

Absence of Persistent Spreading, Branching, and Adhesion in GAP-43-depleted Growth Cones

Ludwig Aigner and Pico Caroni

Friedrich Miescher Institute, CH-4002 Basel, Switzerland

Abstract. The growth-associated protein GAP-43 is a major protein kinase C substrate of growth cones and developing nerve terminals. In the growth cone, it accumulates near the plasma membrane, where it associates with the cortical cytoskeleton and membranes. The role of GAP-43 in neurite outgrowth is not yet clear, but recent findings suggest that it may be a crucial competence factor in this process.

To define the role of GAP-43 in growth cone activity, we have analyzed neurite outgrowth and growth cone activity in primary sensory neurons depleted of GAP-43 by a specific antisense oligonucleotide procedure. Under optimal culture conditions, but in the absence of GAP-43, growth cones adhered poorly, displayed highly dynamic but unstable lamellar

extensions, and were strikingly devoid of local f-actin concentrations. Upon stimulation, they failed to produce NGF-induced spreading or insulin-like growth factor-1-induced branching, whereas growth factor-induced phosphotyrosine immunoreactivity and acceleration of neurite elongation were not impaired. Unlike their GAP-43-expressing counterparts, they readily retracted when exposed to inhibitory central nervous system myelin-derived liposomes. Frequency and extent of induced retraction were attenuated by NGF. Our results indicate that GAP-43 can promote f-actin accumulation, evoked morphogenic activity, and resistance to retraction of the growth cone, suggesting that it may promote regulated neurite outgrowth during development and regeneration.

THE specific arrangement of neuronal processes and synapses that is essential to the functioning of the nervous system is established during development as the result of specific pathway selection by growing neurites. These are guided by soluble, cell-bound, and matrix-bound molecular cues. Translation of guidance cues into steering decisions is performed locally, at the level of the growth cone. Reflecting its central function in regulated neurite outgrowth, the growth cone is provided with abundant and dynamic cytosolic membrane pools, and with complex sets of signal transduction components. The mechanisms involved in regulated growth cone activity are only beginning to be clarified, but some central features have been established. Thus, an early response in growth cone steering is a local increase in the contents of f-actin towards the side of future growth (O'Connor and Bentley, 1993), suggesting that the actin-based cytoskeleton may be the initial structural substrate in this process (O'Connor and Bentley, 1993; Lin and Forscher, 1993). Microtubules and the vesicle-containing central domain of the growth cone then connect with the initial f-actin accumulation, leading to redirected neurite elongation. At the signaling level, local changes in free calcium concentration (Lankford and Letourneau, 1989; Davenport

and Kater, 1992; Rehder and Kater, 1992; Bedlack et al., 1992) and tyrosine phospho- and dephosphorylation reactions (Atashi et al., 1992; Williams et al., 1994) have emerged as a likely major mean of transducing signals from the local environment into growth cone activity.

A successful approach to identify growth cone proteins that play a specific role in neurite outgrowth has been to isolate fast axonal transport proteins whose reinduction in adult neurons coincides with regeneration after a lesion (Skene and Willard, 1981; Skene, 1989). The biological significance of this approach was strengthened by the observation that the induction of some of these proteins after peripheral lesion is apparently required to confer growth competence to the central projection of dorsal root ganglion (DRG) neurons (Richardson and Issa, 1984; Schreyer and Skene, 1991). Subsequent related studies on DRG, rubrospinal and retinal ganglion cell neurons have established that growth competence depends on cell body changes that include the induction of the growth-associated proteins GAP-43 and tubulin- α (Tezlaff et al., 1991; Schreyer and Skene, 1991; Doster et al., 1991). Actual growth, on the other hand, involves a second set of neuronal responses, including elevated β -tubu-

Address correspondence to P. Caroni, Friedrich Miescher Institute, P.O. Box 2543, CH-4002 Basel, Switzerland. Tel: (41) 61-697-6699. Fax: (41) 61-697-3976.

1. *Abbreviations used in this paper:* CNS, central nervous system; DRG, dorsal root ganglion; IGF-1, insulin-like growth factor-1; IR, immunoreactivity; PKC, protein kinase C; PORN, polyornithine; PNS, peripheral nervous system.

lin and actin expression, as well as increased slow axonal transport activity (McKerracher et al., 1993).

GAP-43 is a major growth cone and nerve terminal protein kinase C (PKC) substrate that is reversibly acylated and associates with the cortical cytoskeleton (Basi et al., 1987; Benowitz and Routtenberg, 1987; Skene, 1989; Skene and Virag, 1989; Liu and Storm, 1990; Meiri and Gordon-Weeks, 1990; Moss et al., 1990; Coggins and Zwiars, 1991; Strittmatter et al., 1992). Its role in neurite outgrowth is controversial. Thus, although the presence of this growth cone protein is not required for neurite growth per se (Baetge and Hammang, 1991; Aigner and Caroni, 1993), its overexpression potentiates the neuritic growth response of PC12 cells in the presence of NGF (Yankner et al., 1990), and the introduction of antibodies to GAP-43 in neuroblastoma cells (Shea et al., 1991) or of GAP-43 antisense oligonucleotides in PC12 cells (Jap Tjoen San et al., 1992) specifically impairs neurite outgrowth in these cells. Extending these previous studies, we recently reported that primary sensory neurons depleted of GAP-43 by an antisense oligonucleotide approach regenerate neurites on laminin/polyornithine, but they fail to form growth cones and extend neurites on a polyornithine-coated substratum (Aigner and Caroni, 1993). In addition, GAP-43-depleted neurites growing on laminin in the presence of NGF displayed dramatically diminished growth cone spreading (Aigner and Caroni, 1993). Given the fact that the growth cone must convert local signals into neurite steering, these findings suggested that GAP-43 may play an essential role in regulated growth cone activity.

To explore this possibility, we have now analyzed neurite outgrowth in the absence of GAP-43. As a specific test for a possible role of this protein in growth cone activity, we have determined whether the presence of GAP-43 affected the responses of primary sensory neuron growth cones to growth-promoting or retraction-inducing agents. We report that NGF-induced growth cone spreading and insulin-like growth factor-1 (IGF-1)-induced growth cone branching depend on the presence of GAP-43. In addition, in the presence of GAP-43, DRG neurites were effectively protected against neurite retraction induced by the addition of central nervous system myelin-derived proteins. Our findings indicate that regulated growth cone activity depends on the presence of GAP-43. At the mechanistic level, our observations suggest that GAP-43 plays a major role in stabilizing growth cone structures and promoting growth cone adhesion. Finally, in terms of biological function, these findings suggest that the reinduction of GAP-43 expression by neurons in the adult may promote axonal regeneration, both in the peripheral nervous system and possibly in the adult central nervous system of higher vertebrates.

Materials and Methods

Cell Culture and Oligonucleotides

The three different oligonucleotide pairs of chick GAP-43 cDNA described in a previous study (Aigner and Caroni, 1993) were used. Antisense oligonucleotide-1 (-8+9) consists of the sequence GCACAGCATGATCGTAT. Antisense oligonucleotide-2 (+9+26) has the sequence TTTGTTCTTCTCATACAG. Antisense oligonucleotide-3 (+643+659) consists of the sequence ACAGCATCTGCTTCTC. The corresponding sense oligonucleotides were the inverse complements of the antisense oligonucleotides. The oligonucleotides were synthesized on a synthesizer (380B; Applied Biosystems, Inc., Foster City, CA), ethanol precipitated, desalted over a

column (NAP™-25; Pharmacia LKB Biotechnology AB, Uppsala, Sweden), and dissolved in glass-distilled water to a concentration of 10 $\mu\text{g}/\mu\text{l}$.

The trituration method to introduce oligonucleotides into DRG neurons was as described in a previous study (Aigner and Caroni, 1993). Briefly, two to four chick lumbar embryonic day 16 DRGs were trypsinized for 35 min at 37°C, washed with DME/10%FCS and with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's buffer, and dissociated by trituration (20–25 cycles through a yellow tip [Gilson Medical Elec., Inc., Middleton, WI]) in 50 μl of 200 mM oligonucleotides in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's buffer. Neurons were then preplated for 2 h in 2 ml DME/10%FCS (37°C/5% CO_2) in a 35-mm tissue culture dish. Nonadherent cells were collected, pelleted for 5 min at 120 g, and resuspended in the presence of 20 ng/ml 2.5 S NGF (Harlan Bioproducts for Science, Inc., Indianapolis, IN), in either DME/10%FCS, or, when video time-lapse microscopy was performed, in L15/10%FCS. Cells were plated on coated glass coverslips (18 mm) at a density of 500–1000 cells per coverslip, and grown in a humidified incubator at 37°C/5% CO_2 (DME/10%FCS) or at 37°C (L15/10%FCS). Coverslips were coated with 200 μl poly-L-ornithine (Sigma Immunochemicals, St. Louis, MO) (0.25 $\mu\text{g}/\text{ml}$ in 50 mM borate buffer, pH 8) and with 200 μl laminin (Collaborative Biomedical Products, Bedford, MA) (17.5 $\mu\text{g}/\text{ml}$ in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS) as described (Aigner and Caroni, 1993). IGF-1 (LONG-R3-IGF-1) was purchased from Gropep Pty. Ltd. (Sterling, VA).

Preparation of Liposomes Containing Myelin Proteins

Myelin-derived liposomes were prepared from adult rat spinal cord (CNS myelin) or sciatic nerve (PNS myelin), as described (Caroni and Schwab, 1988). Myelin membrane proteins were extracted with chloroform/methanol 2:1, dried, and resuspended at 1 mg/ml in 100 mM sucrose, 30 mM HEPES/Tris, pH 7.4, and 2.5% cholate. Liposomes were formed from phosphatidylcholine/phosphatidylserine 10:1 (Sigmas) by passage of the cholate-dissolved phospholipid/myelin mixture on a Sephadex G-50 column. Liposomes containing PNS myelin proteins were used for control experiments. In further control experiments, CNS myelin inhibitors were inactivated with trypsin; after a 10-min digestion step at room temperature with 0.1% trypsin (Worthington Biochemical Corp., Freehold, NJ), 0.2% trypsin inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added, and liposomes were subsequently washed free of protease with 100 mM sucrose, 30 mM HEPES/Tris, pH 7.4.

Time-Lapse Video Microscopy

For the observation of living neurites and growth cones, neurons were grown on coated glass coverslips fitted to a corresponding hole in the bottom of 35-mm tissue culture dishes. Cells in 3 ml L15/10%FCS were monitored with a microscope (Axiovert-10; Carl Zeiss, Inc., Thornwood, NY) with a 40 \times Neofluar phase objective on a heating plate (TRZ 3700; Carl Zeiss) at a constant temperature of 37°C. Images were taken either with a video graphic printer UP-860CE (Sony, Tokyo, Japan) connected to a S/W-CCD camera (AVT-BC2; AVT-Horn, Aalen, Germany), or with a Pentax LX camera (Asahi Optical Co., Tokyo, Japan). Time-lapse imaging was obtained with IMAGE-1 software (Universal Imaging Corp., West Chester, PA), a microscope (IM35; Carl Zeiss), a 40 \times Neofluar phase objective, a C2400 video camera (Hamamatsu Photonic Sys. Corp., Bridgewater, NJ), a computer-driven shutter system, and an optical disc recorder TQ-2028F (Panasonic; Matsushita, Osaka, Japan). Images were collected every 20 s during a period of two to several hours.

