Activation of ErbB2 during Wallerian Degeneration of Sciatic Nerve

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We used anti-phosphopeptide-immunodetecting antibodies as immunohistochemical reagents to define the location and activity state of p185^{erbB2} during Wallerian degeneration. Nerve damage induces a phosphorylation event at Y1248, a site that couples p185^{erbB2} to the Ras–Raf–MAP kinase signal transduction pathway. Phosphorylation of p185^{erbB2} occurs within Schwann cells and coincides in time and space with Schwann

cell mitotic activity, as measured by bromodeoxyuridine uptake. These visual images of receptor autophosphorylation link activation of p185^{erbB2} to the Schwann cell proliferation that accompanies nerve regeneration.

Key words: neuregulin; erbB2; receptor tyrosine kinase; Schwann cell; Wallerian degeneration; phosphotyrosine

Unlike elements of the CNS, peripheral nerves can regenerate when damaged. Understanding the regulation of this process has practical implications for treatment of peripheral neuropathies such as those secondary to diabetes, cancer chemotherapeutic agents, and other toxins. Moreover, insights into peripheral nerve regeneration may be transferable to treatment of spinal cord injuries. After peripheral nerves are damaged, they initially undergo Wallerian degeneration. Axons distal to the site of injury degenerate, and their myelin sheaths break down. Schwann cells then proliferate, providing a context for axonal regrowth and nerve regeneration (Waller, 1851; Fawcett and Keynes, 1990). Although Schwann cell proliferation is a prominent feature of nerve regeneration, the molecular signals driving the mitotic response have not been characterized.

One viable candidate for regulating the Schwann cell proliferation that accompanies regeneration of peripheral nerves is the transmembrane tyrosine protein kinase p185^{erbB2}. Schwann cells express p185^{erbB2} both in culture and *in vivo* (Jin et al., 1993; Marchionni et al., 1993). The tyrosine kinase activity of p185^{erbB2} is activated by a family of ligands known collectively as the neuregulins (glial growth factor, acetylcholine receptor-inducing activity, Neu differentiation factor, and heregulin) that are encoded as splice variant transcripts of a common gene. Neuregulins are expressed by neurons in the peripheral nervous system (Marchionni et al., 1993; Dong et al., 1995), and they promote the

proliferation of Schwann cells *in vitro* (Marchionni et al., 1993; Marchionni, 1995; Morrissey et al., 1995).

Activation of the p185^{erbB2} tyrosine kinase results in the autophosphorylation of specific tyrosines on the intracellular domain of the receptor. This autophosphorylation can be monitored with anti-phosphotyrosine antibodies. However, reactivity with generic antibodies to phosphotyrosine provides no specific insight into the catalytic or signaling activities of a growth factor receptor. Moreover, anti-phosphotyrosine antibodies cannot be used as receptor-specific histochemical reagents. To determine the cellular location and activity state of p185^{erbB2} during Wallerian degeneration, we exploited the fact that synthetic tyrosine phosphopeptides, corresponding to major autophosphorylation motifs, can be used to raise anti-phosphopeptide-immunodetecting (APHID) antibodies (Bangalore et al., 1992; Epstein et al., 1992). These APHID antibodies report the phosphorylation state of specific tyrosine residues within a targeted growth factor receptor or phosphoprotein. In the present study, we use an APHID antibody to monitor the activation state and signaling functions of p185^{erbB2} in an injured sciatic nerve. We show that p185^{erbB2} becomes phosphorylated in proliferating Schwann cells during Wallerian degeneration. Moreover, phosphorylation occurs at a position that couples p185^{erbB2} to the Ras–Raf–MAP kinase signal transduction pathway.

MATERIALS AND METHODS

Cell culture. The G8/DHFR cell line was a generous gift from the laboratory of Robert Weinberg (Massachusetts Institute of Technology). These murine fibroblasts, which overexpress the rat *c-erbB2* gene product, were cultured, and cell lysates containing either unactivated or activated p185^{erbB2} were prepared as described previously (Epstein et al., 1992).

