in Dulbecco's modified Eagle's media (DMEM) plus 10% FBS. The SV-40 immortalized SC line MT4H1 (Peden et al., 1989), a gift from Dr. G. Tennekoon (University of Pennsylvania), was grown in DMEM containing 10% FBS, 100 µM ZnSO<sub>4</sub>, and 100 µg/ml G418, while S16, S16Y, and S42, all generated by repetitive passaging of rat SC in the laboratory of Dr. R. Quarles (National Institutes of Health, Bethesda, MD; Sasagasaki et al., 1996), were grown in DMEM, 10% FBS, and 100 µg/ml gentamycin. The SV-40 immortalized oligodendroglial cell lines N19 and N20 (Verity et al., 1993), supplied by Dr. A. Campagnoni (University of California at Los Angeles, Los Angeles, CA), were grown in Ham's F-12 media plus 10% Nu-serum (Collaborative-Becton Dickinson, Bedford, MA).

SC obtained from SN of 3-day-old Sprague Dawley rats (Harlan-Sprague Dawley, Indianapolis, IN; Brockes et al., 1979) were cultured on poly-L-lysine coated plates and maintained in standard growth medium consisting of DMEM with 10% FBS, supplemented with 6 mM L-glutamine, 2 µM forskolin and 10 µg/ml pituitary extract (Sigma; Brockes et al., 1979; Porter et

al., 1986). Cells were passaged upon confluency and used for 4 passages. To test the effects of raised intracellular cAMP levels on caveolin-1 expression, SC were grown for 24–72 h in defined medium (Cheng et al., 1998) with or without 2  $\mu$ M forskolin. Rat dorsal root ganglion cells isolated from E15 Sprague-Dawley rat pups (Russell et al., 1998) and SC were co-cultured on poly-L-lysine coated coverslips for 48 h in defined medium plus 10 nM insulin like growth factor-I, which has been shown to promote myelination (Cheng et al., 1998).

### Membrane Fractionation

Rat SN or SC were homogenized at 4°C in phosphate buffered saline (PBS) plus 1 mM phenylmethylsulfonyl-fluoride and protease inhibitor cocktail (Boehringer-Mannheim), then centrifuged at 200,000 *g* for 1 h. After separation, equal aliquots of the supernatant and pellet were analyzed by Western blotting. CEMs were prepared from rat SN or SC by discontinuous density gradient centrifugation in the presence or absence of detergent, using established procedures (see below). All steps were carried out at 4°C. Briefly, ~10–15 adult SN or two 150 mm plates of cultured SC were utilized per centrifuge tube, whether the detergent-based or detergent-free protocol was followed.

For preparation of low density, Triton-insoluble membranes, the method of Lisanti (Lisanti et al., 1995b) was used with minor modifications. The sample was homogenized in 3 ml 25 mM 2-(N-morpholino)ethane sulfonic acid (pH 6.5) plus 0.15 M NaCl (MBS) containing 1% Triton-X-100, 1 mM phenylmethylsulfonylfluoride and protease inhibitor cocktail. After 8–10 strokes with a Dounce homogenizer, 3 ml of lysate was combined 1:1 with 80% sucrose (in MBS) and placed at the bottom of a centrifuge tube. A 5%–15%–25% step gradient (2 ml each in MBS, without detergent) was layered above, and the sample was centrifuged at 200,000 *g* for 20 h in a SW 41 rotor (Beckman Instruments, Palo Alto, CA). Twelve 1 ml fractions were collected (top to bottom) and equal aliquots of these fractions plus the initial homogenate and pellet were analyzed by SDS-PAGE.

Detergent-free extractions were performed essentially as described (Song et al., 1996a). Tissue or cells were homogenized in 0.5 M sodium carbonate (pH 11.0) using a Dounce homogenizer, followed by sonication (three 20 sec bursts). The sample was then combined 1:1 with 80% sucrose (in MBS) and overlaid with a step gradient of 5%–15%–25% sucrose (containing MBS plus 0.25 M sodium carbonate). Centrifugation and collection of aliquots were carried out as above for the detergent-based method.

### Electrophoresis and Western Blotting Analysis

Protein samples were quantified using a Protein Assay kit (Bio-Rad Laboratories, Richmond, CA). Poly-

peptides and pre-stained molecular mass standards (Amersham Life Science, Arlington Heights, IL) were separated by SDS-PAGE (8%, 10%, or 12% acrylamide) using the method of Laemmli (Laemmli, 1970) and electrically transferred to nitrocellulose as previously described (Mikol and Stefansson, 1988). To minimize non-specific binding of antibodies, nitrocellulose sheets were then blocked overnight in 5% nonfat dry milk or 4% bovine serum albumin (each in Tris-buffered saline plus 0.05% Tween 20), for polyclonal and monoclonal antibody immunoblotting, respectively. Antibodies were diluted in the blocking solution to minimize non-specific reactions. Nitrocellulose sheets were immunoblotted with mAb (1:2,000) or affinity-purified pAb (1:4,000) against caveolin-1, pAb against Fyn (1:2,000), MAG (1:10,000) or PMP-22 (1:2,000), or mAb against MBP (1:2,000), followed by peroxidase-conjugated secondary antibodies (1:2,500; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in the appropriate blocking solution. Both monoclonal and polyclonal anti-caveolin antibodies were used to ensure specific binding to caveolin-1. Immunoblots were visualized using enhanced chemiluminescence reagents. To verify specific binding of antibody to caveolin-1, positive (lung lysate) and negative (SY5Y cell lysate) controls were immunoblotted along with the SN or SC samples, and parallel immunoblots were incubated in the absence of primary antibody (conjugate control) for each antibody.

### Immunohistochemistry and Immunocytochemistry

Frozen sections of rat SN were brought to room temperature prior to processing. Adult SN and transected SN sections, teased fibers and SC/dorsal root ganglion neuron co-cultures grown on poly-L-lysine-coated coverslips were either fixed in 4% paraformaldehyde (in PBS) for 15 min or immunostained without fixation. Samples were permeabilized with either 0.1% Triton X-100 (in PBS) for 20 min or acetone at –2°C for 10 min. After a 30 min incubation in blocking solution (2% nonfat dry milk plus 0.1% Triton X-100 in PBS) to minimize nonspecific binding of antibodies, samples were incubated with primary antibody in blocking solution for 24 h at 4°C. Antibodies against caveolin-1, S100 and P<sub>0</sub> were all used at 1:500. After washing with PBS, a 6 h incubation with secondary antisera (1:100) at room temperature followed, using either Cy-3 conjugated anti-rabbit or FITC-conjugated anti-mouse antibodies (Jackson Immunoresearch, Westgrove, PA) in blocking solution. In addition, conjugate controls (no primary antibody) were done in parallel to demonstrate that immunoreactivity was due to binding of primary antibody. Slides were coverslipped and viewed by fluorescence microscopy using a Leitz fluorovert microscope (W. Nuhsbaum Inc., McHenry, IL).

