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INHIBITION OF NERVE GROWTH FACTOR-INDUCED B-50/GAP-43 EXPRESSION BY ANTISENSE OLIGOMERS INTERFERES WITH NEURITE OUTGROWTH OF PC12 CELLS

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Substantial evidence has now been gathered for the involvement of B-50/GAP-43 in neuronal development and regeneration. The precise role of this protein, however, is still debated. In an earlier study, a linear correlation between NGF-induced neurite outgrowth and B-50/GAP-43 levels was observed in PC12 cells. To establish the involvement of B-50/GAP-43 expression in neurite outgrowth in these cells, we interfered with the expression by antisense oligomers and measured the outgrowth. In the present study, a B-50/GAP-43 antisense 5'-oligomer interfered both with the NGF-induced increase in B-50/GAP-43 and with neurite outgrowth, whereas an antisense 3'-oligomer was ineffective. We conclude, that in PC12 cells under normal conditions B-50/GAP-43 expression and neurite outgrowth are or become coupled upon NGF-induction, in contrast to the situation in PC12 clones with no or very low B-50/GAP-43 expression. © 1992 Academic Press, Inc.

The growth-associated, nervous tissue specific phosphoprotein B-50 (also termed GAP-43, F1, pp46 or neuromodulin) is one of the abundant proteins of the neuronal growth cone (1,2). In view of its localization at the cytoplasmic face of the plasma membrane (3), its atypical calmodulin binding (4) and the phosphorylation by protein kinase C, it is thought to be involved in transmembrane signal transduction (5-7). Different experimental approaches have shown enhanced B-50/GAP-43 expression to be highly correlated with neurite outgrowth during development and differentiation (1,2,8,9). In PC12 cells (10), NGF-induced differentiation and development of neurites is paralleled by a 1.5 - 3 fold increase in B-50/GAP-43 expression (11-13) which can be attenuated by dexamethasone

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ABBREVIATIONS

DEX, dexamethasone: NGF, nerve growth factor.

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(DEX) (14-16). However, a causal link between B-50/GAP-43 expression and neurite outgrowth is still under debate. Recently, Baetge and co-workers (17) reported that a PC12 clone, virtually devoid of B-50/GAP-43, displayed seemingly normal neurite outgrowth in response to NGF. In contrast, Yankner et al. (9) showed that increased expression of B-50 GAP-43 in PC12 cells accelerates the NGF-induced outgrowth while expression of B-50 in non-neuronal cells induced filopodia formation (8). Interference studies, introducing anti B-50/GAP-43 antibodies in NB2a/1d neuroblastomas blocked neurite outgrowth (18).

In this study we specifically inhibited B-50/GAP-43 expression using antisense oligomers and monitored B-50/GAP-43 levels and neurite outgrowth quantitatively in the same cells. Upon NGF-treatment, both the rise in B-50/GAP-43 and neuritogenesis were blocked suggesting a coupling between these parameters under the present conditions.

MATERIALS AND METHODS

Oligomer synthesis.

Oligodeoxynucleotides (15-mers) were trityl-on synthesized on a Biosearch 8600 solid phase DNA synthesizer. Large scale purification was performed with OPC cartridges (Applied Biosystems) and purity of the oligomers was checked by polyacrylamide gel electrophoresis. Final purification was performed by column chromatography (NAP column, Pharmacia). Oligomer concentrations were spectrophotometrically determined.

B-50 ELISA and fiber outgrowth determination.

PC12 cells were cultured in polylysin coated 96-wells plates in chemically defined N1 medium (19) at a density of 1×10^4 cells per well. For antisense interference studies, cells were allowed to adhere for 1.5 h before addition of NGF (β type, 5 ng/ml; (13)). After 24 h, cells were fixed and relative B-50/GAP-43 levels were determined by enzyme linked immuno sorbent assay (ELISA) as previously described (13). Next, cells were stained with Coomassie Brilliant Blue R (0.1% in 25% isopropanol/10% acetic acid) and analyzed for neurite length by computer-assisted video image analysis as described elsewhere (13). Briefly, digitized microscopic images are eroded to one-pixel skeletons describing cell features. Cell bodies are extracted from the digitized image by gray-level erosion and counted. After dilation, cell bodies are subtracted from the skeletons resulting in a number of pixels representing the total length of processes present in the image. Three fields of each well were analyzed for neurite length (averaging 120 cells per well). Results are expressed as mean neurite length per cell (pixels per cell). Note that the same cultures are analyzed both for B-50 immunoreactivity and neurite length. Photographs were taken from the Coomassie Brilliant Blue stained cultures viewed with bright field optics using an Olympus IM inverted microscope and a 20x objective.

