CD44 Enhances Neuregulin Signaling by Schwann Cells

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Abstract. We describe a key role for the CD44 transmembrane glycoprotein in Schwann cell–neuron interactions. CD44 proteins have been implicated in cell adhesion and in the presentation of growth factors to high affinity receptors. We observed high CD44 expression in early rat neonatal nerves at times when Schwann cells proliferate but low expression in adult nerves, where CD44 was found in some nonmyelinating Schwann cells and to varying extents in some myelinating fibers. CD44 constitutively associated with erbB2 and erbB3, receptor tyrosine kinases that heterodimerize and signal in Schwann cells in response to neuregulins. Moreover, CD44 significantly enhanced neuregulin-induced erbB2 phosphorylation and erbB2–erbB3 heterodimerization. Reduction of CD44 expression in vitro resulted in loss of Schwann cell–neurite adhesion and Schwann cell apoptosis. CD44 is therefore crucial for maintaining neuron–Schwann cell interactions at least partly by facilitating neuregulin-induced erbB2–erbB3 activation.

Key words: CD44 • erbB2 • erbB3 • Schwann cell • neuregulin

Introduction

Schwann cells are neural crest derivatives that ensheathe and myelinate axons of peripheral nerves (for review see Bunge and Fernandez-Valle, 1995; Jessen and Mirsky, 1998). Migrating Schwann cell precursors enter nerves after growth cones of axons have begun to extend towards their targets (Carpenter and Hollyday, 1992; Bhattacharyya et al., 1994). The timing of Schwann cell differentiation and the ratio of Schwann cells to neurons must be strictly controlled as peripheral nerves develop. Schwann cells proliferate until early postnatal development, then either become quiescent (Brown and Asbury, 1981; Stewart et al., 1993) or undergo apoptosis (Grinspan et al., 1996; Syroid et al., 1996; Nakao et al., 1997; Zorick et al., 1999). Signals that influence developing Schwann cell proliferation, differentiation, and survival are derived from axons (Bunge and Fernandez-Valle, 1995; Jessen and Mirsky, 1998). Elucidating the molecular mechanisms governing Schwann cell responses to these axon-derived signals is crucial for understanding how nerves develop.

Axon-derived signals that influence Schwann cell proliferation and survival include members of the neuregulin protein family (for review see Mirsky and Jessen, 1999; Topilko et al., 1996). Neuregulins are encoded by alternatively spliced transcripts of the neuregulin-1 (*NRG-1*) gene (Burden and Yarden, 1997; for review see Gassmann and Lemke, 1997). Neuregulins, including glial growth factors (GGFs),[1] are either membrane bound or soluble, each with domains homologous to epidermal growth factor (EGF). Mice with targeted *NRG-1* deletions have dramatically reduced numbers of Schwann cell precursors (Meyer and Birchmeier, 1995). In vitro, neuregulin blocking antibodies inhibit the mitogenic effects of dorsal root ganglion (DRG) neurons on Schwann cells (Levi et al., 1995; Morrissey et al., 1995; Rosenbaum et al., 1997), while GGF and other neuregulins promote mitogenesis of mature Schwann cells and Schwann cell precursors (Baek and Kim, 1998; Raff et al., 1978; Marchionni et al., 1993; Dong et al., 1995). In addition, neuregulins can rescue Schwann cell precursors (Dong et al., 1995; Syroid et al., 1996) and Schwann cells in damaged neonatal nerves (Trachtenberg and Thompson, 1996; Grinspan et al., 1996; Kopp et al., 1997) from apoptosis. Collectively, these data indicate that neuregulins are critical for Schwann cell differentiation, survival, and proliferation at different stages of peripheral nerve development.

In Schwann cells, neuregulins function through the transmembrane receptor tyrosine kinases erbB2 and erbB3 (Morrissey et al., 1995; Vartanian et al., 1997; Rahmatullah et al., 1998). Mice with targeted mutations at *erb*B2 or

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[1]*Abbreviations used in this paper:* AS, antisense; DRG, dorsal root ganglion; E, embryonic day; GGF, glial growth factor; P, postnatal day; rh, recombinant human; SAS, scrambled antisense.

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*erb*B3 lack Schwann cells, underscoring the importance of these receptors in peripheral nerve development (Riethmacher et al., 1997; Britsch et al., 1998; Morris et al., 1999). Although erbB2 has no known ligands, neuregulins bind erbB3 with varying affinities (Peles et al., 1993; Carraway and Cantley, 1994; Kita et al., 1994). However, erbB3 lacks intrinsic kinase activity (Guy et al., 1994). After ligand binding, erbB3 and erbB2 must heterodimerize in order to signal (Sliwkowski et al., 1994). It is unclear from these studies how erbB2–erbB3 heterodimerization is achieved. Furthermore, it is not known whether neuregulins reach erbB2 and erbB3 by simple diffusion, or if accessory proteins are required to sequester neuregulins to the Schwann cell membrane.

We have investigated the possibility that CD44 plays a role in mediating neuregulin signaling in the peripheral nervous system. The CD44 family of transmembrane glycoproteins has been implicated in cell–cell and cell–matrix adhesion, cell migration, and growth factor signaling (for review see Sherman et al., 1996; Naor et al., 1997). Different CD44 proteins are generated from a single gene by alternative RNA splicing of up to 10 variant ("v") exons and by extensive posttranslational modifications. These variant exons encode amino acid sequences in the extracellular portion of CD44, near the transmembrane domain (Screaton et al., 1992). Standard CD44 is an 85–90-kD protein that lacks variant sequences and is expressed in many cell types, whereas higher molecular weight variants are expressed in a limited number of tissues and in certain tumors. CD44 is expressed by subpopulations of rat neural crest cells (Ikeda et al., 1996) and by embryonic and neonatal rat (embryonic day [E]18 to postnatal day [P]2; Sherman et al., 1995) and adult human Schwann cells (Vogel et al., 1992; Sherman et al., 1997). However, the function of CD44 in the peripheral nervous system has not been studied.

Bourguignon et al. (1997) found that CD44 coimmunoprecipitated with erbB2 in an ovarian carcinoma cell line, suggesting that CD44 may be linked to erbB2 signaling. We investigated whether CD44 contributes to erbB2 and erbB3 function in Schwann cells. We found that CD44 associates with erbB2 and erbB3 in rat Schwann cells and that reducing CD44 expression prevents GGF-induced erbB2–erbB3 heterodimerization and signaling. Blocking CD44 expression also results in the release of Schwann cells from neurites in cocultures of Schwann cells and sensory neurons, and in Schwann cell apoptosis. These data indicate that CD44 facilitates neuregulin signaling in Schwann cells, and demonstrate a novel role for CD44 in mediating growth factor receptor function.

Materials and Methods

Reagents

Recombinant human GGF2 was provided by Mark Marchionni (Cambridge Neuroscience, Cambridge, MA). Tyrphostin AG825 was purchased from Calbiochem. Antisense (AS1: 5′-GAAAAGGGTCGCGGGGG-3′, see Lamb et al., 1997; AS2: 5′-CTTGTCCATGATGTGCA-3′) and scrambled control antisense (SAS1: 5′-GGAGAGAGAGGCGGCT-3′; SAS2: 5′-ACCTGTTGGTTCACGAT-3′) CD44 phosphorothioate-protected oligonucleotides were synthesized at the University of Cincinnati DNA Core Facility.

Schwann Cell Culture

Primary Schwann cell cultures were established from neonatal Sprague-Dawley rat (Harlan) sciatic nerves as previously described (Kim et al., 1997). Cells were initially grown on poly-L-lysine–coated (Sigma-Aldrich) tissue culture plastic in DMEM supplemented with 10% FBS, 5 ng/ml recombinant human (rh)-GGF2, and 2 μM forskolin (Calbiochem-Novabiochem), and then either seeded onto neurons (see below) or switched for 24 h to a serum-free defined medium (N2; see Kleitman et al., 1991) either with or without rh-GGF2 and oligonucleotides as described. All assays were performed on cultures at passage 2 or 3. Apoptosis assays were performed using a Tdt-FragEL DNA fragmentation detection kit (Oncogene Research Products) according to the manufacturer's instructions.

