Biological Activities of Nerve Growth Factor Bound to Nitrocellulose Paper by Western Blotting

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We have previously developed a cell-blot technique to visualize directly in tissue extracts molecules that display the biological activity of ciliary neurotrophic factors (CNTFs). This technique involves SDS-PAGE of the tissue extract, Western blotting on nitrocellulose paper, neuronal cell culture on the paper, and, using a vital dye, visualization of the neurons that selectively survive on the trophic factor band. In this report, we show that (1) NGF, either purified or in a crude extract from submaxillary glands, can also be successfully recognized using a slightly modified cell-blot technique; (2) a variety of ganglionic neurons can respond to distinct nitrocellulose-anchored trophic factors; (3) while CNTF and NGF can both support the survival of their common target cells, only NGF also promotes neuritic extension; and (4) both the dimeric and the monomeric forms of immobilized β -NGF are active.

Different types of neurons from the CNS and PNS require different types of trophic factors for their survival and functional maintenance *in vivo* and *in vitro* (Varon and Adler, 1981; Varon 1985; Levi-Montalcini, 1987). Such neuronotrophic factors were identified and purified using *in vitro* bioassays, in which monolayer cultures of the target neuronal cells are incubated in medium containing the factor. For one of them, the ciliary neurotrophic factor (CNTF), a method was developed to determine the molecular mass of the active molecule from a crude preparation. This method, termed the "cell-blot" technique (Carnow et al. 1985; Rudge et al., 1987), involves SDS-PAGE of the extract containing the active molecule followed by electrophoretic transfer of the resolved proteins to nitrocellulose paper. The target cells [in this case, embryonic day eight (E8) chick ciliary ganglionic motor neurons] were then cultured on the paper and neuronal cell survival after 24 hr was visualized by the cell's ability to internalize the vital dye MTT and transform it into a blue water-insoluble product. The cell-blot technique showed that the ciliary ganglionic neurons survive only on 2 protein bands with apparent molecular masses of 24 and 28 kDa, in agreement with the molecular properties of the purified CNTFs (Barbin et al., 1984; Manthorpe et al., 1986; Rudge et al., 1987). Besides permitting the determination of the mo-

lecular mass of the active molecule without prior purification, these results indicate that CNTF can exert its effect on cell survival from a surface-anchored position, as well as from the humoral environment.

The present study was undertaken to extend this technique to another neuronotrophic factor, NGF, and to other probe cells such as sensory and sympathetic neurons. The 26 kDa active dimer of NGF was successfully visualized using a slightly modified cell-blot technique. Nitrocellulose-bound NGF and CNTF are recognized by their respective target neurons in the same way as when the factors are in solution in a culture medium. As with CNTF, the NGF activity can be seen in crude extracts of source tissue. Furthermore, an aldehyde-fixation and staining of the cell-blot culture for neurofilament proteins permits the visualization of neuritic outgrowth, as well as the neuronal survival. In this way, it was possible to demonstrate a differential effect of NGF and CNTF on the same probe neurons. NGF supports survival and stimulates neuritic outgrowth of neurons derived from E10 chick dorsal root ganglia, while CNTF is only able to support their survival. Depending on specific sample treatments preceding the electrophoresis, one can resolve the loaded NGF into either the dimeric form of β -NGF (26 kDa) or its monomeric form (13 kDa), and both species express biological activity after being blotted onto nitrocellulose paper.

Materials and Methods

Preparation of tissue extracts and purified neuronotrophic factors. The CNTF-containing extract of E15 chick intraocular tissues—including the choroid, iris-ciliary body, and pigment epithelium (CIPE)—was prepared as reported (Manthorpe et al., 1980) and purified rat nerve CNTF was prepared as described (Manthorpe et al., 1986). Mouse submaxillary gland extract and purified NGF (7S form or β form) were prepared as described (Varon et al., 1972).

