Depletion of 43-kD Growth-associated Protein in Primary Sensory Neurons Leads to Diminished Formation and Spreading of Growth Cones

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Abstract. The 43-kD growth-associated protein (GAP-43) is a major protein kinase C (PKC) substrate of growing axons, and of developing nerve terminals and glial cells. It is a highly hydrophilic protein associated with the cortical cytoskeleton and membranes. In neurons it is rapidly transported from the cell body to growth cones and nerve terminals, where it accumulates. To define the role of GAP-43 in neurite outgrowth, we analyzed neurite regeneration in cultured dorsal root ganglia (DRG) neurons that had been depleted of GAP-43 with any of three nonoverlapping antisense oligonucleotides. The GAP-43 depletion procedure was specific for this protein and an antisense oligonucleotide to the related PKC substrate MARCKS did not detectably affect GAP-43 immunoreactivity. We report that neurite outgrowth and morphology depended on the levels of GAP-43 in the neurons in a substrate-specific manner. When grown on a laminin substratum, GAP-43–depleted neurons extended longer, thinner and less branched neurites with strikingly smaller growth cones than their GAP-43–expressing counterparts. In contrast, suppression of GAP-43 expression prevented growth cone and neurite formation when DRG neurons were plated on poly-L-ornithine.

These findings indicate that GAP-43 plays an important role in growth cone formation and neurite outgrowth. It may be involved in the potentiation of growth cone responses to external signals affecting process formation and guidance.

The growth of axons during development and regeneration is associated with the expression of specific growth-associated proteins (GAPs)[1] that are thought to play essential roles in the growth process (Skene, 1989; Skene and Willard, 1981). Prominent among the GAPs is the highly hydrophilic, cortical cytoskeleton- and membrane-associated 43-kD-protein GAP-43 (Skene, 1989; Benowitz and Routtenberg, 1987; Skene and Virag, 1989; Moss et al., 1990; Meiri and Gordon-Weeks, 1990). GAP-43 is a major protein kinase C (PKC) substrate of the axonal growth cone and nerve terminal (Meiri et al., 1986; Skene et al., 1986). This protein is an early marker for postmitotic neurons and its expression correlates with axonal growth during development and regeneration (Skene and Willard, 1981; Basi et al., 1987; Skene, 1989; Coggins and Zwiers, 1991). Downregulation of GAP-43 during development frequently coincides with the activity-sensitive elimination of collaterals, and with the establishment of the adult pattern of synaptic connectivity (Neve and Baer, 1989; Caroni and Beker, 1992). However, in certain areas of the central nervous system (CNS) of higher vertebrates that have been associated with plasticity, substantial levels of GAP-43 persist in the adult (Benowitz and Routtenberg, 1987). In addition to neurons, the protein is also expressed at restricted developmental stages in Schwann cells, astrocytes, and oligodendrocytes (Vitkovic et al., 1988; da Cunha and Vitkovic, 1990; Deloulme et al., 1990; Curtis et al., 1991; Curtis et al., 1992; Woolf et al., 1992).

In neurons, GAP-43 associates with Golgi membranes shortly after synthesis and is rapidly transported to growth cones and nerve terminals, where it accumulates. In vitro, GAP-43 binds to calmodulin in the absence of calcium (Andreason et al., 1983), and to the GTP-binding protein G_o in a palmitoylation-sensitive manner (Strittmatter et al., 1990; Sudo et al., 1992). Intracellular application of antibodies to GAP-43 inhibits evoked neurotransmitter release (Dekker et al., 1989), suggesting that it may play a role in exocytosis. Finally, transfection experiments carried out in non-neuronal cell lines have shown that GAP-43 affects morphogenetic processes at the cell surface (Zuber et al., 1989; Widmer and Caroni, 1993). Spreading cells expressing high levels of GAP-43 were strikingly rich in filopodia, and the effects of GAP-43 on cell spreading and morphology could be modulated by mutating the PKC phosphorylation site of GAP-43 (Widmer and Caroni, 1993).

1. *Abbreviations used in this paper*: CNS, central nervous system; DRG, dorsal root ganglion; GAP, growth-associated protein; IR, immunoreactivity; PKC, protein kinase C.

Analysis of a PC12 mutant cell line that does not express GAP-43 suggested that GAP-43 is not required for neurite outgrowth in these cells (Baetge and Hammang, 1991). In cultured hippocampal neurons, suppression of kinesin expression after delivery of antisense oligonucleotide prevented the transport of GAP-43 from the cell body to the neurites, but did not seem to alter axonal outgrowth (Ferreira et al., 1992). These experiments therefore indicate that the presence of GAP-43 in neurites may not be essential for their outgrowth. On the other hand, however, experiments with specific antibodies against GAP-43 in neuroblastoma cells (Shea et al., 1991), and with antisense oligonucleotides in PC12 cells (Jap Tjoen San et al., 1992) have suggested that GAP-43 enhances neurite outgrowth rates in these cells.

To define the possible role(s) of GAP-43 in neurite outgrowth in primary neurons, we analyzed neurite regeneration in cultured chick dorsal root ganglion (DRG) neurons that had been dissociated in the presence of antisense oligonucleotides to GAP-43. Using this approach it was possible to reduce specifically the expression of GAP-43 in ~40% of the cultured neurons to levels below the limit of detection by immunocytochemistry.

We report that in the absence of detectable GAP-43, neurite elongation of DRG neurons grown on laminin/poly-L-ornithine in the presence of NGF was in fact accelerated. However, neurons depleted of GAP-43 extended thin, infrequently branched neurites with extremely small growth cones. On the other hand, when grown on polyornithine, GAP-43–depleted DRG neurons failed to form growth cones and hence neurites failed to emerge from the initial symmetric lamellae.

Our results reveal that GAP-43 plays an important role in spreading, branching, and possibly formation of growth cones. GAP-43 may be a local nodulator of axonal cortex and growth cone activity to transduce external signals into the formation and guidance of neurites.

Materials and Methods

Oligonucleotides

For the GAP-43 experiments three different oligonucleotide pairs were used. Antisense oligonucleotide-1 (−8+9) consisting of the sequence GCACAGCATGATCGTAT is the inverse complement of nucleotide −8 to +9 (Baizer et al., 1990) and overlaps the start codon AUG. Antisense oligonucleotide-2 (+9+26) has the sequence TTTGTTCTTCTCATACAG. Antisense oligonucleotide-1 and -2 were chosen for their possible disruption of the translation start. Antisense oligonucleotide 3 (+643+659) is a 17-mer of the sequence ACAGCATCTGTCTTCTC. It is the inverse complement of nucleotide +643 to +659, covering a 35-base-long unmatched stretch of a bifurcation loop as suggested by the RNA fold program of Zuker and Stiegler (Zuker and Stiegler, 1981). For the MARCKS experiment the antisense oligonucleotide had the sequence CACCCATGCTGGCTTA (−9 to +7 of chicken MARCKS; Graff et al., 1989). The corresponding sense oligonucleotides were the inverse complements of the antisense oligonucleotides. Biotinylated oligonucleotides were generated by adding six Biotin-Phosphoramidite residues (Amersham International, Buckinghamshire, England) to the 5′ end. The oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer, ethanol precipitated, desalted over an NAP™-25 column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and taken up in glass-distilled water to a concentration of 10 μg/μl.

Cell Culture and Application of Oligonucleotides: Trituration Method

The method used to introduce the oligonucleotides into neurons was based upon the trituration method described by Borasio et al. (1989). Two to four chick lumbar embryonic day (E)16 DRGs were trypsinized for 35–40 min at 37°C (0.05% trypsin [GIBCO-BRL, Gaithersburg, MD] in Ca^{2+}/Mg^{2+}-free Hank's buffer). The ganglia were washed once with DME/10% FCS and then with Ca^{2+}/Mg^{2+}-free Hank's buffer. Between wash steps ganglia were allowed to settle down. The medium was then aspirated while leaving a residual volume of 45–50 μl. 5 μl of an oligonucleotide solution (10 μg/μl in H_2O) were then added and the ganglia were dissociated by trituration (20–25 cycles through a Gilson yellow tip). The suspension was then left for 5 min at room temperature after which the cells were suspended in 2 ml DME/10% FCS and preplated for 2 h in a 35-mm tissue culture dish (Corning Inc., Corning, NY) at 37°C/5% CO_2 in a humidified atmosphere (McCarthy and Partlow, 1976). Nonadherent cells were collected, pelleted for 5 min at 120 *g*, resuspended in DME/10% FCS containing 20 ng/ml 2.5 S NGF (Harlan Bioproducts for Science, Inc., Indianapolis, IN), and plated on coated glass coverslips (18 mm) at a cell density of ~1,500 cells per 200 μl and coverslip. Cells were grown at 37°C/5% CO_2 in a humidified incubator.

For coating, coverslips were first treated with 1 N HCl for 6 h at 65°C (Tanaka and Kirschner, 1991) then extensively washed with deionized water and stored in ethanol. Coverslips were coated with 200 μl poly-L-ornithine (Sigma Immunochemicals, St. Louis, MO), washed extensively with water, dried and either used or further coated with 200 μl laminin (Collaborative Biomedical Products, Waltham, MA) (17.5 μg/ml in Ca^{2+}/Mg^{2+}-free PBS) for 1 h at 37°C.

