

Differential Patterns of ERK and STAT3 Phosphorylation after Sciatic Nerve Transection in the Rat

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Peripheral nerve injury induces a specific pattern of expression of growth factors and cytokines, which regulate injury responses and regeneration. Distinct classes of growth factors and cytokines signal through specific intracellular phosphorylation cascades. For example, the ERK phosphorylation cascade mediates signaling through transmembrane tyrosine kinase receptors and the JAK/STAT cascade mediates signaling through the GP130 receptor complex. We tested whether specific phosphorylation patterns of ERK and STAT3 result from nerve injury and whether such phosphorylation correlates with the expression of specific growth factors and cytokines. At sites adjacent to a nerve transection, we observed that ERK phosphorylation peaked early, persisted throughout 16 days, and was equally intense at proximal and distal sites. In contrast, STAT3 phosphorylation peaked later than ERK but did not persist as long and was stronger in the proximal than in the distal segment adjacent to the injury. In addition, in distal segments further away from the injury site, ERK became phosphorylated with a delayed time course, while STAT3 remained unphosphorylated. These patterns of phosphorylation correlated well with the expression of neurotrophin and interleukin-6 mRNAs in the distal stump. In addition, we found that the pattern of SAPK phosphorylation is similar to the pattern observed for STAT3, while the pattern of macrophage infiltration into the transected nerve was distinct from all the phosphorylation patterns observed. Together, these observations suggest that ERK activation is important in the establishment of a regeneration-promoting extracellular environment in the far distal stump of transected nerves and that STAT3 activation is important in the control of cellular responses close to the site of injury.

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INTRODUCTION

Injury to the peripheral nerve initiates a well-defined pattern of cellular changes distal to the site of damage (for reviews, see 12, 14). In the first few days following injury, axonal degeneration proceeds distally from the site of injury and the myelin sheaths that surround the degenerating axons are broken up into ovoids. Macrophages invade the nerve soon after injury and phagocytose the debris (1, 27, 28). Schwann cells dedifferentiate and divide while staying within their basal laminae, producing cordons of Schwann cells known as bands of Büngner (9, 26, 32). These changes modify the nerve into an environment suitable for the regeneration of injured axons from areas just proximal to the damage. Specific sets of growth factors and cytokines are thought to regulate these cellular changes and to promote axonal regeneration.

Nerve injury induces the expression of neurotrophic factors such as NGF (15, 22), BDNF (13, 23), and GDNF (35, 37), of Schwann cell mitogens and survival factors such as neuregulins (4), and of proinflammatory cytokines such as IL-1 (31), IL-6 (2, 3, 18, 29), and LIF (7, 18). In addition to the factors induced by injury, normal peripheral nerves already contain cytoplasmic stores of neurotrophic factors and Schwann cell mitogens such as FGF1 (10, 11, 24) and CNTF (30, 36) that are poised to act when the nerve becomes injured. How this complex array of existing and upregulated factors interacts to regulate axonal regeneration and other injury responses is not entirely clear, especially as there appears to be considerable overlap between the biological activities of some of the factors.

Specific intracellular protein kinase phosphorylation cascades mediate how cells respond to distinct growth factors and cytokines (16). Although a particular kinase cascade is not used exclusively by a single growth factor, factors that stimulate related responses preferentially activate similar cascades. For example, the extracellular signal regulated kinase (ERK) members of the MAP kinase cascade are activated by growth factors that bind to transmembrane protein kinase receptors. This class of factors includes the neurotro-

phins, FGFs, and GDNF (5, 6, 33, 34, 38, 39). Factors that bind the GP130 receptor complex—such as IL-6, LIF, and CNTF—activate the JAK/STAT pathway (8, 17, 19). In addition, proinflammatory cytokines and cellular stress (such as heat shock or UV irradiation) transduce their signals through the stress-activated protein kinase (SAPK) pathway (21, 25). The observation of the phosphorylation state of these distinct cascades in the injured nerve likely will reveal the integrated action of different classes of factors as they shape the cellular response to nerve injury.

Peripheral nerve transection represents an ideal model to determine whether the injury results in distinct spatiotemporal activation of different phosphorylation cascades and whether such patterns correlate with known cellular changes or the expression of specific sets of growth factors and cytokines. In order to address these questions, the present study quantified the extent of phosphorylation of ERKs, STAT3, and SAPK at the different sites along transected rat sciatic nerve at postinjury times ranging from 30 min to 16 days. The number of activated macrophages present and the levels of multiple growth factor (the neurotrophin family and GDNF) and cytokine (CNTF, IL-1 β , and IL-6) mRNAs were measured in parallel samples to allow a comparison of intracellular signaling, regulation of gene expression, and cellular responses. The results demonstrate that injury induces distinct spatial and temporal patterns of activation of the individual signaling cascades. In the distal stump, these patterns of activation correlated well with the regulation of expression of the growth factors and cytokines assayed, but no such correlation was observed in the proximal stump.

METHODS

Animal Surgery

Female Long-Evans rats (180 to 200 g body wt) were anesthetized with a cocktail of ketamine (55.6 mg/ml), xylazine (5.6 mg/ml), and acepromazine (1.1 mg/ml), at a dosage of 1 ml cocktail per kilogram of body weight. Sciatic nerves on both sides of the body were exposed at the upper thigh level, approximately 3 mm below the sciatic notch, and a 3-mm segment of the sciatic nerve fragment was removed on both sides to complete the transection. Sham operations were also performed by exposing the sciatic nerves and stripping away the connective tissues surrounding the nerve. Following surgery, the wound sites were closed with wound clips (Fine Science Instruments) and the animals were returned to animal care facility until sacrifice.