In vitro translations.

Wheat germ translations were performed as previously described (20) in 10 μl volumes with [^{35}S]methionine and 0.2 μg B-50 cRNA, in the presence or absence of 450 pmol oligomer. Radiolabelled protein products were analyzed by SDS polyacrylamide gel electrophoresis on 12.5% acrylamide gels and by autoradiography. Molecular weight markers and purified B-50/GAP-43 were run on the same gel and their positions are indicated. The position of B-50/GAP-43 was double-checked by immuno blotting (not shown).

RESULTS AND DISCUSSION

In a previous study (13) we designed a system to quantify B-50/GAP-43 levels and fiber outgrowth of PC12 cells. Treatment of PC12 cells with increasing concentrations of NGF, showed a linear correlation between B-50/GAP-43 expression and neurite outgrowth while dexamethasone treatment attenuated B-50/GAP-43 expression and impaired neuritogenesis of PC12 cells (16). To determine whether B-50/GAP-43 expression is necessary for neurite outgrowth of PC12 cells we selectively interfered with B-50/GAP-43 synthesis by addition of an antisense oligomer (asB-50-5'; 5'-dCAT ACA GCA CAG CAT-3'), complementary to the first 15 nucleotides of the coding region of B-50/GAP-43 mRNA. To check for the effects of oligomer addition *per se* two oligomers were designed as controls. A nonsense oligomer not directed to a sequence of known importance to neurite outgrowth (5'-dCTA TAG TAT TAT TAA-3'), but part of the sequence of bacteriophage Φx-174, and a 15-mer oligo (asB-50-3': 5'-dGCA TGT TCT TGG TCA-3') directed to the 3-primed end of the B-50/GAP-43 mRNA coding region (bases 663-677, open reading frame ends at base 678)(20) and similar in GC content as the antisense B-50/GAP-43 oligomer.

The effects of the oligomers were tested in a wheat-germ, cell-free translation system. Antisense B-50/GAP-43 5'-oligomer inhibited translation of B-50 cDNA by 90% (Fig. 1), the presence of nonsense oligomer did not affect the B-50 synthesis (Fig. 1). Thus, the antisense B-50/GAP-43 oligomer is able to specifically interact with B-50/GAP-43 translation.

After a single NGF addition, B-50/GAP-43 levels in PC12 cells start increasing within 8-12 h and peak at 24 h (13). Because oligomers are rapidly degraded intracellularly (21),

