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Lateral diffusion of nerve growth factor receptor: modulation by ligand-binding and cell-associated factors

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We compared the properties in human melanoma cell line A875 and rat pheochromocytoma cell line PC12 of nerve growth factor receptor (NGFr). We also analyzed NGFr and a truncated NGFR lacking the cytoplasmic domain, which were transiently expressed in COS cells. The full-length NGFR expressed in COS cells bound nerve growth factor (NGF) with positive cooperativity, but A875 NGFr and truncated NGFr in COS cells did not display positive cooperativity. The anti-human NGFr monoclonal antibody NGFR5 was characterized and found not to compete with NGF for binding to NGFr. Fabs were prepared from NGFR5 and 192, an anti-rat NGFR monoclonal antibody that was previously shown not to compete with NGF for binding. Fluorescein-labeled Fabs were used to measure the distribution and lateral diffusion of the NGFr. NGFr expressed on COS and A875 cells are diffusely distributed, but NGFr on the surface of PC12 cells appeared, for some cells, to be patched. In A875 cells, 51% of the NGFr was free to diffuse with diffusion coefficient (D) $\approx 7 \times 10^{-10}$ cm^2/s. In COS cells, 43% diffused with D $\approx 5 \times 10^{-10}$ cm^2/s. There was no significant difference in diffusibility between the full-length NGFr and the truncated NGFr. We compared NGFr diffusion on PC12 cells in suspension or adherent to collagen-coated coverslips. For suspension cells, we obtained 32% recovery with D $\approx 2.5 \times 10^{-9}$ cm^2/s. On adherent cells, we obtained 17% recovery with 6×10^{-9} cm^2/s. Binding of NGF enhanced lateral diffusion of NGFr in A875 cells and in PC12 cells in suspension but did not alter lateral diffusion of NGFr in COS cells or in adherent PC12 cells. NGF had no effect on the diffusing fraction or the distribution of NGFR for any cell line.

Introduction

Nerve growth factor (NGF)[1] is a 26 000-Da polypeptide neurotrophic factor that acts as a survival factor for sympathetic and sensory neurons (Levi-Montalcini and Aloe, 1987). NGF also modulates differentiation of rat pheochromocytoma PC12 cells to sympathetic neuronlike cells and induces a rapid but transient expression of *fos* mRNA and protein (Milbrandt, 1986). All these actions of NGF are mediated by specific cell-surface receptors (NGFr). Molecular cloning of NGFr indicates that the receptor gene is single copy and that the receptor is a highly conserved protein (Chao *et al.,* 1986; Johnson *et al.,* 1986; Radeke *et al.,* 1987). The human NGFr is a 75 000-Da glycoprotein (gp75) with a hydrophobic signal sequence, a single N-linked glycosylation site, 4 cysteine-rich repeat units in the extracellular domain, a single transmembrane domain, and a 155-amino-acid cytoplasmic tail (Johnson *et al.,* 1986).

Recent studies suggest that both gp75 and additional factors are required for a fully functional NGFr. Responsive cell lines express both low- and high-affinity NGFr, but nonresponsive cell lines express only low-affinity NGFr (Green *et al.,* 1986). Chemical crosslinking studies have demonstrated that both receptor types include gp75 (Green and Greene, 1986). Expression of recombinant gp75 in nonneuronal cell types resulted in low-affinity nonresponsive NGFr (Chao *et al.,* 1986; Radeke *et al.,* 1987). Expression of gp75 in an NGFr-minus variant of PC12 (Hempstead *et al.,* 1989) and in a human brain tumor cell line (S.J. Pleasure, U.R. Reddy, G. Venkatakrishnan, A.K. Roy, J. Chen, A.H. Ross, J.Q. Trojanowski, D.E. Pleasure, and V.M.Y. Lee, unpublished observations) resulted in low- and high-affinity NGFr that mediated NGF-induced upregulation of *fos* oncogene expression.

