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Identification of Gas6 as a Growth Factor for Human Schwann Cells

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Schwann cells are one of the principal components of the peripheral nervous system. They play a crucial role in nerve regeneration and can be used clinically in the repair of injured nerves. We have established serum-free, defined culture conditions that rapidly expand adult human Schwann cells without fibroblast growth. We find that Gas6, a ligand for the Axl and Rse/Tyro3 receptor protein tyrosine kinase family, stimulates human Schwann cell growth, increasing both cell number and thymidine incorporation. Gas6 has synergistic effects with the other known human Schwann cell mitogens, heregulin/glial growth factor and forskolin. Addition of Gas6 causes phosphorylation of Axl and Rse/Tyro3 simultaneously and results in ERK-2 activation. A combination of Gas6 with heregulin and forskolin, on a defined background, supports maximal Schwann cell proliferation, while preserving the typical Schwann cell morphology and expression of the Schwann cell markers S-100, glial fibrillary acidic protein, and low-affinity nerve growth factor receptor. Gas6 mRNA is present in both spinal motor neurons and large neurons of the dorsal root ganglia, and neural injury has been reported to upregulate Rse/Axl in the Schwann cell. This is the first demonstration of a potentially important biological role for the human Gas6/Rse–Axl system.

Key words: human Schwann cells; Gas6; heregulin; serum-free medium; mitogen; Axl; Rse

Schwann cells are one of the principle components of the peripheral nervous system (PNS). These cells originate from the neural crest during early embryonic development and migrate with the axons into the periphery. During this phase, Schwann cells undergo rapid proliferation producing an adequate number of cells to accommodate the growing axons. Subsequently, Schwann cells become quiescent and differentiate to ensheath and myelinate the axons. In the adult there is a reciprocal relationship between axons and Schwann cells in that each supports the survival and maintenance of differentiated phenotype in the other. However, under pathological conditions, Schwann cell proliferation can be stimulated and plays a crucial role in nerve regeneration after injury. When a peripheral nerve is transected, Schwann cells at the site of the injury begin to demyelinate and reenter the cell cycle. The Schwann cells produce neurotrophic factors and extracellular matrix proteins that facilitate the regrowth of the transected axons and finally remyelinate the axons (for review, see Bunge, 1993).

The capacity of Schwann cells to promote nerve fiber regeneration has been demonstrated in the CNS and in the PNS by nerve grafting and the implantation of guidance channels impregnated with Schwann cells (Paino and Bunge, 1991; Guenard et al., 1992; Paino et al., 1994; Xu et al., 1995). A cellular prosthesis containing autologous human Schwann cells has been proposed for transplantation to the site of spinal cord injury to influence the regeneration of central axons and for the repair of complex peripheral nerve injuries (Levi et al., 1994). The clinical success of these procedures depends on the ability to expand *in vitro* a pure Schwann cell population from a biopsy.

Gas6 was initially cloned as a growth arrest-specific protein from fibroblasts (Manfioletti et al., 1993). Recently, Gas6 has been identified as a ligand for protein tyrosine kinase receptors of the Axl/Rse family (Godowski et al., 1995; Stitt et al., 1995; Varnum et al., 1995). We detected Rse and Axl mRNA in human Schwann cells and demonstrated tyrosine phosphorylation of both of these receptors after incubation with human Gas6. A mitogenic response to human Gas6 in these cultures, allowing a significant expansion of the human Schwann cells *in vitro*, is demonstrated. Finally, the identification of Gas6 message in dorsal root ganglion (DRG) neurons, spinal motoneurons, and the developing embryo suggests that Gas6/Axl–Rse plays a significant role in the development of the PNS and in spinal cord repair after injury.

MATERIALS AND METHODS

Materials. Recombinant human heregulin (rhHRG$\beta_{177-244}$) and recombinant human Gas6 were prepared at Genentech as described previously (Holmes et al., 1992; Godowski et al., 1995). Bovine pituitary extract (3 μl/ml) was prepared as described by Roberts et al. (1990). Each lot was tested for growth-promoting activity on the ESC rat Schwann cell line (Li et al., 1995) and the optimal amount (3–10 μl) used in subsequent experiments with that lot. Insulin (recombinant human, Novo Nordsk, Denmark), aprotinin (Boerhinger Mannheim, Indianapolis, IN), forskolin (Calbiochem, San Diego, CA), interleukin-1α (IL-1α; R&D Systems, Minneapolis, MN), and platelet-derived growth factor (PDGF; Collaborative Research, Bedford, MA) were obtained from the indicated suppliers. Recombinant transforming growth factor-β (h-TGF-β1) was obtained through the Genentech Research Collaborations Program. Chemically defined lipids, basic fibroblast growth factor (bFGF), transferrin, and F12/DME medium powder were obtained from Gibco (Grand Island, NY), and progesterone and α-tocopherol were from Sigma (St. Louis, MO). Chemically defined lipid concentrate (Gibco) contained the following: cholesterol, α-DL-tocopherol, arachidonic, linoleic, linolenic,

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myristeric, oleic, palmitric, palmitic, and stearic acids in ethyl alcohol with pluronic F-68 and Tween 80.

Primary culture of Schwann cells in serum-free defined conditions. Peripheral nerve tissues were obtained from the University of Miami School of Medicine, with appropriate patient consent, as described previously (Levi et al., 1995). Pieces of nerve fibers were placed in Belzer's UW solution and shipped to Genentech. Because the culture medium is highly selective for Schwann cells and because fibroblast contamination is not a concern, untrimmed tissue can be used to minimize damage to the Schwann cells. The nerve should not be minced or titurated at this stage. After receipt, the tissue was washed with fresh F12/DME (1:1), incubated with collagenase/dispase (10 mg/ml collagenase/dispase, Boehringer Mannheim cat# 1097-113) in serum-free F12/DME at 37°C for 30–60 min, then washed three times by transferring to fresh culture medium. Intact enzyme-treated nerve fibers were plated in 100 mm petri dishes in F12/DME (1:1) medium supplemented with the following: insulin (10 μg/ml), transferrin (10 μg/ml), α-tocopherol (5 μg/ml), recombinant human heregulin (10 nM), forskolin (5 μM), progesterone (3×10^{-8} M), and bovine pituitary extract (BPE; 3 μl/ml). In later experiments, the BPE was omitted and Gas6 was added in the initial preincubation and plating. The tissue was cultured in suspension for 48–96 hr to allow partial demyelination. Nerve fibers were pooled by centrifugation at 1000 rpm for 5 min, resuspended, and dispersed by gentle pipetting. The dispersed tissue was replated on laminin (Gibco)-coated tissue culture dishes in the above medium (or with Gas6 and without BPE) with the addition of the protease inhibitor aprotinin (25 mg/ml) and 0.5× chemically defined lipid concentrate (Gibco). This was designated the "primary culture." Medium was changed every 5 d. Confluent primary cultures of pure Schwann cells were obtained within 1–2 weeks. Cells harvested from the nerves of four different donors gave similar results. Two samples were carried with Gas6 (without BPE), in addition to the other six factor (6F), continuously from initial plating.