Schwann Cell–Neuron Cocultures

Dissociated rat E15 DRG were cultured on collagen-coated 8-well chamber slides (Fisher Scientific) in the presence of antimitotic drugs to kill dividing cells (Kleitman et al., 1991). Neurons were maintained in DMEM plus 10% human placental serum and 50 ng/ml 2.5 S NGF (Harlan) for 14 d. Approximately 10^5 primary rat Schwann cells were then seeded onto the established neurons. 2 d later, cultures were analyzed by microscopy to confirm that the seeded cells preferentially bound to neurites. Cultures were then treated with AS or SAS oligonucleotides, then examined every 24 h by phase–contrast microscopy. In a separate set of experiments, Schwann cells were pretreated with AS or SAS CD44 oligonucleotides and then plated onto neurons, as described. In each culture, >50 microscopic fields of neurites were examined at each time point.

Immunocytochemistry and Laser Confocal Microscopy

For studies of nerve sections, sciatic nerves were dissected from Sprague-Dawley rat pups (P1, P3, P5, and P7; Harlan) and adults that had been perfused with 4% paraformaldehyde (in 0.1 M phosphate buffer). Nerves were post-fixed overnight and then incubated in 20% sucrose for 24 h. Frozen 5-μm sections were cut on a cryostat (Carl Zeiss, Inc.), fixed again in paraformaldehyde for 10 min, rinsed in phosphate buffer, and then incubated with 0.5% hydrogen peroxide to block endogenous peroxidases. Sections were blocked in 10% normal goat serum in phosphate buffer for 1 h, then incubated with the mouse anti–rat CD44 monoclonal antibody 5G8 (total hybridoma supernatant) overnight at room temperature (Sleeman et al., 1996). Slides were then developed using either the Vectastain ABC immunocytochemistry kit according to the manufacturer's instructions (Vector Laboratories) or by incubation with FITC-conjugated goat anti–mouse IgG (1:100; Jackson Immunoresearch Laboratories). Sections then either were rinsed three times with buffer and mounted in Fluoromount G (EM Sciences) or were processed further. For double labeling, CD44 antibody-labeled sections were fixed again with paraformaldehyde, rinsed, permeabilized in 0.1% Triton X-100 for 15 min, blocked in 10% goat serum for 1 h, and then incubated with rabbit antineurofilament (1:50; Parysek and Goldman, 1987; provided by Linda Parysek, University of Cincinnati), rabbit anti-S100 (1:200; Dako), rabbit anti-erbB2 (1:10; Upstate Biotechnology), or rabbit anti-erbB3 (1:10; C-17; Santa Cruz Biotechnology) antibodies overnight. Next, sections were rinsed, incubated with goat anti–rabbit TRITC (1:100; Jackson Immunoresearch Laboratories) for 1 h, and mounted as above. Sections were analyzed either with a Zeiss Axiophot microscope (Carl Zeiss, Inc.) with epifluorescence or by confocal microscopy using a Zeiss LM-10 (Carl Zeiss, Inc.) or a Bio-Rad MRC-600 laser confocal microscope (Bio-Rad Laboratories).

For analysis of teased nerves, adult rats were killed and sciatic nerves were removed and placed into L15 medium. Nerves were cut to 0.55-mm lengths and teased to single fibers using 20-gauge needles in PBS, then dried onto gelatin-coated glass slides. Slides were stored at −80°C until used. For immunostaining, slides were warmed to room temperature, fixed in methanol at −20°C for 10 min, rinsed in PBS, and blocked in 10% normal goat serum for 1 h. Sections were incubated with mouse monoclonal anti-CD44 (5G8) at a 1:1 dilution overnight, rinsed, and incubated with goat anti–mouse FITC (1:100) for 1 h, then rinsed again. For double labeling with S-100 protein, nerves immunostained with the CD44 antibody were fixed in 4% paraformaldehyde, rinsed, permeabilized in Triton X-100, and blocked in normal goat serum. Nerves were then incubated in S-100 antibody (Dako) overnight, rinsed, incubated with goat anti–rabbit TRITC (1:100) for 1 h, rinsed again, and mounted in fluoromount G and analyzed as described above.

For immunocytochemical localization of CD44, erbB2, and erbB3 in cultured Schwann cells, primary rat Schwann cells were plated onto poly-L-lysine–coated 8-well chamber slides (Fisher Scientific) and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Cells were permeabilized and blocked by incubation in PBS with 10% normal goat serum and 0.2% Triton X-100 for 30 min. Cells were then incubated with a mixture of the anti-CD44 mouse monoclonal antibody 5G8 (1:50) and either anti-erbB2 rabbit polyclonal antibody (1:100) or anti-erbB3 rabbit polyclonal antibody (1:100) as above for 1 h at room temperature. Next, cells were washed three times with blocking buffer and incubated for 30 min with a 1:50 dilution of FITC-conjugated goat anti–mouse IgG and a 1:50 dilution of rhodamine-conjugated goat anti–rabbit IgG (Jackson Immunoresearch Laboratories). Labeled cells were washed three times with blocking buffer and then mounted in Fluoromount G (EM Sciences). Cells were viewed and photographed as above.

Coimmunoprecipitation and Western Blotting

Cells were washed twice with ice-cold PBS and lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 1% Triton X-100 buffer with 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin, 50 mM NaF, and 1 mM sodium orthovanadate (all obtained from Sigma-Aldrich). Cellular debris was pelleted by centrifugation at 10,000 rpm for 10 min, then lysates were incubated overnight at 4°C with 5 μg/ml of anti-erbB2 (Upstate Biotechnology or Oncogene Research Products, Ab-4), anti-erbB3 (Santa Cruz Biotechnology, Inc.), or anti-CD44 antibodies (as described above), or isotype-matched control Igs (Jackson Immunoresearch Laboratories). By Western blotting, we observed only single bands at 185 kD with these erbB antibodies. Protein complexes were immunoprecipitated by adding a 50% slurry of protein A–Sepharose (Amersham Pharmacia Biotech), equilibrated in lysis buffer, for 1 h. Beads were washed four times in lysis buffer, then incubated with Laemmli buffer at 95°C for 10 min.

For Western blotting, proteins were separated on 7% SDS-PAGE gels, blotted onto nitrocellulose, then blocked in PBS with 0.1% Tween 20 and 3% nonfat dry milk for 1 h. Blots were incubated with 5G8 (1:250), anti-erbB2 (1:1,000), or anti-erbB3 (1:1,000) antibodies in blocking buffer, washed three times, then incubated with HRP-conjugated goat anti–mouse or anti–rabbit IgG antibodies (1:2,500; Bio-Rad Laboratories). Blots were washed an additional three times in blocking buffer, then developed using enhanced chemiluminescence (ECL)+ (Amersham Pharmacia Biotech). For assays of phosphorylated erbB2, the antiphospho erbB2 antibody (Upstate Biotechnology) was used at 1:1,000.

Results

CD44 Expression Decreases with Age during Postnatal Peripheral Nerve Development

As an initial test of our hypothesis that CD44 plays a role in peripheral nerve development and function, we analyzed CD44 distribution in developing and adult rat sciatic nerves. We previously demonstrated that CD44 is present in embryonic and adult nerves (Sherman et al., 1995, 1997), but relative levels of expression and detailed localization were not defined. Cryostat sections of rat sciatic nerve were stained with a monoclonal antibody raised against the extracellular domain of CD44 (5G8). This antibody recognizes all forms of CD44, but not other proteins, by Western blotting, immunoprecipitation, and immunohistochemistry (Sleeman et al., 1996). Nerves from P3 animals showed robust staining. CD44 expression was detected in axon–Schwann cell bundles, in endoneurium, and in the perineurium (Fig. 1 A). In adult nerves, however, staining was greatly reduced both in nerve bundles (Fig. 1 B) and in perineurium (data not shown). In a second set of experiments, we stained paraffin sections of P1, P3, P5, and P7 nerves. Strong staining persisted through P5 but was diminished by P7, and, as above, was very low in adult nerves (data not shown). This pattern of expression is consistent with CD44 playing a role in Schwann cell proliferation and/or differentiation.