The ability of receptor molecules at low density to interact with one another and with other membrane proteins depends on the ability of these receptors to diffuse within the plane of the membrane. The lateral diffusibility of membrane proteins and lipids has been widely studied by the use of the technique of fluorescence recovery after photobleaching (FRAP) (Axelrod *et al.,* 1976; Edidin, 1981; Peters, 1981; Wolf, 1989). Early on, it became apparent that the diffusion rates of most membrane proteins are

[1] Abbreviations: FRAP, fluorescence recovery after photobleaching; D, diffusion coefficient; %R, percent recovery; NGF, nerve growth factor; NGFr, nerve growth factor receptor.

not governed by lipid viscosity. Fluid dynamic theory predicts that, in the quasi-two-dimensional lipid bilayer, proteins should diffuse only slightly slower than lipids (Saffman and Delbruck, 1975). However, membrane proteins typically diffuse one to three orders of magnitude slower than membrane lipids, with a significant fraction not diffusing at all (Edidin, 1981; Peters, 1981). Elucidation of the constraints to membrane protein diffusion thus became an important issue. Webb and colleagues (Tank *et al.,* 1981; Wu *et al.,* 1982) demonstrated that blebbing cells resulted in complete release of constraints to diffusion for a number of membrane proteins. They pointed out that these constraints could be either cyto- or ecto-skeletal. With the application of molecular biological techniques to membrane proteins, dissection of this problem became tractable. Livneh *et al.* (1986, for EGF receptor), Edidin and Zuniga (1984, for H-2 L^d antigen), and Scullion *et al.* (1987, for VSV glycoprotein) demonstrated that deletion of the cytoplasmic membrane protein domains had no effect on the protein diffusion coefficient or on the diffusing fraction. Subsequently, Weir and Edidin (1988) demonstrated that deletion of the ectoplasmic domains of H-2, which contain carbohydrate or point mutation of N-linked glycosylation sites, allows free protein diffusion. In contrast, lateral diffusion of class II major histocompatibility antigens was greatly enhanced by deletion of the cytoplasmic domain (Wade *et al.,* 1989). Furthermore, because it is clear that surface phenomena such as patching and capping are sensitive to cytoskeletal disrupting agents (Schreiner and Unanue, 1976) and that specific membrane proteins are attached to the cytoskeleton (Carraway and Carraway, 1989), the nature of cytoskeleton-membrane interactions and the role of these interactions in surface events remain to be defined.

In the case of peptide hormone and other surface receptors, there is the further question of how the binding of ligand alters the physical properties of these receptors and initiates signal transduction. Previous studies of hormone receptor diffusibility have employed fluorescently labeled hormones (Schechter *et al.,* 1978; Schlessinger *et al.,* 1978; Rees *et al.,* 1984). Thus, measurements of receptor diffusibility in the presence and absence of ligand were precluded. It is clear that, in many instances, ligand binding induces aggregation and endocytosis of receptor and that these surface effects (as well as other physiological effects of hormone), can be induced by bivalent anti-receptor antibodies (Schechter *et al.,* 1979; Detmers *et al.,* 1987).

In the present study, we utilize a fluorescent Fab fragment of anti-NGFr, which does not compete with NGF for binding to NGFr. We report the cell-surface distribution and lateral diffusion of NGFr that are transiently expressed in COS cells and those that are endogeneously present on human melanoma A875 cells and rat pheochromocytoma PC12 cells, and we compare these results with an earlier study utilizing fluorescent NGF (Levi *et al.,* 1980). We address several questions about the relationship of the physical and physiological properties of the NGFr: 1) Do the physical properties of NGFr differ in neuronal and nonneuronal cells that are NGF responsive and nonresponsive? 2) Does the addition of NGF to the cells affect the rate of diffusion and the percent recovery (%R) of the receptors? 3) What is the distribution of NGFr on these cells, and does it change on the addition of NGF? and 4) Does the cytoplasmic domain of NGFr regulate NGF binding and diffusibility in the membrane?

Results

Binding and internalization of ^{125}I-NGF to COS cells expressing NGFr

We studied the properties of human NGFr in three cell types: COS cells, which transiently express NGFr after transfection with an expression vector; A875 cells, which naturally express NGFr but lack an apparent response; and NGF-responsive PC12 cells. In the case of the COS cells, we have compared the properties of full-length NGFr (expression vector pCMVX-7) and a truncated NGFr lacking the cytoplasmic domain (pCMVPvu). Equilibrium-binding studies indicate that the full-length receptor binds ^{125}I-NGF with apparent positive cooperativity (Figure 1, A–C). The positive cooperativity was observed in four out of four experiments, but the extent of positive cooperativity varied. The Hill coefficient varied between 1.1 and 1.6 (not shown). In contrast, the binding curve for COS cells expressing the truncated NGFr did not exhibit any cooperativity (Figure 1D). The number of ^{125}I-NGF binding sites of the two forms of receptors per cell and their dissociation constants were determined by Scatchard analysis of the same data and are summarized in Table 1. ^{125}I-NGF was efficiently internalized by PC12 cells but not by A875 or COS cells (Table 1), and there was no induction of *fos* mRNA when NGF was added to COS cells expressing the full-length NGFr (not shown). PC12 cells, but