Tissue Collection and Homogenization

At appropriate postlesion times, the rats were deeply anesthetized with the anesthesia cocktail. The sciatic

nerves were exposed and 3-mm segments of the sciatic nerve were collected at four different distances from the site of transection (Fig. 1). Nerve segments collected from identical distances from the lesion from both sides of the same animal were pooled and immediately frozen on dry ice. For control tissue, nerves were collected from non- or sham-operated rats. The nerve fragments were then stored at -80°C until further processing. Nerve fragments from each animal were homogenized in 400 μl of SDS sample buffer (62.5 mM Tris-HCl, 2% (w/v) SDS, 10% glycerol, 50 mM DTT, and 0.1% (w/v) bromophenol blue). The homogenized tissue was divided into aliquots and stored at -80°C until use.

Western Blotting

Each lane of a 10% SDS-acrylamide gel was loaded with 20 μg nerve fragment protein derived from a single animal. A set of four gels was processed simultaneously, in order to analyze all time points and nerve fragments under identical conditions. The same control samples (noninjured and appropriate injured nerve fragments) were loaded on all gels within an individual experiment, in order to normalize band intensities across different gels. Three independent sets of animals and Western blot experiments were analyzed as follows:

After electrophoresis, gels were electroblotted at room temperature for 1 h onto polyvinylidene fluoride (PVDF) blotting membranes (BioTrace PVDF, Gelman Sciences). The membranes were briefly washed in PBS and then incubated for 2 h at room temperature with blocking buffer (5% (w/v) dry skim milk (Carnation), 0.1% (v/v) Tween 20 in PBS (10 mM sodium phosphate, 150 mM sodium chloride), pH 7.2). The membranes were then incubated overnight at 4°C in primary antibody (rabbit anti-phospho-ERK, rabbit anti-phospho-STAT3, and rabbit anti-phospho-SAPK (all from New England Biolabs, diluted 1:1000 in 0.05% Tween 20, 5% BSA, and 0.02% sodium azide in PBS)). An additional set of membranes was incubated in a mouse monoclonal antibody to neurofilament (68 kDa, from Boehringer Mannheim) at a concentration of 0.2 $\mu\text{g}/\text{ml}$. The membranes were then washed three times for 10 min each with blocking buffer and then incubated in alkaline phosphatase-conjugated secondary antibodies (goat anti-rabbit or anti-mouse; New England Biolabs; diluted 1:1000 in blocking buffer) for 1 h at room temperature. The membranes were washed as above, followed by washing for two times with 10 mM Tris-HCl, 10 mM NaCl, 1 mM MgCl_2 , pH 9.5, and incubation in CDP-Star (1:500 dilution, New England Biolabs) diluted in 0.1 M diethanolamine, 1.0 mM MgCl_2 , pH 9.5. The membranes were placed in a sheet protector and exposed to Kodak autoradiography films for various lengths of time (1–15 min).

Immunocytochemistry

Sciatic nerves were immersion fixed in 5% formalin for 24 h at room temperature. Following fixation, tissues were washed in PBS overnight and subsequently equilibrated in 15% (w/v) sucrose in PBS and then in 30% (w/v) sucrose in PBS. Ten-micrometer sections were cut in a cryostat and collected onto gelatin-coated slides. The sections were washed in PBS for 10 min, incubated in blocking solution (10% horse serum, 0.5% Triton-X in PBS) for 1 h at room temperature, and then incubated overnight at room temperature in mouse monoclonal ED-1 antibody (from Accurate, diluted 1:1000 in blocking solution). Sections were washed four times for 10 min with PBS and incubated with fluorescein-conjugated secondary antibodies (Cappel, diluted 1:200 in blocking solution) for 1 h at room temperature, washed, and coverslipped. The number of ED-1-positive cells was estimated by counting labeled cells in three fields of view each (160 \times final magnification) at sites 1 and 4 mm proximal and distal to the lesion.

Multiprobe Ribonuclease Protection Assay (RPA)

Total RNA from rat sciatic nerves was isolated using an acidified phenol guanidinium method (Tri Reagent, from Molecular Research Center). Riboprobes were transcribed from multiprobe template sets using the Riboquant *in vitro* transcription kit (PharMingen). All enzymes and buffers used in the probe synthesis and in the RPA were from PharMingen and the RPA was conducted according to the manufacturer's specifications. After hybridization and RNase digestion, samples were run on a 5% polyacrylamide/urea sequencing gel. The gel was then dried and exposed to a Kodak phosphorimager screen for quantification.

Quantification of Western Blot and RPA Results

Digital image files were obtained by scanning the exposed films (Western blots) or acquiring the phosphorimager outputs (RPA) and analyzed using the NIH Image software package. Care was taken to analyze only signals within the linear response range of the films. The mean labeling intensity of specific bands was measured after subtraction of background density measurements.

RESULTS

The goal of the present study was to determine whether specific patterns of activation of distinct intracellular kinase cascades are induced by nerve injury and whether such patterns correlate with changes in the expression of growth factors and cytokines. Antibodies that specifically recognize the phosphorylated forms of ERK, STAT3, and SAPK were used to quantify by Western blot the spatial and temporal pattern of

activation of these three distinct kinase cascades after sciatic nerve injury. For this, sciatic nerves of adult rats were transected, and four 3-mm-long segments of nerve were harvested at different postinjury times. The segments are (see Fig. 1) proximal segments 3–6 mm from the injury (segment A), the proximal stump (B), the distal stump (C), and the distal segment 3–6 mm from injury (D). In addition, multiprobe RPA analyses were conducted to measure the levels of neurotrophin, CNTF, GDNF, IL-1, and IL-6 mRNA levels in the injured nerve.