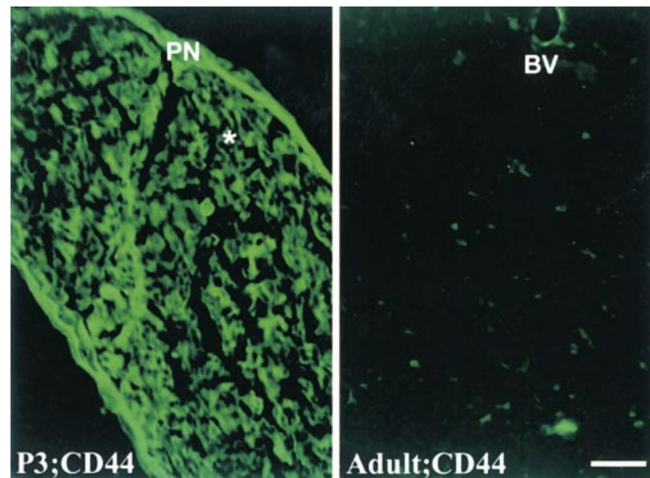

Figure 1. CD44 expression is high in early postnatal peripheral nerve and decreases in adult nerve. Neonatal (P3; left) or adult (right) rat sciatic nerves were cryosectioned and immunolabeled with an antibody (5G8) that recognizes all forms of CD44. In P3 nerves, CD44 expression was high in the perineurial cells (PN) and throughout nerve bundles (*). In adult nerves, however, CD44 expression was dramatically reduced, with only occasional bright staining within nerve bundles and by endothelial cells of blood vessels (BV). Note that both of the above panels had identical exposure and printing times to allow for direct comparisons of signal intensity. Bar, 10 μm.

CD44 Colocalizes with erbB2 and erbB3 in Schwann Cells

Like CD44, erbB2 expression is high during perinatal Schwann cell proliferation and much lower as Schwann cells become quiescent during the postnatal period and in adult animals (Cohen et al., 1992; Jin et al., 1993; Carroll et al., 1997). To test whether CD44 and erbB2 or erbB3 colocalize in peripheral nerve tissue, we examined cryostat sections of P3 and adult rat sciatic nerves by double labeling immunohistochemistry with anti-CD44 and anti-erbB2 antibodies using laser confocal microscopy. In P3 nerves, perineurial cells expressed CD44 but not erbB2 (Fig. 2, top left). Some domains of axon–Schwann cell bundles expressed both erbB2 and CD44, whereas other domains were positive for one but not both proteins (Fig. 2, top left). Sections stained with anti-erbB3 and anti-CD44 antibodies gave identical results (data not shown). P3 nerve sections were also double labeled with antibodies recognizing CD44 and S100 protein to mark Schwann cells (Fig. 2, top right) or neurofilament to mark axons (Fig. 2, top center). Both neurofilament and S100 protein demonstrated patchy expression within axon bundles but the degree of colocalization with CD44 was significantly less than with erbB2. These data are consistent with CD44 being expressed at P3 in some Schwann cell membrane domains that contain erbB2 and erbB3.

Double labeling of adult sciatic nerve cross-sections with CD44 and erbB2 antibodies revealed patches where the two proteins colocalized (Fig. 2, middle left). Consis-

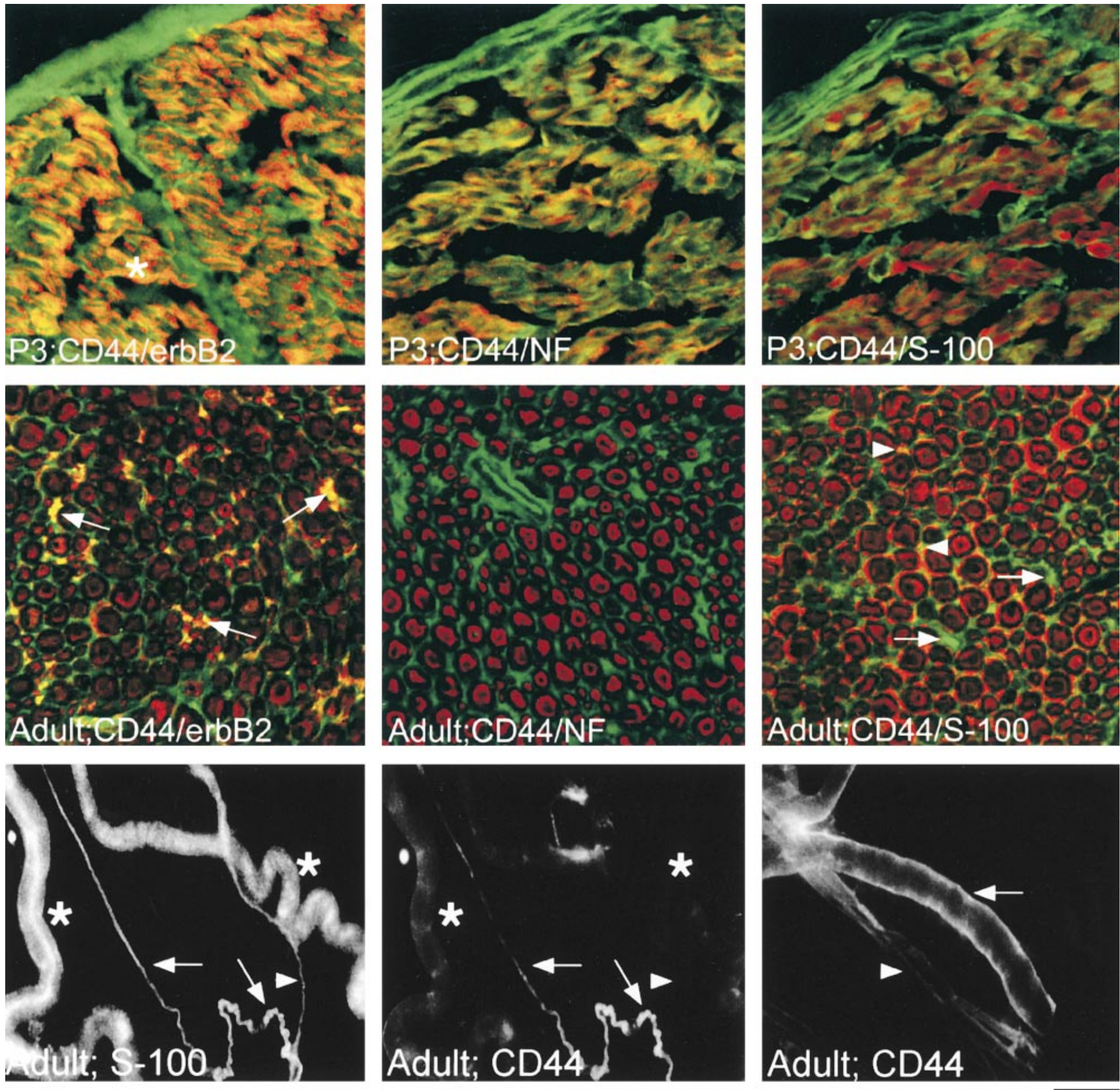

Figure 2. CD44 expression as compared with erbB2, neurofilament, and S-100 protein in peripheral nerve. Cryostat sections of P3 (top row) and adult (middle row) rat sciatic nerves were labeled with the 5G8 mouse anti–rat CD44 antibody (followed by an FITC-conjugated secondary antibody; green) and anti-erbB2, anti-neurofilament M+H (NF) or anti-S100 protein antibodies (followed by a rhodamine-conjugated secondary antibody; each in red), then analyzed by laser confocal microscopy. CD44 was expressed in the perineurium (upper left of each panel in top row), in blood vessels (data not shown), and in many regions of axon–Schwann cell bundles in P3 nerves. CD44 overlapped extensively with erbB2 in P3 nerve (*, yellow, top left), but less so with neurofilament or S100 protein. In adult nerves, CD44 was much more weakly expressed (see Fig. 1), as was erbB2. The center row shows enhanced signals as compared with the top row, so that staining can be easily visualized. Some CD44 colocalization with erbB2 was evident in probable unmyelinated fiber bundles (middle left, arrows) and at the outer part of some Schwann cell abaxonal surfaces. CD44 did not colocalize with neurofilament protein, a marker of axons. Very little colocalization of CD44 with S100 protein was noted except outside a few myelin sheaths (middle right, arrowheads). Arrows denote probable examples of unmyelinated fiber bundles. The bottom row shows photographs of adult rat teased nerve preparations stained with designated antibodies. Shown at the lower left, anti-S100 marked thin unmyelinated fibers (arrows and arrowhead) and thicker myelinated fibers (asterisks). Anti-CD44 labeling of the same field shows expression in one unmyelinated fiber (arrows) but not another (arrowhead). Myelinated fibers show weak labeling by anti-CD44. In the bottom right, rare myelinated fibers with bright anti-CD44 labeling at the abaxonal Schwann cell surface are shown (arrow) with adjacent lightly labeled fibers (arrowhead). Bar: (top six panels) 10 μm; (bottom three panels) 69.4 μm.

tent with findings in teased nerves (see below), most patches of CD44-erbB2 colocalization are probably unmyelinated Schwann cell bundles. There was also colocalization of CD44 and erbB2 in the abaxonal Schwann cell membrane, but only in some myelinated fibers. CD44 but not erbB2 expression was in rings outside myelin sheaths. This CD44 may be in the abaxonal Schwann cell membrane and/or the endoneurium. Finding CD44 in the endoneurium would be consistent with previous studies showing that, under certain circumstances, CD44 can be shed from cell membranes (Gunthert et al., 1996). Electron microscopy will be necessary to determine if this CD44 is localized to one or both of these compartments.