Electrophoresis. Samples containing the trophic factors were mixed with electrophoresis sample buffer (0.0625 M Tris-HCl, 2.3% SDS, 10% glycerol, and 0.05% bromophenol blue, pH 6.8). In some cases only, the samples were further boiled for 5 min in the presence or absence of 5% β -mercaptoethanol. Samples were applied on a 15–25% linear gradient SDS-polyacrylamide slab gel (gel buffer = 0.75 M Tris-HCl, 0.1% SDS, pH 8.3) with a 4.5% stacking gel (gel buffer = 0.125 M Tris-HCl, 0.1% SDS, pH 6.8). Electrophoresis was carried out for 16–18 hr at 250 V in a Bio-Rad dual vertical slab gel apparatus with cooling, in an electrophoresis buffer consisting of 50 mM Tris and 192 mM glycine, 0.1% SDS, pH 8.3. The apparent molecular masses of the separated proteins were determined using prestained molecular weight markers (Rainbow, Amersham Corporation, UK) containing myosin 200 kDa, phosphorylase b 92.5 kDa, BSA 69 kDa, ovalbumin 46 kDa, carbonic anhydrase 30 kDa, trypsin inhibitor 21.5 kDa, and lysozyme 14.3 kDa, as well as unstained molecular-weight marker proteins (Bio-Rad Laboratories: same proteins as above except without myosin).

Electroblotting and staining. After electrophoresis, proteins were electroblotted onto nitrocellulose paper essentially according to the method of Towbin et al. (1979). The blotting buffer consisted of

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25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3. In some cases, 0.1% SDS was added to this buffer. The transfer was performed in a Hoefer blotting apparatus at 100 mA for 16 hr at 4°C. Some of the resulting Western blots were used for (1) staining of the blotted proteins with Aurodyne (Janssen) or (2) immunostaining for NGF antigen. For the immunostaining procedure, the blots were washed with PBS containing 0.1% Tween 20 (PBS-Tween); unoccupied protein-binding sites on the paper were blocked by incubating the blots with 1% BSA in PBS-Tween for 1 hr at room temperature; the blots were then incubated overnight at 4°C with primary antibodies directed against 2.5 S NGF (rabbit anti-NGF, Collaborative Research, Inc.) diluted 1:200 in PBS-Tween + 1% BSA; after 3×10 min washes with PBS-Tween, the NC papers were incubated for 2 hr at room temperature with peroxidase-conjugated goat anti-rabbit immunoglobulins (Cappel, Cooper Biomedical) diluted 1:200 in PBS-Tween + 1% BSA; after 3×10 min washes with PBS-Tween, the bound peroxidase was revealed using 4-chloro-1-naphthol (50 mg in 100 ml of 0.9% NaCl) as the substrate in the presence of H_2O_2 (20 μ l).

Cell blots. The blots were rinsed with PBS. The position of the molecular-weight markers was marked on the sample lanes with a pin, using as guides the lanes of prestained marker proteins run on both ends of the gels. The free protein-binding sites were blocked by incubating the nitrocellulose papers for 1 hr at room temperature with 1% ovalbumin in PBS containing antibiotics (100 μ g/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml fungizone) to prevent subsequent contamination when the paper is placed in culture. The blots were then cut into strips corresponding to individual lanes or groups of lanes and transferred to rectangular culture chambers. Last, the blots were washed with culture medium (DMEM + 10% fetal calf serum + antibiotics) and incubated with 5 ml of the same medium for 20 min before cell seeding. Neurons from 4 types of embryonic chick ganglia were used in these studies: E8 ciliary (CG8), E8 and E10 dorsal root (DRG8, DRG10), and E12 sympathetic (SG12) ganglia. In all cases, ganglia were dissociated into single-cell suspensions, and neurons were enriched to more than 90% purity by differential attachment to tissue culture plastic dishes (Varon et al., 1979; Varon and Adler, 1981; Selak et al. 1983; Davis et al., 1985). Neurons were diluted in culture medium to 6×10^4 /ml and 5 ml added to each blot strip for a seeding density of 10^4 neurons/cm² of paper. Cell blots were cultured for 48 hr instead of the 24 hr previously reported (Carnow et al., 1985; Rudge et al., 1987) to decrease the number of "background" surviving neurons. For the last 2 hr, the cell blot cultures were incubated with a solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], at a final concentration of 0.5 mg MTT/ml of medium. Alternatively, the 48 hr cultures were fixed for 30 min in a solution of 4% paraformaldehyde in PBS and immunostained for neurofilament proteins, using a mouse monoclonal antibody (RT 97, kindly provided by Dr. Frank Walsh) and a HRP conjugated secondary antibody as described by Davis et al. (1987).