Surgical procedures. To obtain nerve samples, male Sprague Dawley rats (~250 gm) were anesthetized with sodium pentobarbital (50 mg/kg). Using aseptic technique, the right sciatic nerve was exposed 1.0 cm distal to the sciatic notch, doubly ligated, and transected. The rats were allowed to recover from surgery. Later, the animals were anesthetized to harvest the sciatic nerves.

Immunoprecipitation and immunoblotting. Immunoprecipitates of

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Figure 1. Tyrosine phosphorylation indicative of p185^{erbB2} activation in sciatic nerve during Wallerian degeneration. p185^{erbB2} immunoprecipitates were prepared from uncut sciatic nerve (*cnt*) or from the distal stump of nerves from 5 to 28 d into Wallerian degeneration as described in Materials and Methods. The immunoprecipitates were size-fractionated on 7.0% SDS-polyacrylamide gels and immunoblotted with a monoclonal antibody to phosphotyrosine. The arrow on the left indicates position of p185^{erbB2}.

p185^{erbB2} were prepared from uncut sciatic nerve or from the distal stump of the nerve at 5–28 d into Wallerian degeneration. To obtain samples for immunoprecipitation and immunoblotting, the proximal and distal stumps of the cut nerve and the opposite uncut nerve were excised and snap frozen in liquid nitrogen. To prepare lysates, frozen nerve samples were minced with a razor blade on top of dry ice. The samples were then homogenized with eight strokes in a Dounce homogenizer in lysis buffer (1% NP-40, 20 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 4 gm/l NaF, 8.8 gm/l sodium pyrophosphate decahydrate, 1 mM PMSF, 10 μ g/ml aprotinin, and 20 μ M leupeptin) and clarified by centrifuging for 10 min in a microcentrifuge at 4°C. One milligram aliquots of each sample were immunoprecipitated with an antibody to p185^{erbB2} [polyclonal antibody 1 (pAb-1) rabbit polyclonal from Zymed Laboratories, Inc., South San Francisco, CA] using established protocols (Harlow and Lane, 1988). The immunoprecipitates were size-fractionated on 7.0% SDS polyacrylamide gels and immunoblotted with a monoclonal antibody to phosphotyrosine (4G10; a generous gift from Tom Roberts, Harvard Medical School).

Antibody specificity was determined by competition experiments in immunoblots of G8/DHFR cells. Competing peptides (14 mer at 100 nM) were preincubated with antibody at 4°C for 2 hr before immunoblotting. The negative and positive specificity controls were, respectively, the peptide and phosphopeptide counterparts of the sequence used to raise the p185^{erbB2} APHID antibody (tyrosine 1248 in the sequence AENPEpYLGDLVPV). Other specificity controls were tyrosine phosphopeptides containing the closely related NPXY motifs in the C-terminal domain of epidermal growth factor (EGF) receptor (phosphotyrosine 1197 in the sequence AENAEpYLRVAPOS) and the erbB4 gene product (phosphotyrosine 1284 in the sequence AENPEpYLSEFSLK). The C-terminal portion of p180^{erbB3} has no positional equivalent of the p185^{erbB2} NPXY motif.

Immunohistochemistry. For immunohistochemistry, anesthetized animals were killed by intracardiac perfusion with 4% paraformaldehyde in PBS with 1 mM sodium orthovanadate added to inhibit endogenous

Figure 2. Schwann cell proliferation is associated with phosphorylation of p185^{erbB2} on tyrosine. BrdU uptake was monitored in parallel with immunoprecipitation studies using separate animals. BrdU-labeled nuclei (mean $\pm$ SD) were counted in five high-powered fields or nerve segments (2 animals per time point; *W*, weeks after lesion). The highest number of labeled nuclei was found in the distal nerve stump (*D*) 2 weeks after transection, coinciding with the peak of p185^{erbB2} activation shown in Figure 1. Nuclear labeling increased to a lesser extent in the proximal stump (*P*). By immunoblotting, lesser amounts of activated p185^{erbB2} also were detected in the proximal stump of the lesioned nerve (*inset*). *C*, Control.