## RESULTS

### Caveolin-1 Expression in SC and SC Derived Lines

We analyzed caveolin-1 expression in a number of different neural cell lines derived from neuroblastoma (N2a, SH-SY5Y, SHEP, NIE-115), pheochromocytoma (PC12), oligodendrocytes (N19, N20), or SC (MT4H1, S16, S16Y, S42) by immunoblotting cell lysates with an affinity-purified anti-caveolin-1 pAb (Fig. 1A). The apparent molecular weight of caveolin-1 is ~24 kD (size markers not shown). Three exposures of the same immunoblot are presented (with intentional overexposure of some lanes at 30 s and 8 min) to emphasize relative caveolin-1 levels. Of the neural cell lines tested, SC derived cell lines express the greatest levels of caveolin-1. Within a class of comparable cell lines, the cell line with the more differentiated phenotype, S16 compared to S16Y and S42 (Sasagasako et al., 1996) and N20 compared to N19 (Verity et al., 1993), expresses relatively more caveolin-1. SHEP and SH-SY5Y cells are derived from the same parental neuroblastoma (Biedler et al., 1988). SHEP cells, which are more differentiated and have been described as having a SC phenotype, express abundant caveolin-1, whereas the less differentiated SH-SY5Y cells, which have a neuroblastic phenotype, express no caveolin-1.

We next sought to identify caveolin-1 in rat SC. Figure 1B shows that caveolin-1 is present both in a rat SN homogenate, which contains a variety of cell types, and in a pure lysate from cultured rat SC. A rat lung lysate, which contains abundant caveolin-1, serves as a positive control. In all of our experiments caveolin-1 from SN and cultured SC has a molecular weight of ~24 kD and co-migrates with caveolin-1 from rat lung.

### CEMs from SC and SN

Two established protocols were used to isolate CEMs from rat SN and cultured SC. The first protocol utilizes detergent, while the second does not. In each case, thin flocculent bands are visible with the naked eye at the 5%–15% and 15%–25% sucrose interfaces. Figure 2A demonstrates that caveolin-1 from rat SN detergent preparations is enriched in fractions 2 through 5, near the 5%–15% and 15%–25% sucrose interfaces, which is consistent with the buoyant characteristics of caveolin from other tissues (Scherer P et al., 1996; Song et al., 1996a,b). However, a considerable portion of caveolin-1 remains in the soluble 40% sucrose layer (fractions 7–12).

Because compact myelin membranes have a light density (Norton and Poduslo, 1973) comparable to that of CEMs, the above SN fractions were immunoblotted with antibodies against several myelin proteins, including MAG, MBP, P<sub>0</sub>, and PMP-22 (Fig. 2A), in order to determine the usefulness of CEM preparations when applied to nervous tissue. There is some variability in the staining patterns, presumably reflecting differen-

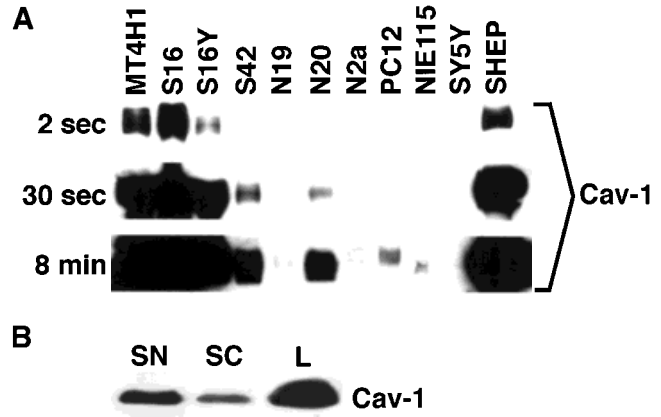


Fig. 1. Caveolin-1 expression in SC derived cell lines and SC. **A:** Cell lysates (50  $\mu$ g) from a number of neural cell lines were immunoblotted with anti-caveolin-1 (Cav-1) pAb. Three exposures (increasing exposure time from top to bottom) are shown in order to illustrate the relative levels of caveolin-1 expression. The SC lines (MT4H1, S16 and S16Y, and S42) express caveolin-1, although there is some variability in the amounts expressed. The SC-like cell line SHEP expresses abundant caveolin-1, whereas SH-SY5Y cells, derived from the same neuroblastoma, have no detectable caveolin-1. One oligodendrocyte cell line (N20) expresses caveolin-1, while N19 and several neuronal-like cell lines (PC12, N2a, NIE-115, SH-SY5Y) have minimal to no detectable caveolin-1. **B:** Rat SN (20  $\mu$ g), SC (50  $\mu$ g) and lung (L) (0.2  $\mu$ g) were immunoblotted with anti-caveolin-1 pAb. The results demonstrate the presence of caveolin-1 in all lysates. The apparent molecular weight of caveolin-1 is ~24 kD (not shown).

tial detergent-solubilities of the myelin proteins. MAG is largely present in the 40% sucrose layer (fractions 7–12), i.e., the original lysate layer, but is also present to a lesser extent in fractions 3 and 4. In contrast, MBP and P<sub>0</sub> are present in most fractions. While these results show that caveolin-1 and the compact myelin protein PMP-22 are enriched in similar fractions, this is likely due to the similar buoyant characteristics of CEMs and myelin membranes. We do not detect significant caveolin-1 in compact myelin membranes isolated by the method of Norton and Poduslo (1973; data not shown). Thus, density-based methods used to enrich for CEMs from nervous tissue are limited by the comparable densities of CEMs and myelin membranes, which results in their co-purification.

Detergent-free preparation of CEMs from SN and SC lysates again reveals enrichment of caveolin-1 within fractions around the 5%–15% and 15%/25% sucrose interfaces (Fig. 2B). These SC cultures are not myelinating, implying that the CEMs here are equivalent to typical caveolae-like membranes (Song et al., 1996a). Once again, caveolin-1 is detected in the lower sucrose fractions from both SN and SC, which indicates that a fraction of caveolin-1 is soluble in buffer alone. Figure 2C illustrates how SN and SC caveolin-1 partitions into soluble and membrane-associated forms.