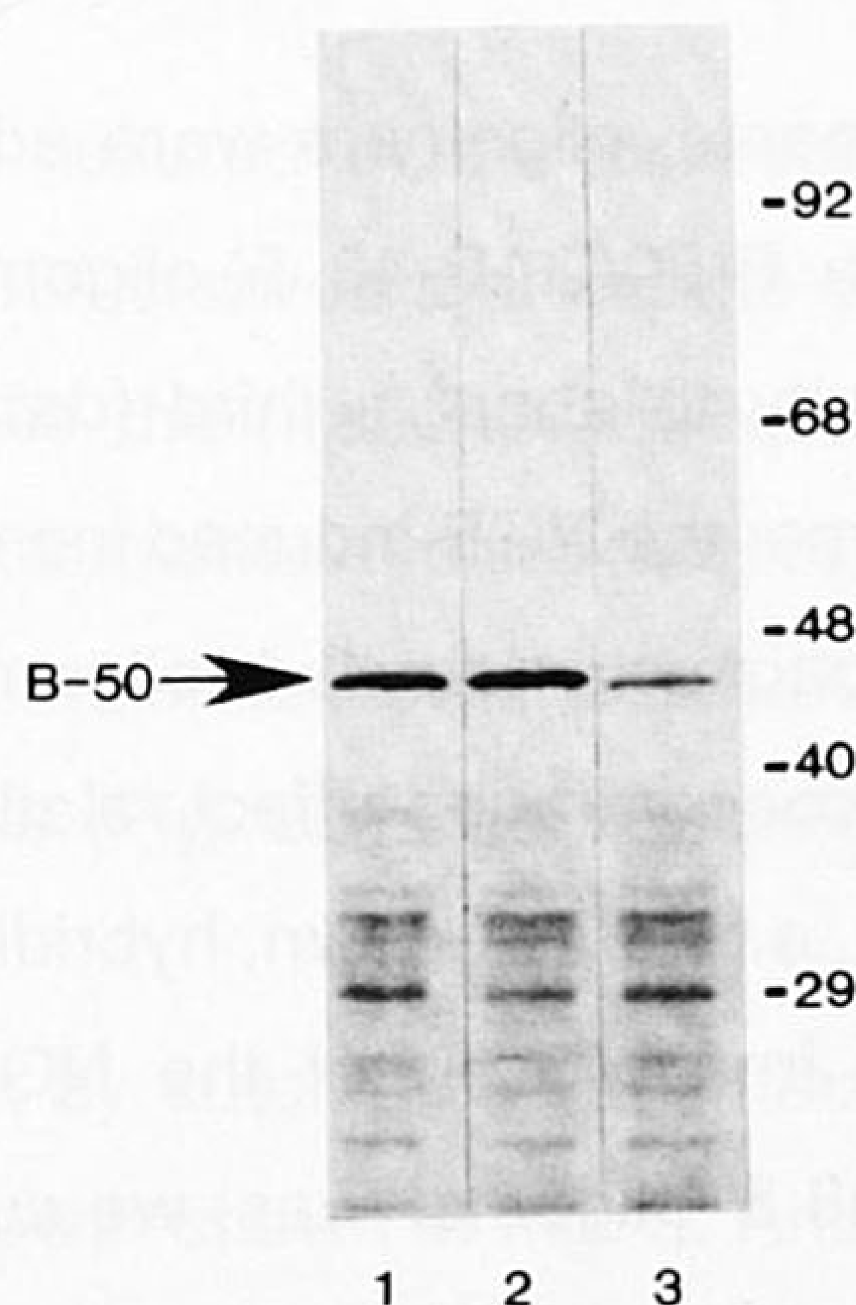

Fig.1. Inhibition of B-50 cDNA translation by antisense B-50/GAP-43 oligomers. The autoradiograph shows the analysis of in vitro translation of B-50 cRNA. Lanes: 1, in the absence of oligomers. 2, in the presence of nonsense oligomer. 3, in the presence of antisense B-50 oligomer. The right margin shows the positions of the marker proteins. The position of B-50/GAP-43, as identified on Western blot is indicated in the left margin.