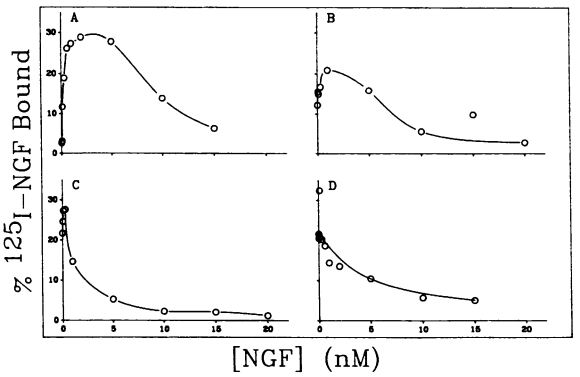

Figure 1. **Binding of ^{125}I-NGF to COS cells expressing NGFr.** ^{125}I-NGF and varying concentrations of unlabeled NGF were incubated for 30 min at 37°C with COS cells (2.5×10^6/ml) transfected with pCMVX-7 (encoding full-length NGFr) or pCMVPvu (encoding an NGFr lacking the cytoplasmic domain). Nonspecific binding was determined in the presence of excess unlabeled NGF and was subtracted. Three independent experiments for pCMVX-7 (A–C) and one experiment for pCMVPvu (D) are shown.

Table 1. Binding and internalization of NGF

Cell line	Plasmid	Kd (nM)[1]	n[1]	I/E[2]
A875	none	3.3	390 000	0.36
COS	pCMVX-7	2.4	230 000[3]	0.31
COS	pCMVPvu	3.2	130 000[3]	0.38
PC12	none	0.3[4]	15 000	4.2
		5.2	57 000	

[1] The dissociation constant and number of cell surface NGFr were determined by Scatchard analysis of ^{125}I-NGF binding. The conditions used for binding (30 min incubation at 37°C) were sufficient for the attainment of the steady state (not shown).

[2] I/E is the ratio of internalized ^{125}I-NGF cpm to externally bound ^{125}I-NGF cpm after a 30 min incubation at 37°C.

[3] Because transfection is a relatively inefficient procedure, only 10%–15% of the COS cells actually express NGFr. Hence, for pCMVX-7 transfected cells the number of cell surface NGFr per expressing COS cell is $1.5–2.3 \times 10^6$.

[4] The n and Kd for PC12 are taken from Buxser *et al.* (1983).

not A875 cells, have already been reported to show NGF-induced upregulation of *fos* expression (Milbrandt, 1986).

Lack of competition for antibody binding to NGFr

Anti-human NGFr monoclonal antibodies (MAb) ME20.4 and ME82-11 compete with NGF for binding to NGFr (Ross *et al.*, 1984) and hence were unsuitable for analysis of effects of NGF on the physical properties of NGFr. At the suggestion of Dr. Mark Bothwell, we analyzed a newer MAb, NGFR5, which was prepared by immunization with intact melanoma cells followed by a boost with purified NGFr (Marano *et al.*, 1987). MAb NGFR5 binds to A875 cells with 1.5×10^5 binding sites per cell and a dissociation constant of 28 nM, as determined by Scatchard analysis (Figure 2). Binding of ^{125}I-NGF to A875 cells was not reduced even in the presence of 50 nM NGFR5 IgG. Furthermore, the addition of 500 nM NGF only slightly reduced (28%) the binding of ^{125}I-NGFR5-IgG to A875 cells, indicating that NGFR5-IgG and NGF bind at different sites on the receptor.

Cell-surface distribution and lateral diffusion of NGFr

Using fluorescein-labeled Fab (FITC-Fab), we have shown that the NGFr expressed on COS cells and A875 cells are diffusely distributed (Figure 3). For A875 cells, there appeared to be a localization of the NGFr at cell-cell junctions. For PC12 cells (Figure 4), the distribution was heterogeneous. Addition of NGF to cells labeled with fluorescein isothiocyanate (FITC)-Fab did not displace the fluorescence from the cell surface, and there was no apparent change in the distribution of fluorescence over a period of 30 min at room temperature (not shown). Moreover, no punctate cytoplasmic fluorescence was observed, suggesting that little or no internalization of the FITC-Fab had occurred during the period of observation.