Immunocytochemistry

Cells were fixed for 30 min with prewarmed (37°C) 4% paraformaldehyde in phosphate buffer (0.1 M sodium phosphate, pH 7, 50 mM sucrose, 0.4 mM CaCl_2), and antibody incubations were performed at room temperature in PBS with 0.1% BSA, 1% FCS, 50 mM glycine, 0.02% NaN_3 , and 0.2% saponin (Burry et al., 1991). Chick GAP-43 was either visualized with the specific mAb 5F10 (1:1000 dilution of ascites fluid; Widmer and Caroni, 1990) or with a specific rabbit polyclonal antiserum against the synthetic COOH-terminal peptide CEEESKADQENA of chick GAP-43 (1:1000 dilution; Aigner and Caroni, 1993). A polyclonal rabbit antiserum against chicken gizzard integrin that specifically detects $\beta 1$ -integrin (1:300 dilution; Hofer et al., 1990) was a kind gift of Dr. R. Chiquet-Ehrismann (Friedrich Miescher Institute, Basel, Switzerland). Phosphotyrosine was detected with the mouse monoclonal 4G10 antibody (2 $\mu\text{g}/\text{ml}$) (Upstate Biotechnology, Inc., Lake Placid, NY). Washes were in PBS. Secondary antibodies were biotinylated goat anti-mouse (4 $\mu\text{g}/\text{ml}$), rhodamine-labeled goat anti-mouse (4 $\mu\text{g}/\text{ml}$), biotinylated goat anti-rabbit (13 $\mu\text{g}/\text{ml}$), and rhodamine-

labeled goat anti-rabbit (4 $\mu\text{g/ml}$). Biotinylated antibodies were visualized by lucifer yellow-coupled streptavidin (3.5 $\mu\text{g/ml}$). Secondary antibodies, lucifer yellow-coupled streptavidin and RITC-phalloidin, were from Molecular Probes, Inc. (Eugene, OR.) Unless indicated otherwise, GAP-43 was detected with streptavidin-lucifer yellow. Stained cultures were mounted in Gelvatol (Monsanto Chemical Co., St. Louis, MO), and fluorescence was detected with an Axiocvert-10 microscope (Carl Zeiss) equipped with the appropriate filters (lucifer yellow: excitation = 395–440 nm, emission = 460 nm; RITC: excitation = 510–560 nm, emission = 580 nm).

Analysis of Data

Growth cone sizes (area distal from the neck of the growth cone) were measured using NIH Image 1.41 software fed with a photo video camera (PHVA7E; Sony). Neurite length was determined by measuring the length of a cell's longest neurite from the soma to the tip. Branching extent was defined as the ratio between the number of branchpoints of a cell's longest neurite and its length. For statistical analysis, the Mann-Whitney U test was applied.

For classification according to GAP-43 immunoreactivity (IR) levels, cells were distributed into three categories, as described previously (Aigner and Caroni, 1993). No GAP-43 immunoreactivity (GAP-43 IR) is a stringent criterium, and corresponds to the background level of fluorescence of nonneuronal cells present in the culture. The assignment of a cell to strong or intermediate GAP-43 IR was controlled by monitoring with an S/W CCD camera (AVT-Horn) and a television (KV-M1430B; Sony): a fixed brightness level was selected to include $\sim 40\%$ of the GAP-43-expressing cells in mock-treated cultures, and cells with GAP-43 signal not detected on the screen at that brightness level were attributed to the intermediate GAP-43 IR category.

For the analysis of f-actin distribution in growth cones and neurites, photographs of RITC-phalloidin-labeled cultures were analyzed with NIH Image 1.41 software, as described above. Growth cone values (arbitrary units \pm SEM; $n = 70$) were defined as the highest pixel value in the growth cone region; neurite values were defined similarly (first 30 μm behind the growth cone). Analysis of phosphotyrosine immunoreactivity in the growth cone was performed on photographs of antibody-labeled cultures, as described above. In this case, however, mean growth cone pixel values are given in the figure (averages \pm SEM; $n = 70$).

Results

Growth Cones of Cultured DRG Neurons Depleted of GAP-43 Adhere Poorly, and Have Shranked Central Domains and Highly Dynamic Peripheral Lamellae

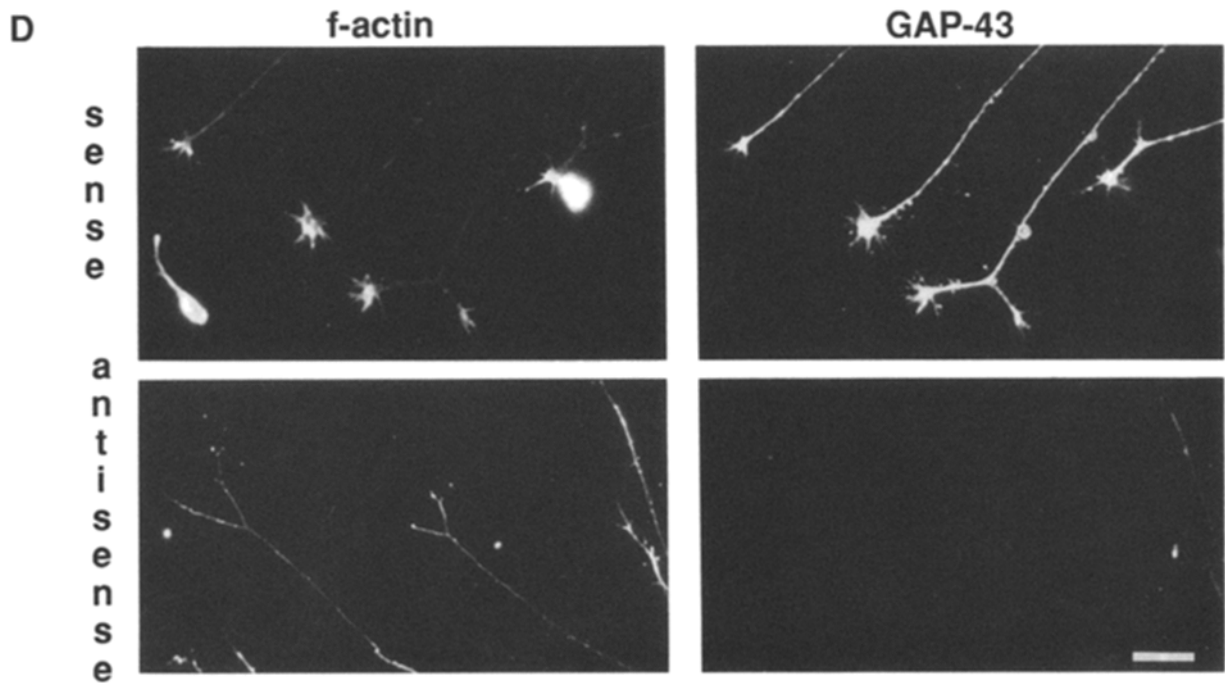
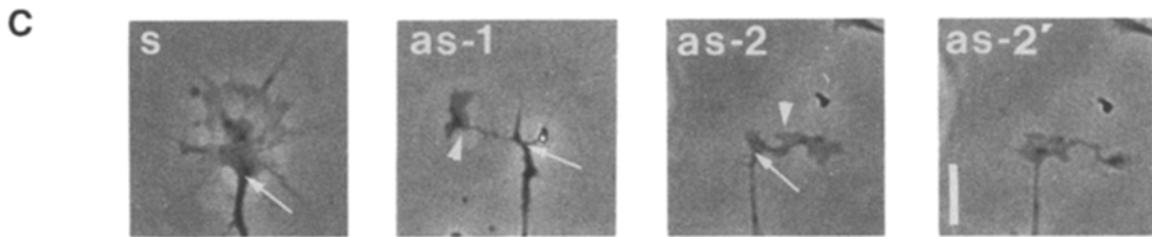
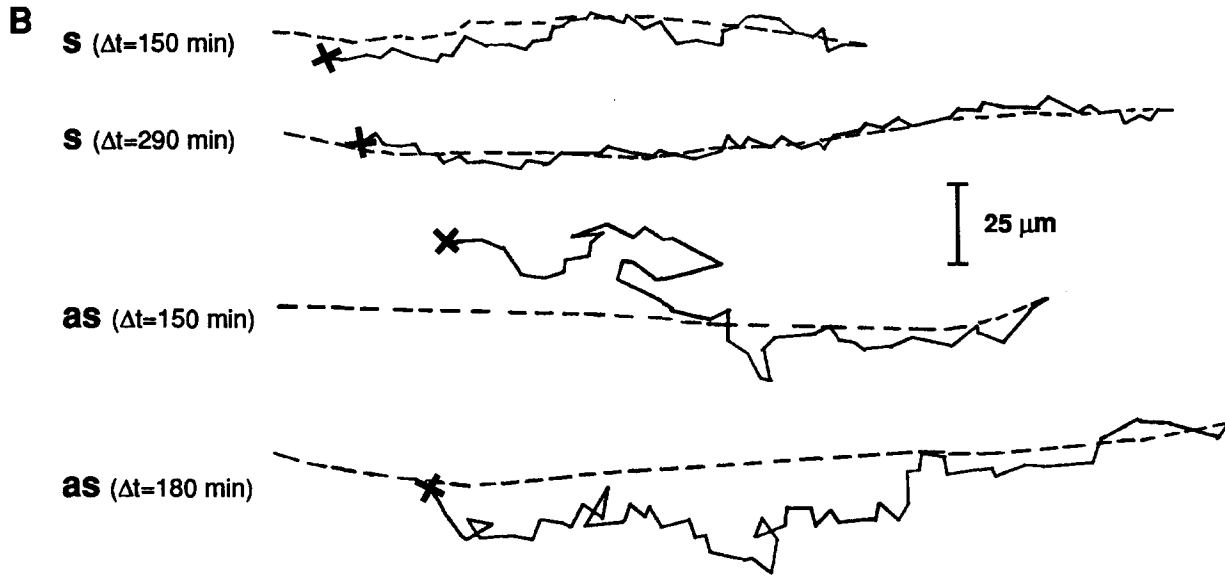
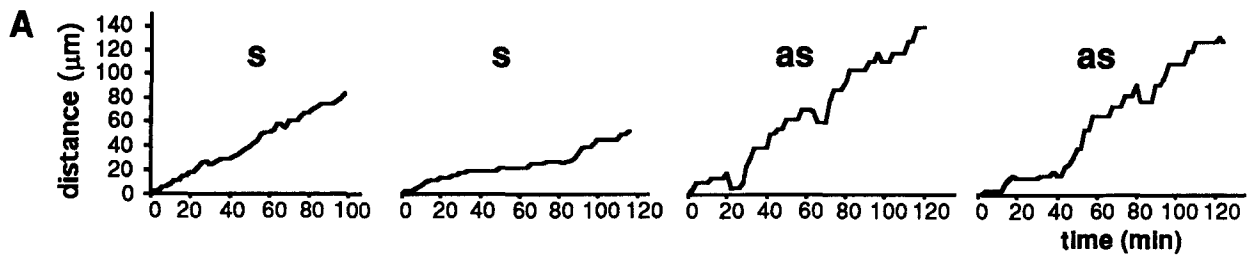
To assess the role of GAP-43 in growth cone activity and neurite outgrowth, we analyzed neurite regeneration by cultured DRG neurons in the presence or absence of GAP-43. Embryonic day 15–17 chick DRGs were triturated in the presence of sense or antisense oligonucleotides to GAP-43 mRNA, preplated on tissue culture plastic for 2 h in the absence of NGF, and finally plated on a polyornithine (PORN)/laminin-coated substratum in the presence of 2 ng/ml of NGF. As demonstrated in a previous study, this antisense oligonucleotide procedure specifically depletes $\sim 50\%$ of the neurons of any detectable GAP-43 immunoreactivity (Aigner and Caroni, 1993). That study also demonstrated that the antisense procedure only altered the relative contents of GAP-43-containing and GAP-43-depleted neurites in the culture, and that the properties of single neurites and growth cones specifically correlated with their GAP-43 contents, not with the particular type of pretreatment (Aigner and Caroni, 1993). The same specific association with GAP-43 contents was found for the growth cone properties analyzed in this study. For consistency, however, a comparison of sense- and antisense-treated neurons is presented throughout this study. When neurite outgrowth was monitored by time-lapse video microscopy, characteristic qualitative differences were con-