Results

Adaptation of the cell-blot method to the study of NGF

When applied to 7S NGF, the cell-blot method developed for CNTF did not work. We studied in detail each step of the procedure and found that 2 of them had to be modified.

1. In the original method, the CNTF was loaded on the polyacrylamide gel in a "sample buffer" containing a reducing agent, β -mercaptoethanol, which inactivates NGF (data not shown, but see below). Therefore, 7S NGF was loaded on the gel in a sample buffer containing no reducing agent.

2. In the original method, the CNTF was electrophoretically transferred from the gel to the nitrocellulose paper in a "blotting buffer" containing 0.192 M glycine, 0.025 M Tris, and 20% methanol, pH 8.6. We found that, to get an efficient transfer of the basic NGF to the nitrocellulose paper, we had to introduce 0.1% SDS into the blotting buffer [data not shown; see also Aebersold et al. (1987)]. Table 1 summarizes the conditions used in the present cell-blot procedures for NGF and CNTF.

Applying the appropriate blotting conditions for each factor, blots were used as substrata for cultured neurons as described in Materials and Methods. The 7S NGF blot was probed with

Figure 1. Western blots of NGF and ciliary neuronotrophic factor (CNTF), cultured for 48 hr with E8 chick ciliary ganglionic (CG8) and E8 chick dorsal root ganglionic (DRG8) neurons, and stained with a vital dye. *a*, Blot of standard proteins stained with Amido black. Blots of 10 ng of rat nerve CNTF, seeded with (*b*) CG8 or (*c*) DRG8 neurons. Blots of 10 ng of mouse submaxillary gland NGF, seeded with (*d*) DRG8 or (*e*) CG8 neurons. Higher magnification of areas outside (*f*, *h*) or on (*g*, *i*) the indicated region of the protein bands shown in *b* and *d*. Scale bar, 100 μ m.

Figure 2. Numerical distribution of probe neurons along the cell blots of Figure 1. Peaks of surviving neuronal numbers identify the apparent molecular mass of the corresponding trophic factor protein.

DRG8 neurons, while the CNTF blot was probed with CG8 neurons. The results are shown in Figure 1. At low magnification (Fig. 1, *b* and *d*), the trophic band can be recognized in both cases by naked eye inspection. The NGF band is generally wider than the CNTF band. When the cell blots are examined at a higher magnification (Fig. 1, *f-i*), there is an unequivocal contrast between the low cell density of MTT-stained neurons in the off-band regions (Fig. 1, *f* and *h*) and their high density in the on-band areas (Fig. 1, *g* and *i*). At this higher magnification, the number of cells can be counted throughout the total length of the blot paper. Such quantitative scans, illustrated in Figure 2, can be used to determine apparent molecular masses of CNTF and NGF, which were found to be 28 kDa and 24–27 kDa,

Table 1. Different conditions required for the cell-blot detection of rat CNTF and mouse NGF^a

Additives		Survival of probe cells on cell-blot of	
Sample buffer	Blotting buffer	CNTF	NGF
SDS + DTT	0	++	–
SDS	0	++	–
SDS	SDS	+/-	++
SDS + DTT	SDS	+/-	–

^a Electrophoretic gel loads = 10 ng/lane.

respectively. The CNTF molecular mass of 28 kDa agrees well with that already assigned to rat CNTF by the blot technique (Rudge et al., 1987). The NGF values agree with the 26,500 kDa molecular mass determined by other means for the β -NGF dimer (Angeletti and Bradshaw, 1971), demonstrating that (1) the active dimer form is retained through SDS exposures (at room temperature) during both electrophoretic and blotting steps, and (2) the NGF dimer can express its trophic action in a surface-anchored position as well as in solution. Cell counts were also used to determine the sensitivity of the method, which for both factors allows the detection of as little as 10 ng loads per lane (data not shown).