Growth studies. After the first 2 week period, cultures were passaged using a 1:4 split ratio. Cells were removed from the stock plate using collagenase/dispase (Boehringer Mannheim), washed with medium containing 3% BSA, and plated on laminin-coated dishes as described. 6F medium was prepared by supplementation of F12/DME (1:1, 1.2 gm/l sodium bicarbonate) with insulin (10 μg/ml), transferrin (10 μg/ml), α-tocopherol (5 μg/ml), progesterone (3×10^{-8} M), aprotinin (25 μg/ml), and 50 μl/ml chemically defined lipid concentrate (Gibco, cat# 11905). Eight F (8F) medium contained 6F and recombinant human heregulin (10 nM) and forskolin (5 μM). Cells were incubated at 37°C in a 5% CO_2 atmosphere with saturating humidity. Cells were counted at the indicated time by removing the cells from the plate with trypsin and counting using a Coulter counter. For measurements of thymidine incorporation into DNA, [^{3}H]methyl thymidine (0.5 mCi/ml) was added at 48 hr of culture. Cells were harvested at 96 hr of culture, and radioactive DNA was measured as described previously (Li et al., 1995). Cells were used between the second and fourth passage *in vitro*. Cells from three different patients gave similar results.

Fluorescence immunocytochemistry. Cells were cultured for 24 hr on laminin-coated chamber slides and fixed in 10% formalin/PBS. Fixed cells were blocked with 10% goat serum and incubated with rabbit anti-GFAP (ICN, Cleveland, OH) and anti-S-100 protein (ICN) as recommended by the manufacturer. Specific binding of the primary antibody was visualized with goat anti-rabbit IgG $(Fab')_2$–fluorescein isothiocyanate conjugates. Cell nuclei were counterstained with the DNA dye propidium iodide (PI) for easy detection of any antibody-negative cells. WI-38 human fibroblast cells were used as a non-Schwann cell control cell type. These cells did not stain with S-100 or GFAP but did stain with PI.

Demonstration of Axl and Rse phosphorylation. Schwann cells (10^6) were grown to near confluence in defined media (8F + Gas6), changed to 6F 24 hr before treatment with purified human recombinant Gas6 for 15 min at 37°C, and lysed on ice with 1 ml of lysis buffer [20 mM HEPES, pH 7.4, 135 mM NaCl, 50 mM NaF, 1 mM sodium vanadate, 1 mM sodium molybdate, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Nonidet P-40, 1 μM okadaic acid, 1 mM phenylmethylsulfonyl fluoride, and 1 mM 4-(z-aminoethyl)benzenesulfonyl fluoride (Sigma)]. Cell lysates were clarified by centrifuging at 14000 × *g* at 4°C for 10 min. Immunoprecipitations were performed at 4°C for 2 hr using 1 μg of rabbit anti-hRseFc fusion protein antibody or 2 μl of rabbit anti-hAxl antiserum raised against the 10 amino acids at the C terminus of hAxl (PAAPGQEDGA). Immune complexes were collected with 10 μl of protein A–Sepharose CL-4B beads. Proteins were separated on Novex 4–12% gradient gels and transferred onto nitrocellulose membranes. Anti-phosphotyrosine immunoblots were performed using 4G10 mouse anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), goat anti-mouse horseradish peroxidase conjugate, and an ECL developing kit (Amersham, Arlington Heights, IL).

Phosphorylation of ERK2. The ERK2 protein is part of the downstream signaling pathway for many growth factors the action of which is mediated via tyrosine kinase receptors. This protein is active when phosphorylated. Phosphorylation can be detected by a shift in size on Western blots of SDS gels. To determine whether ERK2 was activated by exposure to Gas6, Schwann cells were stimulated with hGas6 for 15 min and cell lysates were prepared as described above. Cell lysates containing equal amounts of proteins were run on 8% SDS-PAGE gels. Proteins were transferred onto nitrocellulose membrane and immunoblotted with C20 mouse anti-ERK1+2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse horseradish peroxidase conjugate, and an ECL developing kit.

In situ *mRNA analysis.* Rat DRG were obtained from animals perfused with 4% paraformaldehyde. Mouse spinal cord was fresh frozen with powdered dry ice. Embryonic day 15.5 (E15.5) rat embryos were immersion-fixed for 24 hr in 4% paraformaldehyde. All tissues were sectioned at 16 μm and processed for *in situ* hybridization for Gas6 by a modification of a method previously described by Phillips et al. (1990). [^{33}P]uridine triphosphate-labeled RNA probes were generated as described previously (Melton et al., 1984). Sense and antisense probes were synthesized from a 730 bp DNA fragment of mouse Gas6 that included nucleotides 1231–1060 (Varnum et al., 1995) using T7 polymerase.