Double labeling with erbB2 and CD44 antibodies suggested that much of the CD44 expression in adult peripheral nerves could be ascribed to Schwann cells. Indeed, in adult nerve cross-sections, CD44 did not colocalize with neurofilament (Fig. 2, middle). However, expression of the Schwann cell marker S100 protein only partially overlapped with CD44 expression (Fig. 2, middle right). To clarify the distribution of CD44 and S100 protein in adult nerves, individual teased adult sciatic nerve fibers were double labeled with antibodies against S100 protein and CD44. CD44 was easily detected in the outer, abaxonal membrane of a few brightly stained myelinated fibers (2 out of 40 counted; Fig. 2, bottom right). A larger percentage of myelinated fibers (38 out of 40) only weakly expressed CD44 (Fig. 2, bottom middle). As anticipated by the staining in cross-sections, S100 protein was predominantly detected in the adaxonal, inner cytoplasm and much less in the abaxonal Schwann cell surface where CD44 was found.

Although S100 protein is barely detectable in unmyelinating Schwann cells in cross-sections (Mata et al., 1990; see Fig. 2, middle right), anti-S100 antibodies did stain unmyelinated fibers in teased nerve preparations (Fig. 2, bottom left). Only some of these unmyelinated fibers expressed CD44 (11 out of 45; Fig. 2, bottom middle). These data demonstrate that subsets of both myelinating and unmyelinating Schwann cells express detectable levels of CD44 in adult nerve.

These studies confirm that CD44 is present in peripheral nerves and demonstrate that it is enriched in developing nerves. The data are consistent with colocalization of CD44 with erbB2 and/or erbB3 in specific membrane domains of Schwann cells. To confirm this, cultures containing >99% $S100^+$ neonatal rat Schwann cells were prepared and stained with anti-CD44 and anti-erbB2 (Fig. 3) or erbB3 (data not shown) antibodies. Indeed, patches of membrane showed colocalization of the receptors with CD44, while adjacent membrane domains appeared to be enriched for either CD44 or erbB receptors (Fig. 3 C).

CD44 Associates with erbB2 and erbB3

In combination with the observation that CD44 associates with erbB2 in ovarian carcinoma cells (Bourguignon et al., 1997), the finding that erbB2 and erbB3 colocalize with CD44 in Schwann cells suggested that CD44 may play a role in erbB receptor function. To determine whether CD44 associates with either erbB2 or erbB3, we cultured rat Schwann cells in a defined medium in the absence of added growth factors for 24 h. Cells were then treated for 5 min with 5 ng/ml of rh-GGF2, a neuregulin previously shown to promote Schwann cell precursor survival and Schwann cell proliferation (Minghetti et al., 1996), or with an equivalent volume of vehicle (culture medium). Cell lysates were immunoprecipitated with either erbB2 or erbB3 antibodies and were analyzed by Western blotting. We found that CD44 coimmunoprecipitated with erbB2 and, in apparently distinct complexes, with erbB3 in the absence of rh-GGF2, indicating that CD44 constitutively

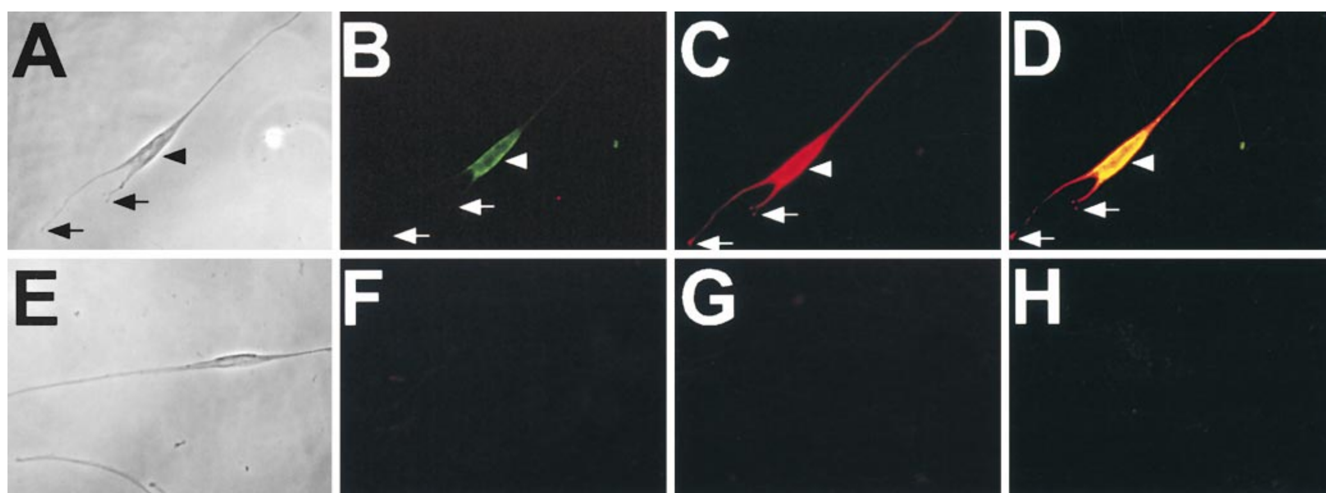

Figure 3. CD44 partially colocalizes with erbB2 in Schwann cells in vitro. Primary rat Schwann cells were fixed and immunostained with the anti-CD44 mouse monoclonal antibody 5G8 and an anti-erbB2 polyclonal antibody. (A and E) Phase–contrast images of single Schwann cells. (B) CD44 staining detected with FITC-conjugated goat anti–mouse IgG. (C) erbB2 staining detected with rhodamine-conjugated goat anti–rabbit IgG. (D) Merged images from B and C, showing where there is extensive overlapping CD44 and erbB2 expression (yellow; arrowhead) and where there is little if any colocalization (arrows). Control labeling using the Schwann cell shown in E included incubation with both secondary antibodies alone (F), incubation with the 5G8 monoclonal antibody followed by the goat anti–rabbit secondary (G), and incubation with the erbB2 polyclonal antibody followed by the goat anti–mouse secondary antibody (H).

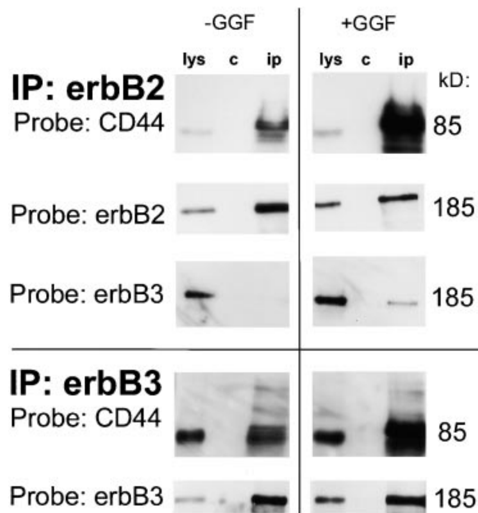

Figure 4. CD44 associates with erbB2 and erbB3. erbB2 and erbB3 protein complexes were immunoprecipitated from subconfluent 100-mm plates of Schwann cells in the presence and absence of rh-GGF2, then analyzed by Western blotting. Lys, aliquot of cell lysate to control for changes protein levels; c, immunoprecipitation with isotype-matched control antibody; ip, immunoprecipitation with erbB2 or erbB3 antibody, as indicated; Probe, antibody used in Western blots. Molecular weights were determined using prestained markers (Bio-Rad Laboratories), and are shown on the right. erbB2 and erbB3 each constitutively associated with CD44 in the absence of rh-GGF2, but only weakly with one another. In the presence of rh-GGF2, erbB2 coimmunoprecipitated with erbB3 and approximately twice as much CD44 (as determined by scanning densitometry) compared with untreated Schwann cells, even though the total level of CD44 did not change (compare levels of lysates in both conditions). Lanes containing lysates were equally loaded with 10 μg of total protein.