Identification of the NGF band

Confirmation of the NGF nature of the 26 kDa band on which DRG8 neurons were surviving was sought by several approaches. A load of 1 μ g of β -NGF, instead of the 7S NGF, was processed through the SDS gel electrophoresis and Western blot steps, and the blots were stained (1) for protein with Aurodye or (2) for NGF antigen with anti-mouse NGF antiserum. Other blots, obtained from 100 ng β -NGF loads, were treated overnight at 4°C with a dilution of 1:100 of rabbit anti-NGF antiserum or with a control antiserum [against rabbit anti-glial fibrillary acidic protein (GFAP)]. The blots were then washed 3 $\times$ 10 min with PBS-Tween and 2 $\times$ 30 min with culture medium and used for cell seeding with DRG8 neurons. Figure 3 illus-

Figure 3. Western blots of 1 μ g β -NGF stained with Aurodyne (A) or immunostained with anti-mouse NGF antiserum (B). C and D, Magnification of the 26 kDa region of blots of 100 ng β -NGF, incubated with either anti-glial fibrillary acidic protein antiserum (C) or anti-mouse NGF antiserum (D), then seeded with DRG8 neurons and probed after 48 hr for cell survival with MTT. (Note the presence of numerous formazan crystals resulting from the reduction of the vital dye, MTT, by the viable cells.) Scale bar, 100 μ m.

trates the results. Both the protein dye (A) and the immunostaining (B) revealed a wide band at 26 kDa. The 26 kDa band from β -NGF incubated with the control anti-GFAP antiserum supported the survival of DRG8 neurons (C), while no survival could be shown after treatment of the blot with anti-NGF antiserum (D).

Evaluation of CNTF and NGF cell blots by neurofilament protein immunostaining

In previous work with CNTF cell blots, the MTT-stained CG8 probe neurons have always appeared round, smooth-contoured, and with no obvious staining of individual neurites (see Fig. 1g). However, the lack of demonstrable neurites from MTT-stained cells does not preclude the possibility that nitrocellulose-bound neurons have grown neurites. Studies with traditional microplate cultures have shown that individual neurites do not stain well with the MTT dye. Therefore, to detect neuritic outgrowth, CNTF and 7S NGF cell blots were fixed without exposing them to MTT and immunostained for neurofilament proteins. The results are shown in Figure 4. Neurofilament protein staining considerably raised the background level of countable cells by adding those cells that exclude MTT (hence, are presumably not viable) but retain the neurofilament protein antigens. The CNTF cell blot, probed with their traditional CG8 neurons, appeared qualitatively similar when stained for neurofilament proteins or with MTT, i.e., no neuritic outgrowth could be seen. In contrast, the NGF cell blot displayed a very robust neurite outgrowth from most of the DRG8 neurons used as probes. Neuritic outgrowth was confined to the "on-band" region. The "off-band" regions showed a higher background

number of neurons than with the MTT stain (as with the CNTF cell blot) but practically no evidence of neurites.

These results indicate that (1) neurites can grow on a nitrocellulose blot and may be visualized with an anti-neurofilament antibody, (2) surface-anchored neuronotrophic factors may also act as neurite-promoting substrata, and (3) neurites are elicited either by blotted NGF but not by blotted CNTF or from DRG8 but not CG8 neurons.

Use of different ganglionic neurons to probe CNTF and NGF cell blots

As the studies described above, CNTF and NGF activities were monitored *in vitro* by testing their effect on the survival of their respective specific target neurons. However, these growth factors can also act on common target cells, such as DRG10 and SG12 neurons. In view of this partially overlapping spectrum of target cells, we studied the ability of these 2 neuronal types to respond to immobilized CNTF or NGF. Cultures were stained for neurofilament protein, rather than with MTT, to evaluate neurite outgrowth as well as survival.