phosphatase activity. Cryostat sections (7.5–10 μ m) from proximal and distal stumps of sciatic nerve were permeabilized with 0.5% NP-40 in Tris-buffered saline (in mM: 20 Tris, pH 7.4, and 150 NaCl) and blocked with 5% normal goat serum. Aliquots of primary APHID antibody (100 μ l) were mixed with BSA (final concentration, 1 mg/ml), solvent control, or competing peptide (100 nM) as indicated in a final volume of 150 μ l. Antibody and competing peptides were mixed with gentle rocking at 4°C for 2 hr before incubating with nerve sections (150 μ l over each sample on a glass slide) for 24–36 hours at 4°C, followed by incubation in biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). Staining was visualized with True Blue (KPL, Gaithersburg, MD) or with DAB per instructions of the Vectastain ABC kit (Vector Laboratories). Identical procedures were used for immunohistochemical staining with commercial antisera to antibody p185^{erbB2} (Triton Biosciences, Alameda, CA) and with antisera to S100 protein (Dako, Carpinteria, CA). For double-labeling experiments, sections were permeabilized, blocked, and incubated with primary APHID antibody overnight at 4°C, followed by biotinylated goat anti-rabbit IgG (Vector Laboratories) and avidin-Cy3 (Jackson Immunochemicals, West Grove, PA). The sections

Figure 3. APHID antibody is specific for phosphotyrosine 1248 within activated p185^{erbB2}. *A*, G8/DHFR cells were treated with phorbol or with solvent control to generate inactive (–) or activated (+) p185^{erbB2} as described in Materials and Methods. Cell lysates were size-fractionated by SDS-PAGE (40 μ g/lane) and immunoblotted with the APHID antibody to p185^{erbB2} (APHID) or with a commercial antibody that recognizes the receptor irrespective of activation state (*pAb-1*). *B*, Lysates containing activated p185^{erbB2} were size-fractionated and immunoblotted with the APHID antibody to p185^{erbB2} as *A*, except that the antibody was preincubated with synthetic peptides or phosphopeptides corresponding to NPXY motifs in p185^{erbB2}, EGF receptor, or p180^{erbB4} as described in Materials and Methods. The double-headed arrow indicates position of 200 kDa molecular weight standard.

Figure 4. Phosphorylation of p185^{erbB2} at Y1248 visualized in sciatic nerve Schwann cells during Wallerian degeneration. *Top panels*, Cryostat sections of control and distal stump of rat sciatic nerve at 2 weeks into Wallerian degeneration were immunostained with the APHID antibody to p185^{erbB2} as described in Materials and Methods. Visualization with True Blue results in blue-green color over areas of immunoreactivity. *Bottom panels*, Controls for specificity of APHID antibody immunohistochemical stain. Sections of the distal stump of rat sciatic nerve at 2 weeks into Wallerian degeneration were stained with APHID antibody preincubated with peptides or phosphopeptides corresponding to NPXY motifs in p185^{erbB2}, EGF receptor, or p180^{erbB4} as described in Materials and Methods. Scale bars, 15 μ m.

Figure 5. APHID antibody immunostaining is specific for activated receptors in cells expressing p185^{erbB2}. G8/DHFR cells were treated with phorbol or solvent control to generate inactive (–) or activated (+) p185^{erbB2} as described in Materials and Methods. The cells were immunostained with a commercial antibody that recognizes p185^{erbB2} irrespective of activation state (*top panels*) or with the APHID antibody to activated p185^{erbB2} (*bottom panels*) and visualized with DAB. For scale reference, G8/DHFR cell nuclei are ~ 10 μ m in diameter.

were then incubated overnight at 4°C with an S100- β monoclonal antibody (Sigma, St. Louis, MO) followed by Cy2-conjugated goat anti-mouse IgG (Jackson Immunochemicals).