Distribution of Phosphorylated ERK, STAT3, and SAPK

Rapid and pronounced phosphorylation of ERK, STAT3, and SAPK was observed in nerve segments immediately adjacent to the site of injury (segments B and C, Figs. 1 and 2). All three molecules showed significant phosphorylation within 30 min after the lesion, but the subsequent time course of phosphorylation revealed some clear differences between the three cascades. For example, ERK phosphorylation reached near-maximum levels at 30 min, while STAT3 and SAPK phosphorylation increased more slowly and did not reach peak level until after 6 h. Also, ERK phosphorylation remained high for up to 16 days postinjury, while STAT3 and SAPK phosphorylation began to decrease 8 days after injury. In addition, the time course and extent of ERK phosphorylation was very similar between segments B and C, while STAT3 and SAPK phosphorylation was more pronounced and prolonged in the proximal (segment B) than in the distal stump (segment C).

Only slight, statistically nonsignificant, increases in phosphorylation of STAT3 and SAPK were observed at sites further away from the injury (between 3 and 6 mm proximal or distal to the injury site, segments A and D). The time course of these small increases paralleled the changes observed in segments adjacent to the injury. In contrast, significant increases in ERK phosphorylation were observed in the far distal segment D, and this increase was delayed by at least 24 h when compared to adjacent segment C. No significant change in ERK phosphorylation was observed in the far proximal segment A.

The increases in phosphorylation observed, especially at later time points, could either be due to an increase in the total amount of the signaling proteins (in which the fraction of the phosphorylated species remain constant while the overall level would increase) or be due to an increase in the specific phosphorylation state of the proteins regardless of whether the amount of total proteins changes. To distinguish between these two possibilities, antibodies that recognized ERK, STAT3, and SAPK, regardless of their phosphorylation state, were used to observe the expression of these