RESULTS

Defined serum-free culture selects for Schwann cells

Cultures of human Schwann cells were established from fragments of peripheral nerve obtained from organ donors 1–2 hr after clamping of the aorta. The nerves were collected and stored as described previously (Levi et al., 1994, 1995). Cells were plated and carried in serum-free medium using a modification of the protocol developed for rat Schwann cells (Li et al., 1996). This protocol was modified from that used for establishing rat Schwann cells as follows. The tissue was incubated for 48–96 hr in hormone-supplemented medium, in the absence of any attachment factor, in bacterial grade Petri dishes. This prevented attachment and allowed time for demyelination of the nerve tissue while providing the factors that promote cell survival. When the tissue pieces were plated after this preincubation, the cells moved out onto the laminin substrate with a high cell yield. The medium was modified by the addition of a protease inhibitor, aprotinin, and a cocktail of chemically defined lipids (see Materials and Methods), and the omission of bovine pituitary extract. In two of the four cases, Gas6 was added throughout the preculture and culture period. The initial growth rate of the cells improved in these conditions, and the cells remained responsive to Gas6 in subsequent passages. These changes allowed us to obtain confluent cultures of pure human Schwann cells within 1–2 weeks of initial plating. In one experiment, with Gas6 used throughout, a 3 cm^3 packed volume of sural nerve was treated as described, and the primary culture was plated into 23 × 150 mm tissue culture dishes. Confluent monolayers of Schwann cells (10^5 cells/cm^2) were obtained within 10 d, giving a total yield of 3.45×10^8 cells.

Gas6 is a mitogen for human Schwann cells

Using the cultures described above, we tested the mitogenic response to two known human Schwann cell mitogens, heregulin and forskolin, and a novel ligand of the Rse/Axl receptor family, Gas6. In the presence of the defined 6F supplement alone, there is good survival but little or no increase in cell number during the 3.5 d culture period shown in Figure 1*A*. Forskolin added in addition to the 6F supplement elicited no further increase in cell number or thymidine incorporation (Fig. 1*A,B*). In contrast, there

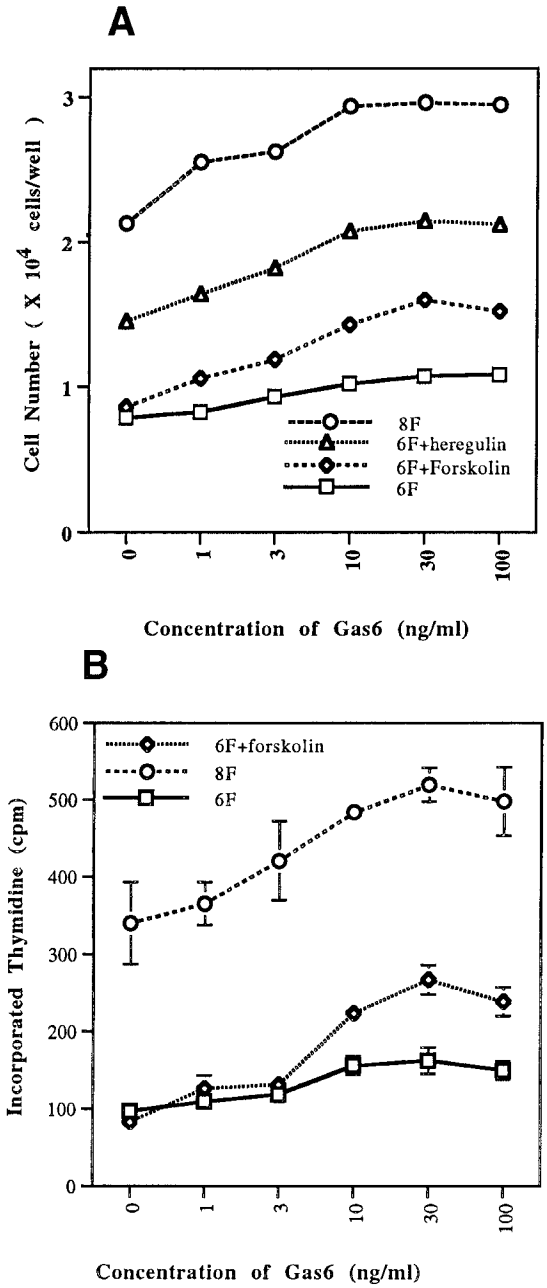

Figure 1. Effect of Gas6 and other growth factors on human Schwann cell growth and DNA synthesis. Schwann cells were plated in laminin-coated 48-well multiplates at 8×10^3 cells/well in defined 6F or 8F medium. *A*, Response of human Schwann cells to the indicated concentrations of Gas6. *B*, Increased thymidine incorporation in Schwann cells cultured as in *A* in the presence of Gas6. All data are presented as mean ± SEM ($n = 4$; error bars smaller than the symbol are not visible).

was a significant stimulation of cell number when heregulin was added to the 6F supplement.

Gas6 stimulates human Schwann cell growth in a dose-dependent manner (Fig. 1*A*) with a significant effect seen at 1 ng/ml (14 pM) and maximal effect at >10 ng/ml. Gas6 alone produces a small, but statistically significant, increase in Schwann cell number compared with control (6F) medium. The stimulation of Schwann cell number by forskolin and Gas6 is synergistic at all concentrations of Gas6. An additive effect is also observed (Fig. 1*A*) between Gas6 and heregulin, another Schwann cell mitogen

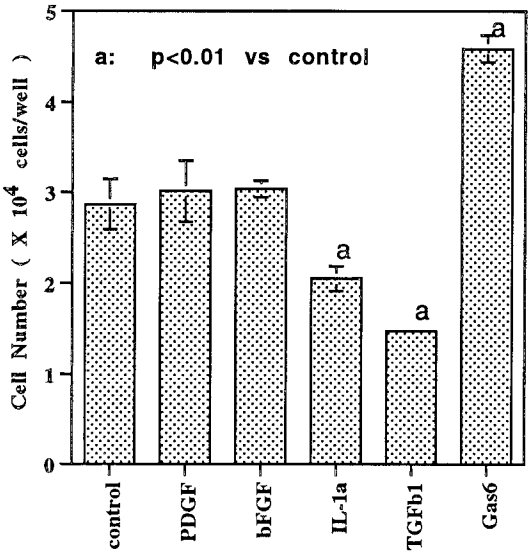

Figure 2. Influence of growth factors on Schwann cell number. Cells were cultured in the presence of 8F for 108 hr with or without the following: *PDGF* (10 ng/ml), *bFGF* (20 ng/ml), IL-1α (1 ng/ml), TGFβ1 (1 ng/ml), and *Gas6* (30 ng/ml). All data are presented as mean ± SEM ($n = 4$; error bars smaller than the symbol are not visible).