Bromodeoxyuridine labeling. For mitotic labeling, animals were injected with bromodeoxyuridine (BrdU; 50 mg/kg, i.p.). After 1 hr, the animals were killed by intracardiac perfusion with 4% paraformaldehyde in PBS. Cryostat sections (7.5 μ m) of the nerves were prepared and stained with a monoclonal anti-BrdU primary antibody and fluorescein-conjugated secondary antibody according to the manufacturer's specifications (Boehringer Mannheim, Indianapolis, IN). Labeled nuclei were counted in five high-powered fields per nerve segment (two animals per time point) viewed by fluorescence optics (Leitz, Wetzlar, Germany; 40 $\times$ objective).

Figure 6. **Top.** Tight positional relationship between p185^{erbB2} activation and BrdU labeling during Wallerian degeneration in sciatic nerve. Sections of proximal and distal stumps of rat sciatic nerve at 2 weeks into Wallerian degeneration were stained for BrdU uptake (*top row*) or with the APHID antibody (*bottom row*). Schematic diagram of sciatic nerve (cell bodies to *left*, nerve termini to *right*) illustrates approximate position of the nerve transection together with relative positions of the *edge*, *center*, and *end* segments. A control, uncut nerve section (*top*) stained for BrdU uptake is shown for comparison. Scale bars, 15 μ M.

RESULTS

Schwann cell proliferation is temporally associated with activation of p185^{erbB2}

As an initial probe into the activation state of p185^{erbB2} during Wallerian degeneration, we surgically transected rat sciatic nerve and ligated both the proximal and distal stumps. We monitored tyrosine phosphorylation of p185^{erbB2} within the nerve segments 5–28 d after nerve transection, the period of Wallerian degeneration (Bradley and Asbury, 1970; Clemence et al., 1989). Sciatic nerve extracts were immunoprecipitated with a pan-p185^{erbB2} antibody, and the immunoprecipitates were immunoblotted with antiphosphotyrosine. Relative to control nerve sections, increased p185^{erbB2} tyrosine phosphorylation was found in the distal nerve stump between 5 and 18 d after transection (Fig. 1). To explore the temporal relationship between p185^{erbB2} tyrosine phosphorylation and Schwann cell mitotic activity we monitored BrdU uptake over the same time frame. The BrdU uptake results (Fig. 2) show, as noted by others (Cohen et al., 1992), that mitotic activity is enhanced within 1 week after surgical transection of the nerve. Mitotic activity remains above baseline levels for at least 14 d but returns to control level by 28 d after injury. Thus p185^{erbB2} tyrosine phosphorylation and Schwann cell mitotic activity occur over the same broad time frame after nerve injury. To explore positional relationships between p185^{erbB2} tyrosine phosphorylation and Schwann cell mitotic activity, we turned to an activation state-specific antibody targeted to p185^{erbB2}.

Receptor specificity and activation state specificity of an APHID antibody to p185^{erbB2}

In previous studies, we raised an APHID antibody directed to Y1248 in human p185^{erbB2} (Epstein et al., 1992). This tyrosine lies within an NPXY motif which is a canonical target for the Shc adapter protein (Campbell et al., 1994; Dilworth et al., 1994; Stephens et al., 1994). Amino acid substitution experiments indicate that Y1248 is necessary for transforming activity of activated p185^{erbB2} (Segatto et al., 1990; Akiyama et al., 1991; Mikami et al., 1992). Moreover, reconstitution experiments indicate that phosphorylation of Y1248 couples p185^{erbB2} to the Ras–Raf–MAP kinase signal transduction pathway and is sufficient for transforming activity (Ben-Levy et al., 1994).