sistently detected between GAP-43-containing and -depleted neurites and growth cones. As shown in the graphs of Fig. 1 A, while GAP-43-containing neurites typically elongated at constant speed and in a steady manner, GAP-43-depleted neurites went through repeated rounds of rapid elongation, followed by phases of no elongation or partial retraction. The frequent retraction events suggested that in the absence of GAP-43, these DRG neurites adhered poorly to the PORN/laminin-coated substratum. This interpretation was also supported by the observation that the growth cones of GAP-43-depleted neurites frequently detached from the substratum, leading to lateral gliding of the corresponding neurite (traces of Fig. 1 B). Analysis of growth cone morphology and activity revealed striking differences between GAP-43-containing and -depleted neurites. In the presence of GAP-43, most growth cones displayed well-spread phase-dense central domains. In addition, the phase-bright peripheral domain usually formed a radially continuous and regular extension of the central domain (Fig. 1 C). In contrast, in the absence of GAP-43, central domains were extremely small, and peripheral lamellae were fragmented and frequently branched (Fig. 1 C, *arrowheads*). In the absence of GAP-43, the dynamic behavior of the peripheral growth cone domain was markedly accelerated, and its ability to form transiently spread lamellar structures was apparently not impaired. Characteristically, shortly after their emergence from the base of the growth cone, peripheral lamellae elongated very rapidly and in an apparently unrestrained manner, leading to the formation of multiple long growth cone branches (Fig. 1 C). In most cases, these branches were short-lived and collapsed within minutes. These observations indicate that basic aspects of growth cone activity are affected by the absence of GAP-43. These include a major reduction in spreading of the phase-dense base of the growth cone, as well as unrestrained extension coupled to dynamic instability of the peripheral lamellae.

In addition to the altered morphological and growth properties discussed above, GAP-43-depleted growth cones displayed a striking absence of peripheral f-actin concentrations (Figs. 1 D and 2). Thus, while staining with RITC-phalloidin revealed the characteristic accumulation of f-actin at the periphery of GAP-43-containing growth cones (see also Forscher and Smith, 1988; Letourneau and Shattuck, 1989), a much more homogeneous distribution of the f-actin signal was detected in GAP-43-depleted neurites (Fig. 2). A quantitative analysis revealed that the intensity of f-actin staining in the neurites of any given culture varied considerably and did not depend on the presence of GAP-43 (Fig. 2). In contrast, GAP-43-depleted neurites consistently lacked peripheral accumulations of f-actin (Fig. 2). Therefore, the absence of GAP-43 is associated with major alterations in the distribution of the actin-based cytoskeleton in growing neurites.

NGF Induces Rapid and Reversible Spreading of DRG Growth Cones on Laminin in the Presence but Not in the Absence of GAP-43

The findings described in the previous section indicate that in the absence of GAP-43 growth cone adhesion and central domain spreading are impaired, but the dynamic behavior of the growth cone's peripheral lamellae is potentiated. These observations suggested that in spite of their highly dynamic properties, GAP-43-depleted growth cones may fail to carry



out sustained evoked morphogenic reactions. We therefore searched for a means to induce a relevant and predictable growth response in our culture system. To this end, we exploited the fact that NGF has neurotropic activity *in vitro*, indicating that it can affect local growth cone activity (Phelan et al., 1992). In addition, >50% of the neurons in our cultures displayed stimulated neurite outgrowth *in vitro* in the presence of low concentrations of NGF (data not shown). Therefore, we analyzed growth cone responses to NGF in the presence and absence of GAP-43. After trituration and preplating, DRG neurons were allowed to regenerate neurites for 4 h on a laminin-coated substratum in the presence of 20 ng/ml of NGF, and then in the absence of NGF for the next 2 h. Growth cones were then monitored before and after the readdition of 2 ng/ml of NGF to the culture medium.

As shown in Fig. 3, NGF dramatically stimulated growth cone spreading in GAP-43-containing, but not in GAP-43-depleted neurons. This response to NGF was maximal after ~30 min, but was already prominent 15 min after addition of the neurotrophin (Fig. 3). The reaction was reversible in that growth cones reverted to the initial type of lamellae-poor configuration within 30–60 min after removal of NGF, and could be reinduced to spread upon its readdition (data not shown). While most GAP-43-containing growth cones spread in response to NGF, this reaction was never detected in GAP-43-depleted neurons (Figs. 3 and 4). In addition, upon addition of NGF, growth cones with high levels of GAP-43 immunoreactivity spread more extensively than those with intermediate levels of this growth-associated protein (Fig. 4). The data in Fig. 4 were deduced from time-lapse recordings, but similar conclusions were reached when growth cone sizes were determined in cultures fixed before and 60 min after the addition of NGF: in the presence of GAP-43, growth cones >250 μm were 24.4% of total ($n = 300$) without, and 73.8% with the addition of NGF; without GAP-43, the corresponding contents were 5.6 and 5.7%. Significantly, GAP-43-depleted neurites were still capable of responding to NGF since a clear increase in the rate of neurite elongation could be detected in these neurons upon addition of the neurotrophin. Thus, the mean elongation rates of GAP-43-depleted neurites were $24.3 \pm 2.92 \mu\text{m}/\text{h}$ before NGF addition, and $43.6 \pm 2.67 \mu\text{m}/\text{h}$ after NGF addition ($n = 24$; $P < 0.0005$, paired Mann-Whitney U test); for comparison, the corresponding rates in the presence of GAP-43 were $21.7 \pm 2.30 \mu\text{m}/\text{h}$ before NGF addition, and $33.5 \pm 2.38 \mu\text{m}/\text{h}$ after NGF addition ($n = 25$; $P < 0.005$). Similar conclusions were reached when growth cone responses to

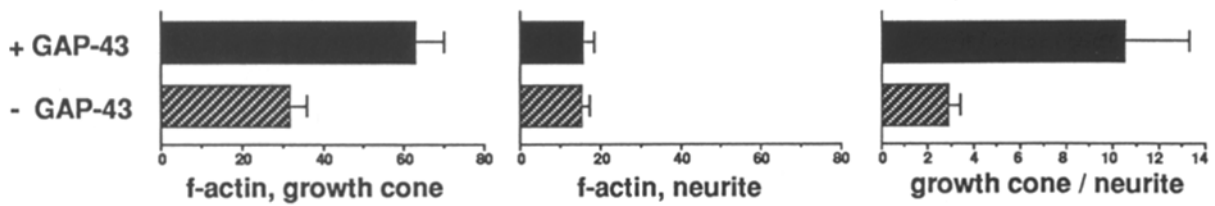
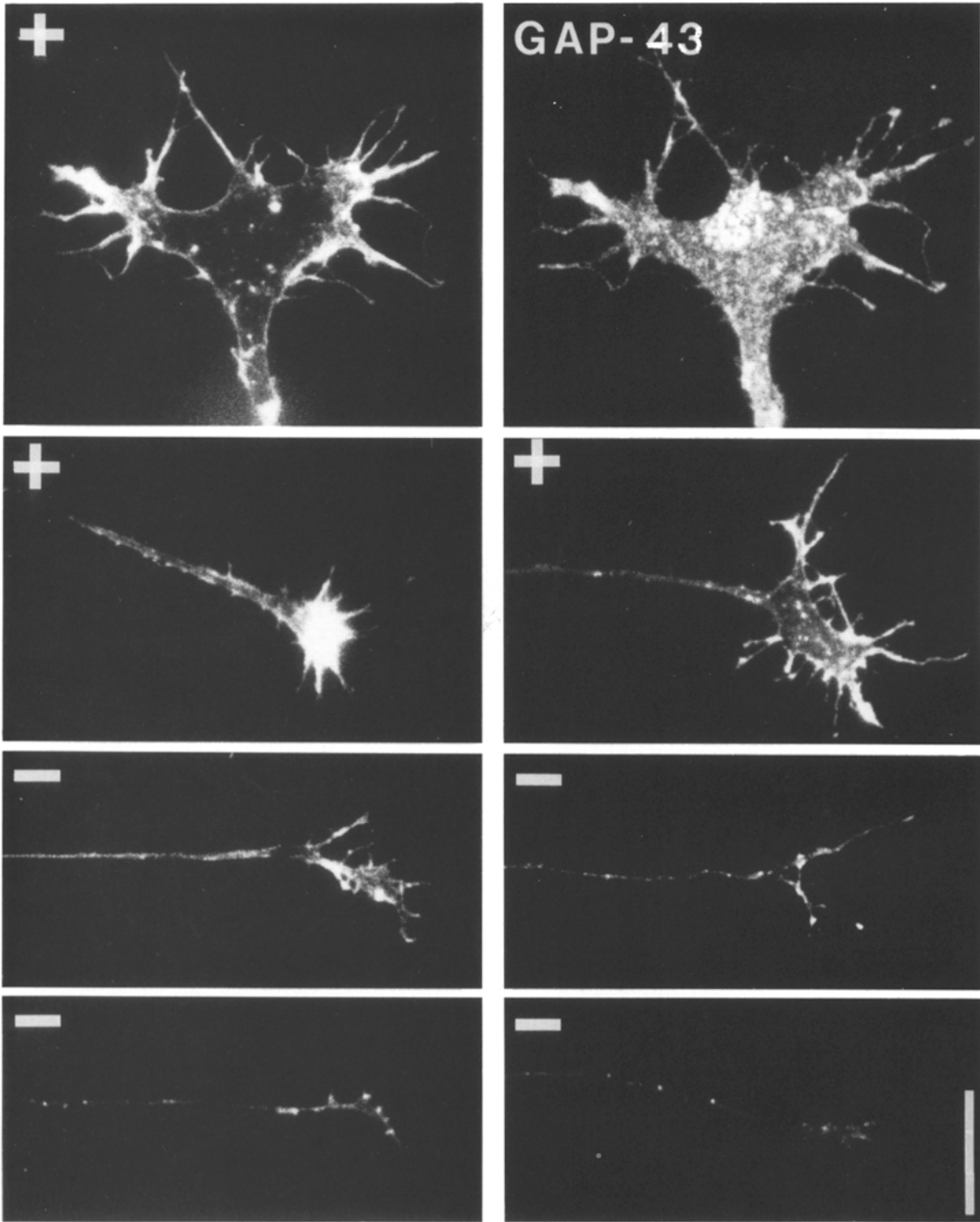
NGF were analyzed using time-lapse recordings. The most striking reaction of GAP-43-containing growth cones to NGF was a large increase in the spreading area of the phase-dense central domain (data not shown). This reaction was accompanied by accelerated central domain and neurite elongation. In contrast, essentially no NGF-induced central domain spreading and no obvious increase in frequency, speed, and size of peripheral domain protrusions were detected in GAP-43-depleted neurons after the addition of NGF. Rather, NGF appeared to promote the stability and retention of phase-bright lamellar structures in these neurons, leading to more steady net elongation. In summary, an evoked growth cone response, spreading in response to NGF, depends on the presence and levels of GAP-43 in the growth cone. In addition, these results indicate that the presence of GAP-43 is required for a specific aspect of NGF-induced growth cone activity, i.e., spreading, but not for NGF-induced acceleration of neurite elongation.