associates with each receptor (Fig. 4). This association did not depend on erbB2–erbB3 activation, since there was little erbB2–erbB3 heterodimerization under these conditions (Fig. 4) and because CD44–erbB interactions were not disrupted by treating cells with 10 μM tyrphostin AG825, a pharmacological inhibitor of erbB2 phosphorylation (data not shown). Less than 10% of total erbB3 immunoprecipitated with erbB2 in the absence of rh-GGF2 (as determined by scanning densitometry, comparing Westerns blots of total cell lysates to immunoprecipitates), whereas >40% immunoprecipitated in the presence of rh-GGF2 ($n = 3$). The low level of heterodimerization in untreated cultures may be the result of autocrine activation of erbB2 and erbB3 by Schwann cell–derived neuregulins, as previously described (Rosenbaum et al., 1997). CD44–erbB2 and CD44–erbB3 interactions were also observed when cell lysates were immunoprecipitated with CD44 antibodies (data not shown). In the presence of rh-GGF2, CD44, erbB2, and erbB3 were all coimmunoprecipitated (Fig. 4), indicating that CD44 remains associated with erbB2 and erbB3 in neuregulin-induced erbB2–erbB3 heterodimers. The level of CD44 in the heterodimeric complexes was approximately twice as high as the levels observed in the absence of rh-GGF2, even though the total levels of CD44 were not significantly changed in the cell lysates. These data are consistent with the notion that rh-GGF2 induces the formation of CD44–erbB2–CD44–erbB3 complexes in Schwann cells.

Antisense CD44 Oligonucleotides Inhibit Schwann Cell–Neurite Adhesion in Schwann Cell–Sensory Neuron Cocultures

To address the possible functions of CD44 in peripheral nerves, we analyzed Schwann cell–sensory neuron cocultures that can be used to study how Schwann cells interact with axons (Salzer and Bunge, 1980; Kleitman et al., 1991). To reduce Schwann cell CD44 expression, we used previously described antisense CD44 oligonucleotides that effectively reduce total CD44 protein levels in rat cells (Lamb et al., 1997). We chose this approach because there are no antibodies that block all of the functions of the CD44 proteins expressed by rat Schwann cells, and because antisense strategies have been used extensively to block CD44 expression in numerous systems in vitro and in vivo (Merzak et al., 1994; Kaya et al., 1997, 1999; Lamb et al., 1997; Chow et al., 1998; Reeder et al., 1998). After 24 h, Schwann cell cultures treated with 5 μM of either of two phosphorothioate-protected antisense CD44 oligonucleotides (AS1 or AS2) expressed 40–70% less CD44 protein (range in seven separate experiments as determined by scanning densitometry of Western blots) than did cells treated with the same concentration of a oligonucleotides with the identical base composition in a random sequence (SAS1 or SAS2; Fig. 5 A; see also Fig. 8 A) or untreated controls. In contrast, erbB3 demonstrated no detectable changes in expression after 24 h and minimal (20–30% using AS1; range in three separate experiments) or no reduction (using AS2) in expression after 36 h (Fig. 5 A). This minimal reduction is consistent with the increased level of cell death observed in CD44 AS-treated cultures (see below).

Having determined that the AS CD44 oligonucleotide effectively reduced CD44 expression in Schwann cells, we examined the effects of antisense CD44 on Schwann cell–neuron interactions. Cultures of E16 rat DRG neurons, from which endogenous Schwann cells and fibroblasts had been removed with antimitotic drugs, were seeded with Schwann cells. By 48 h, Schwann cells aligned with and covered the majority of neurites. Cultures were then treated with either SAS or AS CD44 oligonucleotides as above. Schwann cells remained associated with neurites in SAS-treated cultures (Fig. 5 B, arrows), but lost adhesion to neurites in AS CD44–treated cultures within 48 h (Fig. 5 C). Clumps of dead cells were observed floating in these cultures (Fig. 5 C). If Schwann cells were pretreated with AS CD44 for 24 h and then added to neuron cultures, they failed to adhere to neurites and died soon after seeding (data not shown). These experiments were repeated three times with similar results and suggest that CD44 is required to maintain Schwann cell–axon adhesion.

Reducing CD44 Expression in Schwann Cell Cultures Results in Apoptosis

We next tested the effects of antisense CD44 oligonucleotides on purified cultures of primary rat Schwann cells grown in the presence of 5 ng/ml rh-GGF2. After

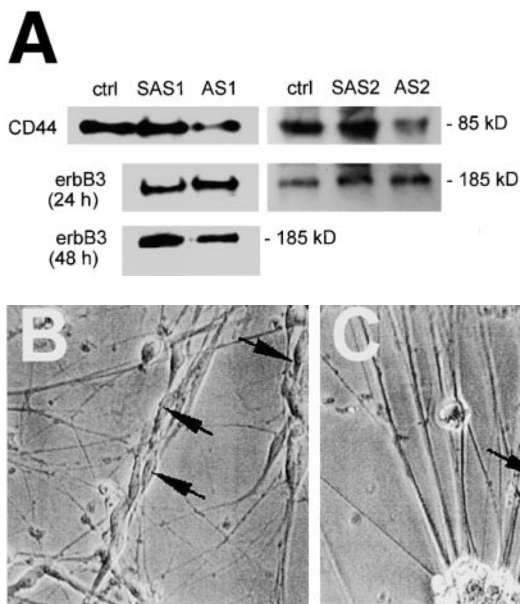

Figure 5. CD44 is required for Schwann cell–neurite adhesion in vitro. (A) Primary cultures of rat Schwann cells were incubated with 5 μM antisense (AS1 and AS2) CD44 or scrambled antisense (SAS1 and SAS2) oligonucleotides, or left untreated (Ctrl) for 24 h. Cultures were then lysed and 20 μg of total protein was assayed for CD44 expression by Western blotting with an antibody that recognizes all forms of CD44 (5G8) or for erbB3 expression. Equal protein loading was confirmed by staining blots with Ponceau S before probing with antibodies. CD44 expression was not altered by the SAS oligonucleotide, but was reduced by 40–70% in AS oligonucleotide–treated cultures, as determined by scanning densitometry. Treatment with AS1 and AS2 CD44 oligonucleotides did not influence erbB3 expression at 24 h. Treatment with AS1 did result in a slight reduction in erbB3 expression (20–30%) after 36 h. This reduced expression probably was due to increased levels of Schwann cell apoptosis in these cultures (see below). (B and C) Schwann cell–neuron cocultures were treated with 5 μM SAS (B) or 5 μM AS (C) oligonucleotides for 48 h. Cultures treated with SAS oligonucleotides demonstrated numerous Schwann cells adhering to neurites (B, arrows). Cultures treated with the AS oligonucleotide lacked Schwann cells attached to neurites, demonstrating only naked neurites. Clumps of dead cells were observed in these cultures (C, arrow).

~48 h, 94 ± 5% (as determined by cell counts) of Schwann cells treated with the AS1 CD44 oligonucleotide became rounded, lifted off the culture dish, and died (Fig. 6 C). SAS1 oligonucleotide–treated and untreated control cultures grew to confluence during the same time period (Fig. 6, A and B). Similar results were obtained using the AS2 and SAS2 oligonucleotides (Fig. 6, A–C, insets). After 36 h, many cells grown in the presence of the AS1 CD44 oligonucleotide were still attached to the culture substrate, but 20–30% (range of three separate experiments) of the cells were undergoing apoptosis as determined by a nuclear fragmentation assay (Fig. 7). These findings are consistent with the notion that CD44 plays a crucial role in promoting Schwann cell survival.