(Levi et al., 1995). Gas6 further increases both cell number and thymidine incorporation even in the presence of optimal concentrations of both forskolin and heregulin (*8F*) (Fig. 1*A,B*).

Interestingly, under the optimized serum-free conditions described above [*8F*: F12/DME supplemented with insulin, transferrin, α-tocopherol, progesterone, aprotinin, chemically defined lipids, recombinant human heregulin, and forskolin], other growth factors previously reported to stimulate Schwann cell growth (Peulve et al., 1994; Watabe et al., 1994) either have no effect (PDGF, bFGF) or reduce (IL-1α, TGFβ1) cell number (Fig. 2). Because protein S, a Gas6 homolog, had been reported to bind to Rse (Stitt et al., 1995), we next tested purified human and bovine

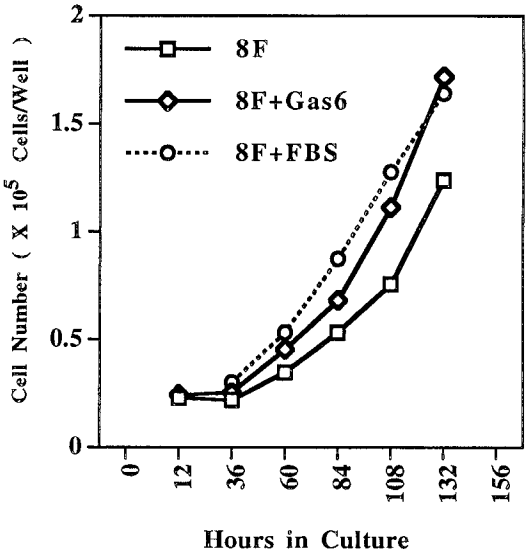

Figure 3. Time course of human Schwann cell growth in culture. Cells were plated at 2×10^4 cells/well in 24-well multiplates in F12/DME (1:1) supplemented with 8F with or without Gas6 or 10% fetal bovine serum (*FBS*). Culture wells were counted every 24 hr. Data shown are mean ± SEM ($n = 4$; error bars smaller than the symbol are not visible).

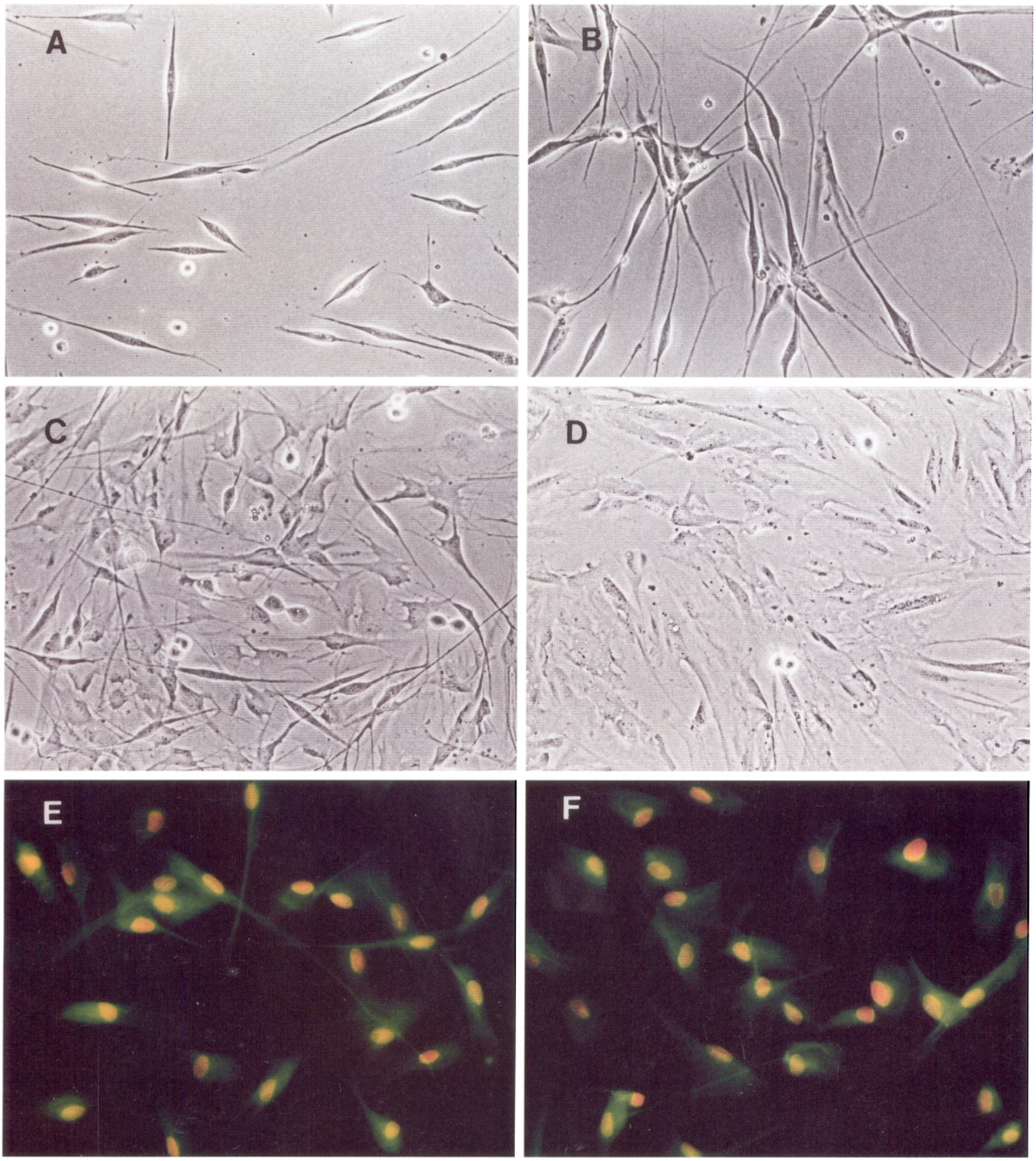

Figure 4. Micrographs of human Schwann cells in culture. *A–D*, Phase-contrast micrographs of human Schwann cells grown for 96 hr in 6F + heregulin (*A*), 6F + heregulin + Gas6 (*B*), 8F + Gas6 (*C*, *E*, *F*), and 8F + 10% fetal bovine serum (*D*) (*A–D* magnification 200×). *E*, *F*, Immunofluorescent staining of passage 4 cultures for the Schwann cell markers GFAP (*E*, *green* fluorescence) and S-100 (*F*, *green* fluorescence). Cultures in *E* and *F* (magnification 300×) were also stained with propidium iodide, which binds nuclei and has *red* fluorescence, aiding in detection of non-Schwann cell contaminants in the cultures. (Cells exhibiting both *red* and *green* fluorescence appear to have *yellow* nuclei.)

protein S. Neither human nor bovine protein S (0.001–5 μg/ml) produced significant growth stimulation in these cultures (data not shown).