IGF-1 Induces Growth Cone Branching and Filopodia Formation in the Presence but not in the Absence of GAP-43

To assess the range of growth cone responses that depend on the presence of GAP-43, we searched for factors that would induce a distinct type of growth cone reaction in these acute stimulation experiments. One such factor is IGF-1. This growth factor is expressed in the nervous system, and it has neurite-promoting activity for a variety of neurons, including those from DRGs *in vitro* (Recio-Pinto et al., 1986), and presumably also *in vivo* (Near et al., 1992). Like NGF, IGF-1 activates a specific high-affinity receptor with tyrosine kinase activity. We therefore characterized the responses of DRG growth cones and neurites to IGF-1, and determined whether these reactions were affected by the presence of GAP-43.

To allow for a direct comparison between the experiments with IGF-1 and NGF, oligonucleotide-treated DRG neurons were precultured in the presence and then in the absence of NGF, as described above. As shown in Fig. 5 A, and in contrast to the response to NGF, when 2 ng/ml of IGF-1 were added to the NGF-depleted culture medium, growth cones did not spread, but they branched extensively and elaborated long filopodia. Branching in the presence of IGF-1 was a sustained phenomenon and led to the growth of numerous long side branches over the course of several hours, as shown in Fig. 5 B. Significantly, however, and like in the case of NGF,

Figure 1. Comparison of neurite outgrowth in the presence and absence of GAP-43. Oligonucleotide-treated chick sensory neurons were plated onto a PORN/laminin-coated substratum in the presence of NGF. Data are from 4–6-h cultures. (A) Rapid but discontinuous elongation of neurites in the absence of GAP-43. Graphs show two representative examples each of neurites growing in the presence (*s*, sense) or absence of GAP-43 (*as*, antisense). (B) Growth tracks of representative GAP-43-containing (*s*) and -depleted (*as*) growth cones. The position of the base of the growth cone (*C*, arrow) was plotted during the indicated time period. The dashed lines indicate the actual outline of the corresponding neurite at the end of the plot. Crosses indicate the position of the growth cones at the beginning of the analysis. Note sideways deviations and absence of corresponding persistent neurite attachment in antisense neurons. (C) Representative examples of GAP-43-containing (*s*) and GAP-43-depleted (*as-1*, *as-2*, *2'*) living growth cones (phase contrast optics). *as-2'*, same growth cone as in *as-2* 10 s later. Note well-spread phase-dense central domain in the presence but not in the absence of GAP-43. Also note irregularly shaped peripheral lamellae in GAP-43-depleted growth cones (arrowheads). The arrows point to the transition zone between neurite and growth cone (base of the growth cone). Bar, 10 μm . (D) Absence of f-actin polarization in GAP-43-depleted neurites. Double labeling of 4-h cultures for f-actin (RITC-phalloidin) and GAP-43. GAP-43-depleted neurites consistently displayed an homogenous distribution of f-actin signal, whereas most GAP-43-containing neurites showed marked accumulation of f-actin at the distal end of the growth cone and weak f-actin signal along neurites, as described in previous studies. Bar, 20 μm .



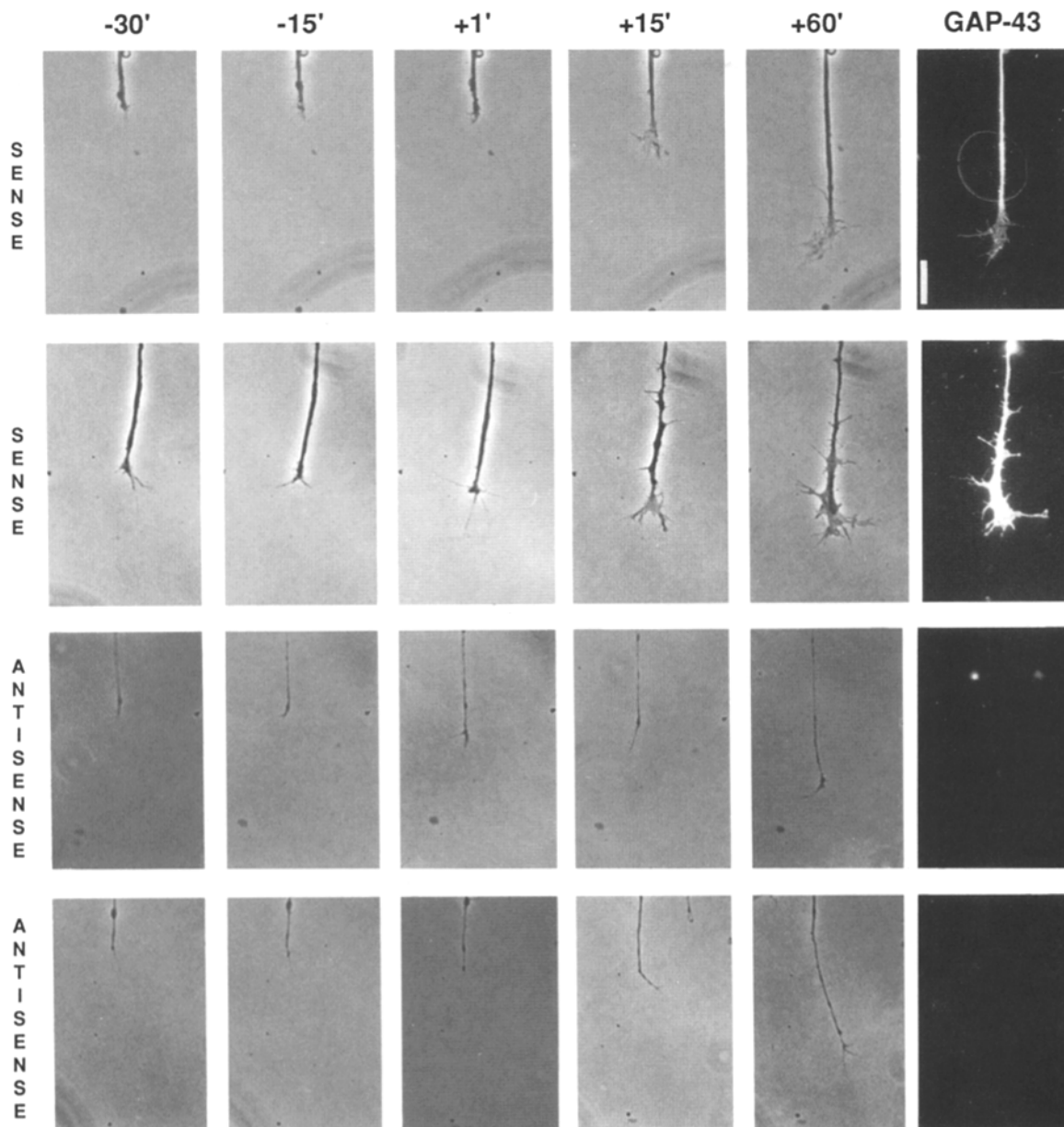


Figure 3. NGF induces growth cone spreading in the presence but not in the absence of GAP-43. Neurons were first grown for 4 h in the presence of 20 ng/ml NGF, and then for 2 h in its absence. At time 0, 2 ng/ml of NGF were added to the culture medium. The behavior of identified growth cones was monitored before and after the readdition of NGF to the culture medium. Two representative examples of GAP-43-expressing and -depleted growth cones are shown in the figure. Immediately after the last recording, the same growth cones were fixed and stained for GAP-43. Bar, 20 μ m.

these reactions of DRG growth cones and neurites to IGF-1 were not detected when the neurons were depleted of GAP-43 (Fig. 5, *A* and *B*; see legend to Fig. 5 for a quantitative analysis of neurite branching data). In contrast, stimulation of neurite elongation by IGF-1 was not impaired in the ab-

sence of GAP-43; in 12-h experiments (last 6 h with or without IGF-1), neurite lengths (micrometers) with GAP-43 were 225 ± 11 (without IGF-1) and 309 ± 16 (with IGF-1), $n = 300$ each, $P < 0.0005$; corresponding values without GAP-43 were 341 ± 10 (no IGF-1) and 385 ± 9 (with IGF-1),

Figure 2. Diminished accumulation of f-actin in GAP-43-depleted growth cones. Experimental conditions as described in Fig. 1 *D*. RITC-phalloidin signals for representative GAP-43-containing (+) and -depleted (-) growth cones are shown in the figure. (*Top right*) GAP-43 immunoreactivity for growth cone shown in top left panel. A quantitative analysis of f-actin distribution in growth cones and neurites is shown in the bottom part of the figure (see also Materials and Methods). The growth cone/neurite values are averages of the ratios for single neurites. They reflect the average degree of polarization of the f-actin signal between neurite and growth cone. The analysis demonstrates that f-actin accumulation in the growth cone is specifically impaired in the GAP-43-depleted neurites. Bar, 15 μ m.