Figure 5 illustrates the appearance, on CNTF and 7S NGF cell blots, of probe neurons susceptible to trophic support by both factors. Beside the expected higher background, the CNTF cultures revealed that, in all cases, the DRG10 or SG12 neurons surviving on the CNTF band exhibited little if any neuritic outgrowth. Once again, the same neurons displayed considerable neuritic growth over the blotted NGF band, demonstrating that the neuritic behavior was dictated more by the type of trophic factor presented on the nitrocellulose paper than by the type of neurons used as probes. DRG10 neurons responded as

Figure 4. Photographs of CNTF (*A, B*) and NGF (*C, D*) cell-blot fields, *outside* (*A, C*) or *on* (*B, D*) the trophic protein band. Cell blots, carried out under the same conditions as in Figure 1, were fixed and stained by immunohistochemistry for neurofilament protein. Scale bar, 50 μ m.

vigorously to NGF as the DRG8 neurons. With SG12 neurons the neuritic outgrowth elicited by NGF was less robust than that observed with DRG10 neurons.

Cell blots of crude extracts

To test the selective properties of the cell-blot systems, we cultured CG8 and DRG8 neurons on blots of crude extracts known to contain one or the other factor, i.e., for the CNTF, CIPE extract and rat nerve extract and for the NGF, mouse submaxillary gland extract. Each crude extract was submitted to the 2 different gel-blot conditions (i.e., with or without SDS; see Table 1) necessary to recognize NGF and CNTF. The results are shown in Table 2. CG8 neurons survived on CIPE and rat nerve extract protein bands having apparent molecular masses of 24 and 28 kDa, respectively, as shown by Rudge et al. (1987). DRG8 neu-

rons survived only in the 24–27 kDa region of the submaxillary gland extract. Thus, the cell-blot method allows (1) the detection of trophic factors in complex mixtures of proteins and (2) the specific identification of the trophic factors contained in an extract. Our observations show also that the differential conditions required for each type of blot apply to the crude as well as to the purified factors.

Dimeric and monomeric forms of β -NGF

Beta-NGF normally occurs in its dimeric, 26 kDa form. Disulfide bond reduction in the absence of denaturing agents does not convert the dimer to its monomers (Greene et al., 1971). Conversion is also not noticeable with SDS in the mild conditions used here (30 min at room temperature in the sample buffer, plus 16 hr at 4°C during SDS gel electrophoresis). On

Figure 5. Neurofilament-stained fields of CNTF and NGF blots seeded with the same target cells, neurons from (*A, C, E*) E12 chick sympathetic ganglia or from (*B, D, F*) E10 chick dorsal root ganglia. Fields *A* and *B* outside the trophic protein bands; fields *C* and *D* on the CNTF protein band; fields *E* and *F* on the NGF protein band. Scale, 100 μ m.

Figure 6. Western blots of (a) 1 μ g, (b) 100 ng, or (c) 20 ng β -NGF. Before electrophoresis, samples were (A) incubated at room temperature in the absence of β -mercaptoethanol, (B) boiled for 10 min in the absence of β -mercaptoethanol, or (C) boiled for 10 min in the presence of 0.6 M β -mercaptoethanol. a, Blots immunostained with anti-mouse NGF; b and c, blots seeded with DRG8 neurons for 48 hr and incubated with MTT to visualize cell survival.

the other hand, exposure to SDS at higher temperatures with or without reduction induces the formation of monomers. Formation and trophic competence of dimer and monomer were examined in Western blots obtained from β -NGF after a 10 min exposure to 90°C in 2.3% SDS, in the absence or the presence of 0.6 M β -mercaptoethanol as a reducing agent. In each case, β -NGF was introduced in the sample buffer and loaded on gels in 3 different amounts: 1 μ g/lane for immunostaining of the resulting blots, 100 and 20 ng/lane for cell-blotting evaluations. The results are compared in Figure 6 with corresponding loads of β -NGF under standard conditions (2.3% SDS, 4°C, no reducing agent).