The nonreceptor tyrosine kinase Fyn is concentrated within caveolae (Lisanti et al., 1995a). Caveolin-1 interacts directly with Fyn and other Src-like kinases, inhibiting their activity (Li et al., 1996a). Because Fyn mediates integrin downstream signaling and anchorage-dependent growth in fibroblasts (Wary et al., 1998) and

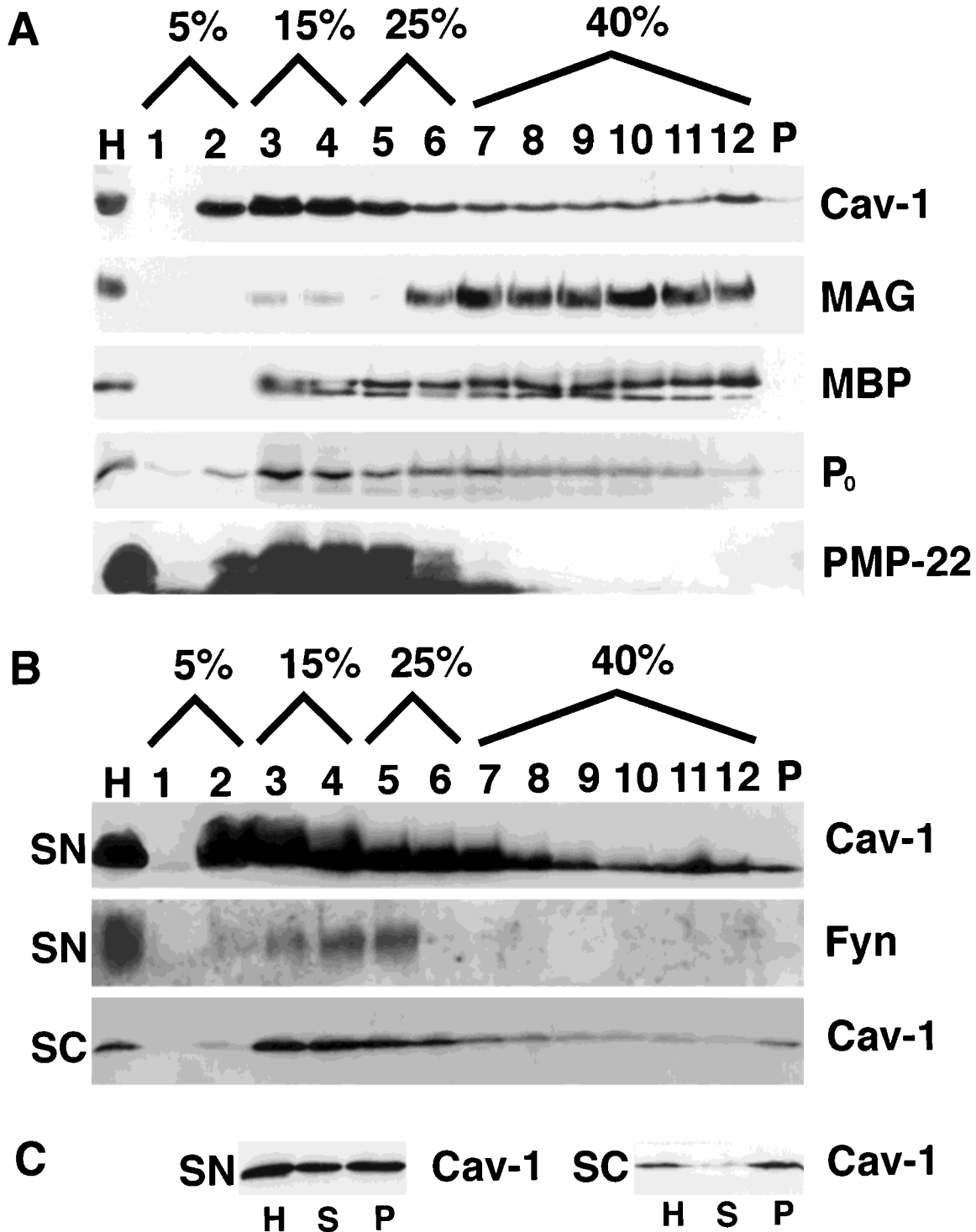


Fig. 2. CEMs from rat SN. **A:** Rat SN was homogenized and subjected to discontinuous sucrose gradient centrifugation in the presence of Triton X-100. Equal aliquots of the initial homogenate (H), insoluble pellet (P), or fractions 1 through 12 (top to bottom) were analyzed by SDS-PAGE, followed by immunoblotting. Caveolin-1 (Cav-1) is enriched in low density membranes (fractions 2 through 5), although it is also detected in the higher density sucrose and pellet fractions. For comparison, the same fractions were immunoblotted with antibodies against several myelin proteins: MAG, MBP, P<sub>0</sub>, and PMP-22. **B:** Rat SN or SC was homogenized and subjected to sucrose gradient centrifugation in the absence of detergent. Equal aliquots of the initial homogenate (H), insoluble pellet (P), or fractions 1 through

12 were analyzed by SDS-PAGE, followed by immunoblotting with anti-caveolin-1 or anti-Fyn pAb (Fyn). Again, caveolin-1 is enriched in the low density membranes (fractions 2 through 5), although small amounts are again detected in the higher density sucrose and pellet fractions. Fyn is enriched in similar buoyant membrane fractions from SN. **C:** Rat SN or SC was homogenized in PBS and centrifuged at high speed in order to identify soluble and membrane-associated forms of caveolin-1. Equal volume aliquots of the initial homogenate (H), or 200,000 g supernatant (S), or pellet (P) from rat SN or SC were separated by SDS-PAGE and immunoblotted with anti-caveolin-1 pAb. A soluble form of caveolin-1 is detected in both SN and SC, although it appears to be more prevalent in the SN sample.

some of the downstream signaling events necessary for myelination in the central nervous system (Umemori et al., 1994), it is possible that Fyn plays a role in PNS myelination. We were unable to detect Fyn in cultured SC (data not shown). However, using either method to prepare CEMs from SN, Fyn is highly concentrated within the buoyant fractions that are also enriched in caveolin-1. Figure 2B demonstrates an anti-Fyn immunoblot of a detergent-free CEM preparation from SN.