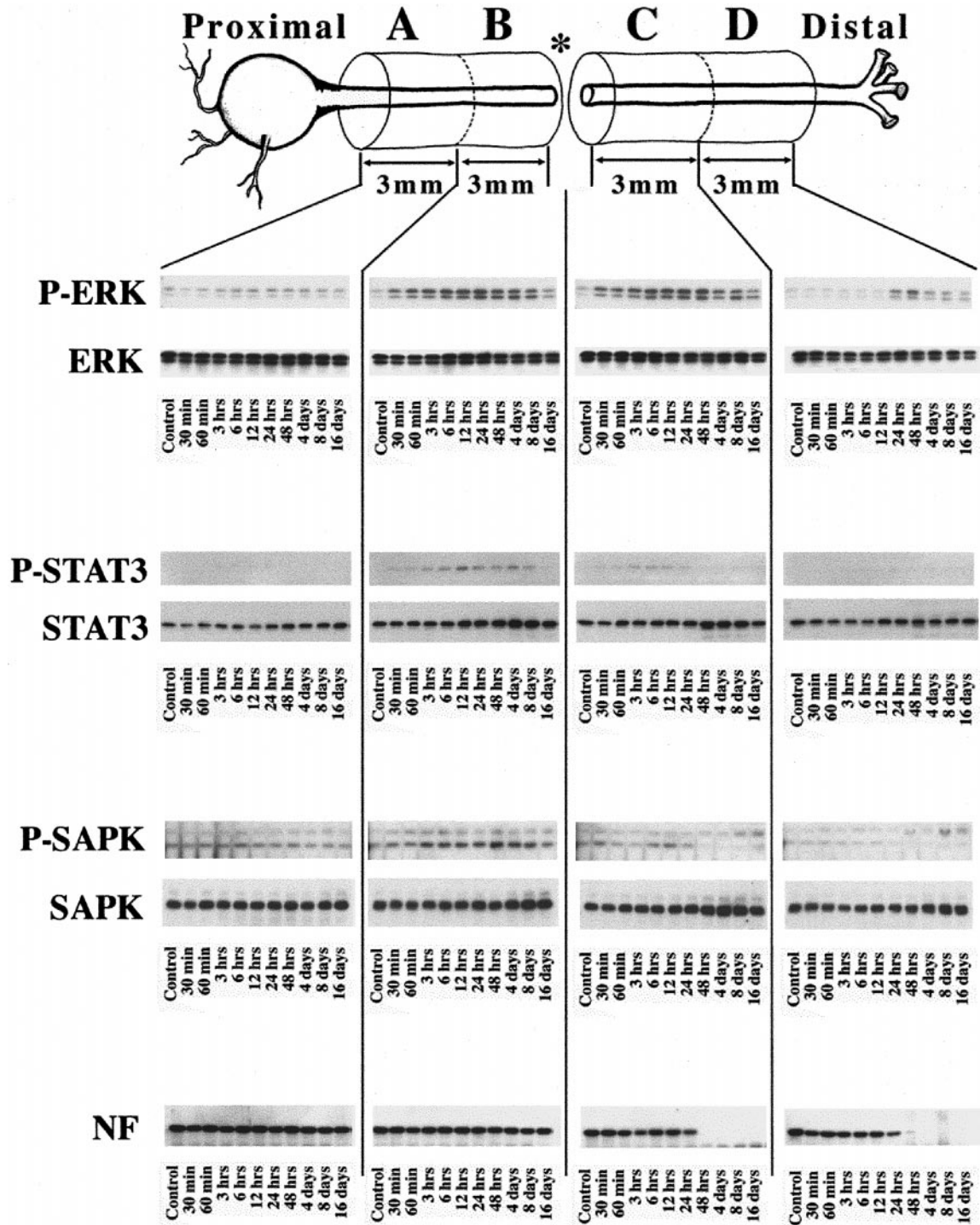


FIG. 1. Western blot analysis of ERK, STAT3, and SAPK phosphorylation along transected sciatic nerve. (Top) Schematic diagram of transected nerve showing the segments collected for analysis. The asterisk denotes the location of the transection. (Bottom) Results of Western blot analysis using antibodies that specifically detect phosphorylated ERK (P-ERK top row, first set), phosphorylated STAT3 (P-STAT3, top row, second set), and phosphorylated SAPK (P-SAPK, top row, third set). Each row demonstrates the changes in phosphorylation levels at different time points (0–16 days) following the injury at the four locations analyzed. The bottom rows (labeled ERK, STAT3, and SAPK) in each set show Western blots probed with antibodies that recognize the three signaling molecules irrespective of their phosphorylation status. Each lane on the Western blot represents a single animal. Note that the total levels of ERK, STAT3, and SAPK remain relatively constant after injury, while specific patterns of signaling molecule phosphorylation are induced. Overall, the pattern of STAT3 and SAPK phosphorylation are similar to each other and distinct from the pattern of ERK phosphorylation. The row of Western blots shown at the bottom was probed with antibodies for the axonal marker neurofilament, and results demonstrate that axonal degeneration in the distal stump is complete after 48 h and that axonal regeneration is not detectable at later time points.

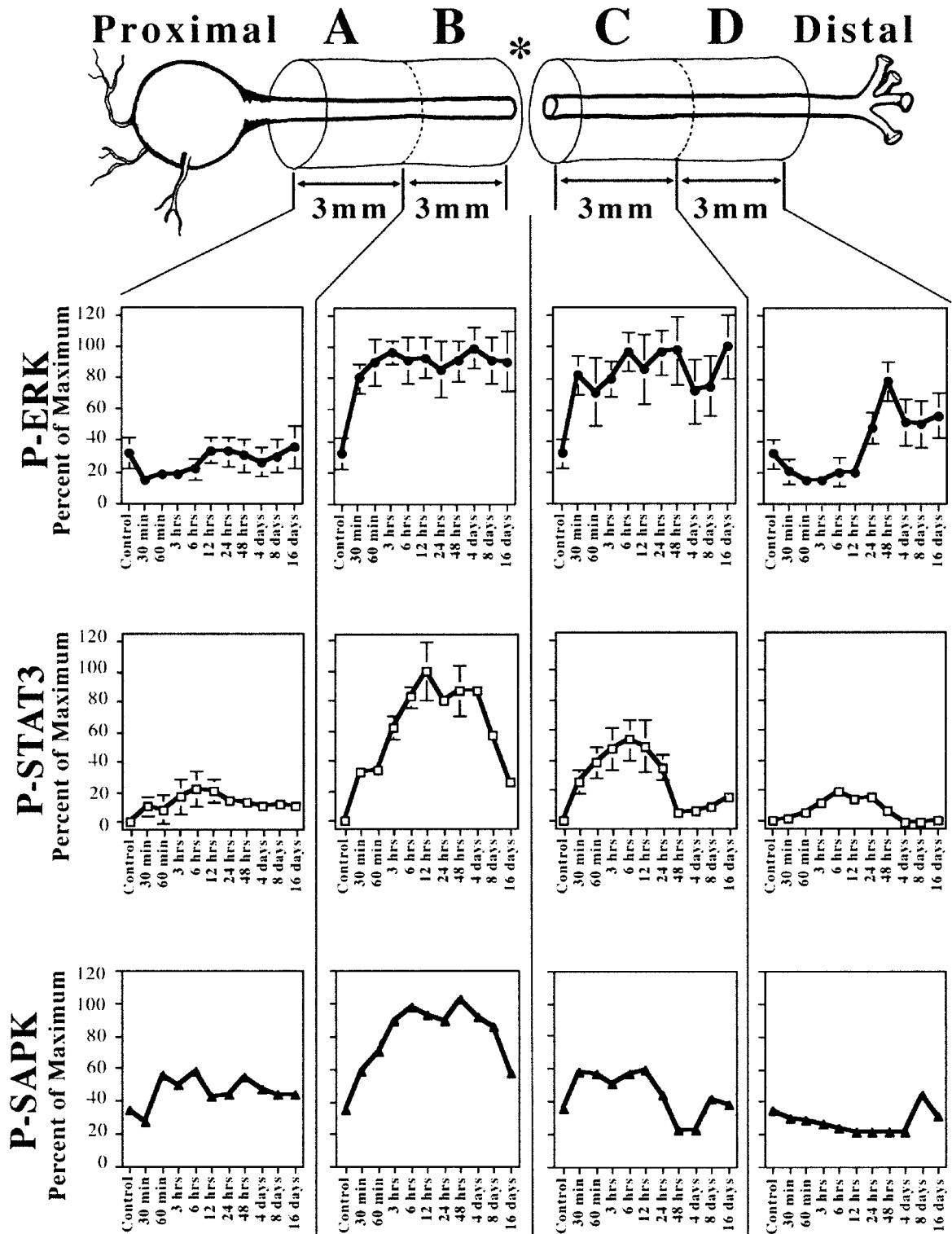


FIG. 2. Quantitative analysis of ERK, STAT3, and SAPK phosphorylation after nerve injury. (Top) Schematic diagram of transected nerve showing the segments collected for analysis. The asterisk denotes the location of the transection. (Bottom) Intensity of signaling molecule phosphorylation at different time points (0–16 days) following the injury at the four locations indicated. The intensity of phosphorylation was measured by densitometry of Western blots (see Fig. 1) as described under Methods. Values shown are the means (\pm SEM) of independent analyses of multiple animals ($n = 4$ animals (p-ERK, p-STAT3) or 2 animals (p-SAPK)). For each molecule analyzed, the mean data point with the highest densitometric measurement was assigned a value of 100% in order to present data in a unified format. Note that nerve transection initiates a rapid phosphorylation of ERK, STAT3, and SAPK in nerve segments adjacent to the injury (segments B and C), which is sustained for several days. At later time points, however, STAT3 and SAPK phosphorylation decline, while ERK phosphorylation remains near maximal for at least 16 days. Both STAT3 and SAPK are phosphorylated to a greater extent and more prolonged in the proximal segment (B) than the distal segment (C) adjacent to the injury. In addition, only ERK shows a delayed and sustained phosphorylation in the far distal segment (D).

signaling molecules. In each of the four segments ERK, STAT3, and SAPK levels remained fairly constant throughout the time course studied (Figs. 1 and 2). There were slight increases in the levels of STAT3 and SAPK at the later time points (e.g., 4 days in segment C), but these increases did not correlate with the increases in the phosphorylation level. This demonstrates that the changes reported above reflect changes of the phosphorylation state of ERK, STAT3, and SAPK.