In the standard conditions (Fig. 6A), all the immunostainable protein was in the 26 kDa dimer form (A,a), as already seen in Figure 3B. Cell-blot activity was expressed in the 26 kDa region as a wider band (100 ng/lane, A,b) or a more restricted one (20 ng/lane, A,c), and no activity was detected in the 13 kDa region at either load. When β -NGF was boiled in sample buffer with no reducer before loading (Fig. 6B), all the immunostainable protein migrated to the 13 kDa monomer region (B,a), and the cell blot activity was similarly transferred to that region (B,b and c). When the boiling of β -NGF was carried out in the presence of β -mercaptoethanol (Fig. 6C) conversion to the 13 kDa form was equally complete (C,a), but trophic activity was no longer detectable (C,b and c).

Conceivably, the 13 kDa monomer may have redimerized during its transfer to the blot (which is carried out in the presence of 0.1% SDS in the blot buffer), and this blotted NGF might occupy the 13 kDa position despite its new 26 kDa size. We eluted the 13 kDa monomer from the SDS-containing gel into

Table 2. Survival of CG8 and DRG8 neurons cultured on blots of different tissues crude extracts

Extract	NGF gel-blot conditions		CNTF gel-blot conditions	
	CG8	DRG8	CG8	DRG8
CIPE	+/-	-	++	-
Nerve	+/-	-	++	-
Submaxillary gland	-	++	-	-

100 trophic units were applied on the gel and corresponded to protein loads of 25, 5, and 0.2 μ g for extracts from CIPE, nerve, and submaxillary gland, respectively.

0.2 ml of SDS-free blot buffer. (Thus, if all the SDS eluted out of the gel with the NGF, the final concentration of SDS in the eluate from the gel would be 0.03%.) We then incubated the gel eluate containing the NGF monomer overnight to allow any potential redimerization, mixed it with sample buffer (without boiling or reduction), and then reran it on the standard SDS gel. Silver staining of this gel showed that all the protein still ran in the 13 kDa position (data not shown). This suggests that the biological activity localized within the 13 kDa band of the cell blot was expressed by a truly monomeric immobilized NGF species.

Discussion

In this study, we report that (1) the cell-blot technique, initially established for CNTF, is also applicable to the study of one other neurotrophic factor, NGF; (2) bound NGF has both survival and neuritic outgrowth effects; (3) the same target cells, the DRG10 neurons, will respond to bound CNTF by survival, and to bound NGF by survival plus neuritic outgrowth; (4) both the dimer and the monomer forms of β -NGF appear to be active.

Concerning point 1, it was not initially evident that other neurotrophic factors beside CNTF (which is very stable) would retain biological activity through treatments such as SDS-PAGE and electrophoretic transfer and that, conversely, other cells beside the ciliary ganglion neurons would be able to benefit from a surface-bound trophic factor. The extension of the cell-blot technique to NGF and its target cells encourages further attempts to use this technique with other types of factors, for example, trophic factors acting on CNS neurons or mitogens. The principal property of the cell-blot method is to allow, in a single experiment, the determination of both the apparent molecular mass and the level of activity of a substance, even if this substance is mixed with many others in a crude extract. This property could be exploited in studies such as (1) the comparison of the molecular weight and the activity of trophic factors contained in crude extracts from different tissues and different animal species; (2) the identification of one or more peptide fragments which retain activity after enzymatic digestion or chemical cleavage; (3) the simultaneous characterization of size and/or activity of mRNA translation products, before and after manipulation of the genes; or (4) the demonstration that an antibody or other interfering substance not only reacts with a precise molecule, but also blocks its biological activity.

Concerning point 2, our study corroborates, using a different approach, the observations made by Frazier et al. (1973), Gunderson (1985), and Sandrock and Matthew (1987). The first group has shown that NGF bound to Sepharose beads retains its effects on the neuritic outgrowth from ganglionic explants;

the second author has shown that bound NGF can guide the fibers growing from a ganglionic explant; the third showed that NGF can be bound to and express neurite outgrowth activity from degenerated but not normal peripheral nerve. None of these studies, nor the present one, provides any information as to whether the cell that encounters the anchored NGF needs to remove it from its anchorage in order to respond to it.