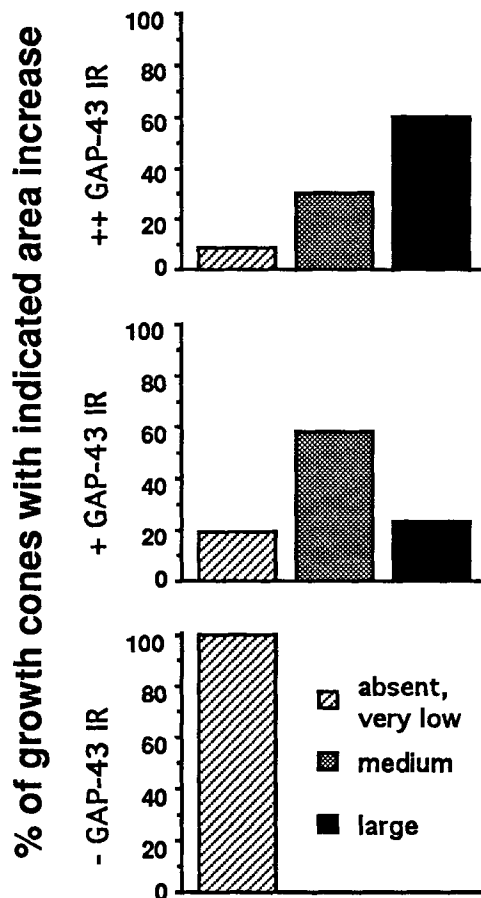


Figure 4. Quantitative analysis of NGF-induced growth cone spreading and its dependence on growth cone GAP-43 immunoreactivity. Experiments were performed as described in Fig. 3. Growth cone sizes of identified neurons were determined shortly before and 1 h after the readdition of NGF. Cultures were then immediately fixed and stained for GAP-43 immunoreactivity. Increases in growth cone area were defined as follows: absent; very low, $0 < x < 10 \mu\text{m}^2$; medium, $10 < x < 150 \mu\text{m}^2$; large, $x > 150 \mu\text{m}^2$. For these experiments, the number of analyzed neurons was 33 (++) , 31 (+), and 60 (-). In parallel experiments, final growth cone sizes 1 h after the readdition of NGF were determined for a larger sample of neurons. The resulting data were comparable to those presented in the figure, and the results of the analysis are given in the text.

$n = 300$ each, $P < 0.005$. Therefore, in further analogy to the responses to NGF, GAP-43-depleted neurites did respond with accelerated elongation to the addition of IGF-1, indicating that they did not lack the capacity to detect and transduce IGF-1-mediated signals. In conclusion, the presence of GAP-43 is not only required for NGF-induced growth cone spreading, but also for IGF-1-induced growth cone branching and stimulated filopodia formation.

Biochemical evidence indicating that GAP-43-depleted neurons express functional NGF and IGF-1 receptors at the level of the neurite and growth cone is provided by the experiments shown in Fig. 6. GAP-43-containing and -depleted DRG neurons were treated as described above, and were fixed 5 min after the addition of either NGF, IGF-1, or medium alone. Activation of tyrosine kinase activity was then detected indirectly by incubating the neurons with a

monoclonal antibody that specifically binds to phosphorylated tyrosine. Representative examples of such labeling experiments are shown in the figure. As expected, growth factor-treated growth cones displayed elevated phosphotyrosine immunoreactivity. Significantly, this response could be detected in both GAP-43-containing and GAP-43-depleted neurites and growth cones. A quantitative analysis of growth cone phosphotyrosine immunoreactivity in these experiments yielded the following values (with or without GAP-43; arbitrary units): $48.3 \pm 7.6/45.7 \pm 6.7$ (no growth factor added); $163.2 \pm 11.5/148.5 \pm 10.9$ (with NGF); $156.4 \pm 10.8/146.2 \pm 10.5$ (with IGF-1). Therefore, these findings are consistent with the neurite elongation data, and they indicate that the presence of GAP-43 in neurites and growth cones is not necessary for general transduction of signals induced by NGF or IGF-1. Rather, the presence of GAP-43 is necessary for at least two specific morphogenic reactions of the growth cone, and possibly also of the neurite, i.e. spreading in response to the addition of NGF, as well as branching and formation of filopodia in the presence of IGF-1.

GAP-43-depleted Neurites Are Highly Susceptible to Neurite Retraction Induced by CNS Myelin-derived Proteins

In addition to growth-promoting signals, growth cones respond to agents that prevent neurite outgrowth. Characteristic *in vitro* reactions to such inhibitory agents include growth arrest, growth cone collapse, and neurite retraction (Bandtlow et al., 1990). GAP-43 is not reinduced when a myelinated CNS nerve is lesioned at a distance of $>1-2$ mm from the corresponding neuronal cell bodies (Doster et al., 1991). It was, therefore, of particular interest to determine the sensitivity of GAP-43-depleted growth cones and neurites to the arrest- and retraction-inducing activity of inhibitors of neurite outgrowth specifically associated with oligodendrocytes and central myelin (Caroni and Schwab, 1988).

For these experiments, oligonucleotide-treated DRG neurons were precultured as described above, except that for some experiments, the NGF was not removed from the culture medium. GAP-43-containing and -depleted neurites were monitored before and after the addition of CNS myelin protein-derived liposomes (Caroni and Schwab, 1988). In agreement with published data (Bandtlow et al., 1990, 1993), and as shown in Fig. 7, when exposed to comparatively high amounts of inhibitor-containing liposomes, DRG growth cones collapsed. Under these experimental conditions, however, DRG neurites with high contents of GAP-43 immunoreactivity did not retract (Fig. 7, mean retraction length ($t = 20$ min): $-0.8 \pm 1.17 \mu\text{m}$, $n = 35$). In addition, when treated with fivefold lower amounts of inhibitory liposomes, GAP-43-containing neurites ceased to grow, but the growth cones failed to collapse completely (Fig. 7, *second row*). In marked contrast, GAP-43-depleted neurites consistently displayed neurite retraction, even in the presence of the lower dose of CNS myelin-derived liposomes (Fig. 7, mean retraction length ($t = 20$ min): $16.8 \pm 2.37 \mu\text{m}$, $n = 36$). Interestingly, the susceptibility of GAP-43-depleted neurons to neurite retraction was reduced when NGF was added to the culture medium (Fig. 7, *third and fourth rows vs fifth and sixth rows*; mean retraction length with NGF ($t = 20$ min): $3.7 \pm 1.15 \mu\text{m}$, $n = 39$). On the other hand, in analogous experiments, essentially no retraction was de-

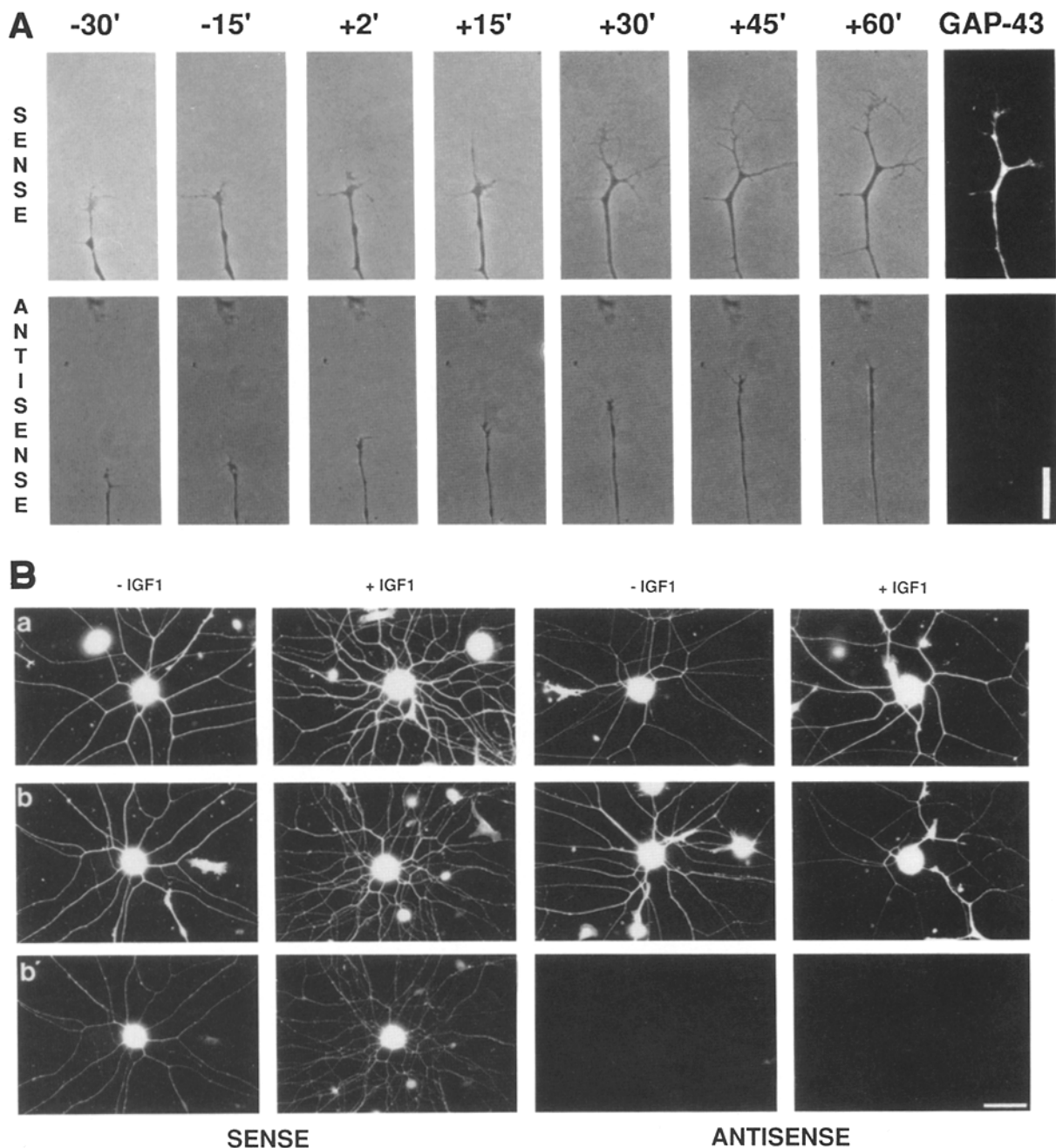


Figure 5. IGF-1 induces growth cone and neurite branching in the presence but not in the absence of GAP-43. (A) Time course of IGF-1-induced growth cone branching. Experimental conditions were like those of Fig. 3, except that 2 ng/ml of IGF-1, instead of NGF, was added to the NGF-depleted culture medium at $t = 0$ min. Note branching and filopodia formation upon addition of IGF-1 in the GAP-43-containing but not in the -depleted growth cone. Neurons were fixed and stained for GAP-43 immediately after the last recording. Bar, 20 μ m. (B) Experimental conditions as in Fig. 5 A, but starting from $t = 0$ min, parallel cultures were maintained for 6 h, either in the presence or in the absence of 2 ng/ml of IGF-1. Cultures were then fixed and processed for immunocytochemistry. Neurons were double-labeled for β 1-integrin (a and b) and GAP-43 (b'), and the corresponding RITC and lucifer yellow channel photographs are shown in the figure. Counting the number of branchpoints per 100 μ m of main neurite (see Materials and Methods) yielded the following values: with GAP-43, 1.14 ± 0.05 (without IGF-1) and 1.50 ± 0.07 (with IGF-1), $n = 300$ each, $P < 0.0005$; without GAP-43, 1.04 ± 0.05 (without IGF-1) and 0.97 ± 0.05 (with IGF-1), $n = 300$ each, not significant. Bar, 50 μ m.