We next compared the growth of these cells in defined 8F medium, 8F medium with the addition of Gas6, and with the addition of serum. The combination of Gas6 with forskolin and heregulin results in maximal cell growth comparable with that seen in the combination of 6F + forskolin, heregulin, and 10% FBS at 5 d of growth (Fig. 3). Based on DNA quantitation, the cells at the fourth subculture grown as described in 8F + Gas6 represent a 500-fold increase in total DNA over the original tissue. This is a minimal estimate of Schwann cell expansion because non-Schwann cells in the original tissue are lost at the early stages of culture. When Gas6 was added initially at the plating of the primary culture and the doubling time measured at the first subculture, the doubling time for the human Schwann cells was 24 hr (data not shown), a significant increase over that reported previously for other culture systems.

Gas6 has a marked effect on cell morphology. The addition of Gas6 to Schwann cells (grown in 6F + heregulin) stimulates the growth of the bipolar processes typical of Schwann cells (Fig. 4*A,B*). The length of the processes was quantified using the NIH Image 1.57 program. The average length of the long axis of the cells treated with Gas6 is significantly ($p < 0.001$) increased (184%) over that of the control. Mitotic figures are clearly seen in the 8F + Gas6 cultures (Fig. 4*C*), even in cells that have maintained the Schwann cell spindle morphology. In contrast, the addition of serum to the 8F cultures causes the cells to flatten, spread, and eventually become vacuolated (Fig. 4*D*). Cells grown as described were characterized at the fourth passage for Schwann cell markers. The WI-38 control cells stained only with PI (not shown). The Schwann cell cultures show 100% staining for the Schwann cell markers GFAP and S-100 protein (Fig. 4*E,F*), as well as p75 low-affinity nerve growth factor receptor (data not shown). There are no fibroblasts or neurons remaining in these cultures by the fourth passage.

Gas6 causes Axl and Rse phosphorylation

Gas6 stimulates human Schwann cell proliferation through the Axl/Rse family of tyrosine kinase receptors. Using Rse- and Axl-specific antibodies, we have detected both Rse and Axl receptor tyrosine kinases in human Schwann cells (data not shown). Addition of human Gas6 to human Schwann cells causes phosphorylation of both Axl and Rse receptors on tyrosine residue(s) (Fig. 5). Activation of Axl and Rse could be detected at 1.4–14 nM Gas6. The ERK2 protein is part of the ras/raf/MEK downstream signaling pathway for many growth factors that act by binding to tyrosine kinase receptors (Yamauchi and Pessin, 1994). We sought to determine whether binding of Gas6 to Rse/Axl causes phosphorylation of ERK2 in this signaling pathway. Phosphorylation can be detected by a shift in size on Western blots of SDS gels. Activation of Axl and Rse receptors induces a characteristic gel mobility shift of p42 ERK2 (Fig. 4*C*) consistent with the activation of ERK2 by the binding of Gas6 to these receptors (Yamauchi and Pessin, 1994).

Gas6 is expressed in neurons

If the Gas6/Axl–Rse system is to play a role in *in vivo* regulation of Schwann cell function, it is important to determine the *in vivo* source of the Gas6 ligand. Using a radioreceptor assay and Northern blot, we detect neither Gas6 expression nor Gas6 activity in cultured Schwann cells or conditioned medium (data not shown).

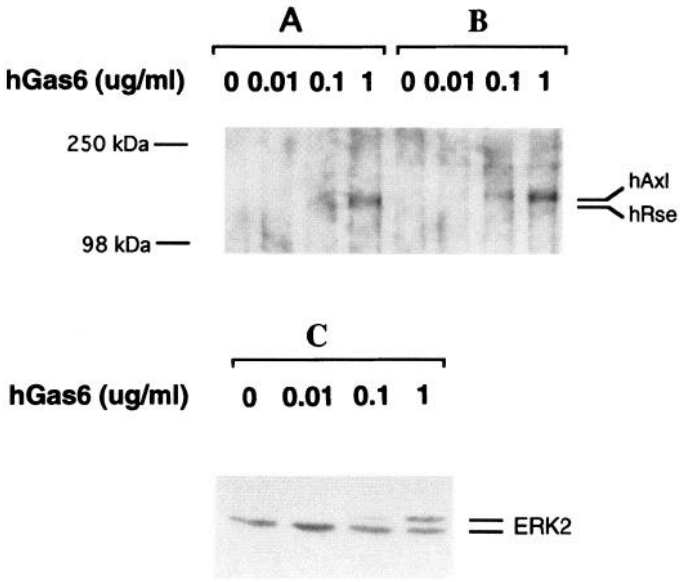

Figure 5. Human Gas6 activation of hRse and hAxl receptor tyrosine kinase in human Schwann cells. Cultures were stimulated with the indicated amount of *hGas6* for 15 min at 37°C. Cell lysates were prepared and immunoprecipitated with rabbit anti-hRseFc fusion protein antibody (*A*) and rabbit anti-hAxl antibody (*B*). Tyrosine phosphorylation of *hRse* and *hAxl* receptor was detected with 4G10 anti-phosphorylation antibody. Activation of p42 ERK2 in human Schwann cells after *hGas6* treatment (*C*) was detected in cells prepared as described above. Cellular proteins were separated by SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with anti-ERK1+2 antibody (*ERK2*).