Figures 5 and 6 summarize the diffusion coefficients (D) and %R of NGFr on COS, A875, and PC12 cells. The full-length NGFr and the truncated NGFr lacking the cytoplasmic domain diffuse in the membrane of COS cells with similar diffusion

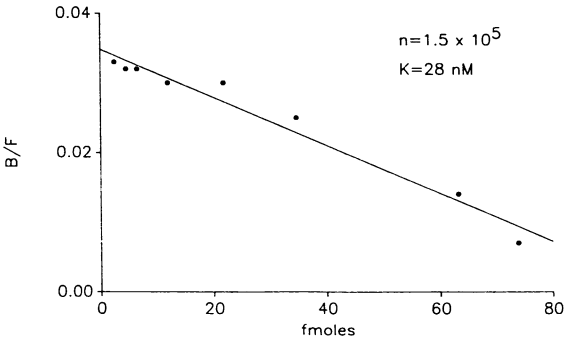

Figure 2. **Scatchard plot for equilibrium binding of anti-NGFr IgG to a human melanoma cell line.** A875 cell were incubated for 30 min at 37°C with NGFR5 ^{125}I-IgG (0.8 nM) and varying concentrations of unlabeled IgG. The cells were pelleted through a 0.15-M sucrose layer, and the pellets and supernatants were assayed for radioactivity.