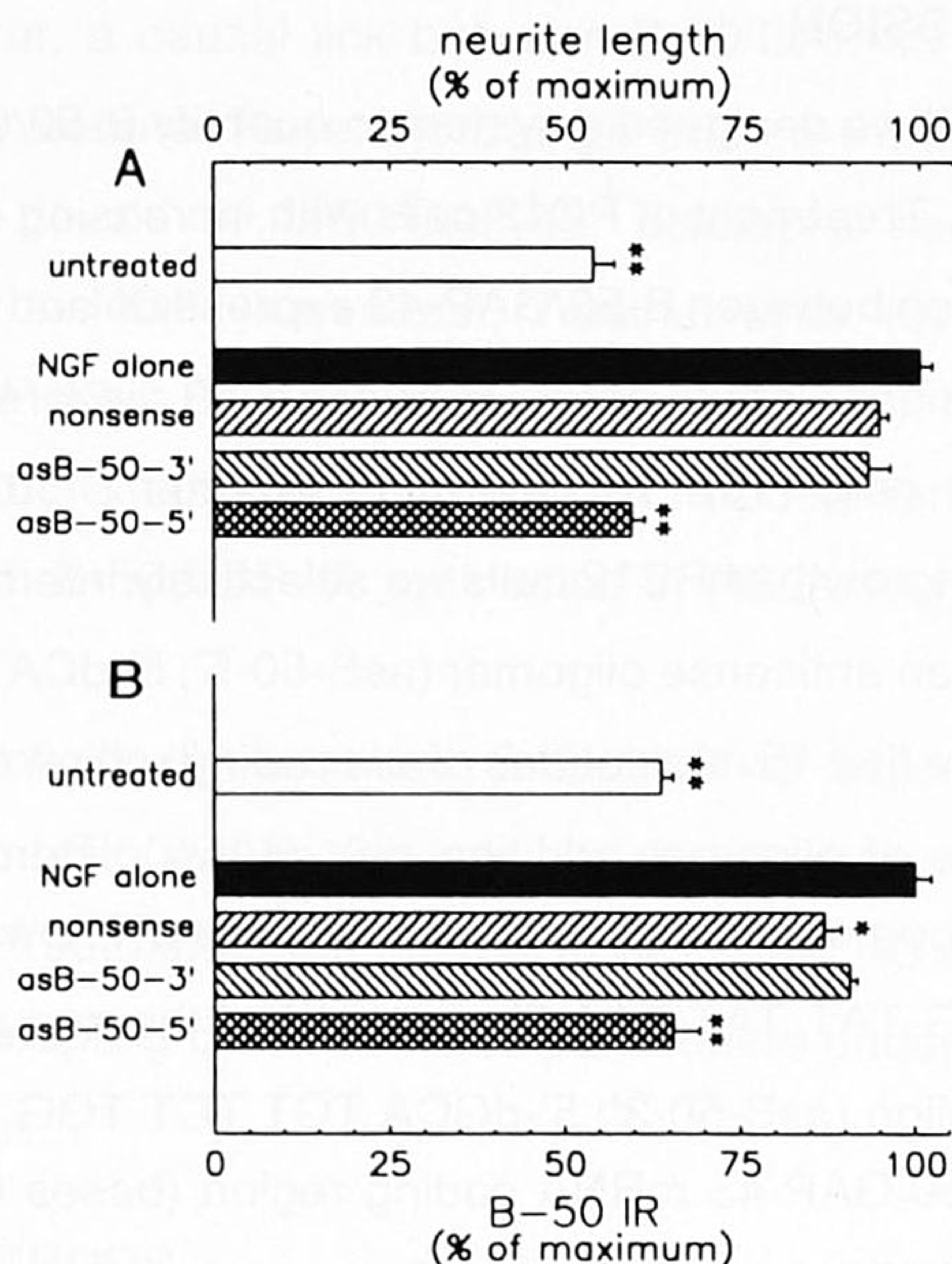

Fig. 2. Quantification of effects on neurite formation (A) and B-50/GAP-43 immunoreactivity (B-50 IR; B) in PC12 cells 24 hours after NGF addition. Results are expressed as percentage of the NGF-induced value (100%). All oligomer treatments were done in the presence of NGF as well. Mean ± SEM (n=8). * $p<0.005$ versus NGF alone, ** $p<0.001$ versus NGF alone, Tukey-Kramer HSD test.

careful timing of oligomer addition is important. In a first experiment, addition of 30 or 50 μM antisense B-50/GAP-43 5'-oligomer, 4 h prior to NGF, showed no inhibition of NGF-induced B-50/GAP-43 synthesis. Administration of 70 μM antisense B-50/GAP-43 5'-oligomer 4 h prior to NGF resulted in a 25% inhibition of B-50/GAP-43 levels (data not shown).