Concerning point 3, the fact that the same target cells will respond by only survival when seeded on CNTF and by both survival and neuritic outgrowth when seeded on NGF blots implies that the neuritic outgrowth response is dictated by the type of factor rather than by the type of cell. NGF is able to exert by itself both survival and neuritic outgrowth effects. In contrast, cells allowed to survive by CNTF will need the presence of another molecule, like laminin, to support the growth of their neurites (Davis et al., 1985, 1987). The cell-blot technique will allow us to address, in a new way, the role of NGF in neurite growth by cells that do not depend on NGF for their survival. It has been reported by Collins (1984) that NGF is able to accelerate the neuritic outgrowth of CG8 neurons cultured on a laminin-containing substratum. These studies had to be restricted to the first few hours in culture because the cells were cultured in the absence of any surviving factor. The cell-blot approach may give the opportunity to determine whether the survival and the neuritic outgrowth effects of NGF can be dissociated.

Concerning point 4, NGF activity migrated in the 26 kDa region under our standard conditions, whether NGF was loaded as its 7S complex or its β -subunit form. The 26 kDa band was recognized by antibodies against NGF, and its cell blot activity was blocked by the same antibody, demonstrating the NGF identity of the active blot. Conversion of the 26 kDa dimeric form of β -NGF to its monomeric one is completely achieved when NGF is heated in an SDS-containing sample buffer prior to electrophoresis. The cell-blot technique has allowed us to demonstrate that the 13 kDa band is biologically competent with regard to both neuronotrophic and neurite-promoting activities and that it loses these activities after sulfhydryl reduction. The fact that neuronotrophic factors can exert their effects in an immobilized form raises the question of their active location *in vivo*. The presentation of a trophic factor to its target cells in an anchored conformation may afford advantages compared to the humoral form, such as an increased stability of the activity, a better control of the topographical distribution, and a more specific action of the factor. It would also imply that the producing cell (or the storage cell) can be situated in the immediate proximity of the target cell, so that the anchored factor can be recognized by its receptors. The idea that a growth factor may occur in a bound position *in vivo* has been proposed by Gospodarowicz et al. (1986) for a class of mitogens, the heparin-binding growth factors. The physiological "heparin-presenting" material is the extracellular matrix. It has been therefore suggested that these factors could, *in vivo*, be sequestered by heparan sulfate proteoglycans embedded within the extracellular matrix.

The observation that NGF can promote neuritic outgrowth in a bound form may also offer possibilities to improve nerve regeneration. For example, it has been shown that NGF can prevent the disappearance of septal cholinergic neurons after transection of the fimbria-fornix pathways (Hefti, 1986; Williams et al., 1986; Kromer, 1987). However, even under NGF administration, axons are unable to cross the lesion space between the projecting neurons from the septum and the receiving neurons from the hippocampus, either because some com-

pounds necessary for the regrowth are lacking or because the axons are able to grow but are not guided through the gap. The latter possibility could be studied by using, as a bridge across the gap, a piece of nitrocellulose paper presoaked in an NGF solution or, perhaps better, NGF anchored by electrophoretic transfer.