Excess rh-GGF2 Rescues Schwann Cells with Low CD44 Expression from Apoptosis

The finding that reducing CD44 expression leads to Schwann cell apoptosis in purified Schwann cell cultures suggests that CD44 is involved in mediating signals that promote Schwann cell survival. As mentioned above, rh-GGF2 is the chief added factor that can promote Schwann cell survival in our cell culture system. If CD44 is involved in promoting signaling by GGF or other neuregulins, then one might expect that the effects of lowering CD44 expression by Schwann cells could be overcome by adding high concentrations of such survival factors. When Schwann cells were cultured in the presence of a 10-fold excess of rh-GGF2 (50 ng/ml) and treated with AS1 CD44, we observed significantly less cell death after 48 h (42 ± 12%; Fig. 6, D–F) compared with cultures treated with only 5 ng/ml rh-GGF2 (Fig. 6 C). These data are consistent with the idea that CD44 is involved in facilitating neuregulin signaling.

Reducing CD44 Expression Inhibits erbB2 Phosphorylation in Schwann Cells

To test directly whether reducing CD44 expression influences erbB2–erbB3 signaling, we cultured Schwann cells in the presence of 5 ng/ml rh-GGF2 and either AS CD44 or SAS oligonucleotides for 24 h. 20 μg of protein from each condition were examined for levels of erbB2 phosphorylation by Western blotting with a phospho-specific erbB2 antibody. As expected, we observed a high level of phosphorylated erbB2 in cultures treated with 5 μM SAS oligonucleotides after GGF addition (Fig. 8 A). However, there was a dose-dependent decrease in erbB2 phosphorylation in cells treated with increasing concentrations (from 1 to 5μM) of AS CD44 oligonucleotides, such that phosphorylation was barely detectable in cells treated with 5 μM AS CD44 (the same concentration used in the experiments described above). The levels of total erbB2 were either unchanged in these experiments (using AS2) or reduced by ~20–30% (using AS1; range in three separate experiments), as shown above for erbB3 (Fig. 5). As above, we believe that this reduction probably is due to the fact that a significant proportion of the Schwann cells are already undergoing apoptosis at this time point (Fig. 7). Nonetheless, the degree of reduction in phosphorylation is far greater (55–78%, range in three separate experiments) than the reduction in total erbB2 protein. These data suggest that CD44 is involved in signaling by erbB2–erbB3 receptor complexes in Schwann cells.

Reducing CD44 Expression Blocks erbB2–erbB3 Heterodimerization in Schwann Cells

In light of our finding that CD44 forms complexes with erbB2 and with erbB3, the observation that lowering CD44 expression blocks erbB2 phosphorylation suggested that CD44 might be required for efficient erbB2–erbB3 heterodimerization. To test this idea, we grew Schwann cells in defined medium (N2) alone with either SAS1 or AS1 CD44 oligonucleotides for 24 h, as above. Cells were then treated with 5 ng/ml rh-GGF2 for 30 min and assayed for erbB2–erbB3 heterodimerization by immunoprecipitation with an erbB2 antibody, followed by Western blotting with an erbB3 antibody. erbB3 coimmunoprecipitated with erbB2 in SAS1 oligonucleotide–treated cultures, but either barely or not at all in AS1 CD44–treated cultures

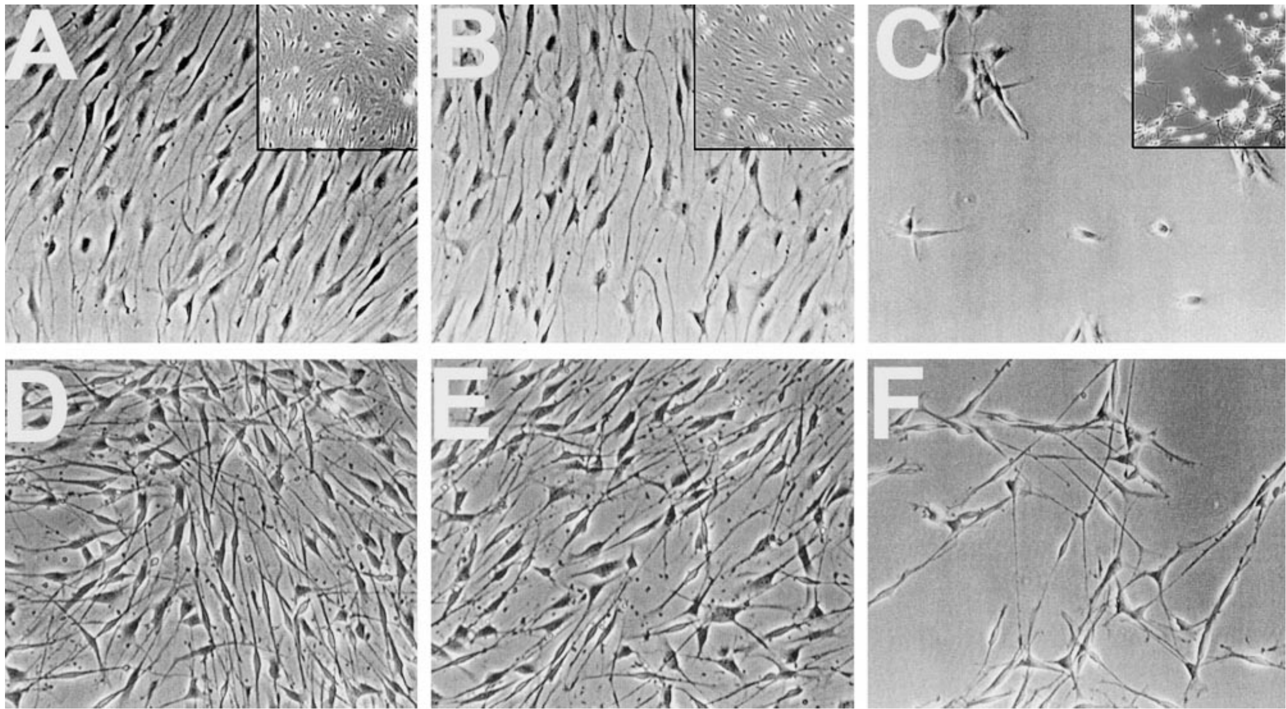

Figure 6. Antisense CD44 induces Schwann cell rounding and death. Primary rat Schwann cells were grown in N2 in the presence of 5 ng/ml (A, B, and C) or 50 ng/ml (D, E, and F) rh-GGF2, then treated with AS1 (C and F), AS2 (C, inset), SAS1 (B and E), or SAS2 (B, inset) oligonucleotides, or left untreated (A, D). After 48 h, SAS oligonucleotide–treated cultures were unaltered compared with untreated controls (compare A with B, D with E). Schwann cells grown in 5 ng/ml rh-GGF2 and treated with AS CD44 rounded up and died within 48 h (compare B with C). This effect was partly reversed in cultures grown in the presence of 50 ng/ml rh-GGF2 (compare E with F).

(Fig. 8 B). These experiments were performed three times with identical results, and indicate that lowering CD44 expression in Schwann cells significantly interferes with erbB2–erbB3 heterodimerization and signaling in response to neuregulins.

Discussion

We have demonstrated a novel role for CD44 in mediating neuregulin signaling in Schwann cells. A number of previous studies established the importance of GGF and related neuregulins in promoting the survival of embryonic