tected when GAP-43-containing neurons were exposed to the higher dose of liposomes in the presence or in the absence of NGF. From these results, we conclude that in the absence of GAP-43, DRG neurites growing on a PORN/laminin-coated substratum are particularly susceptible to the inhibitory activity in CNS myelin-derived protein frac-

tions. In addition, this susceptibility is further enhanced if NGF is removed from the culture medium. This latter finding is further evidence that depletion of GAP-43 does not suppress all local effects of NGF on growth cones and neurites. In addition, it suggests that NGF and possibly also other neurotrophic factors may have a protective effect

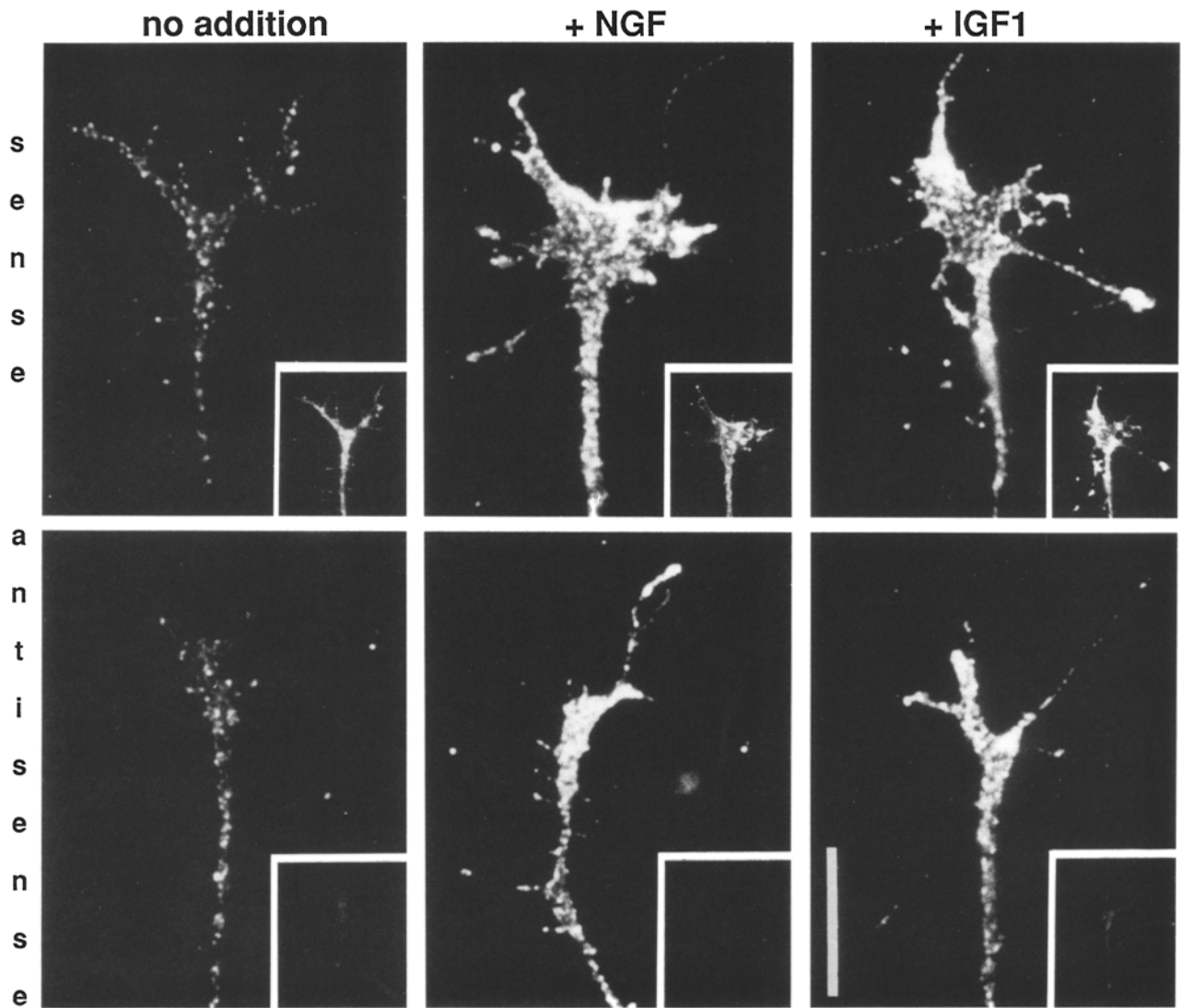


Figure 6. Absence of GAP-43 does not prevent elevation of phosphotyrosine immunoreactivity upon addition of NGF or IGF-1. Representative examples of GAP-43-containing and -depleted growth cones before and after the addition of NGF or IGF-1 are shown in the figure. Experimental conditions were like those of Fig. 3 (NGF) or Fig. 5 (IGF-1). Cultures were fixed 5 min after growth factor readdition and were then processed for immunocytochemistry. Double labeling for phosphotyrosine (*larger panels*) and GAP-43 (*insets*) immunoreactivity. All photographs were processed in the same manner. The results of a quantitative analysis of phosphotyrosine immunoreactivity values for the same type of experiments is given in Results. Bar, 10 μ m.

against the growth-suppressing actions of CNS myelin-derived inhibitors.

Discussion

We have analyzed the role of the growth-associated protein GAP-43 in neurite outgrowth and growth cone activity. Characteristic features of GAP-43-depleted sensory neurons plated on PORN/laminin in the presence of NGF were the rapid elongation of poorly adhesive thin neurites that had growth cones with strikingly dynamic and unstable peripheral lamellae, rudimentary central domains, and no detectable accumulation of *f*-actin. When tested for evoked morphogenic activity, the growth cones of GAP-43-depleted neurites failed to display NGF-induced growth cone spreading or IGF-1-induced growth cone and neurite branching. On the other hand, NGF and IGF-1 stimulated neurite elongation

and tyrosine phosphorylation in GAP-43-depleted neurons, indicating that the corresponding receptors and at least some signal transduction pathways were still functional in the absence of GAP-43. When challenged with liposomes formed from CNS myelin-derived proteins, GAP-43-depleted neurites were particularly susceptible to retraction. Thus, addition of low amounts of inhibitor-containing liposomes induced growth arrest and only minor growth cone shrinkage in GAP-43-containing neurites, but induced major neurite retraction in their GAP-43-depleted counterparts. In addition, and interestingly, NGF had a protective effect against inhibitor-induced neurite retraction in GAP-43-depleted neurons.

In summary, our findings indicate that GAP-43 plays a major role in neurite outgrowth in vitro, where its presence promotes neurite and growth cone adhesion, is necessary for sustained morphogenic responses to growth factors, and

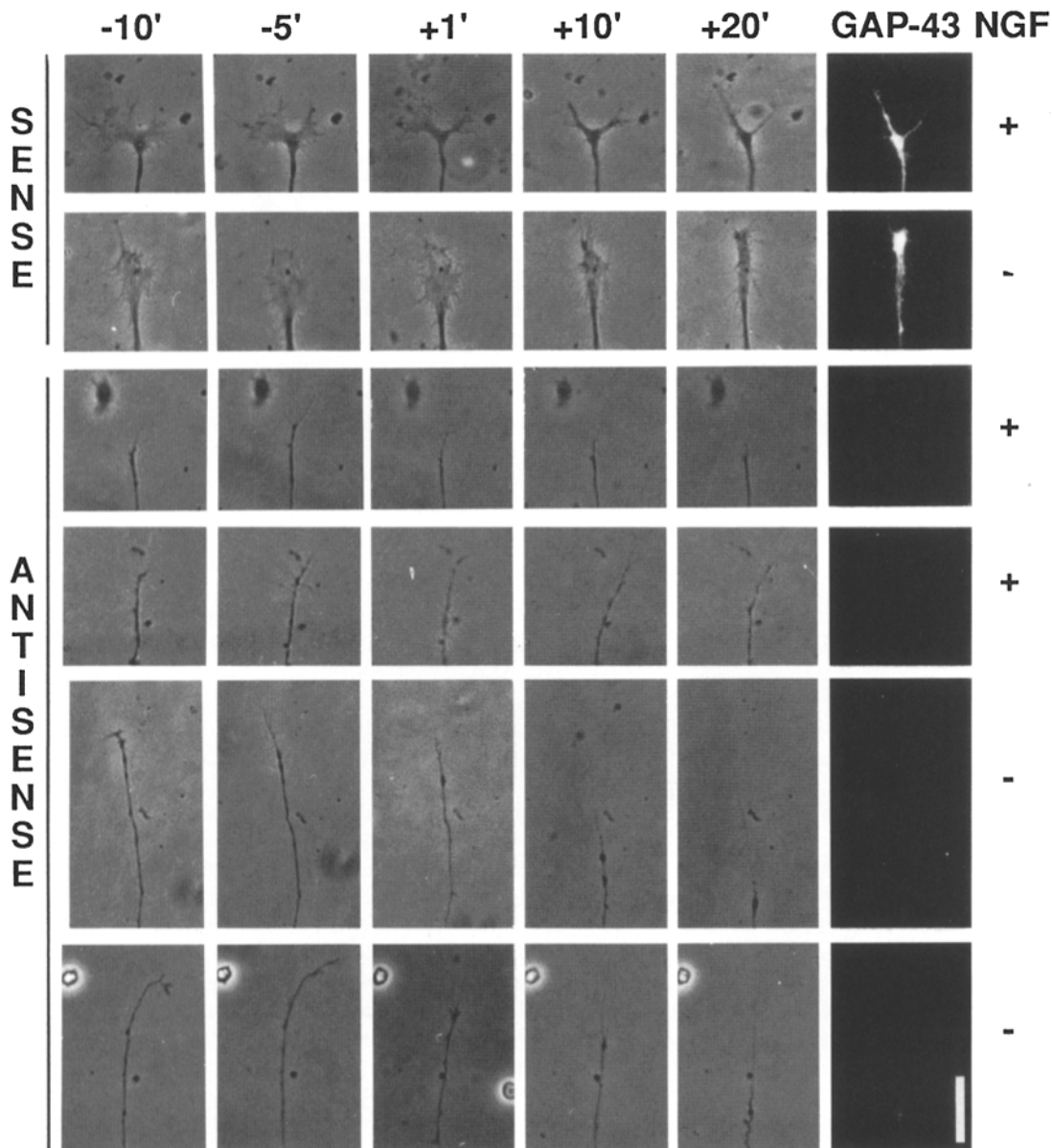


Figure 7. Retraction of GAP-43-depleted neurites in the presence of CNS myelin-derived liposomes, and protective effect of NGF. Cultures were processed in the same manner as in the growth factor addition experiments described above, except that inhibitor-containing liposomes were added to the culture medium at $t = 0$ min. In addition, where indicated, 2 ng/ml of NGF were added to the culture medium at $t = -30$ min. 30 μ l of inhibitory liposomes were added per milliliter of culture medium (except for the experiment shown in the top row, where the liposome quantity was fivefold higher). Shortly after the 20-min recording, cultures were fixed and processed for GAP-43 immunoreactivity, and the corresponding photographs are shown in the rightmost lane. Note growth cone collapse, but no retraction in GAP-43-containing neurites. Also note protective effect of NGF against inhibitor-induced retraction in GAP-43-depleted neurites. A quantitative analysis of analogous data yielded the following values for 20-min retraction lengths (micrometers): with GAP-43, -0.8 ± 1.17 ($n = 35$); without GAP-43, 3.7 ± 1.15 (with NGF, $n = 39$), and 16.8 ± 2.37 (without NGF, $n = 36$). Bar, 20 μ m.

protects against retraction-inducing agents. In addition, our findings suggest that the presence of GAP-43 in adult neurites may be a major determinant of stimulus-induced plasticity and regeneration, both in the PNS and possibly in the CNS.