In order to check the success of the transection injury, the amount of neurofilament, an axon marker, was measured on Western blots (Fig. 1). The level of neurofilament remained relatively constant throughout proximal segments A and B. In contrast, in the distal segments C and D, neurofilament levels began to diminish after 24 h, were not detectable after 48 h postinjury, and did not reappear at the later time points, demonstrating that the transection was complete and that no axonal regeneration occurred in the distal stump.

Correlation of Signaling Activation with Changes in Gene Expression

The expression patterns of a number of neurotrophic factors and cytokines in the injured nerve were studied in order to correlate the expression pattern of potential intercellular signals with the activation of intracellular signaling pathways. Messenger RNA levels for IL-1 β , IL-6, CNTF, NGF, BDNF, and GDNF were measured using multiprobe RPA. The amounts of RNA isolated from nerves were relatively small; thus segments A and B (the 6 mm of nerve proximal to the injury) and segments C and D (the distal 6 mm) were combined for analysis. All of the mRNAs species studied were upregulated following nerve injury, except CNTF mRNA, which decreased after the injury. Like the signaling molecule activations, these changes in mRNA showed distinct time courses spatial distributions, with changes in the distal stump being markedly larger than those seen in the proximal stump (Fig. 3).

Three temporally distinct waves of gene regulation were observed in the distal stump. The mRNAs coding for IL-1 β , IL-6, and NGF were upregulated very rapidly following injury with a shared time course reaching maximum expression at 6 h. This peak was short-lived and began to decrease by 24 h. In contrast, GDNF mRNA levels increased with an intermediate time course starting at 24 h, peaked at 2 days, and began to decline at 4 days. BDNF mRNA levels increased late (8 days after the injury) and remained high for at least another week (the last time point studied, Fig. 3).

An interesting correlation emerged when the pattern of ERK phosphorylation was compared with the time course of induction of trophic factors known to activate the ERK cascade (NGF, GDNF, and BDNF). Although

none of the upregulated factors individually matched the prolonged activation of the ERK signaling cascade, together, the sum of the peaks of mRNA upregulation seem to fit the persistent ERK phosphorylation curve (Fig. 4). Similarly, an examination of STAT3 phosphorylation and CNTF and IL-6 regulation revealed a striking correlation (Fig. 4, distal stump): STAT3 phosphorylation increased just when IL-6 mRNA was induced (starting at 60 min), and STAT3 phosphorylation decreased when both CNTF and IL-6 mRNA levels diminished (beginning at 24 h and completely by 2 days).

The proximal stump showed limited changes in the expression of the mRNAs studied. Only NGF, IL-1 β , and IL-6 mRNAs were upregulated. These mRNAs still retained the rapid upregulation time course seen in the distal stump; however, the magnitude of expression in the proximal stump was reduced to about 50% of those seen in the distal stump (Fig. 3). The mRNAs coding for GDNF and BDNF were not significantly induced in the proximal stump. This limited upregulation of trophic factor and cytokine mRNAs did not correlate with the robust and persistent phosphorylation of relevant signaling molecules. For example, when the pattern of ERK activation in the proximal segment was compared to the patterns of trophic factor regulation, ERK phosphorylation persisted throughout the 16 days while the trophic factor and cytokine mRNAs were only upregulated during the first day (Fig. 4). Similarly, STAT3 phosphorylation in the proximal stump was more persistent than the duration of IL-6 expression and remained high while both IL-6 and CNTF decreased at the later time points. It is thus likely that growth factors and cytokines not included in the study (such as neuregulin and LIF (4, 7, 20)) may be responsible for ERK and STAT3 activation in the proximal stump.

Correlation of Signaling Activation with Macrophage Invasion

The distribution and time course of macrophage invasion were observed in the injured nerve in order to see if macrophage invasion correlated with the phosphorylation of ERK, STAT3, or SAPK. Macrophages were identified by immunofluorescence, using the macrophage-specific antibody ED-1, and counted at four distinct locations along the nerve, at 1 and 4 mm away from the injury site in both the distal and the proximal direction (Fig. 5).

The number of macrophages did not increase in the nerve at 30 min after injury, which contrasts with the robust ERK, STAT3, and SAPK phosphorylation by this early time (Figs. 1 and 2). The number of macrophages had begun to increase at 6 h at all four of the locations studied and generally continued to increase throughout 8 days after injury.