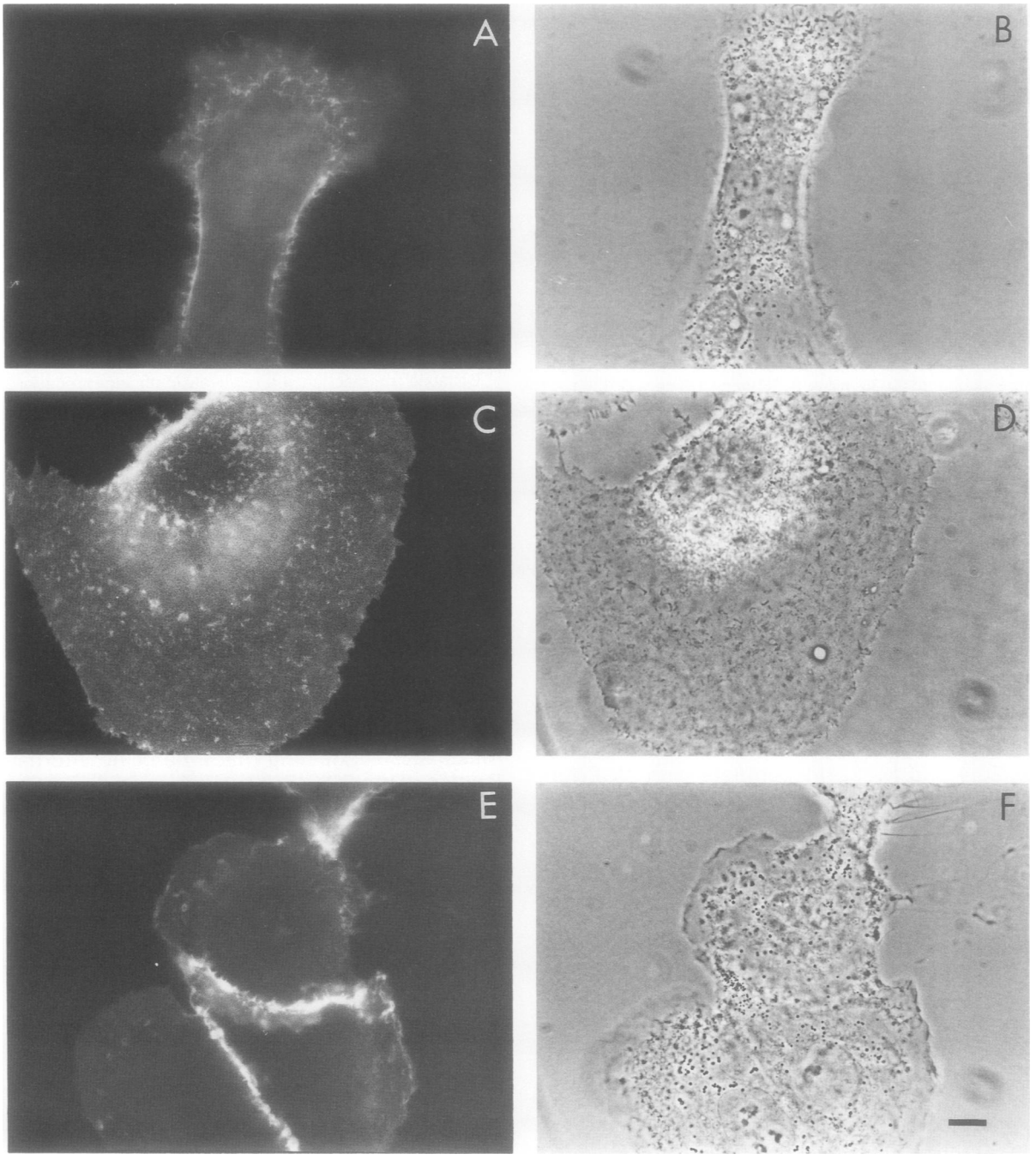

Figure 3. **Cell-surface distribution of NGFr.** Fluorescence (A, C, and E) and phase-contrast (B, D, and F) microscopy of COS cells transfected with pCMVX-7 (A and B) or with pCMVPvu (C and D) and A875 cells (E and F) incubated for 30 min at 4°C with FITC-Fab (MAb NGFR5) and washed. Bar equals 10 μm.

coefficients, $D \approx 5 \times 10^{-10}$ cm^2/s at room temperature. The diffusing fractions for the two receptors were also the same, %R $\approx$ 44. NGFR present in A875 cells diffused with $D \approx 7 \times 10^{-10}$ cm^2/s and a diffusing fraction of 51%. There was no significant difference in diffusion properties between NGFr localized at the cell-cell boundaries and NGFr on the remainder of the cell surface (not shown). The diffusion parameters of NGFr in PC12 cells in suspension or adherent to a collagen substratum were $D \approx 2.5 \times 10^{-9}$ cm^2/s and 6.2×10^{-9} cm^2/s with diffusing fractions of 32% and

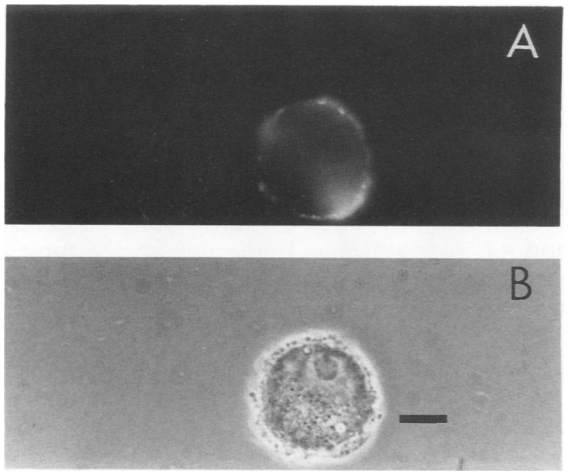

Figure 4. **Cell-surface distribution of NGFr.** Nonadherent PC12 cells were stained with FITC-Fab (MAb 192) and washed. Bar equals 10 μm.

17%, respectively. The difference between diffusing fractions for suspension and adherent cells was statistically significant (Student's *t* test, $p < 0.005$).

To check the specificity of the labeling, we treated control cell lines—such as COS cells, which lack NGFr—with the fluorescent Fab. There was no detectable staining. To check that the FRAP curve truly represents cell-surface diffusion, we carried out two control experiments. A875 cells were crosslinked with glutaraldehyde and then stained with NGFR5 Fab. There was no significant recovery after bleaching. For PC12 cells, an additional control experiment was carried out. An anti-mouse immunoglobulin antibody was added after staining with fluoresceinated 192 Fab. There was no significant recovery after bleaching. These control experiments prove that the anti-NGFr Fabs specifically label the NGFr and that the resulting FRAP curves are due to movement of the NGFr on the cell surface.

We have measured the diffusion of NGFr on the three cell types in the presence and absence of NGF (Figures 5 and 6). For A875 cells, we considered 7.6, 25, or 100 nM NGF. Only at 7.6 nM was there a statistically significant difference ($p < 0.005$), an ~50% increase. At higher concentrations, no statistically significant effects were observed. For PC12 cells, addition of NGF to cells in suspension, but not to adherent cells, enhanced the rate of diffusion of NGFr. This increase was significant for 25 ($p < 0.05$) and 100 nM NGF ($p < 0.005$). For COS cells the addition of NGF at 100 nM had no effect on D for pCMVX-7 or pCMVPvu. In no instance, for A875, PC12, or COS cells, was a statistically significant NGF-induced change in %R observed.