Immunocytochemistry

Cells were fixed for 30 min with prewarmed (37°C) 4% paraformaldehyde in phosphate buffer (0.1 M sodium phosphate, pH 7, 0.05 M sucrose, 0.4 mM $CaCl_2$) and incubated with antibodies diluted in PBS containing 0.1% BSA, 1% FCS, 50 mM glycine, 0.02% NaN_3, and 0.2% saponin at room temperature (Burry et al., 1991). The following primary antibodies were used for this study: chick GAP-43 was either visualized with the specific mAb 5F10 (1:1,000 dilution of ascites fluid; Widmer and Caroni, 1990), or with a specific rabbit polyclonal antiserum raised against the synthetic COOH-terminal peptide CEEESKADQENA of chick GAP-43 (1:1,000 dilution). For CAP-23 staining, the mAB 15C1 (Widmer and Caroni, 1990; 1:10 dilution of supernatant) was used. Chicken MARCKS was visualized with a specific rabbit polyclonal antiserum raised against the synthetic COOH-terminal peptide CSPEGPAEPAE. A polyclonal rabbit antiserum against chicken gizzard integrin that specifically detects β1-integrin (Hofer et al., 1990; 1:300 dilution) was a kind gift of Dr. Chiquet-Ehrismann. After each incubation with antibody, cells were washed in PBS. Secondary antibodies were biotinylated goat anti-mouse (4 μg/ml), rhodamine-labeled goat anti-mouse (4 μg/ml), biotinylated goat anti-rabbit (13 μg/ml) and rhodamine-labeled goat anti-rabbit (4 μg/ml). Biotinylated antibodies were visualized by Lucifer yellow-coupled streptavidin (3.5 μg/ml). Secondary antibodies and Lucifer yellow-coupled streptavidin were from Molecular Probes, Inc., Eugene, OR. Unless otherwise stated, GAP-43 was detected with streptavidin-Lucifer yellow. Biotinylated oligonucleotides were detected with Lucifer yellow-coupled streptavidin. Finally, cultures were washed, mounted in Gelvatol (Monsanto, St. Louis, MO), and fluorescence was detected with an Axiovert-10 microscope (Carl Zeiss Inc., Thornwood, NY) equipped with the appropriate filters (Lucifer yellow: excitation 395–440 nm, emission 460 nm; RITC: excitation 510–560 nm, emission 580 nm).

Immunoblotting and RNA Blots

Cultures in 35-mm dishes were washed once with EDTA and then scraped into 1 ml of EDTA solution. Cells were pelleted at 13,000 *g* for 10 min at 4°C, lysed in 62 mM TrisHCl, pH 6.8, 3% β-mercaptoethanol, 3% SDS, 10% glycerol, homogenized by passing through a syringe, and incubated at 95°C for 5 min. Proteins were fractionated in a 10% SDS polyacrylamide gel and blotted onto polyvinyldendifluoride-membrane (Millipore Corp., Bedford, MA). The membrane was blocked for 15 min in 0.1 M TrisHCl, pH 7.4, 0.1 M $MgCl_2$, 0.5% Tween-20, 1% NP-40, 1% BSA, 5% FCS, and sequentially incubated with anti-chick GAP-43 mAb 5F10 (1:1,000) and monoclonal anti-neurofilament 160 (Sigma Immunochemicals; 1:100) in 1% BSA and 0.05% Tween-20 for 3 h. The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:5,000) in PBS containing 1% BSA, 0.05% Tween-20 for 45 min. Bound antibodies were visualized by a color reaction with nitro blue tetrazolium (Sigma Immunochemicals)

and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim, Mannheim, Germany).

Total RNA from chick E16 lumbar DRGs was prepared by conventional methods and further enriched for poly(A)$^+$ RNA with oligo-(dT)-cellulose (Boehringer Mannheim). 5–10 μg RNA was separated on 1.2% agarose formaldehyde gel and transferred onto positively charged nylon membrane (Boehringer Mannheim). Oligonucleotides were tailed with digoxygenin-conjugated dUTP according to the recommendations of the manufacturer (Boehringer Mannheim). Hybridization was performed at a nucleotide concentration of 5 pmol/ml in 5× SSC, 1% blocking reagent (Boehringer Mannheim), 0.1% *N*-lauroylsarcosine, 0.02% SDS, 100 μg/ml poly (A) (Boehringer Mannheim) for 15 h at 37°C (GAP-43 oligonucleotides 1) or at 43°C (GAP-43 oligonucleotides 3). Blots were subsequently washed 2× 30 min with 5× SSC at 4°C and 2× 20 min with 3 M tetramethylammoniumchloride, 50 mM Tris, 2 mM EDTA, 0.1% SDS, pH 8.0, at 52°C (GAP-43 oligonucleotides 1) or 2× 5 min with 2× SSC, 0.1% SDS at 43°C and 2× 5 min with 0.1% SSC, 0.1% SDS at 43°C (GAP-43 oligonucleotides 3). Hybridization with digoxygenin-labeled GAP-43 antisense riboprobe was carried out at a concentration of 50 ng/ml in 50% formamide, 5× SSC, 2.5% blocking reagent (Boehringer Mannheim), 0.1% SDS, 0.1% *N*-Lauroylsarcosine for 15 h at 68°C and washing was done 2 × 5 min in 2× SSC, 0.1% SDS at room temperature, and 2 × 20 min in 0.1× SSC, 0.5% SDS at 68°C. The blots were further processed for chemoluminescence detection of digoxygenin as recommended by the manufacturer.

Evaluation of GAP-43 Staining

All cells from randomly selected fields were analyzed. Cells were distributed into three categories: cells with strong, intermediate, and no specific GAP-43 immunoreactivity (IR). No GAP-43 immunoreactivity (GAP-43 IR) is a stringent criterium, and corresponds to the background staining of non-neuronal cells present in the culture. The assignment of a cell to strong or intermediate GAP-43 IR was controlled by monitoring with a S/W-CCD-Camera (AVT-Horn) and a KV-M1430B TV (SONY): a fixed brightness level was selected to include ~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.

Morphometric Analysis

After processing for immunocytochemistry, cells were selected randomly in the phase channel, photographed in phase (10×) and analyzed using the NIH Image 1.41 program fed with a PHV-A7E Photo Video Camera (SONY). Neurite length was determined by measuring the length from the cell soma to the tip of the longest neurite. Branching behavior was scored by counting the branchpoints of a cell's longest neurite compared with its length. The diameter of the longest neurite was measured in the vicinity of the growth cone, using photomicrographs (100×) of the integrin signal. Statistical analysis of neurite length was performed by applying the Mann-Whitney *U* test, because the distribution of the values was skewed. All other data were analyzed with *t* test.

Results

Depletion of GAP-43 in Cultured DRG Neurons by Antisense Oligonucleotides

To characterize the effect of GAP-43 on neurite outgrowth we generated cultures consisting of neurons that were devoid of GAP-43 from the onset of neurite regeneration in vitro. To achieve an efficient introduction of oligonucleotides into dissociated neurons, E16 chick DRG neurons were triturated in the presence of high concentrations of the oligonucleotides. After trituration the neurons were preplated for 2 h on a tissue culture plastic substratum in the absence of NGF to deplete the culture of rapidly adhering non-neuronal cells.

1,000–2,000 neurons per DRG survived the procedures preceding plating, as estimated by counting of Trypan blue excluding cells after the preplating step. Experiments with biotinylated oligonucleotides showed that 4 h after final plating, ~70% of the neurons still contained significant amounts of oligonucleotides (data not shown). 18 h after plating on a laminin substratum in the presence of NGF 30–50% of the neurons had formed processes that were longer than their cell diameter, irrespective of whether they had been treated with no-, sense-, or antisense oligonucleotides.

As shown in Fig. 1, DRG neurons extended neurites in the absence of GAP-43. Comparable numbers of GAP-43–depleted neurons were detected 1 and 24 h after plating of the neurons on laminin with NGF, and substantial extension of neurites from these DRG neurons was detected as early as 1 h after plating of the neurons on a laminin-coated substratum in the presence of NGF (Fig. 1 *e*). Therefore, under these experimental conditions, regeneration of neurites by DRG neurons does not require the presence of detectable GAP-43.