The distal segments demonstrated a greater increase in macrophage number than the proximal seg-

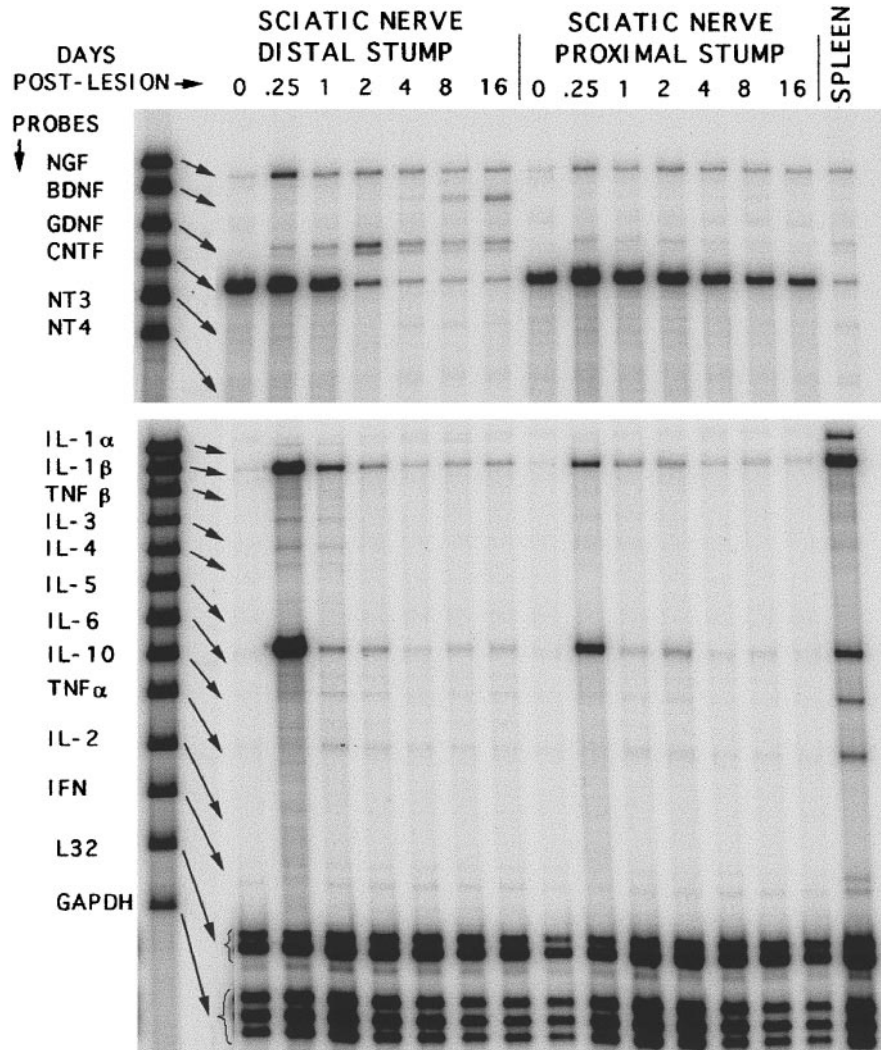


FIG. 3. Expression of neurotrophic factor and cytokine mRNAs in transected sciatic nerve. Total RNA was isolated from a 6-mm-long nerve segment distal and 6 mm proximal to the transection, and expression patterns of the mRNAs indicated were analyzed by multiprobe ribonuclease protection assays (RPA). RNA isolated from the spleen of an animal injected with lipopolysaccharide was used as a positive control. (Top) Multiprobe RPA showing bands corresponding to protected neurotrophic factor (NT) mRNAs. Note that only NGF expression is induced rapidly in both distal and proximal segments and that expression is sustained for at least 16 days. GDNF and BDNF expression is induced at intermediate or late time points, respectively, only in the distal stump. CNTF expression is significantly downregulated in the distal stump and to a lesser degree in the proximal stump. (Bottom) Multiprobe RPA showing bands corresponding to protected cytokine mRNAs. Note that of all the cytokines analyzed, IL-1 β and IL-6 mRNAs show the most marked induction after injury. Both mRNAs are expressed rapidly after transection, in both distal and proximal stumps, and show significant downregulation 1 day later. The expression of mRNAs used for loading controls (L32 and GAPDH) was not affected by the injury.

ments; this is in contrast to STAT3 and SAPK phosphorylation, which is more pronounced in the proximal stump. Overall, the pattern of increase in macrophage number correlated better with the upregulation of trophic factor and cytokine mRNAs than with the time course of signaling molecule phosphorylation.

DISCUSSION

Specific intracellular phosphorylation cascades mediate cellular responses to distinct extracellular stimuli, such as the response to growth factors and cyto-

kines after nervous system injury. Ultimately, an injured cell's decision to grow, survive, differentiate, or die is controlled by the integrated action of all the different phosphorylation cascades. The present study demonstrates that sciatic nerve transection induces distinct spatial and temporal pattern of phosphorylation of ERK, STAT3, and SAPK signaling pathways, as demonstrated on Western blots using phospho-ERK-, phospho-STAT3-, and phospho-SAPK-specific antibodies. These antibodies could not be used successfully for immunohistochemical detection; thus the present study cannot identify the specific cell types (Schwann