Lipid probe analysis

For comparison, we have also measured the diffusion of the fluorescent lipid probe C_{16}dil. C_{16}dil was found to be uniformly distributed on the surface of both COS and A875 cells (not shown). The diffusion coefficient and the percent recovery of C_{16}dil was similar for both the cell types, with $D \approx 8 \times 10^{-9}$ cm^2/s and diffusing fraction 90%–92%. These values are consistent with earlier measurements on other cell types (Peters, 1981). Hence, NGFr in A875 and COS cells is diffusing at a rate slower than the hydrodynamic limit and must be constrained by elements other than membrane "fluidity." The exception is PC12 cells, in which a small fraction of NGFr (20%–30%) diffuses, with $D \approx 3 \times 10^{-9}$ cm^2/s, which is probably at or close to the fluid dynamic limit.

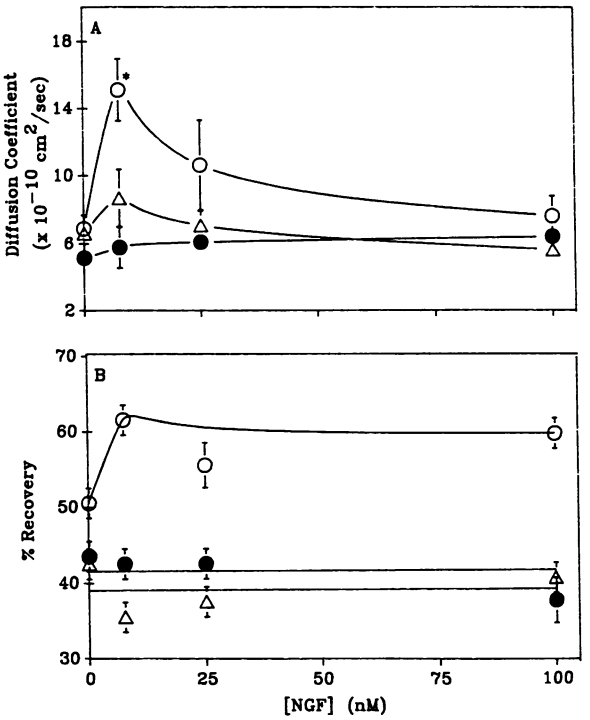

Figure 5. **Diffusibility of NGFr as a function of NGF concentration.** The diffusion coefficient and %R were determined with the use of anti-NGFr FITC-Fab (MAb NGFR5) for human melanoma A875 cells (○), COS cells transfected with pCMVPvu (●), and for COS cells transfected with pCMVPvu (NGFr lacking the cytoplasmic domain, △). The measurements were made immediately after the addition of various concentrations of NGF.

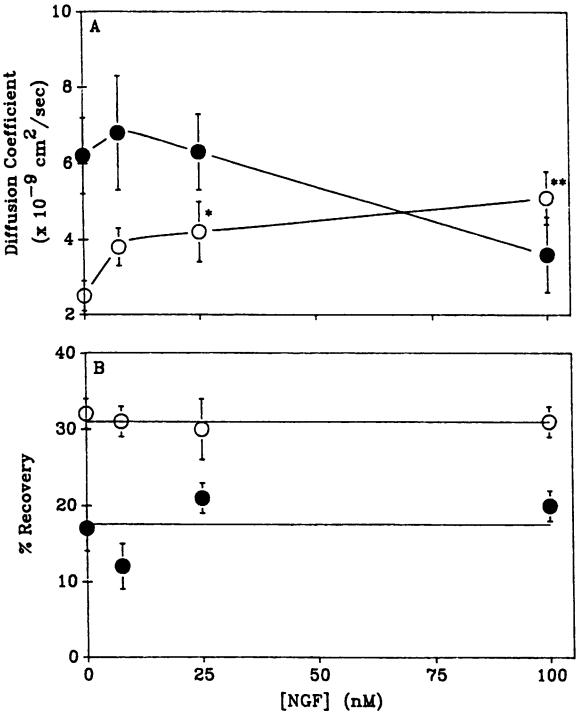

***Figure 6.* Diffusion properties of NGFr on rat pheochromocytoma PC12 cells.** PC12 cells either in suspension (○) or adherent to collagen-coated coverslips (●) were labeled with anti-NGFr Fab FITC-Fab (MAb 192). Various concentrations of NGF were added, and the cells were immediately analyzed by FRAP.