### Caveolin-1 Localization in Rat SN

Vesicles with the appearance of caveolae were identified ultrastructurally in peripheral nerve two decades ago. Most of these studies described such vesicles in perineurium and blood vessels (Akert et al., 1976; Hall and Williams, 1971; Olsson and Reese, 1971; Reale et al., 1975; Sandri et al., 1977; Waggener et al., 1965), although a single report described them in SC (Mugnaini et al., 1977). Using an affinity-purified anti-caveolin-1 pAb to stain SN, SC and abaxonal myelin are strongly immunostained, with additional staining of the perineurium and blood vessels (Figs. 3–6). Cross sections reveal caveolin-1 immunoreactivity of SC cytoplasm, while the abaxonal localization is most apparent on longitudinal sections. Cytoplasmic caveolin-1 immunoreactivity overlaps with the S100 staining pattern (Fig. 3). Double immunostaining with antibodies against caveolin-1 and P<sub>0</sub> demonstrates that there is no caveolin-1 staining of compact myelin (data not shown). Caveolin-1 immunostaining of adult SN teased fibers localizes to SC cytoplasm (including paranodes) and abaxonal myelin, but in addition reveals a stippled appearance (Fig. 4). Such punctate caveolin-1 immunoreactivity suggests the distribution of caveolae (Rothberg et al., 1992). S100 immunostaining of teased fibers shows a similar staining of SC cytoplasm, but without the stippled pattern seen in the case of caveolin-1. Immunofluorescent staining of cultured SC (Fig. 5A) and SC/dorsal root ganglion co-cultures (Fig. 5B), using the same caveolin-1 pAb, intensely labels SC, whereas neurons and their axons do not visibly stain. The staining pattern is punctate over the entirety of the cells, along stress fibers, and at the cell margins. The SC membrane is strongly caveolin-1 immunoreactive where it contacts axons.

We have shown here that caveolin-1 is expressed in PNS myelin. We questioned whether caveolin-1 expression might be affected by *in vivo* axonal cues, such as nerve transection, which down-regulates myelin genes (Mirsky and Jessen, 1990; Scherer et al., 1993), or elevated intracellular cAMP, which induces expression of myelin genes *in vitro* (Jessen et al., 1991; Lemke and Chao, 1988). We chose to look at SC caveolin-1 levels 12 days after nerve transection, as myelin protein expression declines considerably by this time (Scherer S et al., 1995). Figure 6A,C shows that SC caveolin-1 expression decreases significantly after axotomy; perineurium and blood vessels retain intense caveolin-1 immunoreactiv-

ity, which serves as an internal control. P<sub>0</sub> is detected 12 days after axotomy in myelin debris (Fig. 6B,D). SC persist and are S100 positive (not shown).

We also questioned whether caveolin-1 expression by SC was affected *in vitro* by forskolin, which raises intracellular cAMP, mimicking in part axonal contact and thereby inducing expression of myelin genes such as P<sub>0</sub> (Lemke and Chao, 1988). We conducted several experiments exposing SC to 2  $\mu$ M forskolin, with incubation varying between 24 and 72 h. In every case, P<sub>0</sub> expression increases in the presence of 2  $\mu$ M forskolin, whereas caveolin-1 levels are unaltered. Figure 6E shows the effects of forskolin treatment (for 24 h) on caveolin-1 and P<sub>0</sub> expression by cultured SC. Thus, caveolin-1 expression is associated with a myelinating phenotype but does not appear to be coordinately linked with the expression of myelin genes.

### DISCUSSION

The caveolins are increasingly being implicated in such diverse biological processes as signal transduction, cellular transport, differentiation, and apoptosis (for review see Okamoto et al., 1998). However, caveolin has been little studied in the nervous system. We questioned whether caveolin-1 was expressed in SC and their differentiated organelle, myelin. Here, we have identified caveolin-1 in a rat SN homogenate, which contains a number of different cell types, in SC derived cell lines, and in a pure SC lysate (Fig. 1).

Of the neural cell lines tested, the SC derived cell lines (MT4H1, S16, S16Y, S42) express the greatest levels of caveolin-1, and cell lines with more differentiated phenotypes express relatively more caveolin-1 (Biedler et al., 1988; Sasagasako et al., 1996; Verity et al., 1993; Fig. 1A). Although they were both derived from a single human neuroblastoma (Biedler et al., 1988), the SH-SY5Y and SHEP cell lines show differential caveolin-1 expression. SHEP cells, which have some characteristics of SC, tend to be relatively slow growing, have a finite life-span *in vitro*, are anchorage and serum dependent, and do not typically form tumors in nude mice. In contrast, SH-SY5Y cells have neuroblastic characteristics, divide more readily, are anchorage and serum independent, and readily form tumors in nude mice. SHEP cells express abundant caveolin-1 whereas SH-SY5Y cells have no detectable caveolin-1. Of course, caution must be used in interpreting these results, as protein expression in cell lines is not necessarily representative of protein expression in primary cells *in vitro* or *in vivo*. Nonetheless, these results are consistent with previous findings that associate caveolin expression with a differentiated cellular state (Fan et al., 1983; Scherer P et al., 1995; Song et al., 1996b).

The caveolins are most abundantly expressed in terminally differentiated cells such as muscle cells, endothelial cells, and adipocytes. For example, caveolin-1 levels increase as much as 25-fold when NIH3T3 fibroblasts differentiate into adipocytes (Fan et al.,