As shown in Fig. 2 *a*, the antisense treatment produced a substantial number of DRG neurons with undetectable levels of GAP-43 in their cell bodies and neurites. GAP-43 depletion was observed when the trituration was performed in the presence of either of three different antisense oligonucleotides, but not in the presence of the corresponding sense oligonucleotides (Fig. 2 *a*). A quantitative analysis of GAP-43 immunoreactivity contents in populations of DRG neurons treated with the three antisense and sense oligonucleotides is presented in Fig. 2 *a*. Contents of neurons displaying high, intermediate, and undetectable levels of GAP-43 were comparable for the sense oligonucleotides, and for untreated or mock-treated cultures. All these cultures contained comparable amounts of neurons with high and intermediate signal levels, and a very small proportion (1–5%) of neurons with no detectable GAP-43 IR. Treatment with any of the three antisense oligonucleotides dramatically shifted these proportions, resulting in lower numbers of neurons (~10%) with high levels of GAP-43, and ~40% of the neurons with no detectable GAP-43 signal (Fig. 2 *a*). Fig. 2 *b* shows that no differences in the levels of GAP-43 IR were detected 3 d after plating in neurons treated with antisense or sense oligonucleotide, indicating that the effect of the antisense oligonucleotides on DRG GAP-43 IR was reversible. Fig. 2 *c* shows that the antisense, but not the sense oligonucleotides specifically detected GAP-43 mRNA on Northern blots of chick DRG poly(A)$^+$ RNA (two mRNA species, see Baizer et al., 1990). Finally, Fig. 2 *d* demonstrates that the treatment resulted in a strong reduction in the total contents of GAP-43, as determined by immunoblot analysis. However, the corresponding levels of the neurofilament protein NF-160 did not change in a comparable manner (Fig. 2 *d*). Our data indicate that the effect of the antisense oligonucleotides was specific for GAP-43. Thus, three specific antisense oligonucleotides, but not the corresponding sense oligonucleotides, had comparable effects on the levels of GAP-43 in the DRG neurons (Fig. 2 *a*). In addition, integrin immunoreactivity signals in neurons with high or low levels of GAP-43 were comparable (Fig. 1). Similarly, levels of the cortical cytoskeleton-associated protein CAP-23 were apparently unchanged (Fig. 3, *a* and *b*).

Since the latter is a PKC substrate of neurons and developing tissues with subcellular distribution and structural properties similar to those of GAP-43 (Widmer and Caroni, 1990), this observation suggests that in these acute experiments compensatory reactions leading to changes in the lev-

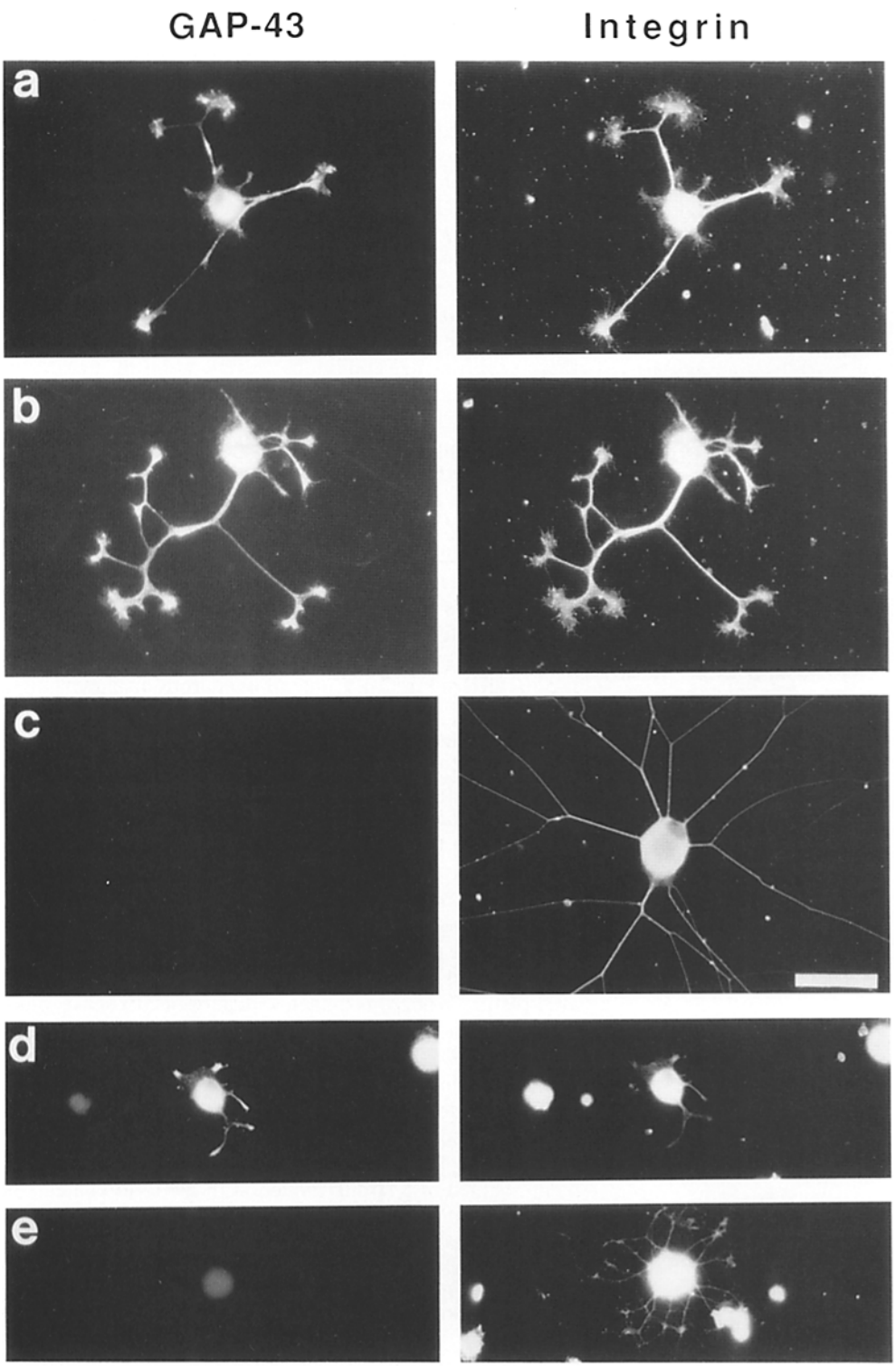

Figure 1. Suppression of GAP-43 expression in E16 chick DRG neurons. Cells were treated with no (*a*), 200 μM sense-3 (*b* and *d*) or 200 μM antisense-3 (*c* and *e*) oligonucleotide, cultured for 18 h (*a–c*) or 1 h (*d* and *e*) on poly-L-ornithine/laminin-coated coverslips, fixed and stained with the mAb 5F10 against GAP-43 and with the antiserum against β1-integrin. The neurons shown in *a*, *b*, and *d* were representative of those expressing high levels of GAP-43. Note thin, longer neurites in GAP-43–depleted neuron (*c*). Also note that already during the initial phase of neurite outgrowth antisense-treated neurons were depleted of GAP-43 IR and displayed the characteristic pattern of neurite outgrowth as detected in older cultures (*e*). Bar, 50 μm.

els of at least one related protein associated with the cortical cytoskeleton did not occur. To rule out the possibility that the effects of the antisense oligonucleotides on GAP-43 IR and neurite outgrowth were simply due to the formation of an RNA–DNA hybrid in the neurons, we triturated the ganglia in the presence of an antisense oligonucleotide to MARCKS (Aderem, 1992). This protein is a PKC substrate with biochemical properties similar to those of GAP-43. As shown in Fig. 3, *c* and *d*, the antisense, but not the corresponding sense oligonucleotide significantly reduced MARCKS IR in the neurons. On the other hand, no detectable changes in GAP-43 IR or neurite morphology (see also Table II) were detected in the presence of the antisense oligonucleotide to MARCKS, indicating that the effects of the GAP-43 oligonucleotides were specific for this protein. Therefore, these combined observations indicate that spe-