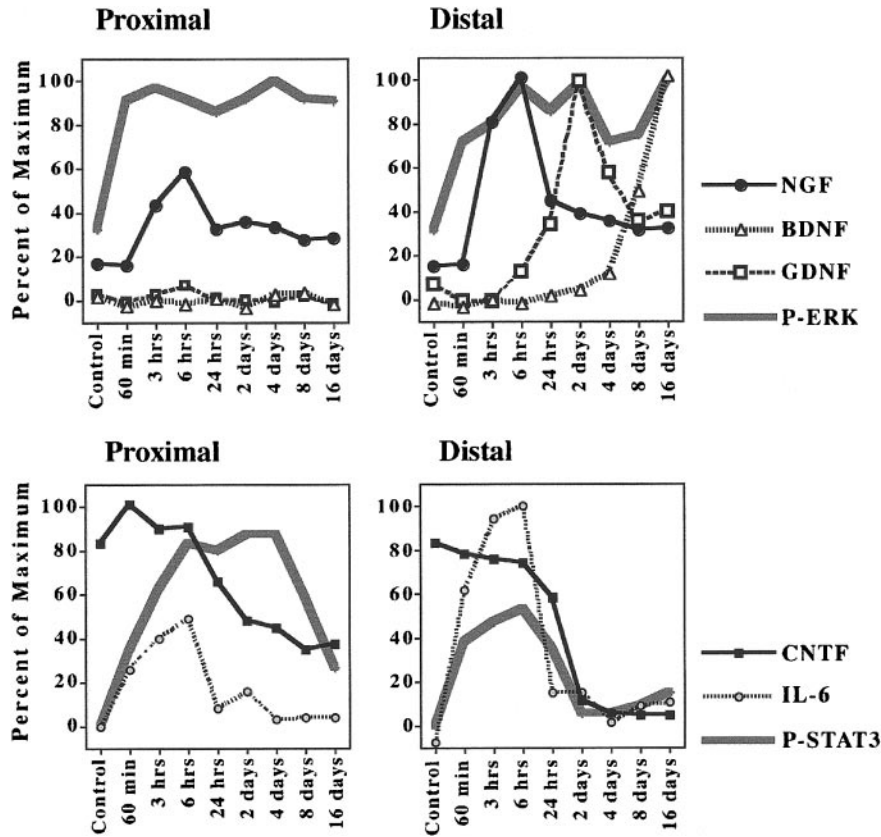


FIG. 4. Quantitative analysis of the expression patterns of neurotrophic factor and cytokine mRNAs and correlation with ERK or STAT3 phosphorylation. Multiprobe RPA data (such as these shown in Fig. 3) were quantified using a phosphorimager and the NIH Image software package. For each mRNA analyzed, the data point with the highest densitometric measurement was assigned a value of 100% in order to present data in a unified format. (Top) The expression patterns of neurotrophic factors that signal through transmembrane tyrosine kinase receptors and the ERK pathway are shown and the phosphorylation pattern of ERK is superimposed. Note that the sequential induction of expression of neurotrophic factors correlates well with the pattern of ERK phosphorylation in the distal stump, while no such correlation can be observed in the proximal stump. (Bottom) The expression patterns of cytokines that signal through the GP130 receptor complex and the STAT3 pathway are shown and the phosphorylation pattern of STAT3 is superimposed. Note that the pattern of IL-6 expression correlates well with the pattern of STAT3 phosphorylation in the distal stump, while STAT3 phosphorylation is more prolonged than IL-6 expression in the proximal stump. In addition, CNTF expression is high in uninjured nerve, but STAT3 is not phosphorylated, demonstrating that CNTF does not signal in normal nerve.

cells, macrophages, endothelial cells, fibroblasts, or neuronal axons) that contain the phosphorylated signaling molecules.

Overall, STAT3 and SAPK showed a similar pattern of phosphorylation that was distinct from the pattern of ERK phosphorylation. An important difference between these signaling cascades is that at sites at least 3 mm distal from the injury, ERK alone showed significant phosphorylation and that the delayed timing of ERK phosphorylation at distal sites correlated with axonal degeneration. In addition, ERK phosphorylation remained high both at the site of injury and at distal sites throughout the time course studied, while STAT3 and SAPK phosphorylation levels returned to almost baseline. Together, these observations suggest that the extracellular signals that activate the ERK cascade are of importance for inducing the cellular changes that transform the distal nerve tube into an

excellent environment for regeneration. On the other hand, signals that activate the STAT3 and SAPK cascades likely are important regulators of cellular responses at the site of injury. These local responses include inflammatory mechanisms and the removal of debris. Interestingly, the level of STAT3 and SAPK phosphorylation was significantly higher and longer lasting in proximal than in distal areas adjacent to the injury. The main difference between these two areas is that the proximal stump contains a large number of axons that attempt, but fail, to regenerate, while all axons in the distal stump degenerate within 48 h after transection. Therefore, the long-lasting high-level of STAT3 and SAPK activation likely is due to the presence of the tips of these axons.

Previous work has identified a significant number of growth factors and cytokines that are involved in regulating the response of peripheral nerves to injury (2,