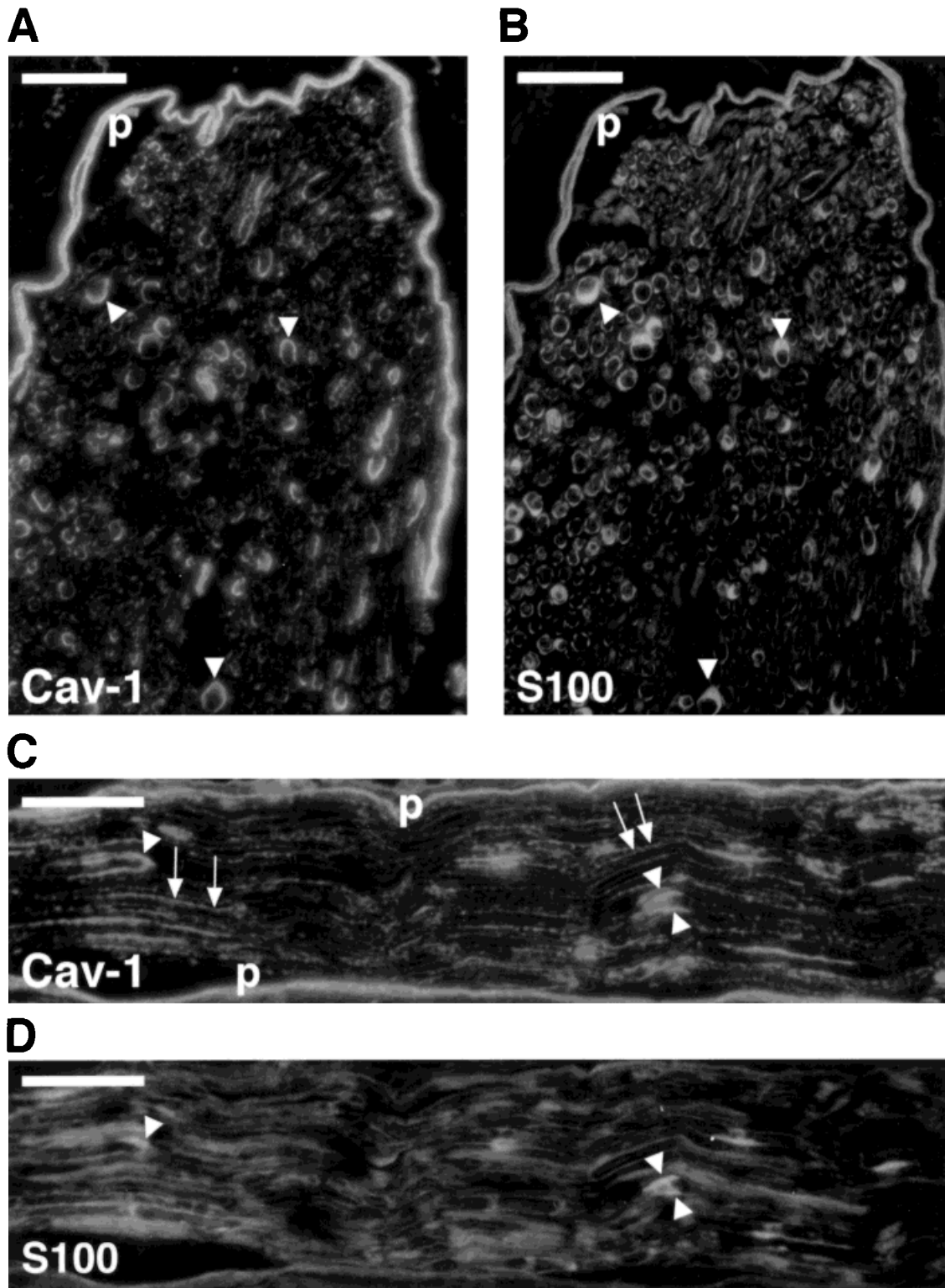


Fig. 3. Caveolin-1 localization in rat SN. Rat SN sections were permeabilized with 0.1% Triton X-100 and double immunostained with pAb against caveolin-1 (Cav-1) (A,C) and mAb against S100 (B,D). The cross sections (A,B) reveal crescent-shaped caveolin-1 immunoreactivity of SC cytoplasm (arrowheads). Longitudinal sections (C,D) demonstrate caveolin-1 staining in a linear fashion that is

consistent with immunoreactivity of abaxonal myelin (arrows) as well as SC cytoplasm (arrowheads). Perineurium (p) is also immunoreactive for caveolin-1. The pattern of S100 immunostaining of SC cytoplasm (arrowheads) overlaps with that of caveolin-1. Scale bar = 20  $\mu$ M.

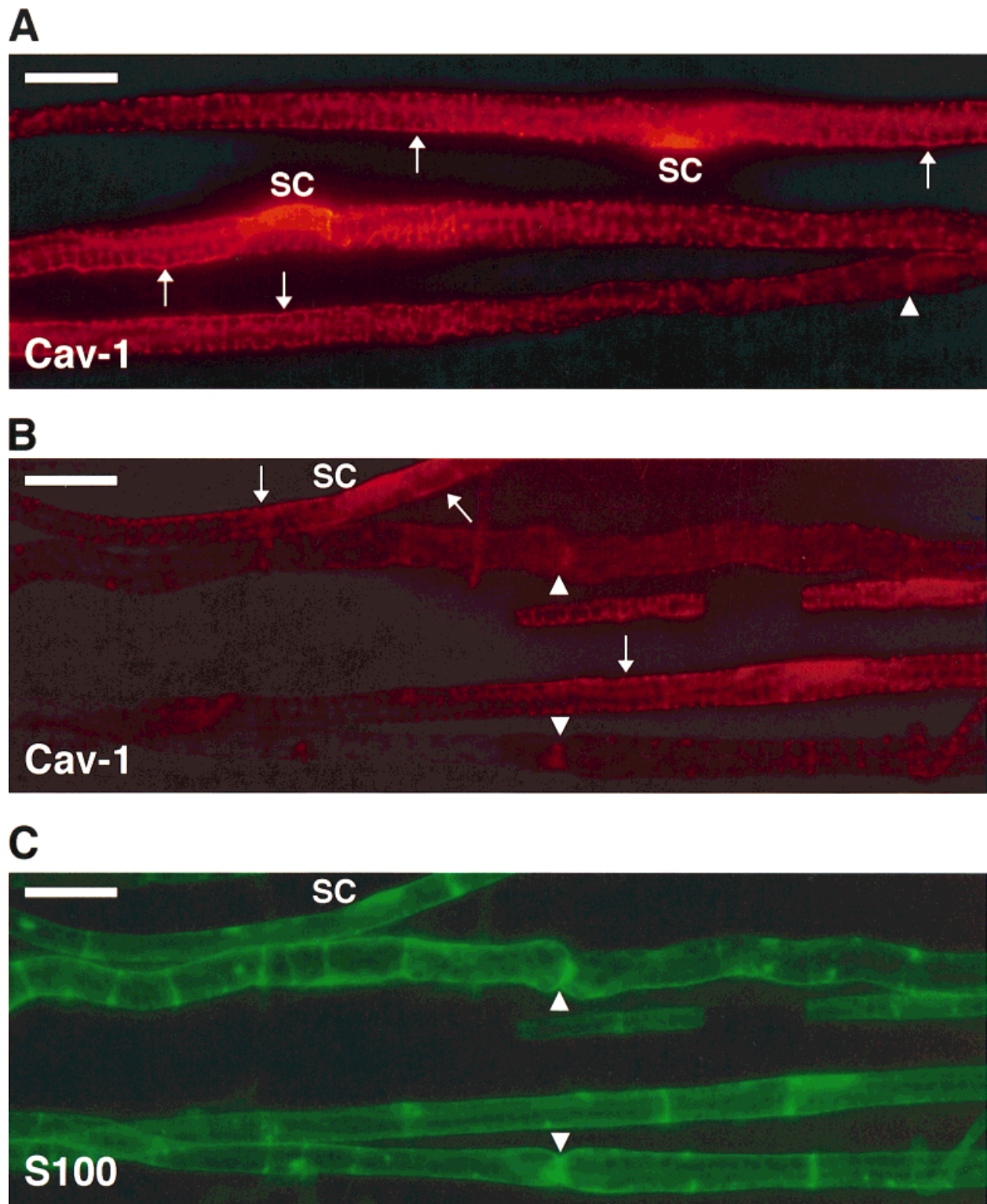


Fig. 4. Caveolin-1 immunostaining of rat SN teased fibers. Rat SN teased fibers were immunostained with pAb against caveolin-1 (Cav-1) (A,B) or mAb against S100 (C). Double immunostaining of the same fibers with caveolin-1 and S100 is illustrated in (B) and (C). Caveolin-1 is strongly localized in the outer myelin membrane (arrows) and SC

cytoplasm (SC). Caveolin-1 is also found at the paranodes (arrowheads). Caveolin-1 immunoreactivity has a stippled appearance, most apparent in (A), which was permeabilized with cold acetone. (B) and (C) were permeabilized with 0.1% Triton X-100. Caveolin-1 and S100 are both detected in SC cytoplasm. Scale bar = 10  $\mu$ M.