References

- Aebersold, R. H., J. Leavitt, R. A. Saavedra, L. E. Hood, and S. B. H. Kent (1987) Internal amino sequence analysis of proteins separated by one or two-dimensional gel electrophoresis after *in situ* protease digestion on nitrocellulose. *Proc. Natl. Acad. Sci. USA* 84: 6970-6974.
- Angeletti, R., and R. A. Bradshaw (1971) Nerve Growth Factor from mouse submaxillary gland: Amino acid sequence. *Proc. Natl. Acad. Sci. USA* 68: 2417-2420.
- Barbin, G., M. Manthorpe, and S. Varon (1984) Purification of the chick eye Ciliary Neuronotrophic Factor (CNTF). *J. Neurochem.* 43: 1468-1478.
- Carnow, T. B., M. Manthorpe, G. E. Davis, and S. Varon (1985) Localized survival of ciliary ganglionic neurons identifies neuronotrophic factor bands on nitrocellulose blots. *J. Neurosci.* 5: 1965-1971.
- Collins, F. (1984) An effect of nerve growth factor on the parasympathetic ciliary ganglion. *J. Neurosci.* 4: 1281-1288.
- Davis, G. E., M. Manthorpe, and S. Varon (1985) Parameters of neuritic outgrowth from ciliary ganglion neurons: Influence of laminin, Schwannoma polyornithine-binding neurite promoting factor and ciliary neuronotrophic factor. *Dev. Brain Res.* 17: 75-84.
- Davis, G. E., E. Engvall, S. Varon, and M. Manthorpe (1987) Human amnion membrane as a substratum for cultured peripheral and central nervous system neurons. *Dev. Brain Res.* 33: 1-10.
- Frazier, W. A., L. F. Botd, and R. A. Bradshaw (1973) Interaction of Nerve Growth Factor with surface membranes: Biological competence of insolubilized Nerve Growth Factor. *Proc. Natl. Acad. Sci. USA* 70: 2931-2935.
- Gospodarowicz, D., G. Neufeld, and L. Schweigerer (1986) Fibroblast growth factor. *Mol. Cell. Endocrinol.* 46: 187-204.
- Greene, L. A., S. Varon, A. Piltch, and E. M. Shooter (1971) Substructure of the β subunit of mouse 7S nerve growth factor. *Neurobiology* 1: 37-48.
- Gundersen, R. W. (1985) Sensory neurite growth cone guidance by substrate absorbed Nerve Growth Factor. *J. Neurosci. Res.* 13: 199-212.
- Hefti, F. (1986) Nerve Growth Factor promotes survival of septal cholinergic neurons after fimbrial transections. *J. Neurosci.* 6: 2155-2162.
- Kromer, F. L. (1987) Nerve Growth Factor treatment after brain injury prevents neuronal death. *Science* 235: 214-216.
- Levi-Montalcini, R. (1987) The Nerve Growth Factor: Thirty years later. *In Vitro* 23: 227-238.
- Manthorpe, M., S. D. Skaper, R. Adler, K. B. Landa, and S. Varon (1980) Cholinergic neuronotrophic factors: Fractionation properties of an extract from selected chick embryonic eye tissues. *J. Neurochem.* 34: 69-75.
- Manthorpe, M., S. D. Skaper, L. R. Williams, and S. Varon (1986) Purification of adult rat sciatic nerve ciliary neuronotrophic factor. *Brain Res.* 367: 282-286.
- Rudge, J. S., G. E. Davis, M. Manthorpe, and S. Varon (1987) An examination of ciliary neuronotrophic factors from avian and rodent tissue extracts using a blot and culture technique. *Dev. Brain Res.* 32: 103-110.
- Sandrock Jr., A. W., and W. D. Matthew (1987) Substrate-bound Nerve Growth Factor promotes neurite growth in peripheral nerve. *Brain Res.* 425: 360-363.
- Selak, I., S. D. Skaper, and S. Varon (1983) Ionic behaviors and neuronal survival in developing ganglia. III. Studies with embryonic chick sympathetic neurons. *J. Cell Physiol.* 114: 229-234.
- Towbin, H., T. Sarhelin, and J. Gordon (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350-4354.

- Varon, S. (1985) Factors promoting the growth of the nervous system. *Discuss. Neurosci.* 2: 1–62.
- Varon, S., and R. Adler (1981) Trophic and specifying factors directed to neuronal cells. *Adv. Cell Neurobiol.* 2: 115–163.
- Varon, S., J. Nomura, J. R. Perez-Polo, and E. M. Shooter (1972) The isolation and assay of the Nerve Growth Factor proteins. In *Methods and Techniques of Neuroscience*, R. Fried, ed., pp. 203–229, Dekker, New York.
- Varon, S., M. Manthorpe, and R. Adler (1979) Cholinergic neuronotrophic factors. 1. Survival, neurite outgrowth and choline acetyltransferase activity in monolayer cultures from chick embryo ciliary ganglia. *Brain Res.* 173: 29–45.
- Williams, L. R., S. Varon, G. Peterson, K. Victorin, W. Fisher, A. Bjorklund, and F. H. Gage (1986) Continuous infusion of Nerve Growth Factor prevents basal forebrain neuronal death after fimbria-fornix transection. *Proc. Natl. Acad. Sci. USA* 83: 9231–9235.