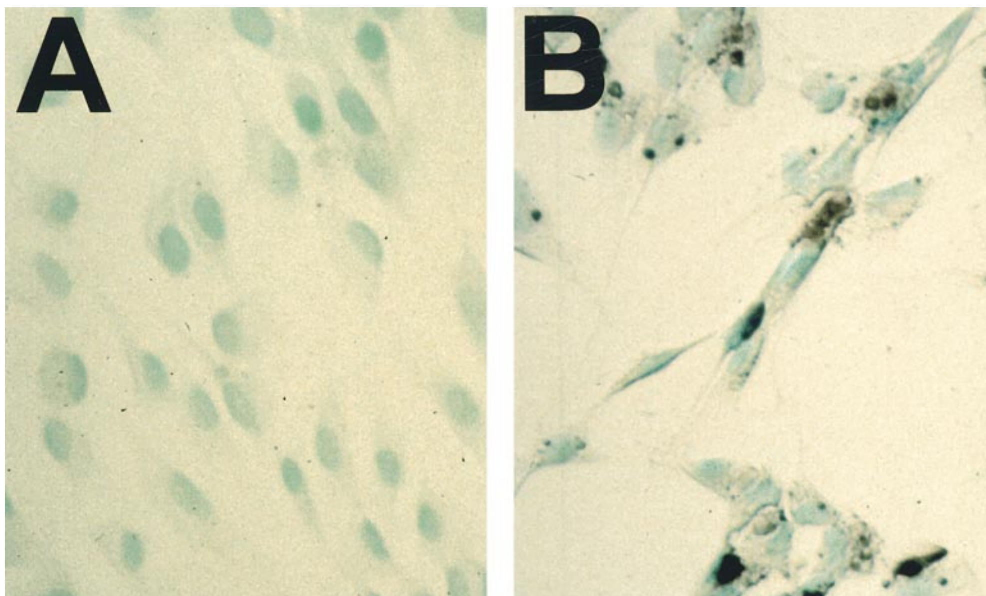

Figure 7. Antisense CD44 induces Schwann cell apoptosis. Primary rat Schwann cells were grown in the presence of SAS (A) or AS (B) oligonucleotides, then assayed at 24 h for apoptosis using a DNA fragmentation detection assay. Cells stained green with counterstain alone were viable, whereas cells with brown nuclei had significant DNA fragmentation. SAS-treated cells demonstrated little detectable DNA fragmentation (A), whereas 20–30% of cells treated with the AS oligonucleotide demonstrated fragmentation. A higher percentage of cells may have had DNA fragmentation, since many of the AS-treated cells lifted off the dish during the course of the fragmentation assay (B).

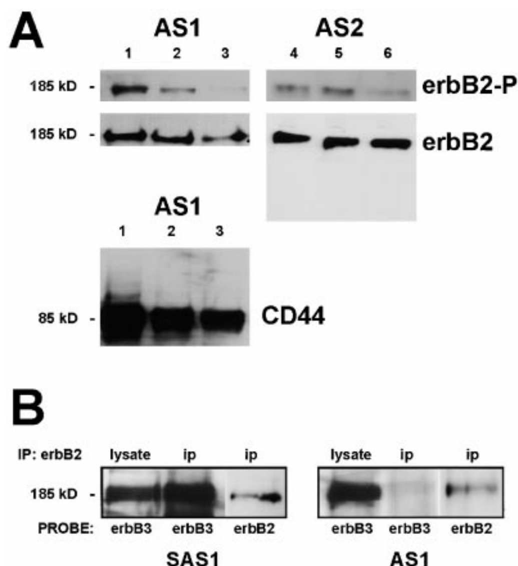

Figure 8. CD44 is required for erbB2 phosphorylation and erbB2–erbB3 heterodimerization. (A) Schwann cells were grown in the presence of 5 ng/ml rh-GGF2 and either 5 μM CD44 SAS1 (lane 1), 1 μM AS1 CD44 (lane 2), 5 μM AS1 (lane 3), 5 μM SAS2 (lane 4), 1 μM AS2 (lane 5), or 5 μM AS2 (lane 6) for 24 h. 20 μg of protein were then separated by SDS-PAGE and analyzed by Western blotting for levels of CD44, erbB2, or phosphorylated erbB2 using a phospho-specific erbB2 antibody. Equal protein loading was confirmed by Ponceau S staining. ErbB2 phosphorylation was significantly reduced in the presence of 1 μM AS1 (lane 2) and nearly absent in the presence of 5 μM AS1 (lane 3), where CD44 expression was reduced by ~50%. Similar results were observed using AS2 (compare lanes 4, 5, and 6). Total erbB2 expression in cultures treated with 5 μM AS2 was unchanged compared with controls and was reduced ~25% in cells treated with 5 μM AS1, probably due to increased levels of Schwann cell apoptosis. These data indicate that reducing CD44 expression by Schwann cells inhibits erbB2 activation. (B) Schwann cells grown in N2 alone were cultured in the presence of either 5 μM SAS 1 or AS1 oligonucleotides for 24 h, then with 5 ng/ml of rh-GGF2 for 30 min. Total cell lysates were immunoprecipitated with an erbB2 antibody, then probed with an erbB3 antibody. In this experiment, the total levels of erbB3 were not significantly different between AS- and SAS-treated cultures. However, erbB2 and erbB3 did not associate with one another in cultures treated with AS CD44. These data indicate that CD44 is required for erbB2-erbB3 heterodimerization. Lys, lysate; ip, immunoprecipitation with an erbB2 antibody.

and early neonatal Schwann cells. Here, we demonstrated that CD44 is also crucial for neonatal Schwann cell survival in vitro, that CD44 is constitutively associated with erbB2 and erbB3 in Schwann cells, and that CD44 is required for erbB2–erbB3 heterodimerization in the presence of rh-GGF2. Lowering CD44 expression resulted in loss of Schwann cell–neurite adhesion and Schwann cell apoptosis, an effect that could be partially rescued by excess rh-GGF2. These data are consistent with neuregulins being crucial for maintaining Schwann cell survival in vitro and in situ (Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996; Kopp et al., 1997), and show for the first time that interactions between CD44 and a growth factor receptor can enhance growth factor signaling.

CD44 Facilitates Neuregulin Signaling through erbB Receptors

erbB2 is the preferred heterodimerization partner of the other erbB receptors (Graus-Porta et al., 1997). However, it is unclear how erbB2 specifically associates with erbB1, erbB3, or erbB4 in the presence of particular ligands. Our data are consistent with the notion that CD44 plays a critical accessory role in bringing erbB2 and erbB3 together in Schwann cells to form heterodimers in the presence of GGF. It is possible that CD44 achieves this function by binding GGF or other neuregulins, forming a ligand bridge between erbB2 and erbB3 that facilitates receptor interactions. CD44 splice variants containing sequences encoded by exon v3 can bind and sequester heparin binding growth factors and present these growth factors to their high affinity receptors (Brown et al., 1991; Faassen et al., 1992; Tanaka et al., 1993; Bennett et al., 1995; Jackson et al., 1995; Sherman et al., 1998; van der Voort et al., 1999). This function of CD44 depends on heparin sulfate modifications to amino acids within the v3 variant sequence. Certain neuregulins, including GGF2, are heparin binding growth factors (Ratner et al., 1988; Peles et al., 1993; Sudhalter et al., 1996). Furthermore, heparin sulfate proteoglycans on the surface of Schwann cells are required for neuregulin signaling (Sudhalter et al., 1996; Loeb et al., 1999). Heparin-binding neuregulins therefore could act to bridge CD44–erbB2 and CD44–erbB3 complexes, resulting in a functional signaling receptor heterodimer (Fig. 9).

Although Schwann cells express CD44v3 variants at low levels (Sherman et al., 1997), the majority of the CD44 that coimmunoprecipitated with erbB2 and erbB3 in Schwann cells was the 85–95-kD standard CD44 protein that lacks variant sequences. Therefore, it is possible that CD44 contributes to erbB2–erbB3 heterodimerization through a mechanism that does not depend on CD44–GGF interactions via heparin sulfate. For example, CD44 may stabilize erbB2–erbB3 heterodimers through its interactions with the actin cytoskeleton, either via ankyrin, annexin II, or binding to members of the ezrin–radixin–moesin family of actin-binding proteins (Kalomiris and Bourguignon, 1989; Tsukita et al., 1994; Oliferenko et al., 1999). We are presently performing experiments that will distinguish between these possibilities.

Our data indicate that CD44 facilitates Schwann cell erbB2–erbB3 heterodimerization and signaling in response to a neuregulin. However, we cannot rule out the possibility that CD44 has additional functions in Schwann cells, including mediating signaling by other growth factors, cell-–cell adhesion, or cell–matrix interactions. For example, Bourguignon et al. (1997) found that hyaluronate binding to CD44 could stimulate erbB2 phosphorylation in an ovarian carcinoma cell line. Hyaluronate could cross-link CD44–erbB2 complexes with other erbB family members also bound to CD44, resulting in erbB2 phosphorylation, or hyaluronate could stimulate cell signaling through CD44, directly influencing erbB2 activity. The relevance of these findings to Schwann cell biology remains to be determined.