We next performed *in situ* mRNA analysis of the nervous system to determine potential sites of Gas6 production. As shown in Figure 6, Gas6 hybridization of rat DRG labels neuronal cell bodies (*A*). Labeling of the epineurium is also observed (*arrow*, *A*). Higher magnification of the DRG under dark-field (*C*) and light-field (*D*) illumination reveals Gas6 expression predominately in larger neuronal cell bodies. Figure 6, *C* and *D* (*closed arrows*), shows examples of labeled large neurons, and *C* and *D* (*open arrows*) show also examples of unlabeled smaller neurons.

Gas6 expression is seen throughout gray matter of the spinal cord (Fig. 6*E*), but is especially intense in the ventral horn, which contains motor neurons (*vh*, *E*). In the E15.5 rat, expression is again most intense in the developing ventral horn of the spinal cord (*vh*, *F*), and a more diffuse labeling is observed in the developing DRG (*drg*, *F*). Extensive labeling was also noted peripherally in the embryo.

DISCUSSION

Defined culture conditions for human Schwann cells

Schwann cells have been successfully grown in culture medium supplemented with serum and growth factors. Fibroblasts are a major contaminant of these preparations, particularly when adult tissues are used. Multiple outgrowth protocols or antimitotic agents (Brockes et al., 1979; Morrissey et al., 1991) select for Schwann cell growth. We have used a defined, serum-free culture system (for review, see Mather and Sato, 1979; Roberts et al., 1990) that stimulates the growth of Schwann cells, but does not support the growth or survival of fibroblasts. Using this approach, we have been able to establish normal Schwann cell lines from embryonic and adult rats in serum-free medium supplemented with growth factors (Li et al., 1996). Here we have adapted and

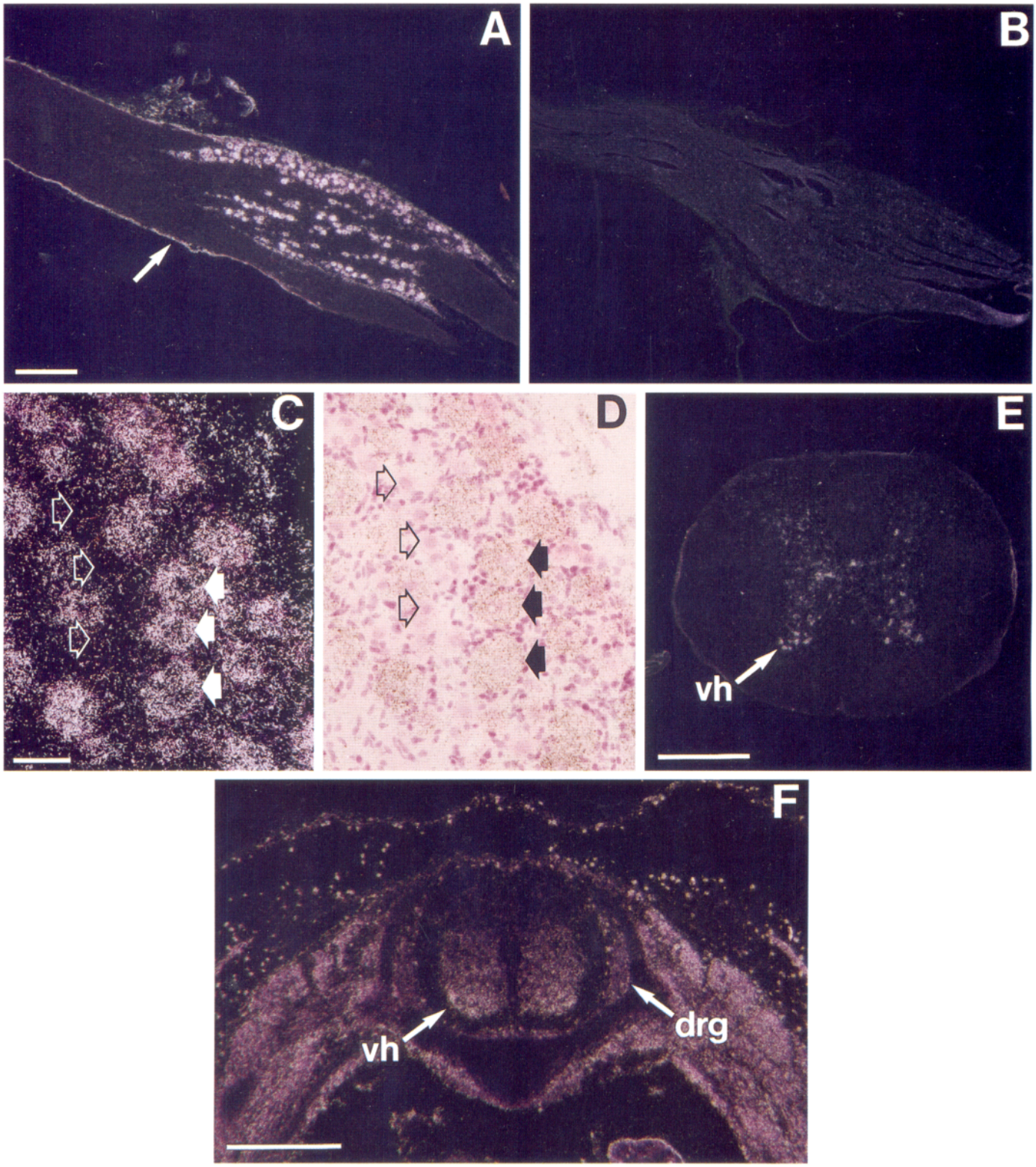

Figure 6. Expression of Gas6 mRNA in adult rat DRG, adult mouse spinal cord, and embryonic E15.5 rat spinal cord and DRG. Emulsion autoradiographs of DRG (*A, C, D*), spinal cord (*E*), and developing DRG and spinal cord (*F*) hybridized with a [^{33}P]uridine triphosphate-labeled Gas6 antisense probe are shown. Hybridization using a Gas6 sense strand control probe is shown in a DRG section (*B*). *vh*, Ventral horn; *drg*, dorsal root ganglion. Scale bars: *A, B, E, F*, 0.5 mm; *C, D*, 50 μm.

extended this medium to support the growth of human Schwann cells. We confirm the importance of forskolin and heregulin/glial growth factor (GGF) (Levi et al., 1995; Rutkowski et al., 1995) as stimulators of human Schwann cell growth. In addition, Gas6 is shown to be an important growth/survival factor for human Schwann cells *in vitro*. Using defined serum-free conditions, and using Gas6, heregulin, and forskolin as mitogens, a significantly expanded pure human Schwann cell population can be obtained within a few weeks.