The erbB2 gene is a member of the subgroup I receptor tyrosine kinase family (Ullrich and Schlessinger, 1990) that includes the EGF receptor, p185^{erbB2} (Peles et al., 1992), erbB3 (Kraus et al., 1989), and erbB4 (Plowman et al., 1993). To document receptor specificity and activation state specificity of the APHID antibody targeted to Y1248, we used an indicator cell line, G8/DHFR. This is a murine fibroblast line that overex-

presses the rat *c-erbB2* gene product. Semiconfluent G8/DHFR cells express an intermediate level of *c-erbB2* receptor tyrosine kinase activity. This intermediate level of activity is suppressed by exposure to phorbol-based tumor promoters, which transmodulate the receptor (Epstein et al., 1990). As shown by immunoblot analysis of G8/DHFR cells (Fig. 3), our APHID antibody recognizes p185^{erbB2} only in the activated state, whereas a conventional antibody reacts with the protein in the activated or inactivated state. Peptide competition experiments (Fig. 3) document receptor selectivity of the APHID antibody directed to Y1248 in p185^{erbB2}. The synthetic tyrosine phosphopeptide used to raise the antibody competes for recognition of activated p185^{erbB2}. An identical peptide without a phosphate at Y1248 does not compete. Phosphopeptides corresponding to positionally equivalent NPXY motifs in the C-terminal domain, the EGF receptor, and p180^{erbB4} do not compete with the APHID antibody for recognition of activated p185^{erbB2}. The C-terminal portion of p180^{erbB3} has no positional equivalent of the p185^{erbB2} NPXY motif and was therefore not tested.

Phosphorylation of p185^{erbB2} at Y1248 visualized in sciatic nerve Schwann cells

To determine whether the APHID antibody can be used as an immunocytochemical reagent to visualize p185^{erbB2} signaling *in vivo*, we stained cryostat sections from control nerve and from the distal stump of sciatic nerve at 2 weeks into Wallerian degeneration, a time when receptor activation is at its zenith, as shown by immunoblot analysis with conventional reagents (Fig. 1). As shown in Figure 4, *top panel*, sections from transected sciatic nerve show much more immunoreactivity with the APHID antibody than control nerve sections. Selectivity of the APHID antibody as an immunocytochemical reagent is established by competition with the subgroup I receptor peptides and phosphopeptides. As shown in Figure 4, *bottom panel*, histochemical staining was completely ablated by competitions with the erbB2 phosphopeptide. Competitions with erbB2 peptide or other subgroup I receptor phosphopeptides had little or no effect on staining. Double labeling with an antibody to S100 protein confirms that cells that react with APHID antibody are Schwann cells (see Fig. 7). In both proximal and distal stump sections, the APHID antibody immunostaining is localized diffusely over the surface of reactive cells, as predicted for a cell surface protein in 7.5 μ m cryostat sections. We obtained a similar pattern of immunostaining using a different APHID antibody directed to tyrosines 1221 and 1222 that also are autophosphorylated in activated p185^{erbB2} (data not shown). As a positive control for immunohistochemistry in sciatic nerve sections, we used the

Figure 7. Middle. Colocalization of activated p185^{erbB2}, Schwann cell marker protein, and BrdU incorporation by double immunofluorescence. *Top panels.* Sections of distal stumps of rat sciatic nerve at 2 weeks into Wallerian degeneration were costained with a monoclonal antibody to S100 protein and the APHID antibody to p185^{erbB2} followed by secondary antibodies conjugated to Cy2 (S100) or Cy3 (p185^{erbB2}). Colocalization of S100 (green) and activated p185^{erbB2} (red) is indicated in the color overlay (yellow). *Bottom panels.* Sections were immunostained with the APHID antibody to p185^{erbB2} and visualized with Cy3. BrdU uptake was visualized in the same sections with an antibody to BrdU followed by a Cy2-conjugated secondary antibody. Colocalization of BrdU uptake (green) and activated p185^{erbB2} (red) is indicated in the color overlay. The somewhat inferior quality of activated p185^{erbB2} immunostaining reflects the fact that the sections must be exposed to partially denaturing conditions for BrdU immunostaining before staining for activated p185^{erbB2}.