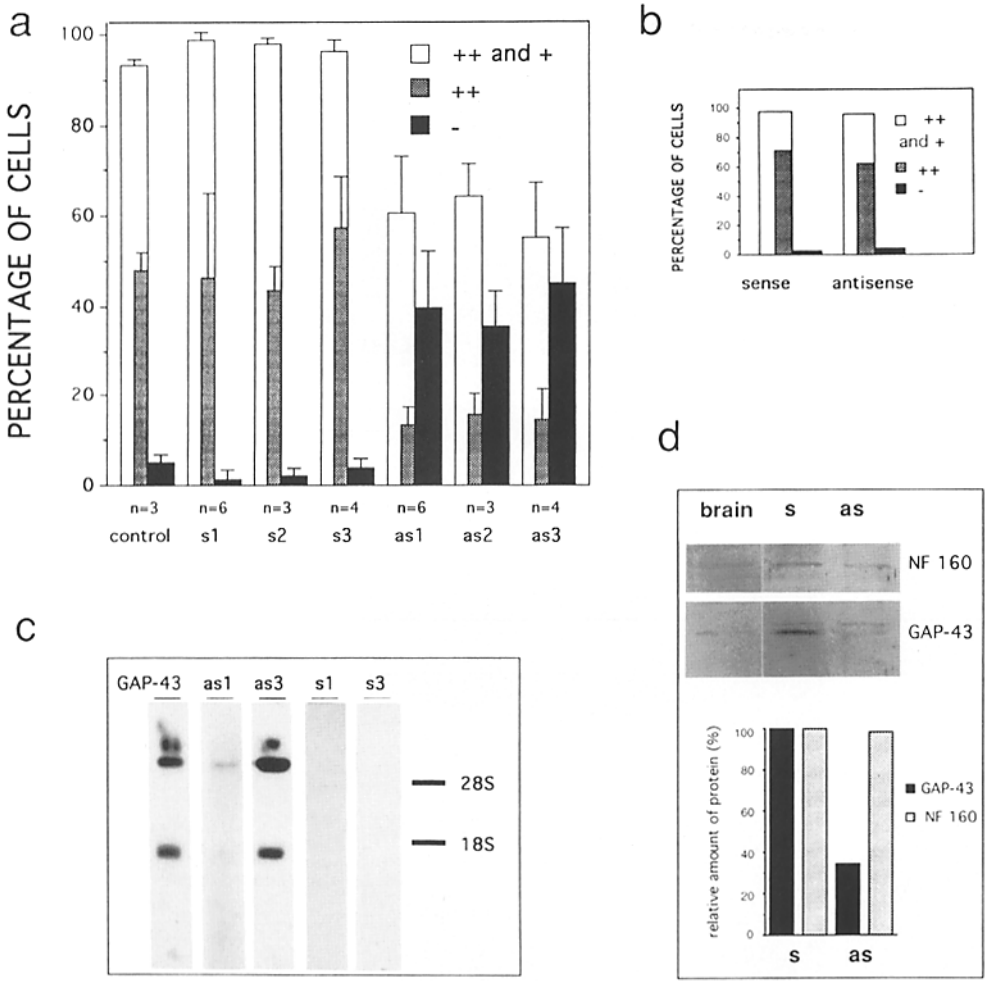

Figure 2. Antisense oligonucleotides to GAP-43 specifically bind to GAP-43 mRNA and reduce GAP-43 levels in DRG neurons. (*a*) Histogram showing the suppressive effect of antisense oligonucleotides on GAP-43 expression. Cells were treated with (*s*, *as*) or without (*control*) 200-μM oligonucleotides (sense [*s*] −1 to −3, antisense [*as*] −1 to −3), cultured for 18 h on poly-L-ornithine/laminin-coated coverslips, fixed and stained for GAP-43 and for β1-integrin. For each experiment 80–100 cells with process(es) longer than two cell diameters from randomly selected fields were distributed into three categories: ++ (strong GAP-43 IR), + (intermediate GAP-43 IR), − (no GAP-43 IR). The column labeled with (++ and +) includes both (++) and (+) neurons. Signal intensity levels were defined as described in Materials and Methods. The number of experiments analyzed (*n*) is indicated. Values represent means ± SD. Note that all three antisense oligonucleotides tested shift the relative content values towards cells with no detectable GAP-43 IR. (*b*) Recovery of GAP-43 immunoreactivity in antisense GAP-43–treated cultures. Data as in *a*, but from 3-d cultures (antisense-3, or sense-3). (*c*) Specific detection of GAP-43 mRNA from E16 chick DRG poly(A)$^+$ RNA by antisense (*as*) oligonucleotides 1 and 3, but not by the corresponding sense (*s*) oligonucleotides. The control lane labeled *Gap-43* was hybridized with a digoxigenin-labeled riboprobe antisense to the coding sequence of chick GAP-43. This probe visualizes the two chick GAP-43 mRNA species, as described (Baizer et al., 1990). (*d*) Immunoblot analysis of GAP-43 in DRG neurons treated as described in *a*. Equal amounts of total protein combined from six separate triturations was analyzed. After GAP-43 detection, the same blot was reprobed for neurofilament-160. The band that migrates slightly more slowly than GAP-43 in the lower panel corresponds to a major species in the homogenate, and the signal is not specific for the GAP-43 antibody. Lane labeled *brain: 15* μg of homogenate protein from chicken E7 brain. Densitometric quantitation of the immunoblot data is shown in the graph. Values were normalized to the intensity of the unspecific band mentioned above.

cific alterations in GAP-43 contents were not accompanied by comparable changes in other major components of neurites and growth cones, and that the effects of the GAP-43 antisense oligonucleotides on GAP-43 IR and neurite morphology were specific for this protein.

DRG Neurons Depleted of GAP-43, Extend Thin Neurites, with Infrequent Branching and Very Small Growth Cones

The most dramatic difference between DRG neurons regenerating neurites on a laminin-coated substratum in the presence or absence of GAP-43 was observed when growth cones were compared. Fig. 4 shows that in the absence of GAP-43 the growth cones of DRG neurons were extremely small. In most cases, a few filopodia pointing in the direction of neurite extension could be observed, but the growth cone region was not significantly wider than the average neurite diameter, and the characteristic well-spread appearance of growth cones growing on laminin was essentially never observed. The panels in Fig. 4 are from 18 h cultures and were representative of all the growth cones from neurons expressing high, respectively undetectable levels of GAP-43. A quantitative analysis of the relationship between growth cone surface area and GAP-43 levels is presented in Table I. Interestingly, variations in growth cone size were much more pronounced among the neurons expressing intermediate levels of GAP-43 (Table I), suggesting that high or undetectable levels of GAP-43 have a dominant effect on growth cone formation and behavior.

Similar observations were made when growth cone sizes were analyzed during the initial phase of neurite elongation

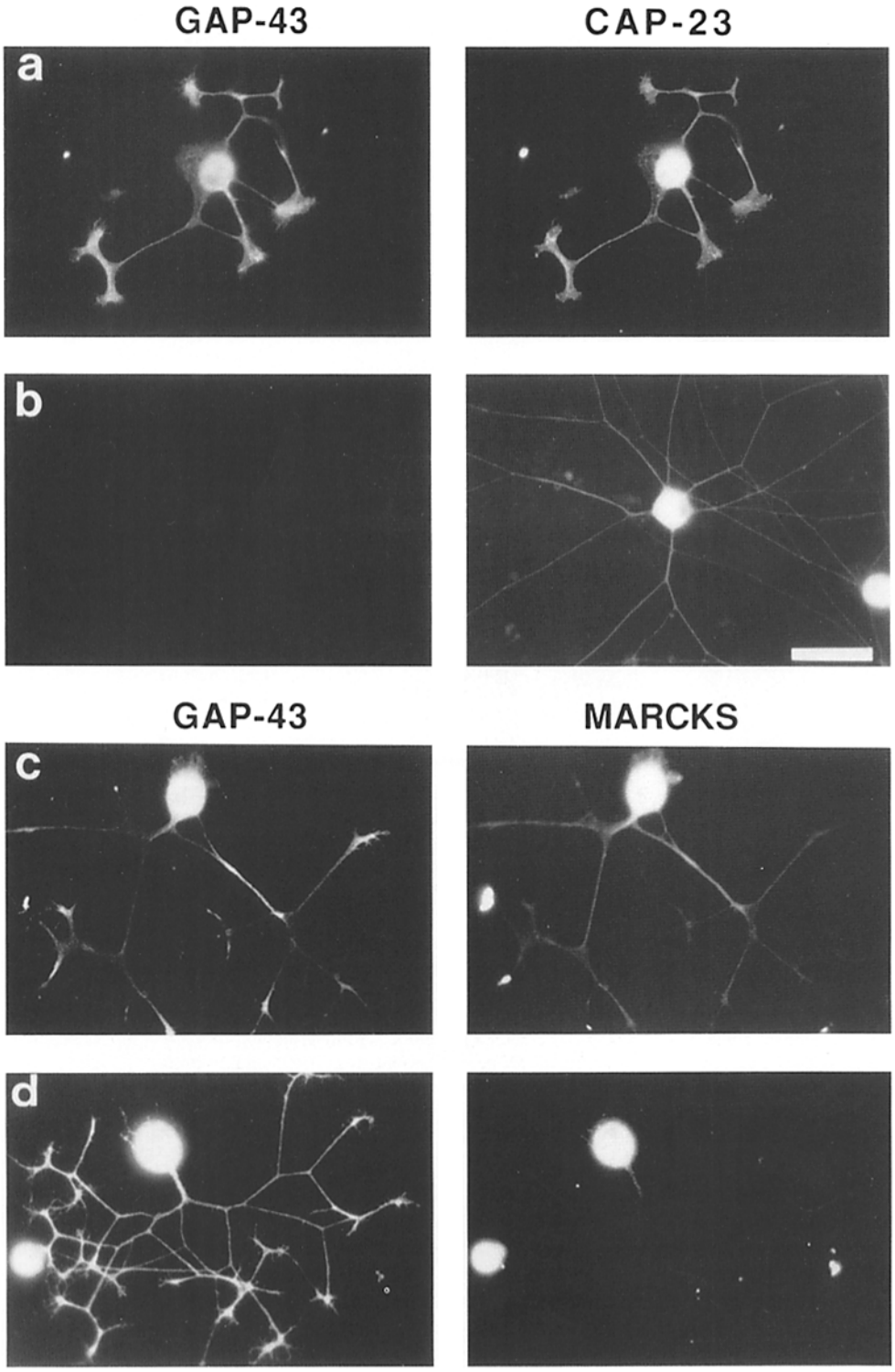

Figure 3. (*a* and *b*) Suppression of GAP-43 expression in DRG neurons does not affect the signal levels for the related cortical cytoskeleton-associated PKC substrate CAP-23. Experiments were carried out as described in Fig. 1, *a–c*. Oligonucleotides: (*a*) sense-3 (*b*) antisense-3. Double-labeling experiments with mAb to CAP-23 and antiserum to GAP-43. (*c* and *d*) Treatment of DRG neurons with an antisense oligonucleotide to chick MARCKS mRNA reduces MARCKS IR without detectable effects on GAP-43 IR or neurite morphology (see also Table II). Cells were treated with 200 μM sense (*c*) or antisense (*d*) MARCKS oligonucleotide, fixed and stained with an antiserum against chick MARCKS and with mAb 5F10 against GAP-43. Bar, 50 μm.

on laminin (data not shown). However, during the first 1–2 h in culture, when neurite lengths were comparable with cell diameters, GAP-43–depleted neurons did display slightly larger growth cones (see Fig. 1 *e*). This residual growth cone activity may be due to a greater tendency to form large growth cones during the very initial phases of neurite regeneration.