Discussion

The development of anti-NGFr MAb NGFR5 was critical for the analysis of the physical properties of human NGFr. The characterization of the initial anti-NGFr MAb (Ross *et al.*, 1984) revealed that these MAb were very similar and probably bound to the same epitope. This result was not surprising because for poorly immunogenic antigens, one or a few epitopes frequently predominate. In the generation of the second group of anti-NGFr MAb, we treated the mice with cyclophosphamide, which may be useful for generating MAb against previously undefined epitopes (Matthew and Patterson, 1983; Marano *et al.*, 1987). Because, unlike MAb ME82-11 and ME20.4, NGFR5 does not compete with NGF for binding to NGFr, we feel confident that NGFR5 does define a new epitope. Because NGFR5 does not bind to the rat NGFR, we utilized MAb 192, which was previously reported not to compete with NGF for binding (Chandler *et al.*, 1984). Both the NGFR5 and the 192 Fab bind strongly to NGFr and should be useful for a variety of studies.

Because NGFr in different cell types differs in binding affinity and responsiveness (Buxser *et al.*, 1983; Vale and Shooter, 1985), we compared the lateral diffusibility and distribution of NGFr in neuronal and nonneuronal cell lines. NGFr in COS cells and A875 cells had similar homogeneous distribution and diffusion properties, with $D \approx 5 \times 10^{-10}$ cm^2/s and %R of 51 and 44, respectively. These values are typical for membrane proteins (Edidin, 1981; Peters, 1981). In contrast, diffusion of NGFr in PC12 cells was very rapid ($D \approx 3 \times 10^{-9}$ cm^2/s) with a low %R (20–30). NGFr was uniformly distributed on COS cells but was associated with cell-cell boundaries for A875 cells. Because the boundary-associated NGFr was not immobilized, this effect is not due to a cytoskeletal attachment. It may be due, in part, to surface amplification at the vertical interface of the two membranes (Wolf and Ziomek, 1983). The distribution of NGFr on PC12 cells was heterogeneous and, in some cells, appeared patchy. Hence, the diffusion properties and distribution of NGFr on the responsive PC12 cells are quite different from the nonresponsive COS and A875 cells. A difference in diffusibility of responsive and nonresponsive receptors has been observed for epidermal growth factor. The high-affinity, biologically responsive binding sites are much less diffusible than the low-affinity, nonresponsive binding sites (Rees *et al.*, 1984; Bellot *et al.*, 1990). We are currently testing this correlation for NGFr with additional responsive and nonresponsive cell lines.

We observed NGF-enhanced diffusion of NGFr for A875 cells and for PC12 cells in suspension, but not for COS cells or for PC12 cells adhered to a collagen substratum. We did not observe any NGF-induced changes in the lateral distribution of NGFr. These results are in disagreement with Levi *et al.* (1980). They reported that NGFr in PC12 cells immediately after binding of fluoresceinated NGF diffuses with $D \approx 8 \times 10^{-10}$ cm^2/s. The NGF-NGFr complexes then aggregated and formed immobile patches. There are two possible explanations for this disagreement. The first is that the Fab used in this study perturb the diffusion properties of the NGFr. We do not think that this is the case because these MAb do not block NGFr function. The MAb do not block NGF binding. Also, 192 MAb bound to NGFr is internalized and transported in a retrograde manner by rat cervical neurons (Taniuchi and Johnson, 1985). Rat PC12 cells in the presence of 192 MAb (Chandler *et al.*, 1984), and human neuroblastoma SHSY5Y cells in the presence of NGFR5 MAb (J. Chen

and A.H. Ross, unpublished observations), differentiate in response to NGF, although a slightly higher concentration of NGF is required. The second explanation is that the fluoresceinated NGF used by Levi *et al.* (1980) was not representative of native NGF. The fluoresceinated NGF was extensively conjugated, with at least 8–10 carboxylic acid sidechains derivatized, and was not freely soluble at neutral pH. The presence of multiple hydrophobic fluorophores replacing carboxylic acid groups could radically alter NGF charge and induce self-aggregation.

The anti-NGFr Fab were used to analyze which domains of the NGFr control lateral diffusion. Deletion of all but five amino acids of the cytoplasmic domain of NGFr expressed in COS cells does not remove constraints to its free diffusion. This result is consistent with studies of Livneh *et al.* (1986) for EGFr, Edidin and Zuniga (1984) for H-2 L^d antigen, and Scullion *et al.* (1987) for VSV glycoprotein but differs with class II major histocompatibility antigen, for which deletion of the cytoplasmic tail enhanced the rate of lateral diffusion (Wade *et al.*, 1989). There are two caveats concerning the role of the NGFr cytoplasmic domain. First, one cannot remove all of the amino acids from cytoplasmic domain and still have it processed to the plasma membrane. The remaining five amino acids still potentially could have a regulatory role in diffusibility. Second, we have shown that the lateral diffusion properties of the NGFR differ between cell types. It is quite plausible that in neuronal cells there is a connection between the cytoplasmic domain of the NGFr and the cytoskeleton.