The Gas6/Axl–Rse family

Axl (also called Tyro7, UFO, or Ark) and Rse (also called Tyro3, Sky, brt, or tif) (see Stitt et al., 1995) are related receptor-like tyrosine kinases (RTKs) that were cloned based on their homology to other tyrosine kinase receptors for known growth factors and their expression in the nervous system (Lai and Lemke, 1991; Mark et al., 1994). Axl and Rse RTK mRNAs are widely expressed in the nervous system, suggesting a role for these RTKs in regulating neuronal function (Mark et al., 1994; Stitt et al., 1995). Recent papers have identified Gas6 as the ligand for the Axl receptor (Stitt et al., 1995; Varnum et al., 1995). Stitt et al. (1995) have reported that protein S, a protein related to Gas6, is the ligand for Rse. However, we have shown that human Gas6 has a much higher affinity for human Rse than does human protein S (Godowski et al., 1995); human protein S does not appear to be able to act as a ligand for human Rse. We confirm that human Schwann cell cultures, which contain both Axl and Rse, show phosphorylation of both receptors in the presence of human Gas6, but not protein S. In addition, these cells show a dose-dependent mitogenic response to Gas6, but not to protein S.

Varnum et al. (1995) report that Gas6 can act as a mitogen for 3T3 cells but not A172 or Wi38 cells. However, this stimulation of 3T3 cells growth is seen at a higher concentration of Gas6 and is a smaller effect than that reported here for Schwann cells. A recent report by Nakano et al. (1995) shows that a growth-potentiating protein isolated from rat vascular smooth muscle cells (VSCM) *in vitro* is rat Gas6. Rat Gas6 potentiates the effects of thrombin on VSCM cell thymidine incorporation, suggesting an autocrine mitogenic activity in this cell type. The above observations suggest that Axl/Gas6 may play a role in regulating some aspect of vascular biology and may be involved in the nervous system.

Gas 6 as a stimulator of Schwann cell growth

Rse/Tyro3 mRNA has been detected previously in rat Schwann cells (Stitt et al., 1995). We have extended these observations by demonstrating the presence of Axl and Rse proteins in our human Schwann cell cultures. Additionally, we demonstrate that Gas6 causes the phosphorylation of both Rse and Axl tyrosine kinase receptors present on human Schwann cells *in vitro*. The difference in concentrations of Gas6 required for half-maximal stimulation of growth and receptor phosphorylation may be attributable to the lengths of the two assays (4 days vs 15 min) or to stimulation of growth without complete receptor occupancy. Such phosphorylation of Axl and Rse is not observed in cultures stimulated with heregulin and, hence, is not a result of transactivation.

Growth factors such as epidermal growth factor (EGF) and PDGF exert their functions by activating their corresponding cell surface receptor tyrosine kinases with signal transduction through cascades of protein kinases. In one of these protein kinase cascades, the mitogen-activated protein (MAP) kinase pathway, p42 ERK2 and p44 ERK1 are specifically activated as an early event after receptor activation (Davis, 1993). Activation of ERK2 by growth factor receptors in other cell types leads to multiple cellular events, including cell proliferation (Davis, 1993). These data suggest that Gas6 exerts its growth and survival effects on human Schwann cells through the MAP kinase cascade by activation of Axl and/or Rse receptors.

The activation of Axl/Rse receptors on Schwann cells by Gas6 is highly specific, because growth factors known to act via other tyrosine kinase receptors, such as PDGF, EGF, and FGF, do not increase Schwann cell proliferation under these conditions. Alternatively, heregulin/GGF, acting independently through the ErbB receptor family, synergizes with Gas6 in stimulating Schwann cell growth (Levi et al., 1995). We do not know whether Gas6 needs Axl, Rse, or both for signal transduction; however, Gas6 stimulates both Axl and Rse phosphorylation in these cells. This is the first demonstration of a biological response after ligand binding and phosphorylation of both of these receptors. Because receptor dimerization or oligomerization is the first step after binding of ligand to many RTKs, it is possible that heterodimerization of these receptors may occur after Gas6 binding. It is not known at this time whether both Axl and Rse are required for, or involved in, the biological response to Gas6 in these cells.

Gas6 is expressed in neurons

The biological activity of Gas6 and the Axl/Rse tyrosine kinase receptors in Schwann cells that has been described here is consistent with the high expression of both receptors and ligand in the nervous system. Gas6 mRNA is specifically expressed both in spinal motor neurons and in large DRG neurons, two cell types with myelinated axonal processes. Extensive expression of Gas6 in the embryo occurs during a period closely associated with the onset of Schwann cell proliferation and myelination. These data, together with the *in vitro* biological effect, are consistent with the interpretation that this factor is involved in neuron/Schwann interactions.

Biological role

Gas6, in combination with the known Schwann cell mitogens forskolin and heregulin, enables us to efficiently expand a pure human Schwann cell population in serum-free defined medium, without fibroblast contaminants. Schwann cells grown in the defined medium express normal Schwann cell markers and support rat DRG neuron survival and neurite outgrowth in coculture (data not shown). Having maintained this morphological and functional phenotype *in vitro*, we would expect these cells to exhibit normal Schwann cell function *in vivo*. Such ability to expand normal human Schwann cells *in vitro* is crucial for autologous transplantation for repair of spinal cord and other peripheral nerve injuries.

Recent evidence has indicated a role for the heregulin–ErbB2 receptor system as a part of the mechanism by which neurons stimulate Schwann cell division (Morrissey et al., 1995). The data reported here suggest that the Gas6/Axl–Rse system is also an important part of that mechanism. The growth response to Gas6, together with the localization of Gas6 message in the neuron and Rse and Axl receptors in the Schwann cells, strongly suggests that the Gas6/Axl–Rse system is involved in Schwann–neuron interaction during development and in repair of injury.