CD44 binds a number of extracellular matrix components in addition to hyaluronate and may cooperate with integrins to mediate cell adhesion (Fujisaki et al., 1999;

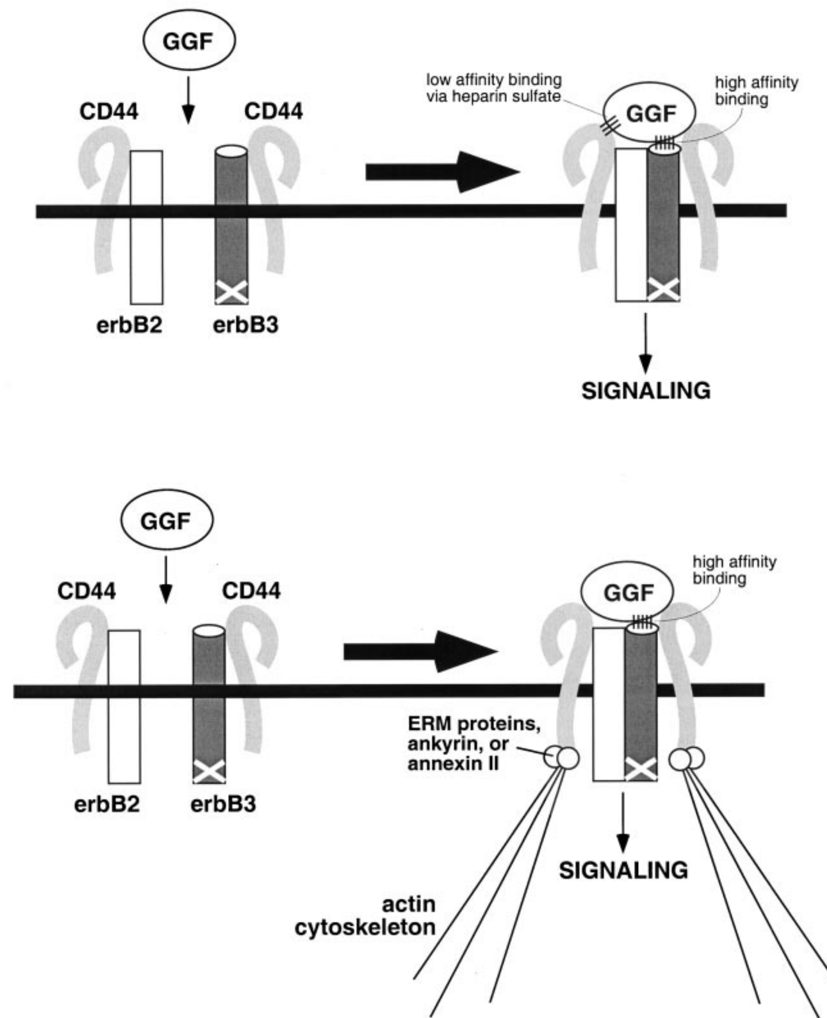

Figure 9. Two models of how CD44 could function in erbB2–erbB3 heterodimerization and signaling in Schwann cells. Our data indicate that CD44 is constitutively associated (either directly or indirectly) with erbB2 and erbB3 in Schwann cells, and that CD44–erbB2 and CD44–erbB3 complexes come together in the presence of GGF. ErbB3, which has no intrinsic kinase activity (as denoted by the white ×), can bind GGF, but erbB2, which has kinase activity, cannot bind GGF (left side of top and bottom models). Certain CD44 proteins can bind heparin binding growth factors, like GGF, either directly through heparin sulfate–modified amino acid sequences, or indirectly through an invariant glycosaminoglycan binding domain that can bind heparin sulfate. One possibility, therefore, is that CD44 facilitates erbB2–erbB3 heterodimerization by acting as a low affinity receptor, anchoring GGF to the heterodimeric complex (top model). Another possibility, however, is that CD44 stabilizes erbB2–erbB3 heterodimers through its interactions with the actin cytoskeleton (bottom model). The cytoplasmic tail of CD44 binds to ezrin, radixin, and moesin (ERM proteins), ankyrin, and annexin II, each of which associate with actin. Our data do not yet allow us to distinguish between these two models.

Katagiri et al., 1999). Numerous studies have implicated integrins and components of extracellular matrix in Schwann cell survival, differentiation and growth (for review see Mirsky and Jessen, 1999). Our own preliminary studies indicate that Schwann cell apoptosis due to reduced CD44 expression is diminished when the cells are cultured on laminin instead of poly-L-lysine (our unpublished observations). The finding that CD44 is expressed at the abaxonal Schwann cell surface in adult myelinated nerves also suggests a role for CD44 in mediating Schwann cell interactions with components of the basal lamina that they themselves synthesize (for review see Bunge, 1993). Furthermore, studies of a transient population of CD44-positive cells in the developing mouse optic chiasm suggested that CD44 may influence the function of the L1 cell adhesion molecule (Sretavan et al., 1994) that has been implicated in Schwann cell–axon adhesion in the peripheral nervous system (Seilheimer and Schachner, 1988; Bixby et al., 1988; Haney et al., 1999). CD44 proteins therefore may function both in the mediation of neuregulin signaling and in Schwann cell–axon adhesion, accounting for the dramatic effects of CD44 antisense oligonucleotides in Schwann cell–neuron cocultures.

If CD44 is critical for erbB2–erbB3 heterodimerization and signaling, then one might predict that mice with targeted mutations in the CD44 gene would have at least some common phenotypes with mice lacking neuregulins, erbB2, or erbB3. However, mice with targeted mutations of the CD44 gene demonstrate only minor hematological abnormalities that include aberrant lymphocyte recirculation (Schmits et al., 1997; Protin et al., 1999). However, mice with such targeted mutations can compensate for the lack of one gene by upregulating the expression of related genes. For example, mice with targeted mutations in the cardiac alpha actin gene dramatically upregulate expression of vascular smooth muscle actin and skeletal alpha-actins (Kumar et al., 1997). Therefore, it is possible that embryos lacking CD44 from very early stages compensate for the lack of CD44. In agreement with this notion, transgenic mice expressing antisense CD44 under the control of the keratin-5-sulfate promoter have a dramatic skin phenotype, and keratinocytes from these animals fail to respond properly to particular growth factors (Kaya et al., 1997).

An alternative explanation for the phenotypic discrepancy between mice with targeted *CD44* mutations and findings from studies, including this one, where CD44 was targeted with antisense strategies, is that additional gene

transcripts are affected by antisense CD44. In the case of our study, we cannot exclude this explanation. However, several lines of evidence are consistent with CD44 being linked to the observed effects of the oligonucleotides: (i) the oligonucleotide sequences we used previously have been shown to specifically reduce CD44 expression in rat cells and to cause phenotypes that were predicted from independent, biochemical data (Lamb et al., 1997); (ii) the oligonucleotide sequences we used do not share homology with other known genes, including the recently cloned CD44 homologue LYVE-1 (Banerji et al., 1999); (iii) the effects of antisense CD44 on Schwann cells are consistent with our findings that CD44 colocalizes and interacts with erbB2 and erbB3; and (iv) antisense strategies have been used successfully to reduce CD44 in a number of in vitro and in vivo systems, often resulting in phenotypes that were predicted by independent means (Merzak et al., 1994; Lamb et al., 1997; Kaya et al., 1997, 1999; Chow et al., 1998; Reeder et al., 1998).

CD44 May Be Required for Other Processes Linked to erbB Receptor Signaling

The observation that CD44 expression is highest in early postnatal peripheral nerves at times when Schwann cells are proliferating and then declines as Schwann cells become quiescent is consistent with the idea that CD44–erbB2/erbB3 interactions mediate Schwann cell proliferation during peripheral nerve development. CD44 may also play a role in conditions characterized by abnormal Schwann cell proliferation, such as Wallerian degeneration and Schwann cell tumorigenesis. Interestingly, *NRG-1* transcripts, including GGF mRNAs, are induced in adult nerves during Wallerian degeneration (Carroll et al., 1997), and Schwann cells themselves produce neuregulins (Raabe et al., 1996; Rosenbaum et al., 1997; Cheng et al., 1998), suggesting that CD44 could be involved in a neuregulin autocrine signaling loop under certain circumstances. Furthermore, we found elevated CD44 expression in schwannomas with mutations in the *NF2* gene (Sherman et al., 1997), whose protein product, merlin, associates with the cytoplasmic tail of CD44 (Sainio et al., 1997). Some of the abnormal growth and survival properties of schwannoma cells are consistent with aberrant cell adhesion and growth and survival signaling (Pelton et al., 1998; Rosenbaum et al., 1998). Therefore, it is intriguing to speculate that CD44–erbB receptor interactions contribute to Schwann cell tumorigenesis and other peripheral nerve pathologies.

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