Figure 8. Bottom. Nerve damage increases the amount of activated p185^{erbB2} per cell. Cryostat sections of uncut sciatic nerve or transected nerve (distal stump) at 2 weeks into Wallerian degeneration were stained with the APHID antibody to p185^{erbB2} (*top row*) or a conventional antibody to p185^{erbB2} (pAb-1, *bottom row*). To aid in visualization of stained cells, photomicrographs were taken at identical exposure settings, scanned, and digitized into the Adobe Photoshop program. Background color was deleted electronically. Weakly stained cells in the processed images from uncut nerve and distal stump (*arrows*) can be matched to positional equivalents in the unprocessed photomicrographs.

G8/DHFR cell line. As shown in Figure 5, the APHID antibody recognizes p185^{erbB2} on G8/DHFR cells only when p185^{erbB2} is active. By contrast, a conventional antibody to p185^{erbB2} (pAb-1) recognizes p185^{erbB2} irrespective of its activation state. Collectively, these data indicate that the staining differential between control and transected nerve sections shown in Figure 4, *top panel*, most likely reflects the activation of p185^{erbB2} in Schwann cells.

Tight linkage between p185^{erbB2} activation and BrdU labeling during Wallerian degeneration in sciatic nerve

Using the APHID antibody as an immunohistochemical reagent, we established a tight positional relationship between Schwann cell mitotic activity and p185^{erbB2} signaling functions during Wallerian degeneration (Fig. 6). In the distal stump of transected nerve, cells that stain with the APHID antibody are distributed uniformly from the edge of the lesion to the end of the nerve segment. Double-labeling experiments indicate that BrdU incorporation occurs predominantly within Schwann cells (Fig. 7). Thus, the distribution of APHID antibody immunostained cells tracks well with Schwann cell mitotic activity, as visualized by BrdU labeling. The proximal stump of the nerves reveals a different immunostaining pattern for both APHID antibody and BrdU label but with the same tight positional coincidence. Here, APHID antibody staining and mitotic activity are seen at the edge of the nerve lesion. However, beyond the edge of the lesion proximal toward the nerve cell bodies, APHID antibody staining and mitotic activity are attenuated to the level seen in uncut nerve.

Enhanced Schwann cell staining with APHID antibody reflects activation state, rather than abundance, of p185^{erbB2}

Both erbB2 mRNA and p185^{erbB2} accumulate in the distal stump of sciatic nerve during Wallerian degeneration (Cohen et al., 1992; Carroll et al., 1997). The number of Schwann cells in the distal stump also increases as a consequence of mitotic activity. Accordingly, we wondered whether the increased staining with APHID antibody reflects a gain in the number of cells that display activated p185^{erbB2} or an increase in the amount of activated p185^{erbB2} per cell or both. To address this question, we compared immunostaining patterns with our APHID antibody with those obtained with a commercial antibody that recognizes p185^{erbB2} irrespective of activation state. To visualize the staining differential more readily, we used image-processing software to subtract background color in the stained sections.

Relative to uncut nerve, distal stump sections stained with APHID antibody show a gain in both number of stained cells and staining intensity per cell (Fig. 8, *top row*). By contrast (Fig. 8, *bottom row*), identical sections stained with conventional antibody show a gain in the number of stained cells (in confirmation of Cohen et al., 1992), but the staining intensity per cell is comparable in uncut nerve and distal stump. This comparison is somewhat subjective, because immunohistochemical staining reactions are not inherently linear. However, the differential intensity of cell staining with the APHID antibody was apparent to the eye in multiple experiments.