In a second series of experiments, oligomers were added simultaneously with NGF. Application of 50 μM antisense B-50/GAP-43 5'-oligomer inhibited the NGF-induced increase in B-50 immunoreactivity to about a third (data not shown), using 70 μM of antisense B-50/GAP-43 5'-oligomer the NGF-induced increase was completely prevented (Fig. 2B). As uptake of radioactively labelled oligomer was linear with oligomer concentration (not shown), the concentration-effect relationship is apparently controlled by factors other than the uptake, e.g. break-down, hybridization rate etc. Since, the asB-50-3' oligomer was ineffective in inhibition of the NGF-enhanced B-50 expression, whereas antisense B-50/GAP-43 5'-oligomer was, we suggest that RNase H activity is not responsible for the antisense effects. This notion is supported by the demonstration of oligomer:mRNA duplexes in PC12 cells by others (22). However, we cannot exclude the possibility that the 3' antisense B-50/GAP-43 oligomer resulted in synthesis of truncated, yet partly functional B-50/GAP-43.

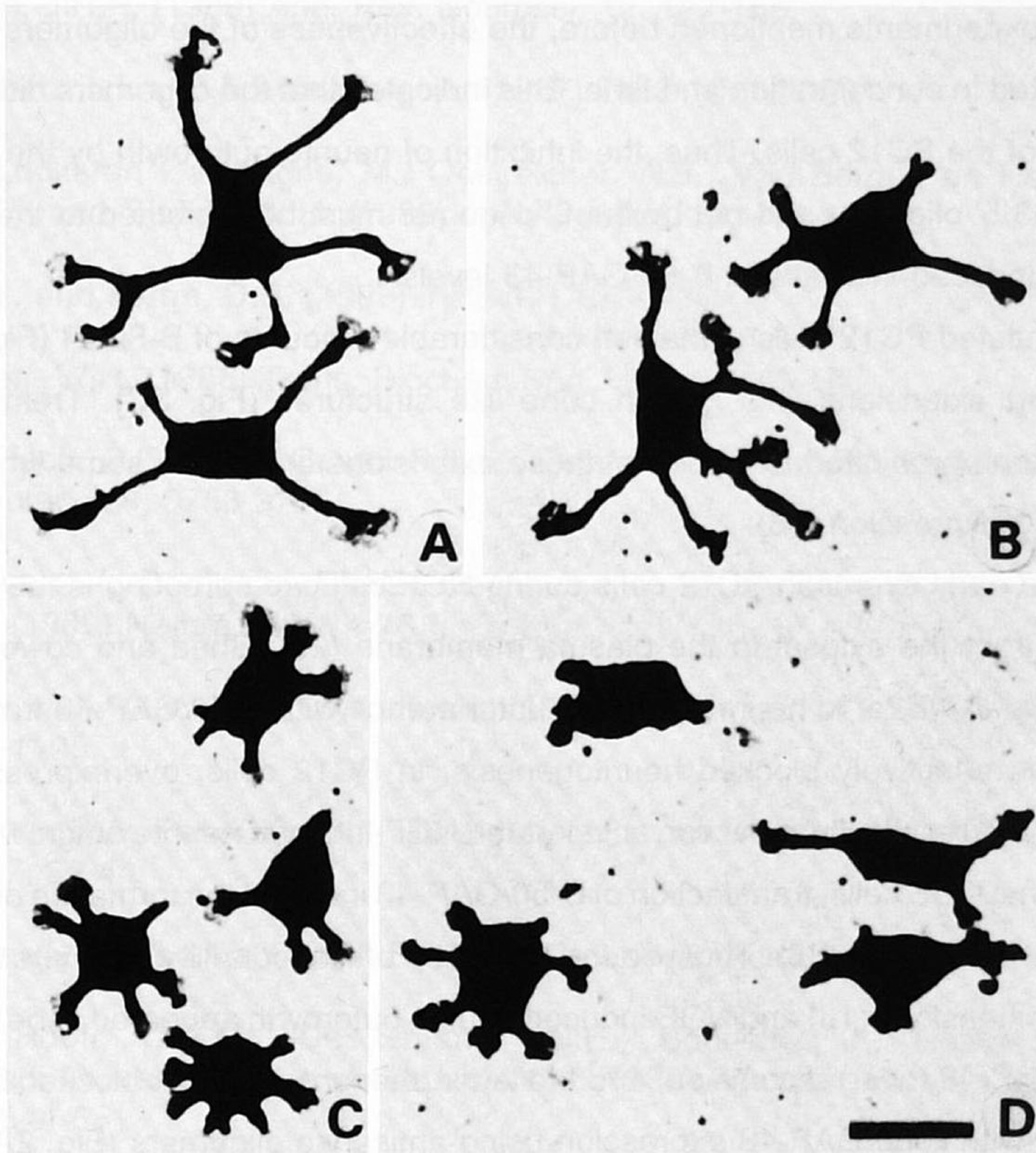

Fig. 3. Effects antisense oligomers on NGF-induced neuritogenesis in PC12 cells after 24 hours. Typical micrographs are shown of cells cultured with NGF alone (A), in the presence of NGF and asB-50-3' oligomer (B), in the absence of NGF (C) and in the presence of NGF and antisense B-50/GAP-43 oligomer (D). Scale bar is 25 μm.

Examination of PC12 cells cultured in the presence of NGF (Fig. 3A) and with antisense B-50 oligomer (Fig. 3D) showed that neurite outgrowth was severely impaired by the latter: Although grown in the presence of NGF (plus antisense B-50/GAP-43 5'-oligomer) (Fig. 3D), PC12 cells maintained the morphology of their untreated (no NGF) counterparts (Fig. 3C). Both the nonsense and the asB-50-3' oligomer permitted the NGF-induced B-50/GAP-43 synthesis (Fig. 2B) and the concomitant change in morphology (Fig. 3B).

Quantitation of neurite length by automated video image analysis (Fig.2A) confirmed the differences in neurite outgrowth shown in Fig. 3. Antisense B-50 completely blocked the NGF-induced increase in neurite length, whereas only one of the control oligomers showed a minor effect. The same specificity of the oligomer effects was shown on B-50/GAP-43 synthesis in cell-free translation (Fig.1) and on intracellular B-50/GAP-43 levels by ELISA (Fig. 2B). Moreover, the overall pattern of changes in neurite length