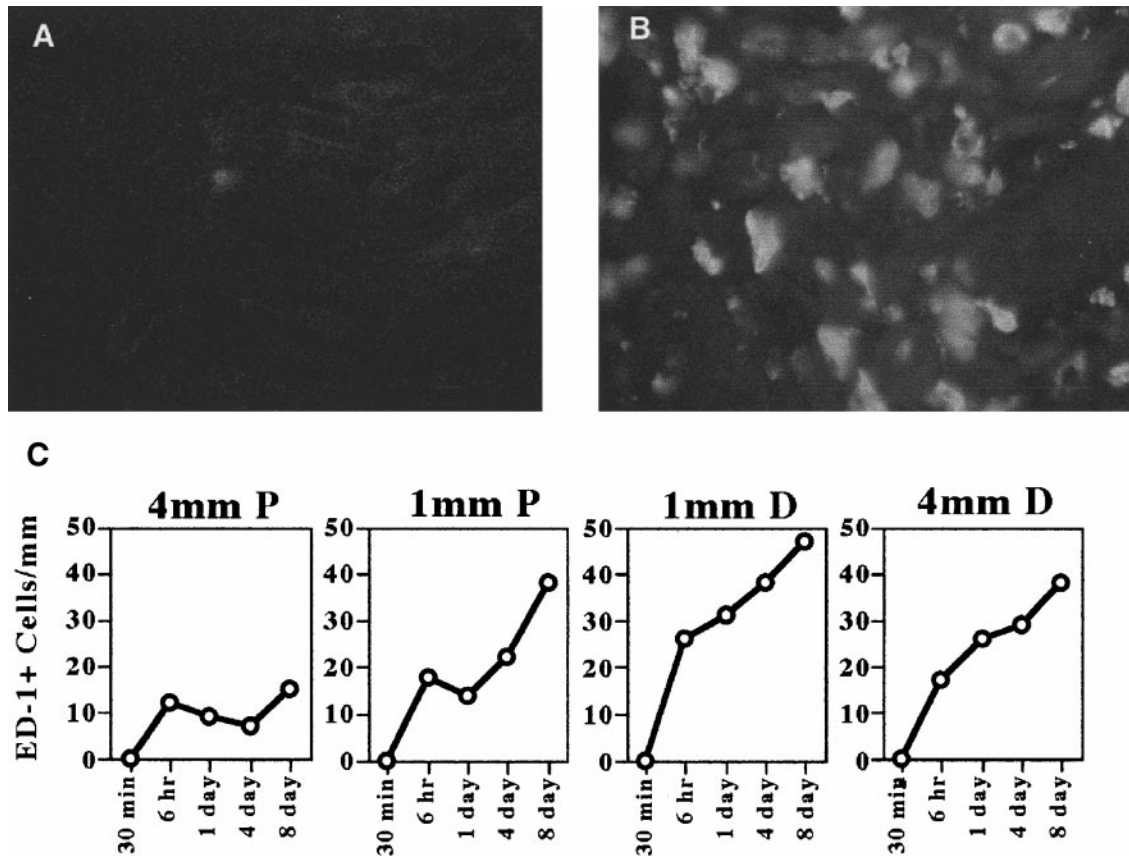


FIG. 5. Macrophage invasion into transected sciatic nerve. (A and B) Examples of ED-1 staining in sections of sciatic nerve. A, noninjured nerve shows no detectable ED-1 staining. B, section from 1 mm distal to the injury, taken 6 h after transection, shows increased presence of ED-1 labeled cells. (C) The number of ED-1-positive cells was estimated by counting labeled cells in three fields of view each ($160\times$ final magnification) at sites 1 and 4 mm proximal (P) and distal (D) to the lesion. Note that no ED-1-positive cells are seen at any location 30 min after injury, but that significant numbers of labeled cells are present at all sites 6 h after injury. In addition, the number of labeled cells continues to increase over the next week at all sites, except for the most proximal site analyzed.

4, 7, 13, 15, 18, 29, 31, 37). It is important to define which classes of factors may activate the signaling pathways described above. Phosphorylation that occurs within 30 min after injury likely is due to nonprotein stress signals or to growth factors and cytokines that are already present in the nerve prior to the lesion and do not require induction of gene expression. Normal adult peripheral nerves contain high levels of FGF1 and CNTF, which appear to be stored in cytoplasmic compartments of axons and Schwann cells, respectively (10, 11, 30, 36). Therefore, the rapid activation of ERK and STAT3 pathways at the site of lesion might be due to action of these factors which may be released after breach of the plasma membrane. In addition, the initial phase of ERK activation in far distal segments might be due to the release of FGF1 from degenerating axons. However, within a short time, disrupted plasma membranes will seal (40) or degeneration will proceed to completion; thus neither FGF1 nor CNTF is expected to be available in the extracellular space for prolonged time periods. On the

other hand, growth factors and cytokines induced by nerve injury are more likely to provide such sustained signals. In this study, the mRNA levels coding for a few of these factors (NGF, BDNF, NT3, GDNF, CNTF, IL-6, and IL-1 β) were quantified in order to test whether the regulation of expression of these potential signals correlated with the activation of corresponding intracellular signaling cascades. In the distal stump, the transient induction of IL-6 expression correlated well with the pattern of STAT3 phosphorylation, and the successive and overlapping induction of NGF, GDNF, and BDNF expression correlated well with the overall sustained level of ERK activation. Clearly, a number of additional factors, including LIF (7), may be involved in STAT3 activation, and neuregulin may be involved in ERK activation in the distal stump (4, 20). In the proximal stump, in contrast, the very moderate and transient induction of expression of the cytokines and growth factors assayed showed a dramatic lack of correlation with the intense phosphorylation of STAT3, SAPK, and ERK. Possible explanations are that the

precise factors and cytokines responsible for the phosphorylation of the signaling molecules (for example, LIF or neuregulin) were not included in the multiprobe RPA or that changes in cytokine protein levels are not reflected in the RPA measurement used here. It is also possible that the cytokines and growth factors responsible for the sustained intense activation of intracellular signaling pathways in the proximal stump are released by the tips of axons that attempt to regenerate. The mRNA coding for these axonal factors would be present only in the neuronal cell bodies and not in the nerve; thus the present study would not have detected them.

None of the phospho-specific antibodies used for the present study allowed the unambiguous immunohistochemical localization of phosphorylated intracellular signaling molecules. This prevents any firm identification of the cell type containing the activated molecules. Nevertheless, the absence of axons from the distal stump 48 h after the injury indicates that the high and sustained levels of ERK phosphorylation in the distal stump are not present within neuronal elements. In addition, the invasion of macrophages 4 mm distal to the injury is already significant at 6 h after the injury, when ERK phosphorylation is still low at this site. This suggests that the delayed phosphorylation of ERK in the distal stump occurs in Schwann cells where activation of the ERK pathway likely controls the expression of genes important for regeneration.

In summary, the present study suggests that a sustained activation of the ERK cascade is important for inducing and maintaining a regeneration-promoting environment in injured peripheral nerve. The sequential induction of expression and action of distinct growth factors may represent the mechanism that results in sustained ERK activation in this tissue. The precise molecular identity of these growth factors remains to be established, but we hypothesize that FGF1 acts immediately after the injury, while neurotrophins, GDNF, and neuregulins provide intermediate and sustained signals. In addition, growing axons themselves likely release or induce the expression of cytokines that activate the JAK/STAT or SAPK pathways. These cytokines might function to signal the arrival of regenerating axons in an injured nerve fragment.

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