The GAP-43–depleted neurons of Figs. 1 and 3 have thinner, longer and more numerous neurites than their GAP-43–expressing counterparts. A quantitative analysis of neuritic morphological parameters as a function of the levels of GAP-43 immunoreactivity is shown in Fig. 5. This analysis shows that in the absence of GAP-43 DRG neurites extending on laminin are particularly long, thin, and with little branch

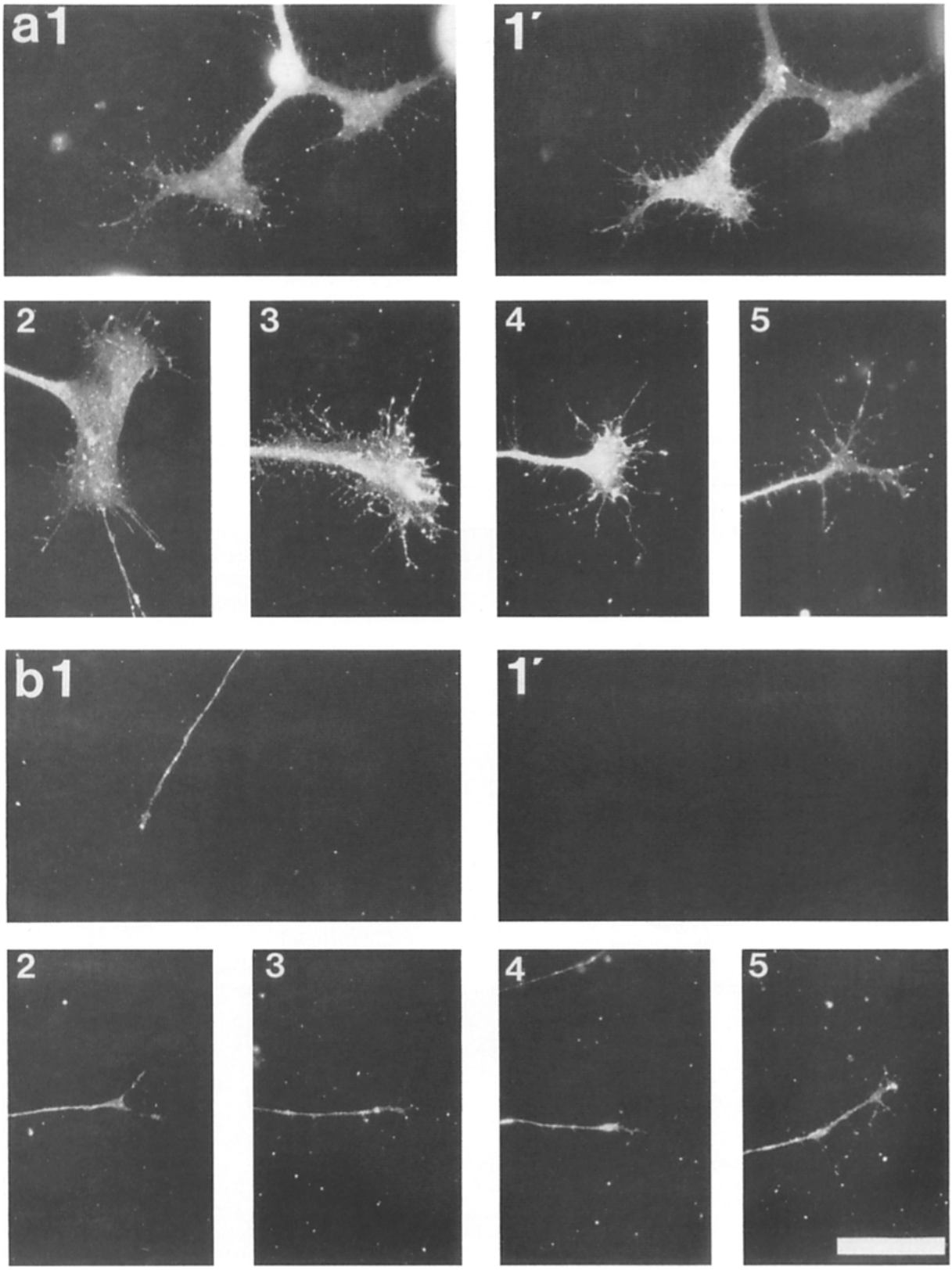

Figure 4. GAP-43–depleted DRG neurons regenerating neurites on laminin have drastically reduced growth cone sizes. Cells were treated with 200 μM sense-3 (*a1–a5*) or 200-μM antisense-3 (*b1–b5*) oligonucleotide, cultured for 18 h on poly-L-ornithine/laminin-coated coverslips, fixed and stained for chick GAP-43 and for β1-integrin. (*a1–5* and *b1–5*) integrin; (*a1'*, *b1'*) GAP-43 signal corresponding to *a1*, *b1*. The growth cones shown in the figure are representative of the range of morphologies detected in the two types of experiments (see also quantitative data of Table I). As in *b1*, growth cones in *b2–b5* displayed no detectable GAP-43 IR. Bar, 25 μm.

Table I. Relationship between Growth Cone Size and GAP-43 IR Levels in DRG Neurons Regenerating Neurites on a Laminin Substratum

	Growth cone size		
	$x \geq 250\ \mu m^2$	$30\ \mu m^2 \leq x \leq 250\ \mu m^2$	$x \leq 30\ \mu m^2$
	% cells	*% cells*	*% cells*
Distribution in the culture			
Sense (n = 92)	43.5	35.9	20.6
Antisense (n = 86)	26.8	27.9	45.3
Distribution according to GAP-43 levels			
Sense ++	28.3	15.2	1.1
Sense +	15.2	20.7	17.3
Sense −	0	0	2.2
Antisense ++	9.3	3.5	0.
Antisense +	17.5	16.3	8.1
Antisense −	0	8.1	37.2

Experimental conditions were as described in Fig. 1. All cells from randomly selected fields with process(es) longer than two cell diameters were scored, and the size of the cell's largest growth cone was estimated. $x > 250\ \mu m^2$: growth cones larger than that shown in Fig. 4, *a4*; $x < 30\ \mu m^2$: growth cones smaller than that shown in Fig.4, *b2*. IR levels were defined as in Fig. 2. *n* is the total number of cells that were analyzed per type of experiment. Each of these total numbers of cells was set as 100% in the sense and antisense experiments. The data indicate the presence of a strong correlation between growth cone size and GAP-43 IR levels.

points. For each separate experiment, antisense- and corresponding sense-oligonucleotide–treated neurons were generated and analyzed, and the neuritic morphology parameters displayed the same dependency on the levels of GAP-43 immunoreactivity, irrespective of whether the analysis encompassed a single set of experiments or all experiments were combined. Significantly, as in the growth cone size analysis, parameter values did not depend on the presence of oligonucleotides during the dissociation procedure, but only on the final levels of GAP-43 IR in the DRG neurons. This observation suggests that dissociation of the DRGs in the presence of antisense oligonucleotides to GAP-43 affected the relative contents of neurons with high, intermediate, and extremely low levels of GAP-43 immunoreactivity, and it was the levels of GAP-43 that produced the differences in growth cone and neuritic morphology. This relationship between a specific shift by the GAP-43 antisense oligonucleotides towards neurons with lower or undetectable GAP-43 IR and the distribution of neuritic morphologies in the culture was confirmed by the morphometric analysis of neurons in the control- and antisense-treated cultures irrespective of GAP-43 IR levels (Table II, see also Table I). The data of Table II also demonstrate that neuritic morphology was specifically affected by antisense oligonucleotides to GAP-43, but not by antisense oligonucleotides to the related PKC substrate MARCKS. These data support our interpretation that variations in growth cone size, neurite length, and branching correlated with GAP-43 IR levels in the neurons, and that the effect of the antisense oligonucleotides was to shift the GAP-43 content distribution towards lower levels of this protein. This interpretation is further supported by the data of Fig. 6: both sense- and antisense-treated cultures contained neurons with high, intermediate and low-to-undetectable GAP-43 IR levels, and GAP-43 contents correlated with neuritic morphology in both types of cultures.