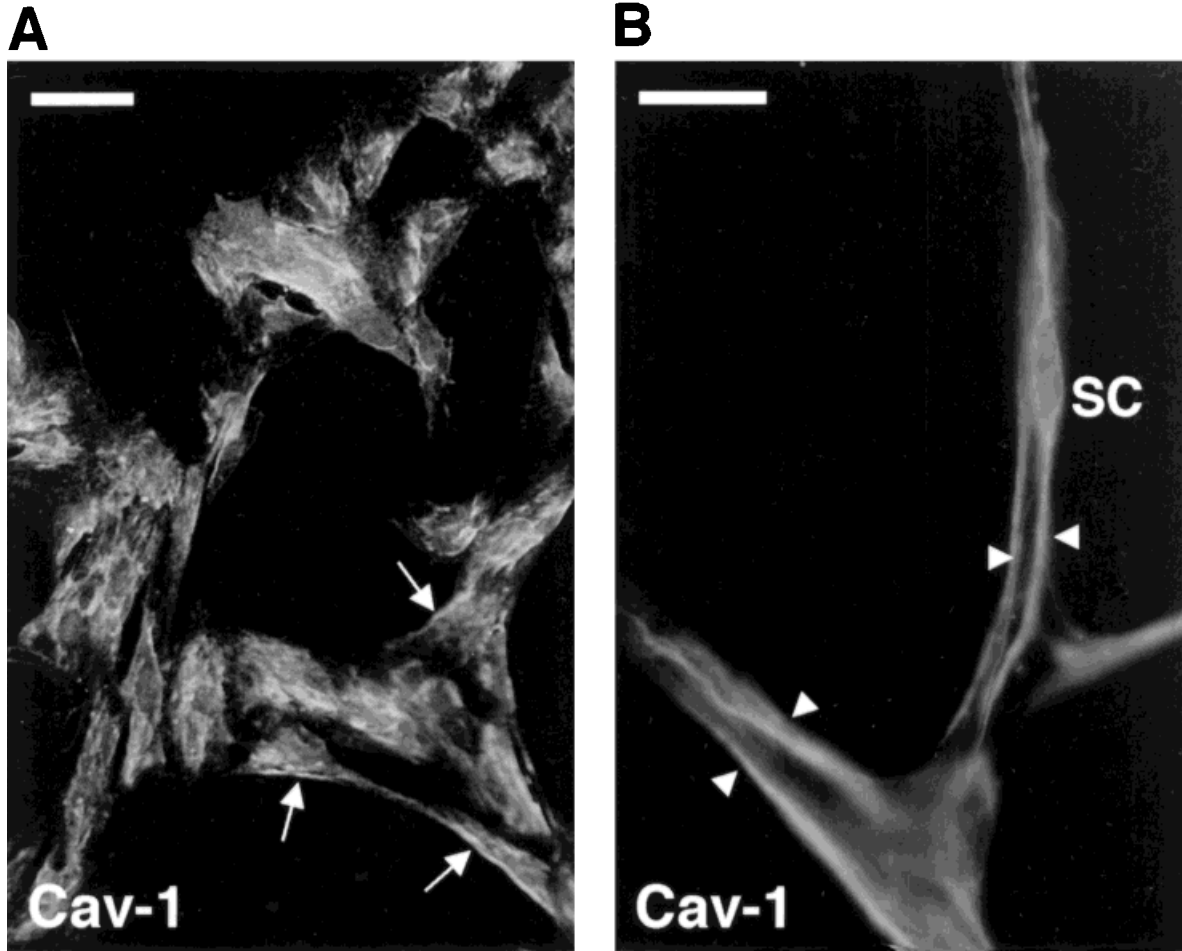


Fig. 5. Caveolin-1 immunostaining of SC in vitro. Rat SC (A) or co-cultures of SC/dorsal root ganglion neurons harvested 48 h after plating in the presence of insulin like growth factor-I (B) were immunostained with anti-caveolin-1 (Cav-1) pAb, after first permeabil-

izing cells with 0.1% Triton X-100. Strong SC staining is evident, especially at cell margins (A, arrows) and where SC membranes abut axons (B, arrowheads), whereas axons do not appreciably stain. In (A), scale bar = 20  $\mu$ M; in (B), scale bar = 10  $\mu$ M.

1983; Scherer P et al., 1995), and caveolin-3 expression increases substantially during differentiation of C2C12 myoblasts into myotubes (Song et al., 1996b). In contrast, cellular transformation results in decreased levels of caveolin and caveolae (Engelman et al., 1997; Koleske et al., 1995). Caveolin expression in transformed cells leads to suppression of the transformed phenotype (Engelman et al., 1998b; Lee et al., 1998), with loss of anchorage independent growth (Engelman et al., 1997). The mechanisms for the effects of caveolins on growth control are being elucidated. It appears that caveolins function largely as negative regulators of signal transduction, e.g., by suppressing MAP kinase activity (Engelman et al., 1997, 1998a) and signaling through the Neu receptor (Engelman et al., 1998b), the epidermal growth factor receptor, and protein kinase C (Couet et al., 1997). However, caveolin may stimulate other signal transducing molecules, such as the insulin receptor (Yamamoto et al., 1998). Thus, regulated caveolin expression by SC may in part determine their responsiveness to growth factors, and ultimately, their phenotype.

Clearly, caveolin is an essential structural protein of caveolae, and its expression is sufficient to drive the formation of caveolae-like vesicles. Cells that contain abundant caveolae, such as adipocytes, endothelial cells, smooth muscle cells, and fibroblasts also express the highest levels of caveolin (Glenney, 1992; Lisanti et al., 1994b). Furthermore, within a given cell type expressing caveolin-1, the degree of caveolin-1 expression correlates with the number of caveolae (Fan et al., 1983; Koleske et al., 1995; Scherer P et al., 1994; 1996). Recombinant expression of caveolin in cell lines without detectable caveolin, or identifiable caveolae, results in caveolin enrichment within caveolae-like membranes and the de novo formation of caveolae (Fra et al., 1995b; Scherer P et al., 1995). Thus, the presence of a caveolin in SC likely drives the formation of caveolae, and we therefore anticipated that caveolin-1 would be present in CEMs from SN and SC (Fig. 2).