DISCUSSION

Targeted disruption studies show that the neuregulin and erbB2 gene products play vital roles in development of the embryonic heart, Schwann cell precursors, and cranial nerve ganglia (Gassman et al., 1995; Lee et al., 1995; Marchionni, 1995; Meyer and Birch-

meier, 1995). However, because null mutants of either gene die *in utero* at day 10.5, the functions of neuregulins and their receptors in adult animals cannot be discerned by gene disruption. The APHID antibody images shown here indicate that these proteins play an active role in the regeneration of injured nerves in adult animals.

Activation of p185^{erbB2} after nerve damage could, in principle, reflect suppression of a negative regulator. In tissue culture model systems, the activity state of p185^{erbB2} is negatively regulated by protein kinase C agonists (Dougall et al., 1994). For several reasons, however, we favor the view that activation of p185^{erbB2} reflects increased availability of p185^{erbB2}-activating ligands, the neuregulins. Neuregulins are produced by neurons (Marchionni et al., 1993; Dong et al., 1995) in a variety of splice variant transcripts, some of which encode a membrane-bound ligand (Marchionni et al., 1993). Immunohistochemical studies demonstrate neuregulins within axons (Sandrock et al., 1995), and neuronal membrane preparations stimulate the growth of cultured Schwann cells (Salzer et al., 1980) through the activation of p185^{erbB2} (Morrissey et al., 1995).

Neuregulins are thought to interact with p185^{erbB2} by inducing the formation of heterodimers between p185^{erbB2} and either p180^{erbB3} or p180^{erbB4}. Neuregulin-induced heterodimer formation between p185^{erbB2} and p180^{erbB3} constitutes an interesting example of a symbiotic relationship in receptor signaling. By itself, p185^{erbB2} cannot interact with neuregulins. Although p180^{erbB3} is a competent neuregulin-binding protein, it appears to have no intrinsic tyrosine kinase activity (Carraway and Cantley, 1994). Formation of a heterodimeric p185^{erbB2}-p180^{erbB3} complex generates a fully competent growth factor receptor (Carraway and Cantley, 1994; Marchionni, 1995; Wallasch et al., 1995). In preliminary studies, we have noted that p180^{erbB3} is present in rat sciatic nerve lysates (data not shown) (Carroll et al., 1997).

The anatomy of sciatic nerve raises an interesting question. If neuregulin synthesis is confined to neurons, what serves as a source of neuregulin for Schwann cells in the distal stump of a regenerating nerve? Neuronal protein synthesis is mainly confined to the nerve cell bodies. Accordingly, only neuregulins synthesized before transecting the nerve would have access to Schwann cells in the distal stump. Fischbach and associates have described a slow release mechanism for neuregulins at the sciatic nerve ending. Here within the neuromuscular junction, neuregulin is bound to extracellular matrix and inhibited from activating p185^{erbB2} on striated muscle until it is released by proteolysis (Goodearl et al., 1995; Loeb and Fischbach, 1995; Sandrock et al., 1995). Conceivably, stored reservoirs of inactive neuregulin are distributed along the length of the axon to be activated and slowly released as axons degenerate after transection of the nerve. Alternatively, neuregulins may be synthesized by non-neuronal cells during Wallerian degeneration, perhaps by Schwann cells themselves in an autocrine growth mode (Carroll et al., 1997). Finally, the phosphorylation of p185^{erbB2} may be triggered by a novel and presently uncharacterized ligand that is released after nerve damage.

Future studies with APHID antibodies targeted to other members of the subgroup I receptor family may identify heterodimeric partners and the source of ligand for p185^{erbB2} during nerve regeneration. In the meantime, it is worth noting that the approach taken here to provide images of p185^{erbB2} activation *in vivo* has general utility. Synthetic phosphopeptides can, in principle, be used to raise APHID antibodies targeted to any growth factor receptor or signal-generating protein that is regulated by

tyrosine phosphorylation events. As immunochemical probes, these APHID antibodies are more selective than conventional reagents, and they can be used as immunohistochemical reagents to display the activation state of specific receptors or signal generators *in situ*.

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