Impaired Formation of Neurites by GAP-43–depleted DRG Neurons Grown on a Polyornithine Substratum

Laminin is a potent promoter of neurite outgrowth for DRG neurons (Rogers et al., 1983), and downstream reactions to

Table II. Depletion of GAP-43 Specifically Affects Neurite Morphology in Cultured Sensory Neurons

	Neurite length (μm)		Branching (branchpoints/100 μm)	
	Found (*n* = 200)	Predicted from data Fig. 5 *a*	Found (*n* = 200)	Predicted from data Fig. 5 *b*
Untreated	316 ± 210*/259 ± 174‡	251 ± 164	1.24 ± 1.16/1.33 ± 1.00‡	1.39 ± 0.88
s 3 GAP-43	286 ± 203/300 ± 202	257 ± 187	1.13 ± 0.90/1.25 ± 1.07	1.10 ± 0.73
as MARCKS	279 ± 166/295 ± 198	ND	1.13 ± 0.72*/1.23 ± 1.08	ND
as 3 GAP-43	381 ± 219*/398 ± 221‡	396 ± 199	0.89 ± 0.64*/0.83 ± 0.60‡	0.87 ± 0.75
Significance range	$p < 0.03$*/$p < 0.0005$‡		$p < 0.025$*/$p < 0.00005$‡	

Neurite length (cell's longest neurite) and branching was determined for control cultures (untreated, sense GAP-43, antisense MARCKS) and for antisense GAP-43–treated cultures. 200 cells with neurites longer than two cell diameters per experiment were scored and data from two experiments each are shown. Values are means ±SD. For neurite length, significance was determined by the Mann-Whitney *U* test, for branching, *t* test was used. Predicted values were determined by pooling data from the three GAP-43 IR levels (Fig. 5 *a*, respectively Fig. 5 *b*) after weighing them for their relative frequency in each type of culture. Note that the average values of neuritic morphology parameters can be derived from the weighted contributions of the neurons with high, intermediate and undetectable GAP-43 IR levels.

* The worst significance values.

‡ The best significance values.

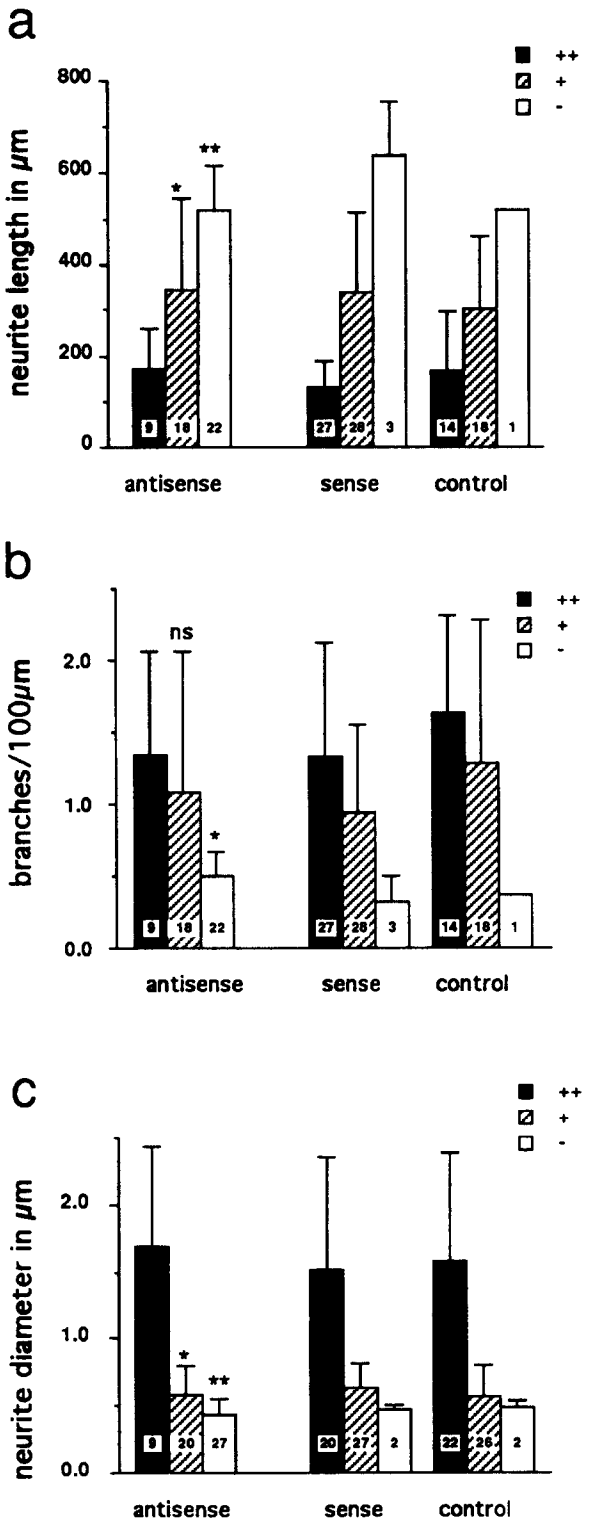

Figure 5. Relationship between neurite morphology and levels of GAP-43 IR. Experiments were carried out as described in Fig. 1. Cells from randomly selected fields with processes longer than two cell diameters were scored, and subdivided into GAP-43 IR levels as described in Fig. 2 *a*. Data from two independent experiments were pooled for each type of culture. (*a*) Neurite length (length of the longest neurite). * $P < 0.02$; ** $P < 0.0005$. (*b*) Branchpoints per 100 µm of neurite length as a function of GAP-43 IR. *ns*, not significant; * $P < 0.001$. (*c*) Neurite diameter. * $P < 0.0005$; ** $P < 0.00005$. Significance of differences as compared with strong GAP-43 IR group are given for the three morphological parameters. For neurite length, the significance was determined by the Mann-Whitney *U* test because the distribution of the values was skewed. The remaining significance values were determined by *t* test. Values are means ±SD. Numbers in the columns represent the number of cells analyzed. Note that antisense oligonucleotides only affected the relative contents in the culture of neurons with high, intermediate, and undetectable levels of GAP-43 IR.

the interaction of the growth cone with laminin involve the activation of PKC (Bixby, 1989). GAP-43 is a major PKC substrate of the growth cone, and it can interact with and modulate the function of components involved in signal transduction, such as calmodulin, G_o and PIP-kinase (Andreason et al., 1983; Strittmatter et al., 1990; van Dongen et al., 1985). To determine whether the characteristic morphological changes in GAP-43-depleted neurons were restricted to the laminin substratum, we plated these neurons in the same culture medium, but on polyornithine without laminin. We further reasoned that if laminin was providing the neurons with a strong stimulus to grow, the absence of such a stimulus may better reveal possible competence impairments in GAP-43-depleted neurons.

As shown in Fig. 7, GAP-43-depleted DRG neurons failed to form neurites when plated on polyornithine in the presence of NGF. Clearly, in the absence of laminin neurite outgrowth was also reduced in neurons expressing high levels of GAP-43 (Fig. 7, *a*, *b*, and *e*). However, and in contrast to the outcome on a laminin substratum, in the absence of GAP-43 no growth cone or neurite emerged from the initial lamellar configuration (Fig. 7, *c*, *d*, *f*, and *g*). Similar observations were made when the neurons were examined 2, 4, or 8 h after plating. Total numbers of fixed neurons at the end of the various experiments were undistinguishable in the sense- and antisense oligonucleotide-treated cultures. Thus, failure to form neurites in the absence of GAP-43 was not due to overall reduced adhesiveness in these neurons. In addition, amounts of GAP-43-depleted neurons did not differ from those expressing GAP-43 when cell attachment was compared on laminin, polyornithine, or glass (data not shown). It therefore appears that GAP-43-depleted neurons did respond to laminin with neurite outgrowth, but that in the absence of laminin an intrinsic impairment in their ability to form growth cones and neurites was revealed. As a consequence, GAP-43-depleted neurons formed lamellae with rudimentary asymmetries and filopodia, but no growth cones nor neurites emerged.

Discussion

We have depleted E16 chick DRG neurons of GAP-43 by an antisense oligonucleotide approach. This allowed us to analyze neurite regeneration by cultured primary neurons in the absence of detectable levels of GAP-43 immunoreactivity. The analysis reveals, to our knowledge for the first time, that the formation, morphology, and growth of growth cones and neurites are affected by the levels of GAP-43 in the neurons. These findings suggest that levels of GAP-43 in growth cones, axons and nerve terminals may affect the local responsiveness of these structures to morphogenetic stimuli, thus modulating their competence to grow.