REFERENCES

Brockes JP, Fields KL, Raff MC (1979) Studies on cultured rat Schwann cells. I. Establishment of purified populations from purified nerve. Brain Res 165:105–118.

Bunge RP (1993) Expanding roles for the Schwann cell: ensheathment, myelination, tropism, and regeneration. Curr Opinion Neurobiol 3:805–809.

Davis RJ (1993) The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268:14553–14556.

Godowski PJ, Mark MR, Chen J, Sadick MD, Raab H, Hammonds RG (1995) Reevaluation of the roles of Protein S and gas6 as ligands for the receptor tyrosine kinase Rse/Tyro3. Cell 82:355–358.

Guenard V, Kleitman N, Morrissey TK, Bunge RP, Aebischer P (1992) Syngeneic Schwann cells derived from adult nerves seeded in semipermeable guidance channels enhance peripheral nerve regeneration. J Neurosci 12:3310–3320.

Holmes WE, Sliwkowski MX, Akita RW, Henzel WJ, Lee J, Park JW, Yansura D, Abadi N, Raab H, Lewis GD, Shepard M, Kuang WJ, Wood WI, Goeddel DV, Vandlen RL (1992) Identification of heregulin, a specific activator of p185erbB2. Science 256:1205–1210.

Lai C, Lemke G (1991) An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. Neuron 6:691–704.

Levi ADO, Guenard V, Aebischer P, Bunge RP (1994) The functional characteristics of Schwann cells cultured from human peripheral nerve after transplantation into a gap within the rat sciatic nerve. J Neurosci 14:1309–1319.

Levi ADO, Bunge RP, Lofgren JA, Meima L, Hefti F, Nikolics K, Sliwkowski MX (1995) The influence of heregulins on human Schwann cell proliferation. J Neurosci 15:1329–1340.

Li R-H, Phillips DM, Mather JP (1995) Activin promotes ovarian follicle development in vitro. Endocrinology 136:849–856.

Li R-H, Sliwkowski MX, Lo J, Mather JP (1996) Establishment of Schwann cell lines from normal adult and embryonic rat dorsal root ganglia. J Neurosci Methods, in press.

Manfioletti G, Brancolini Avanzi G, Schneider C (1993) The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. Mol Cell Biol 13:4976–4985.

Mark MR, Scadden DT, Wang Z, Gu Q, Goddard A, Godowski PJ (1994) Rse, a novel receptor-type tyrosine kinase with homology to Axl/Ufo, is expressed at high levels in the brain. J Biol Chem 269:10720–10728.

Mather JP, Sato GH (1979) The use of hormone-supplemented serum-free media in primary culture. Exp Cell Res 124:215–224.

Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR (1984) Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter (Abstr). Nucleic Acids Res 12:7035A.

Morrissey TK, Kleitman N, Bunge RP (1991) Isolation and functional characterization of Schwann cells derived from adult peripheral nerve. J Neurosci 11:2433–2442.

Morrissey TK, Levi ADO, Niujens A, Sliwkowski MX, Bunge RP (1995) Axon-induced mitogenesis of human Schwann cells involves heregulin and p185erbB2. Proc Natl Acad Sci USA 92:1431–1435.

Nakano T, Higashino K, Kikuchi N, Kishino J, Nomura K, Fujita H, Ohara O, Arita H (1995) Vascular smooth muscle cell-derived, Gla-containing growth-potentiating factor for Ca(2+)-mobilizing growth factors. J Biol Chem 270:5702–5705.

Paino CL, Bunge MB (1991) Induction of axon growth into Schwann cell implants grafted into lesioned adult rat spinal cord. Exp Neurol 114:254–257.

Paino CL, Fernandez-Valle C, Bates ML, Bunge MB (1994) Regrowth of axons in lesioned adult rat spinal cord: promotion by implants of cultured Schwann cells. J Neurocytol 23:433–452.

Peulve P, Laquerrierre A, Paresy M, Hemet J, Tadie M (1994) Establishment of Adult Rat Schwann Cell Cultures: effect of b-FGF, a-MSH, NGH, PDGF, and TGF-β on cell cycle. Exp Cell Res 214:543–550.

Phillips HS, Hains JM, Laramee GR, Rosenthal A, Winslow JW (1990) Widespread expression of BDNF but not NT3 by target areas of basal forebrain cholinergic neurons. Science 250:290–294.

Roberts PE, Phillips DM, Mather JP (1990) Properties of a novel epithelial cell from immature rat lung: establishment and maintenance of the differentiated phenotype. Am J Physiol 3:C415–C425.

Rutkowski LJ, Kirk CJ, Lerner MA, Tennekoon GI (1995) Purification and expansion of human Schwann cells in vitro. Nature Med 1:80–83.

Stitt TN, Conn G, Gore M, Lai C, Bruno J, Radziejewski C, Mattsson K, Fisher J, Gies DR, Jones PF, Masiakowski P, Ryan TE, Tobkes NJ, Chen DH, DiStefano PS, Long GL, Basilico C, Goldfarb MP, Lemke G, Glass DJ, Yancopoulos GD (1995) The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases. Cell 80:661–670.

Varnum BC, Young C, Elliot G, Garcia A, Bartley TD, Fridell YW, Hunt RW, Trail G, Clogston Toso RJ, Yanagihara D, Bennet L, Sylber M, Merewether LA, Tseng A, Escobar E, Liu ET, Yamane HK (1995) Axl receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6. Nature 373:623–626.

Watabe K, FukudaT, Tanaka J, Toyohara K, Sakai O (1994) Mitogenic effects of platelet-derived growth factor, fibroblast growth factor, transforming growth factor-β, and heparin-binding serum factor for adult mouse Schwann cells. J Neurosci Res 39:525–534.

Xu XM, Guenard V, Kleitman N, Bunge MB (1995) Axonal regeneration into Schwann cell-seeded guidance channels grafted into transected adult rat spinal cord. J Comp Neurol 351:145–160.

Yamauchi K, Pessin JE (1994) Insulin receptor substrate-1 (IRS1) and Shc compete for a limited pool of Grb2 in mediating insulin downstream signaling (Abstr). J Biol Chem 269:31107A.