CEMs can be prepared on the basis of their low density and detergent-insolubility. They share many of the features of caveolae (Lisanti et al., 1995b; Song et al., 1996a), including enrichment in cholesterol, as well

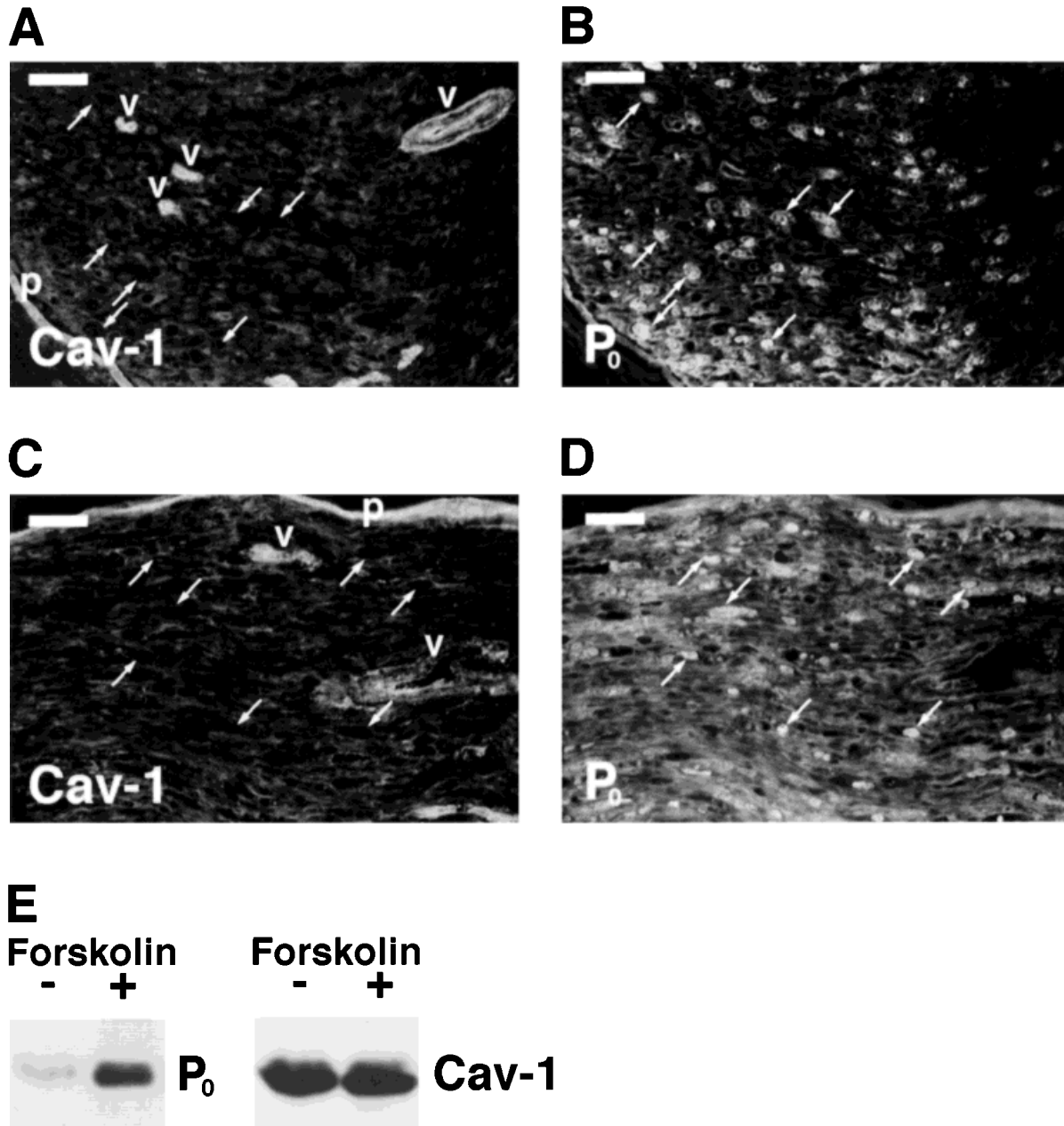


Fig. 6. Axonal influences on caveolin-1 expression. Rat SN collected 12 days after nerve transection were permeabilized with 0.1% Triton X-100 and double immunostained with pAb against caveolin-1 (Cav-1) (A,C) and P<sub>0</sub> (B,D). Cross sections are shown in (A) and (B), while longitudinal sections are shown in (C) and (D). SC caveolin-1 expression is reduced significantly (compare to Fig. 3), while perineurial (p) and blood vessel (v) caveolin-1 staining remain intense. P<sub>0</sub> is detected

in myelin debris (arrows); areas of P<sub>0</sub> immunoreactivity are indicated in panels (A–D, arrows). Scale bars = 20  $\mu$ M. E: SC were cultured in serum-free defined medium with (+) or without (–) 2  $\mu$ M forskolin for 24 h, and cell lysates were immunoblotted with anti-P<sub>0</sub> mAb or anti-caveolin-1 pAb. P<sub>0</sub> increases with forskolin treatment whereas caveolin-1 expression is unaffected.

as glycosphingolipids and a variety of signal-transducing components (Lisanti et al., 1994b; Montesano et al., 1982; Parton, 1994; Sargiacomo et al., 1993). Because detergent-based methods to prepare CEMs are susceptible to potential artifacts, such as apparent enrichment in glycosylphosphatidylinositol-anchored proteins (Schnitzer et al., 1995), we also used an established detergent-free protocol to prepare CEMs from rat SN and SC. Using either method, caveolin-1 is enriched in

a buoyant membrane fraction (fractions 2 through 5) of SN and SC (Fig. 2A,B). The Src-like kinase Fyn, which is known to be enriched in caveolae (Lisanti et al., 1995a), is concentrated within overlapping fractions from SN. While SN preparations are complicated by the presence of compact myelin, which has a comparable light density, nonmyelinating SC cultures also show enrichment of caveolin-1 in buoyant fractions, i.e., CEMs.