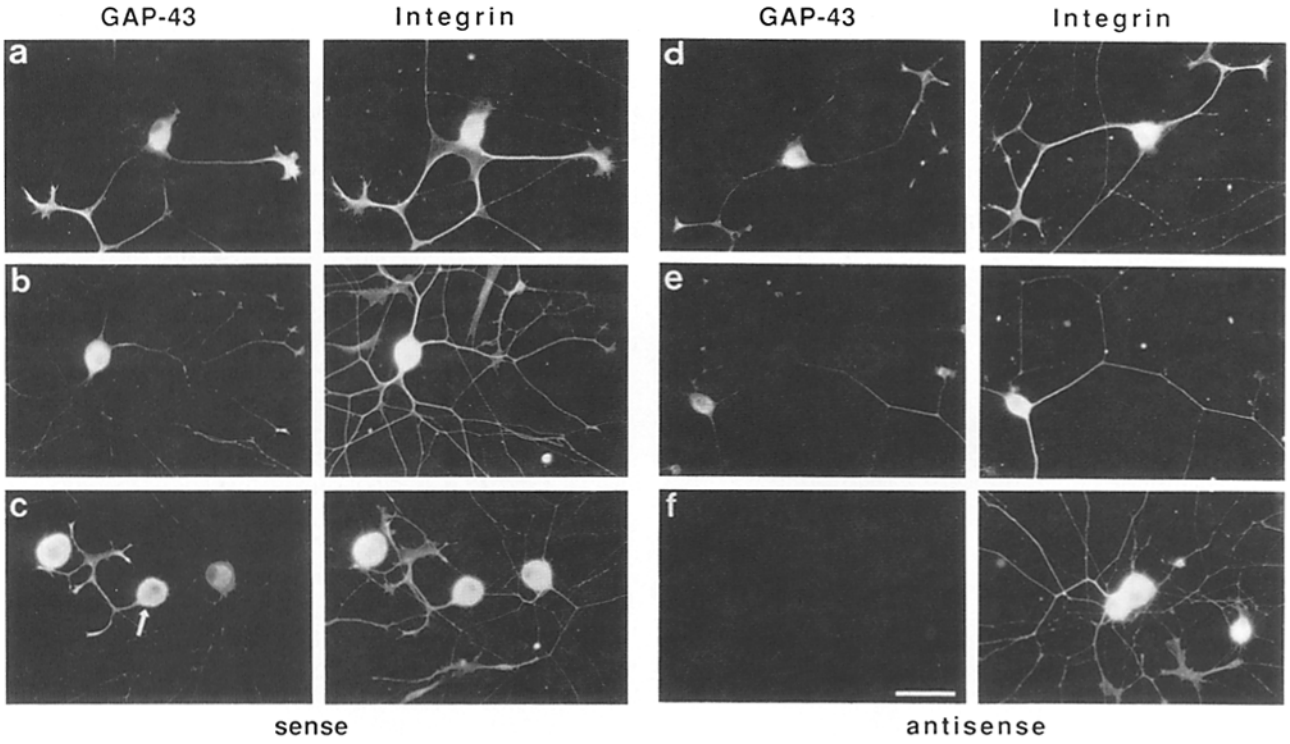

Figure 6. Neurons with high, intermediate, and nondetectable GAP-43 IR levels have similar neuritic morphologies in sense- and antisense-treated cultures. Experiments were carried out as described in Fig. 1. Data are from 18-h cultures. These data support the conclusion that shifts in neuritic morphologies in cultures treated with antisense GAP-43 oligonucleotides were due to GAP-43 depletion in these cultures. Note both, neuron expressing high levels of GAP-43 (*arrow*) and neuron expressing very low levels of GAP-43 in same field of sense-treated culture (*c*). Also note that in neurons with intermediate signal levels GAP-43 IR tended to be lower in neurites than in cell bodies. Bar, 50 μm.

Production of GAP-43–depleted Primary Neurons

The DRG trituration method described in this study was adapted from a procedure developed to introduce the oncogenic form of the ras protein in cultured chick DRG neurons (Borasio et al., 1989). In that study, an efficiency of transfer of close to 100% was estimated, and sufficient amounts of a 21-kD protein could be introduced into the neuronal cytosol to rescue significant numbers of the neurons in the absence of NGF. Our results suggest that the treatment with antisense oligonucleotide against GAP-43 may have affected GAP-43 protein levels in most DRG neurons. To estimate the efficiency of the method we triturated the DRGs in the presence of biotin-coupled oligonucleotide. After 4 h in culture >70% of the cells contained fluorescently labeled oligonucleotide in their cell body and nucleus (data not shown). In addition, the data from the quantitative analysis of Fig. 2 are consistent with the interpretation that the antisense oligonucleotide treatment specifically produced a general shift of GAP-43 levels in most neurons in the culture, possibly leading from high to intermediate and from intermediate to undetectable signal values.

It is not usual to culture DRG neurons from E16 chicks. Our selection of this late stage, as opposed to neurons from younger embryos, that regenerate neurites in culture with greater efficiency was dictated by the difficulties that we encountered in depleting younger chick DRG neurons from GAP-43. Application of the same experimental procedure to E8–E10 DRGs yielded a similar shift in the distribution of GAP-43 signal levels in cultured neurons, but almost no neurons could be depleted of GAP-43 immunoreactivity. This observation is probably due at least in part to the higher initial contents of GAP-43 in these neurons. In addition, preliminary pulse–chase experiments with ^{35}S-labeled amino acid precursors suggested that the metabolic half-life of GAP-43 in E8–E10 chick DRG neurons (>6 h) is longer than in older neurons. It is therefore possible that an additional difficulty in rapidly depleting young DRG neurons of GAP-43 by an antisense approach is the comparably high metabolic stability of the protein in these neurons. As a consequence, we have restricted our presentation to the data with E16 neurons, but a brief discussion of the comparable phenotypic effects caused by reduced GAP-43 levels on E8 DRG neurons is presented below.

Depletion of GAP-43 IR was specifically due to interaction of the antisense oligonucleotides with GAP-43 mRNA (Fig. 2 *c*), and an oligonucleotide complementary to MARCKS mRNA affected MARCKS IR, but not GAP-43 IR (Fig. 3 *d*). In addition, the antisense oligonucleotide procedure specifically affected GAP-43 levels, without obvious modifications of the signal levels for β1-integrin, CAP-23 or neurofilament-160. Integrin-β1 is a subunit of cell surface receptors for extracellular matrix proteins that affect neurite outgrowth (Horwitz et al., 1985; Tomaselli et al., 1986; Letourneau and Shattuck, 1989); CAP-23 is, like GAP-43, a PKC substrate of the cortical cytoskeleton (Widmer and Caroni, 1990); and neurofilament-160 is a major component of the neuritic cytoskeleton. Therefore, although the levels of other neuronal proteins were not assessed, it is clear that the antisense procedure not only specifically affected the lev-

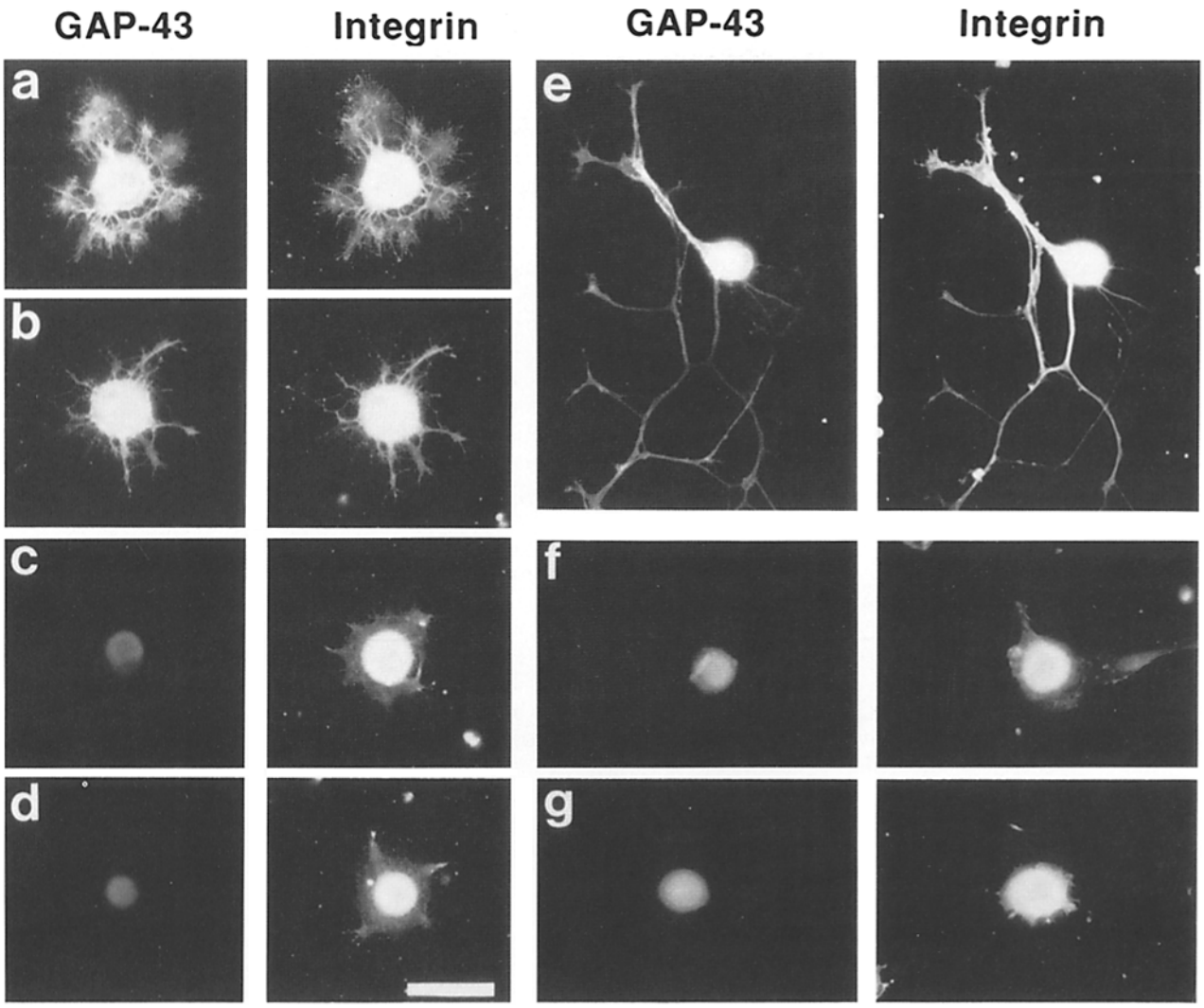

Figure 7. GAP-43–depleted neurons fail to form growth cones and neurites when plated on poly-L-ornithine. Experiments were carried out as described in Fig. 1, except that the poly-L-ornithine substratum had not been additionally coated with laminin. (*a–d*) 15-h culture; (*e–g*) 23-h culture. (*a*, *b*, and *e*) Sense-3 oligonucleotide; (*c*, *d*, *f*, and *g*) antisense-3 oligonucleotide. Bar, 50 μm.

els of GAP-43, but probably did not cause generalized alterations in proteins involved in neuritic outgrowth.