Regulated Growth Cone Activity and Adhesion Depend on the Presence of GAP-43

Addition of NGF to the culture medium consistently induced extensive spreading in GAP-43-containing growth cones.

This response was detectable within 3–5 min, kept augmenting for ≤ 20 –40 min, and was reversed upon withdrawal of the NGF. Very similar observations were made by Greene and co-workers with PC12 cells and sensory neurons (Phelan et al., 1992). Interestingly, activation of a distinct receptor tyrosine kinase with IGF-1 induced growth cone branching instead of spreading. In addition, IGF-1 induced the formation of long filopodia along preexisting neurites. To our knowledge, this is the first report of qualitatively different effects of two neurotropic factors on growth cone morphogenic activity. These observations suggest that diffusible

growth factors may not only promote neurite outgrowth to different degrees, but may also affect its pattern in qualitatively distinct manners.

The characteristic morphogenic responses of DRG growth cones to NGF or IGF-1 were not detected in the absence of GAP-43. Time-lapse analysis revealed that GAP-43-depleted growth cones had highly dynamic phase-bright lamellae that formed extensive transient structures irrespective of the presence or absence of added growth factor. This suggested that the absence of persistent changes in growth cone morphology in the presence of either growth factor was not caused by a general impairment in lamellar formation or dynamics in these growth cones. Furthermore, NGF or IGF-1 induced accelerated neurite elongation and elevated growth cone phosphotyrosine immunoreactivity in GAP-43-depleted neurons, indicating that these could transduce NGF- or IGF-1-induced signals. The absence of sustained evoked morphogenic reactions in GAP-43-depleted growth cones therefore appears to involve specific intrinsic defects in the growth cone. The observations of this study suggest that these may primarily lead to impaired structural stability and/or diminished adhesiveness of the growth cone.

In addition to being unable to carry out sustained morphogenic responses, GAP-43-depleted growth cones were highly susceptible to retraction in the presence of CNS myelin-derived liposomes. Our findings with these liposomes are consistent with previous reports by Schwab and co-workers, who demonstrated that such liposomes induce growth arrest, growth cone collapse, and neurite retraction (Bandtlow et al., 1990, 1993). In control experiments, PNS myelin-derived liposomes and protease-pretreated CNS myelin liposomes were inactive, indicating that the arrest and retraction inducing activity detected in our assay was caused by CNS myelin-associated inhibitors of neurite outgrowth (Caroni and Schwab, 1988). These inhibitors are thought to act locally through a pertussis toxin-sensitive pathway (Igarashi et al., 1993), and our findings indicate that this signaling pathway is not impaired in the absence of GAP-43. The strikingly elevated susceptibility of GAP-43-depleted neurites to retraction may be caused by alterations in signal transduction or structural properties in these growth cones. However, as mentioned above, an attractive interpretation of all findings reported in this study is that GAP-43 depletion leads to a major reduction in growth cone and neurite adhesiveness. This hypothesis is discussed in more detail in the next section.

An additional finding of this study is that in GAP-43-depleted neurites NGF has a protective effect against inhibitor-induced retraction. No such effect was detected for GAP-43-containing neurites, which usually did not retract, even when comparatively high amounts of inhibitory liposomes were added to the culture medium. Like the findings described above, these observations can also be interpreted in terms of GAP-43-dependent adhesiveness. Thus, NGF appears to promote adhesiveness in GAP-43-depleted neurites since we noticed that upon NGF withdrawal GAP-43-depleted neurites and growth cones detached and/or retracted more frequently, whereas this type of behavior was very rare in GAP-43-containing neurites, irrespective of the presence or absence of NGF (data not shown).

Finally, irrespective of the mechanism involved, our observations indicate that the effects of CNS myelin-derived inhibitors are antagonized by GAP-43 and at least one neu-

rotrophin. This finding has important potential implications for sprouting and regeneration in the adult CNS of higher vertebrates.

Possible Role of GAP-43 in Growth Cone Activity

Which growth cone mechanisms are affected by the presence of GAP-43? Perhaps the most characteristic feature of GAP-43-depleted growth cones is the extremely reduced size of a phase-dense central domain. It presumably corresponds to the organelle- and microtubule-rich central domain that plays a key role in growth cone adhesion, neurite elongation, and the transition from growth cone to neurite (Goldberg and Burmeister, 1986; Aletta and Greene, 1988). This domain appears to be the main target of NGF-induced spreading, and its greatly reduced extension may be responsible for the apparent reduction in adhesiveness and the absence of evoked morphogenic activity in GAP-43-depleted growth cones. However, while these interpretations are consistent with recent findings on the mechanism of neurite elongation at the growth cone, further detailed studies will be needed to determine what structural components of the neurite and growth cone are affected by the absence of GAP-43.

The observation that neurite adhesiveness is apparently impaired in the absence of GAP-43 is consistent with previous findings by Baetge and Hammang, and by Meiri and co-workers on GAP-43-deficient PC12B cells (Baetge and Hammang, 1991; Baetge et al., 1991). The latter authors reported that normal adhesiveness could be restored by reintroduction of GAP-43 cDNA in these cells (Baetge et al., 1991). Possibly related results were obtained when phosphorylation site mutants of GAP-43 were transfected in non-neuronal cell lines (Widmer and Caroni, 1993). In those experiments, GAP-43 (Ala42) reduced cell spreading, whereas GAP-43 (Asp42) promoted it. These results indicated that the presence of GAP-43 can affect cell spreading, and that this activity of GAP-43 is affected by phosphorylation at Ser42 (Widmer and Caroni, 1993). Conceivably, therefore, the results of this study with primary sensory neurons, and those of previous studies with PC12B and nonneuronal cell lines may reflect the same cellular mechanism leading to GAP-43-mediated adhesiveness. Furthermore, since NGF induces the phosphorylation of GAP-43 in synaptosomes and growth cone particles (Meiri and Burdich, 1991), PKC-mediated phosphorylation of GAP-43 may be one downstream event in evoked growth cone morphogenic activity. It is not, however, sufficient to induce this activity, since direct activation of PKC by the addition of 15–50 nM phorbol ester to NGF-deprived, GAP-43-containing DRG neurons induced transient filopodia formation and acceleration of neurite elongation, but did not induce growth cone spreading (data not shown). Interestingly, in those experiments, a partial filopodial reaction to phorbol ester was also detected in GAP-43-depleted neurites.

How does GAP-43 affect growth cone activity? One possibility is that GAP-43 may modulate second messenger systems in the growth cone (Skene, 1989). This possibility is supported by the results of cell-free studies indicating that GAP-43 binds calmodulin in a phosphorylation- and calcium-sensitive manner (Liu and Storm, 1990), activates G_o and G_i in a palmitoylation-sensitive manner (Strittmatter et al., 1990; Sudo et al., 1992), and activates the metabolism

of phosphoinositides (van Dongen et al., 1985; Coggins and Zwiers, 1991). Regulated release of GAP-43-associated calmodulin at the leading edge may promote local actin polymerization via the activation of calcium- and calmodulin-dependent kinase (Liu and Storm, 1990; Bamburg et al., 1992). In addition, microinjection of a GAP-43-encoding mRNA in frog oocytes resulted in activation of a G-protein-activated chloride current in these cells (Strittmatter et al., 1993), suggesting that G protein activation by GAP-43 may be a relevant *in vivo* function of this protein. However, because of the limitations of presently available knowledge, it is not possible to construct an integrated view of the interactions between second messenger systems in regulating growth cone behavior. Therefore, the combined implications of these *in vitro* studies for growth cone activity are difficult to evaluate.

A possible indication on the type of growth cone processes affected by GAP-43 is given by the characteristic absence of local f-actin accumulations in growing cultured neurites and growth cones depleted of GAP-43. Local accumulation of f-actin is an early step in growth cone steering (O'Connor and Bentley, 1993; Lin and Forscher, 1993). It has been suggested that substantial accumulation of f-actin in the peripheral domain of growth cones may function as a regulated barrier to invasion by microtubules from the central domain (Forscher and Smith, 1988). Furthermore, f-actin-poor lamellar extensions in lesioned axons (Goldberg and Burmeister, 1992) may resemble the dynamic lamellar extensions of GAP-43-depleted growth cones. It is, therefore, attractive to speculate that the striking reduction in polarized f-actin accumulations that is detected in GAP-43-depleted neurites may be a cause of the unrestrained and highly dynamic extension of transient lamellar and neurite-like structures at the tip of these growth cones. In addition, a defect in local f-actin accumulation may compromise the formation of a well-spread central domain, possibly leading to diminished growth cone adhesiveness. Finally, Raper and coworkers (Fan et al., 1993) recently demonstrated that an early step in growth cone collapse is the loss of f-actin in the anterior part of the growth cone. Conceivably, therefore, protection against inhibitor-induced retraction by GAP-43 may also be mediated by its effects on growth cone f-actin.

Evidence suggesting that GAP-43 may play a role in promoting the accumulation of f-actin at the cell cortex has recently been produced. Thus, the results of transfection experiments in nonneuronal cells indicate that GAP-43 promotes peripheral f-actin formation during spreading and codistributes with f-actin in the pseudopods of spreading cells (Widmer and Caroni, 1993). In addition, direct binding of GAP-43 to f-actin *in vitro* has been claimed in a recent report (Hens et al., 1993). Therefore, when combined with the results of these previous transfection studies, our observations suggest that GAP-43 may be involved in the local accumulation of f-actin at the growing tips of neurites.

In conclusion, the results of this study demonstrate that depletion of GAP-43 in cultured sensory neurons has drastic consequences on the mode of neurite outgrowth, evoked morphogenic activity by the growth cone, and resistance to neurite retraction induced by CNS myelin-derived proteins. Future investigations will determine whether (a) the absence of GAP-43 impairs the ability of the growth cone to carry out steering activity; (b) whether and how these findings can be

applied to other types of neurons from different developmental stages; and finally, perhaps most importantly, (c) whether the relation between GAP-43, neurotropic factors, and CNS myelin-associated inhibitors may also apply to neurite regeneration *in vivo*.

We are grateful to S. Arber, M. Chiquet, J. Kapfhammer, B. Ludin, and D. Monard for critically reading the manuscript, and to A. Matus (Friedrich Miescher Institute, Basel) for allowing us to use his time-lapse video microscopy setup.

Received for publication 11 July 1994 and in revised form 4 November 1994.