(Fig.2A) and in that of changes in B-50/GAP-43 (Fig.2B) were very similar. In the preliminary experiments mentioned before, the effectiveness of the oligomers appeared to be restricted in concentration and time. This indicates that the oligomers did not affect the viability of the PC12 cells. Thus, the inhibition of neurite outgrowth by the antisense B-50/GAP-43 5'-oligomer and not by the 3'-oligomer must be attributed to the inhibition of the NGF-induced increase in B-50/GAP-43 levels.

Undifferentiated PC12 cells contained considerable amounts of B-50 IR (Fig. 2A) and showed short extensions with growth cone like structures (Fig. 3C). Treatment with dexamethasone prohibited formation of these extensions and at the same time lowered B-50/GAP-43 expression (16).

One of the early events in PC12 cells during NGF-induced sprouting is the shift of B-50/GAP-43 from the cytosol to the plasma membrane (23). Shea and coworkers (18) described that in NB2a/1d neuroblastomas, interference with B-50/GAP-43 translocation by antibodies effectively blocked neuritogenesis. In PC12 cells, overexpression of B-50/GAP-43 as a result of transfection accelerated NGF-induced neurite outgrowth (9), and in nonneuronal COS cells, transfection of B-50/GAP-43 induced the formation of filopodia-like membrane structures (8). Thus, so far, formation of filopodia-like structures (8), short neurite-like extensions (16) and NGF-induced neurite outgrowth appeared to be correlated with B-50/GAP-43 (over)expression, and the latter phenomenon was blocked by specific interference with B-50/GAP-43 expression using antisense oligomers (Fig. 2A and B).

We suggest, that in wild-type PC12 cells during NGF-induction B-50/GAP-43 expression and neurite outgrowth are or become coupled. During this phase B-50/GAP-43 -in undifferentiated PC12 cells for a considerable part associated with lysosomal structures (23)- becomes membrane-bound and highly localized within growth cone structures (3,23): This process might be a factor in the coupling. In PC12 clones lacking B-50/GAP-43 but with many other features for neuritogenesis retained, such a coupling is not established, allowing neurite outgrowth (17,24) using more general (i.e. not axon specific) mechanisms (25). However, preliminary data revealed that those cells showed poor growth cone morphology (24). Present research is directed to unravel the role of the membrane-bound B-50/GAP-43 in growth cone morphology and motility.

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