Role of GAP-43 in Neurite Outgrowth

The properties of E16 chick DRG neurites regenerating on a laminin substratum correlated with the levels of GAP-43 in the neurons. In the presence of high levels of GAP-43 neurites were of larger diameter, had larger growth cones, and branched more as compared to those having low amounts of GAP-43. In the absence of detectable levels of GAP-43 IR neurites were longer, very thin, with nearly undetectable growth cone structures and little branching points. The quantitative analysis of Fig. 2, Table I, and Table II indicates that the distribution of neuritic morphologies in the culture correlated with that of neurons with strong, intermediate and undetectable GAP-43 IR levels, and that both were affected by the antisense treatment in a similar manner. These data support the interpretation that the relationship described here between the pattern of neurite outgrowth and GAP-43 levels is a direct outcome of GAP-43 contents in the neurons. These experiments therefore indicate that GAP-43 levels affect growth cone and neurite formation and morphology in primary neurons.

Most GAP-43–sensitive properties of the neurites may be rationalized assuming that the main site of action of GAP-43 is the growth cone. Thus, reduced branching and longer neurites in the absence of GAP-43 are likely to be due to reduced growth cone exploratory activity. This may lead to reduced hindrance of the pushing activity by the microtubule-based elongation apparatus (Letourneau et al., 1987). Time-lapse video microscopic analysis of DRG neurites regenerating in the presence and absence of GAP-43 should indicate whether this interpretation is correct. In the absence of detectable GAP-43 IR, neurites of strikingly thin diameter were already observed during the initial phases of process outgrowth on laminin (Fig. 1 *e*). This may either be a consequence of reduced contents of neurite-forming components in small growth cones, of a diminished laminin-mediated stimulus for neurite formation, or of reduced adhesiveness in GAP-43–depleted neurites. Here again, time-lapse video microscopic analysis may provide indications on whether the reduced neurite diameter of GAP-43–depleted neurons is related to growth cone activity.

We detected a similar relationship between GAP-43 contents and neuritic morphology in E8 DRG neurons growing on laminin in the presence of NGF. Neurons with low contents of GAP-43 displayed an initial tendency to elaborate large extended lamellae that failed to form the characteristic point of transition between growth cone and neurite. Neurites growing in the presence of low levels of GAP-43 were thin, little branched, longer than average, with frequent lateral lamellae and unpolarized growth cones (data not shown). Therefore, like in E16 neurons, low levels of GAP-43 appeared to interfere with growth cone formation and activity in the E8 DRG neurons.

When grown on polyornithine, GAP-43–depleted neurons failed to form growth cones and elongate neurites. This may possibly have been due to the absence of a strong neurite outgrowth stimulus from the substratum. These observations are consistent with recent reports on the outgrowth of neuroblastoma (Shea et al., 1991) and PC12 neurites (Yankner et al., 1990; Jap Tjoen San et al., 1992) in the presence of low or elevated GAP-43 activity. In neuroblastoma cells, introduction of an antibody to GAP-43 into the cytosol by lipofection interfered with the formation of neurites (Shea et al., 1991). Since these cells may have a comparatively low potential of extending neurites, the effect of the antibody may be related to our observations with GAP-43–depleted neurons on polyornithine. Transfected PC12 lines expressing elevated levels of GAP-43 in a constitutive manner responded markedly more vigorously to stimulation of neurite formation by NGF (Yankner et al., 1990). Therefore, like in our experiments, high initial levels of GAP-43 may enhance a neuron's responsiveness to a neurite outgrowth stimulus. Similar conclusions on a possible role of GAP-43 in mediating responsiveness of PC12 cells to the neurite-promoting activity of NGF were reached in a recent depletion study with antisense oligonucleotides (Jap Tjoen San et al., 1992).

Possible Mechanisms

One possible interpretation of our data is that levels of GAP-43 affect growth cone and neurite adhesiveness. Thus, GAP-43–depleted neurons regenerating neurites on laminin may display smaller growth cones, reduced branching, and thinner neurites due to reduced attachment to the substratum (Bray, 1982). Arguing against a simple reduction of the effects of GAP-43 to changed adhesiveness, however, are the observations that, first, attachment of DRG neuronal cell bodies on laminin, polyornithine or glass was not affected by GAP-43 levels, and second, that on a very adhesive substrate like polyornithine growth cone and neurite, but not lamellae formation was impaired in the absence of GAP-43. These results are therefore more consistent with the hypothesis that GAP-43 may affect the responsiveness of the growth cone, and possibly also of the neurite machinery to growth signals. Accordingly, GAP-43 may be an amplifier of local stimuli to the growth cone and neurite (see also Strittmatter et al., 1992). This interpretation is consistent with our observations on the effects of GAP-43 and its phosphorylation-site mutants on spreading and local surface morphology of non-neuronal cell lines (Widmer and Caroni, 1993). In these transfection experiments, GAP-43 appeared to promote the formation of local actin-containing surface structures like filopodia and pseudopods. This effect of GAP-43 was especially prominent during cell spreading and was superimposed onto the intrinsic tendency of different cell types to form such structures. In addition, phosphorylation-site mutants of GAP-43 had opposite effects on cell spreading and morphology: a Ser42 to Ala42 mutant interfered with cell spreading, whereas a Ser42 to Asp42 mutant promoted cell spreading. It is therefore tempting to speculate that GAP-43 may amplify local signals involved in local surface morphogenetic activity, and this function of GAP-43 may be modulated by PKC-mediated phosphorylation. The appearance of neurites regenerating from DRG neurons on laminin in the absence of GAP-43 may be due to altered responsiveness of the growth cone to some of the neurite-promoting activities induced by laminin. Similarly, reduced competence to elaborate neurite-associated structures in GAP-43–depleted neurons may prevent the formation of growth cones and neurites on a polyornithine substratum.

What growth cone components are affected by GAP-43? In neurons and in transfected cells, GAP-43 levels affected the organization of actin-based cell surface structures, at the tip of the growth cone and pseudopods. In addition, during spreading of transfected cells, co-accumulation of GAP-43 and F-actin at surface structures was detected (Widmer and Caroni, 1993). Conceivably, therefore, GAP-43 may affect the local activity of actin-based structures at the cell surface, possibly by interacting with molecular components that control the dynamic properties of these structures. It will therefore be important to determine whether GAP-43 and proteins of potentially related function like CAP-23 (Widmer and Caroni, 1990) and MARCKS (Aderem, 1992) interact with proteins involved in microfilament dynamics.

Function of GAP-43

Our observations can be related to the pattern of expression of GAP-43 during development and regeneration to generate hypotheses on the possible biological role of this protein. The proposed role for GAP-43 in amplifying local environmental signals into growth cone activity implies that this protein may enhance competence in axonal outgrowth and guidance, and its downregulation at the time of the elimination of collaterals may coincide with a reduced susceptibility of axons to local growth stimuli (see also Skene, 1989). Similarly, reinduction of GAP-43 may potentiate regeneration, and its presence in selected adult CNS regions may indicate an elevated susceptibility for local growth. A similar hypothesis was proposed recently by Strittmatter et al. (1992). Further, it should be mentioned in this context that the expression of GAP-43, and presumably its range of action, is not restricted to neurons, as differentiating astrocytes, oligodendrocytes, and Schwann cells express GAP-43 (Vitkovic et al., 1988; da Cunha and Vitkovic, 1990; Deloulme et al., 1990; Curtis et al., 1991; Curtis et al., 1992; Woolf et al., 1992). Like in neurons, GAP-43 expression in glial cells coincides with phases of elevated morphogenetic and motile activity. This relation between GAP-43 expression and morphogenetic activity is particularly striking in terminal Schwann cells at denervated neuromuscular junctions, where reinduction of GAP-43 coincides with a dramatic expansion of this cell's processes in the perisynaptic space (Woolf et al., 1992). In conclusion, GAP-43 may be an important amplifier of local morphogenetic processes at the cell cortex in both neurons and in glial cells.

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