References

- Aigner, L., and P. Caroni. 1993. Deletion of 43-kD growth-associated protein in primary sensory neurons leads to diminished formation and spreading of growth cones. *J. Cell Biol.* 123:417-429.
- Aletta, J. M., and L. A. Greene. 1988. Growth cone configuration and advance: a time-lapse study using video-enhanced differential interference contrast microscopy. *J. Neurosci.* 8:1424-1435.
- Atashi, J. R., S. G. Klinz, C. A. Ingraham, W. T. Matten, M. Schachner, and P. F. Maness. 1992. Neural cell adhesion molecules modulate tyrosine phosphorylation of tubulin in nerve growth cone membranes. *Neuron.* 8:831-842.
- Baetge, E. E., and J. P. Hammang. 1991. Neurite outgrowth in PC12 cells deficient in GAP-43. *Neuron.* 6:21-30.
- Baetge, E. E., L. E. Bickerstaff, K. M. Felsenstein, J. P. Hammang, and K. F. Meiri. 1991. Abnormal neurite extension in GAP-43 deficient PC12 cells stably transfected with mutated (Ser41-Ala41) GAP-43. *Soc. Neurosci.* 17:1309.
- Bamburg, J. R., R. O. Lockerbie, B. W. Bernstein, T. E. Morgan, M. Whitney, and M. E. Adams. 1992. Actin dynamics in neuronal growth cones. *In* The Nerve Growth Cone. P. C. Letourneau, S. B. Kater, E. R. Macagno, editors. Raven Press, Ltd., New York. pp. 27-38.
- Bandtlow, C., T. Zachleder, and M. E. Schwab. 1990. Oligodendrocytes arrest neurite growth by contact inhibition. *J. Neurosci.* 10:3837-3848.
- Bandtlow, C. E., M. F. Schmidt, T. D. Hassinger, M. E. Schwab, and S. B. Kater. 1993. Role of intracellular calcium in NI-35-evoked collapse of neuronal growth cones. *Science (Wash. DC).* 259:80-83.
- Basi, G. S., R. D. Jacobson, I. Virag, J. Schilling, and J. H. P. Skene. 1987. Primary structure and transcriptional regulation of GAP-43, a protein associated with nerve growth. *Cell.* 49:785-791.
- Bedlack, R. S., M.-D. Wei, and L. M. Loew. 1992. Localized membrane depolarizations and localized calcium influx during electric field-guided neurite growth. *Neuron.* 9:393-403.
- Benowitz, L. I., and A. Routtenberg. 1987. A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism, and synaptic plasticity. *Trends Neurosci.* 10:527-531.
- Burry, R. W., J. J. Lah, and D. M. Hayes. 1991. Redistribution of GAP-43 during growth cone development *in vitro*: immunocytochemical studies. *J. Neurocytol.* 20:133-144.
- Caroni, P., and M. E. Schwab. 1988. Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *J. Cell Biol.* 106:1281-1288.
- Coggins, P. J., and H. Zwiers. 1991. B-50 (GAP-43): biochemistry and functional neurochemistry of a neuron-specific phosphoprotein. *J. Neurochem.* 56:1095-1106.
- Davenport, R. W., and S. B. Kater. 1992. Local increases in intracellular calcium elicit local filopodial responses in *Helisoma* neuronal growth cones. *Neuron.* 9:405-416.
- Doster, S. K., A. M. Lozano, A. J. Aguayo, and M. B. Willard. 1991. Expression of the growth-associated protein GAP-43 in adult rat retinal ganglion cells following axon injury. *Neuron.* 6:635-647.
- Fan, J., S. G. Mansfield, T. Redmond, P. R. Gordon-Weeks, and J. Raper. 1993. The organization of f-actin and microtubules in growth cones exposed to a brain-derived collapsing factor. *J. Cell Biol.* 121:867-878.
- Forscher, P., and S. J. Smith. 1988. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. *J. Cell Biol.* 107:1505-1516.
- Goldberg, D. J., and D. W. Burmeister. 1986. Stages in axon formation: observations of growth of *Aplysia* axons in culture using video-enhanced contrast-differential interference contrast microscopy. *J. Cell Biol.* 87:1921-1931.
- Goldberg, D., and D. W. Burmeister. 1992. Microtubule-based filopodium-like protrusions form after axotomy. *J. Neurosci.* 12:4800-4807.
- Hens, J. J. H., F. Benfenati, H. B. Nielander, F. Valtorta, W. H. Gispen, and P. N. E. DeGraan. 1993. B-50/GAP-43 binds to actin filaments without affecting actin polymerization and filament organization. *J. Neurochem.* 61:1530-1533.
- Hofer, U., J. Syfrig, and R. Chiquet-Ehrismann. 1990. Identification and char-

- acterization of a dimeric chicken fibronectin receptor. *J. Biol. Chem.* 265:14561-14565.
- Igarashi, M., S. M. Strittmatter, T. Vartanian, and M. C. Fishman. 1993. Mediation by G proteins of signals that cause collapse of growth cones. *Science (Wash. DC)*. 259:77-79.
- Jap Tjoen San, E. R. A., M. Schmidt-Michels, A. B. Oestreicher, W. H. Gispen, and P. Schotman. 1992. Inhibition of nerve growth factor-induced B-50/GAP-43 expression by antisense oligomers interferes with neurite outgrowth of PC12 cells. *Biochem. Biophys. Res. Commun.* 187:839-846.
- Lankford, K. L., and P. C. Letourneau. 1989. Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments. *J. Cell Biol.* 109:1229-1243.
- Letourneau, P. C., and T. A. Shattuck. 1989. Distribution and possible interactions of actin-associated proteins and cell adhesion molecules of nerve growth cones. *Development (Camb.)*. 105:505-519.
- Lin, C.-H., and P. Forscher. 1993. Cytoskeletal remodeling during growth cone-target interactions. *J. Cell Biol.* 121:1369-1383.
- Liu, Y., and D. R. Storm. 1990. Regulation of free calmodulin levels by neuromodulin: neuron growth and regeneration. *Trends Pharmacol. Sci.* 11:107-111.
- McKerracher, L., C. Essagian, and A. J. Aguayo. 1993. Marked increase in β -tubulin mRNA expression during regeneration of axotomized retinal ganglion cells in adult mammals. *J. Neurosci.* 13:5294-5300.
- Meiri, K. F., and P. R. Gordon-Weeks. 1990. GAP-43 in growth cones is associated with areas of membrane that are tightly bound to substrate and is a component of a membrane skeleton subcellular fraction. *J. Neurosci.* 10:256-266.
- Meiri, K. F., and D. Burdich. 1991. Nerve growth factor stimulation of GAP-43 phosphorylation in intact isolated growth cones. *J. Neurosci.* 11:3155-3164.
- Moss, D. J., P. Fernyhough, K. Chapman, L. Baizer, D. Bray, and T. Allsopp. 1990. Chicken growth-associated protein GAP-43 is tightly bound to the actin-rich neuronal membrane skeleton. *J. Neurochem.* 54:729-736.
- Near, S. L., L. R. Whalen, J. A. Miller, and D. N. Ishii. 1992. Insulin-like growth factor II stimulates motor nerve regeneration. *Proc. Natl. Acad. Sci. USA*. 89:11716-11720.
- O'Connor, T. P., and D. Bentley. 1993. Accumulation of actin in subsets of pioneer growth cone filopodia in response to neural and epithelial guidance cues. *J. Cell Biol.* 123:935-948.
- Phelan, K. A., E. H. Sherr, J. M. Aletta, and L. A. Greene. 1992. Regulation of growth cone formation and function by neurotrophic factors. In *The Nerve Growth Cone*. P. C. Letourneau, S. B. Kater, and E. R. Macagno, editors. Raven Press Ltd., New York. pp. 151-166.
- Recio-Pinto, E., M. M. Rechler, and D. N. Ishii. 1986. Effects of insulin, insulin-like growth factor II, and nerve growth factor on neurite formation and survival in cultured sympathetic and sensory neurons. *J. Neurosci.* 6:1211-1219.
- Rehder, V., and S. B. Kater. 1992. Regulation of neuronal growth cone filopodia by intracellular calcium. *J. Neurosci.* 12:3175-3186.
- Richardson, P. M., and V. M. K. Issa. 1984. Peripheral injury enhances regeneration of spinal axons. *Nature (Lond.)*. 284:264-265.
- Schreyer, D. J., and J. H. P. Skene. 1991. Fate of GAP-43 in ascending spinal axons of DRG neurons after peripheral nerve injury: delayed accumulation and correlation with regenerative potential. *J. Neurosci.* 11:3738-3751.
- Shea, T. B., N. I. Perrone-Bizzozero, M. L. Beerman, and L. I. Benowitz. 1991. Phospholipid-mediated delivery of anti-GAP-43 antibodies into neuroblastoma cells prevents neuriteogenesis. *J. Neurosci.* 11:1685-1690.
- Skene, J. H. P. 1989. Axonal growth-associated proteins. *Annu. Rev. Neurosci.* 12:127-156.
- Skene, J. H. P., and M. B. Willard. 1981. Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. *J. Cell Biol.* 89:86-95.
- Skene, J. H. P., and I. Virag. 1989. Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. *J. Cell Biol.* 108:613-624.
- Strittmatter, S. M., J. Valenzuela, T. E. Kennedy, E. J. Neer, and M. C. Fishman. 1990. Go is a major growth cone protein subject to regulation by GAP-43. *Nature (Lond.)*. 344:836-841.
- Strittmatter, S. M., T. Vartanian, and M. C. Fishman. 1992. GAP-43 as a plasticity protein in neuronal form and repair. *J. Neurobiol.* 23:507-520.
- Strittmatter, S. M., S. C. Cannon, E. M. Ross, T. Higashijima, and M. C. Fishman. 1993. GAP-43 augments G protein-coupled receptor transduction in *X. laevis* oocytes. *Proc. Natl. Acad. Sci. USA*. 90:5327-5331.
- Sudo, Y., D. Valenzuela, A. G. Beck-Sickinger, M. C. Fishman, and S. M. Strittmatter. 1992. Palmitoylation alters protein activity: blockade of Go stimulation by GAP-43. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:2095-2102.
- Tezloff, W., S. W. Alexander, F. D. Miller, and M. A. Bisby. 1991. Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. *J. Neurosci.* 11:2528-2544.
- van Dongen, C. J., H. Zwiers, P. N. E. deGraan, and W. H. Gispen. 1985. Modulation of the activity of purified phosphatidylinositol 4-phosphate kinase by phosphorylated and dephosphorylated B-50 protein. *Biochem. Biophys. Res. Commun.* 128:1219-1227.
- Widmer, F., and P. Caroni. 1990. Identification, localization, and primary structure of CAP-23, a particle-bound cytosolic protein of early development. *J. Cell Biol.* 111:3035-3047.
- Widmer, F., and P. Caroni. 1993. Phosphorylation-site mutagenesis of the growth-associated protein GAP-43 modulates its effects on cell spreading and morphology. *J. Cell Biol.* 120:503-512.
- Williams, E. J., F. S. Walsh, and P. Doherty. 1994. Tyrosine kinase inhibitors can differentially inhibit integrin dependent and CAM stimulated neurite outgrowth. *J. Cell Biol.* 124:1029-1037.
- Yankner, B. A., L. I. Benowitz, L. Villa-Komaroff, and R. L. Neve. 1990. Transfection of PC12 cells with the human GAP-43 gene: effects on neurite outgrowth and regeneration. *Mol. Brain Res.* 7:39-44.