Immunohistochemically, caveolin-1 is detected in SC cytoplasm and abaxonal myelin (Figs. 3 and 4), which may correspond to soluble and insoluble forms of caveolin-1, respectively (Fig. 2C). The cytoplasmic localization of caveolin-1 is readily apparent when compared to the similar staining pattern of S100. Adult SN teased fibers and cultured SC immunostained for caveolin-1 demonstrate a stippled appearance of immunoreactivity (Figs. 4 and 5), which has been noted in other cell types and likely correlates with the distribution of caveolae (Cameron et al., 1997; Rothberg et al., 1992; Scherer, P. et al., 1995). We have previously shown that SC align along axons by 48 h in SC/dorsal root ganglion neuron co-cultures treated with insulin like growth factor-I (Cheng et al., 1998). Here, we find that after 48 h of co-culture, the SC membrane is strongly caveolin-1 immunoreactive where it abuts axons (Fig. 5B), which could reflect compartmentalized signal transduction at the initial stage of myelination. Although Galbiati et al. (1998) have recently identified caveolin expression in the cell bodies and growth cones of dorsal root ganglion neurons, we did not observe caveolin-1 immunostaining of axons.

SC phenotype is controlled by downstream signaling effects of the extracellular matrix (Bunge et al., 1986; Eldridge et al., 1989), cell-cell adhesion events (Owens and Bunge, 1989; Wood et al., 1990), and growth factors (Brookes et al., 1980; Cheng et al., 1998; Davis and Stroobant, 1990; Goodearl et al., 1993; Hansson et al., 1986; Krasnoselsky et al., 1994; Ridley et al., 1989). When premyelinating SC contact axons during development, they align on and ensheath axons (Webster, 1993). Myelin formation follows attainment of a one-to-one ratio between SC and large axons, and is accompanied by SC expression of myelin genes such as P<sub>0</sub>, MBP, and MAG (Jessen et al., 1991) and suppression of SC proliferation (Jessen and Mirsky, 1991). After nerve transection, with loss of axonal signals, SC revert to a premyelinating phenotype and no longer express myelin genes (Mirsky and Jessen, 1990; Scherer et al., 1993, 1996). Figures 6A–D shows that SC caveolin-1 expression decreases substantially after nerve transection. Caveolin-1 staining of blood vessels and perineurium remains intense, while SC staining declines (see Fig. 3 for comparison). Our preliminary results show that caveolin-1 is also developmentally regulated, concordant with the onset of myelination (not shown). In non-neural cells, caveolin-1 expression is associated with differentiation in senescent cells, as well as with cell death in cells that are rapidly dividing (Engelman et al., 1997; Wary et al., 1996). Caveolin-1 might play an analogous role regarding the fate of developing SC, which either cease proliferation and begin to differentiate in response to axonal signals or undergo cell death (Grinspan et al., 1996; Jessen et al., 1994; Nakao et al., 1997; Stewart et al., 1996; Syroid et al., 1996).

Recently, caveolin-1 has been shown to mediate anchorage dependent growth in fibroblasts by serving as a membrane adaptor protein, linking integrins to Fyn (Wary et al., 1998). Fyn is concentrated within caveolar

membranes (Lisanti et al., 1995a) and likely binds caveolin directly (Li et al., 1996a). Although Fyn has not been extensively studied in the PNS, it appears to be involved in the signaling events that occur during the early stages of myelination in the central nervous system (Umemori et al., 1994). Thus, increased caveolin-1 expression in differentiated cells may reflect the effects of the extracellular matrix on growth arrest and differentiation. Extrapolation of these results into SC allows one to speculate that myelination in the PNS might require signaling pathways that involve the extracellular matrix, caveolin-1 and Fyn. Caveolin-1's abaxonal localization is consistent with its possible role mediating integrin signaling.

The effects of axonal contact upon SC can be partially mimicked *in vitro* by elevating cAMP levels (Lemke and Chao, 1988). That is, SC expression of myelin genes such as P<sub>0</sub>, MBP, and MAG increases upon cAMP elevation. We did not find any difference in SC expression of caveolin-1 after treatment with 2  $\mu$ M forskolin for 24–72 h, whereas P<sub>0</sub> expression increased under this condition. Figure 6E shows the effects of forskolin on SC expression of P<sub>0</sub> and caveolin-1 after 24 h. These data suggest that caveolin-1 expression is regulated by other factors and is not directly linked to the transcription of myelin genes.

There is growing evidence that sterol levels control caveolin-1 expression at the level of transcription (Bist et al., 1997; Fu et al., 1998). At the same time, caveolin-1 may itself regulate cholesterol efflux in fibroblasts, with the expression of caveolin-1 varying in response to cellular cholesterol content (Fielding and Fielding, 1995, 1997b; Fielding et al., 1997a). Interestingly, hydroxymethylglutaryl-coenzyme A reductase, the rate-limiting enzyme in cholesterol biosynthesis, is upregulated in parallel with myelination, yet this enzyme is also unaffected by elevated cAMP levels (Fu et al., 1998). Rather, it is regulated primarily by the intracellular sterol concentration (Fielding et al., 1997a; Fu et al., 1998; Holthuizen et al., 1990). Although caveolin-1 is thought to be a membrane protein, a considerable portion of caveolin-1 from SN or SC is soluble in buffer alone (Fig. 2C). These results are interesting in light of the recent findings by Uittenbogaard et al. (1998), who have identified a soluble heat-shock protein-caveolin-chaperone complex in NIH 3T3 cells that transports newly synthesized cholesterol en route to caveolar membranes by a vesicle-independent mechanism. Soluble caveolin-1 in SN and SC might be similarly associated with newly synthesized cholesterol. We detect a larger fraction of soluble caveolin-1 in SN than in cultured SC (Fig. 2C), perhaps reflecting the greater need for cholesterol synthesis in myelin forming SC.

It is unknown whether caveolin-1 mediates cholesterol transport in SC, whose cholesterol is entirely endogenous, but caveolin-1 expression could facilitate cholesterol trafficking and ultimately myelin synthesis. Given that cholesterol binds caveolin-1 (Murata et al., 1995), accounts for roughly 25% of myelin lipids (Dewille and Horrocks, 1992), is entirely derived from locally

synthesized sources in peripheral nerve (Jurevics and Morell, 1994; Jurevics et al., 1998), and is essential for the proper structure and function of caveolae (Hailstones et al., 1998; Li et al., 1996b; Rothberg et al., 1990; 1992), it is plausible that caveolin-1 might regulate cholesterol trafficking during myelination and remyelination.

In summary, caveolin-1 is expressed by SC. Caveolin-1 exists in soluble and membrane associated forms, and is localized to SC cytoplasm and abaxonal myelin membranes. Caveolin-1 is expressed in myelinating SC but decreases after axotomy, when SC revert to a premyelinating phenotype. We postulate that caveolin-1 may regulate signal transduction and/or cholesterol transport in myelin forming SC.

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