In contrast, the cytoplasmic domain does appear to be important in the apparent positive cooperativity that we have observed in COS cells. Positive cooperativity previously has been observed for NGFr in PC12 cells by Woodruff and Neet (1986) but not by other groups (Buxser *et al.*, 1983; Vale and Shooter, 1985). Comparison of diffusion results for A875 cells, which do not show positive cooperativity, and COS cells, which do, suggests that diffusion rate and extent are not the determining factors for positive cooperativity. A consistent model is that a single receptor binds multiple NGF and that NGF binding induces a conformational change in the NGFr, which requires the cytoplasmic domain of the receptor and which increases the affinity of NGFr for binding additional NGF. The stoichiometry of binding of NGF to NGFr is not known, and it is possible that the four cysteine-rich repeat units represent two or four NGF-binding sites. Alternatively, stable NGF binding could be enhanced by binding of ligand to two or more receptors. NGFr oligomers were detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions (Buxser *et al.*, 1985; Grob *et al.*, 1985). Receptors could be complexed with intact cytoplasmic domains and monomeric with the domains deleted. The formation of such oligomers might be enhanced in COS cells because of the extremely high density of NGFr (Table 1).

Levi *et al.* (1980) proposed that NGF binding resulted in aggregation of NGFr into patches that were internalized. The clustering and internalization were suggested to be part of the signal transduction mechanism. Although further experiments are required to fully define the state of aggregation of NGFr and although some differences between our data and those of Levi *et al.* (1980) might be due to differences in methodology and experimental conditions, our data do not seem consistent with ligand-induced aggregation of NGFr or, in particular, with ligand-induced formation of large NGFr patches. We did not observe NGF-induced immobilization of NGFr and in some cell types actually observed enhanced diffusion in the presence of NGF. Also, the direct role of NGFr aggregation in signal transduction seems unlikely because treatment of NGF-responsive cells with anti-NGFr MAb's does not induce a biological response (Chandler *et al.*, 1984; J. Chen and A.H. Ross, unpublished observations). An alternative model of signal transduction is that NGF induces an allosteric conformational change of the NGFr that modulates the association of the NGFr with other proteins. Such an allosteric conformational change might be the origin of the positive cooperativity observed for NGFr expressed in COS cells. Ligand-induced modulation of interactions with other proteins have been observed for platelet-derived growth factor receptor (Morrison *et al.*, 1989) and interleukin-6 receptor (Taga *et al.*, 1989). We are currently carrying out experiments to identify NGFr-associated proteins that might be responsible for limiting the diffusion of NGFr. In addition, we are determining the state of NGFr aggregation in the presence and absence of ligand.

Methods

Cells and cell culture

COS (monkey kidney, Gluzman, 1981) cells were grown in Iscove's modified Dulbecco's medium supplemented with

10% fetal bovine serum (FBS). The human melanoma cell line A875 (Fabricant *et al.,* 1977) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Rat pheochromocytoma PC12 cells (Greene and Tischler, 1976) were grown in DMEM/7% horse serum/7% FBS. Glutamine (2 mM) and gentamycin (50 μg/ml) were added to all culture media, and cultures were maintained in a 5% CO_2 humidified atmosphere.

MAb and Fab fragments

The preparation of MAb 192 and NGFR5 has been described (Chandler *et al.,* 1984; Marano *et al.,* 1987). IgG was purified from mouse ascites using either an Affi-gel Protein A MAPS II kit (Bio-Rad, Richmond, CA), or a Protein G Sepharose 4 Fast Flow column (Pharmacia LKB, Upsala, Sweden). Fab fragments were prepared by brief digestion of the purified IgG with papain (Sigma Chemical, St. Louis, MO) as described (Hudson and Hay, 1976). Fab was purified by passage through a DE-52 column, was concentrated by vacuum dialysis, and was judged pure by SDS-PAGE. The conjugation of FITC (100 μg) (Molecular Probes, Eugene, OR) to Fab (400 μg) was carried out at 4°C in 1 ml 5 mM sodium phosphate, pH 8.0, for 10 h. Free FITC was removed from the FITC-Fab conjugate by repeated washing and centrifugation through a Centricon-10 filter (Amicon, Danvers, MA). The final fluorescein:Fab molar ratio was 1.1–1.4, depending on the batch of Fab.

Transfection of COS cells

Cells were transfected with plasmid DNA (1–2 μg per 35-mm dish or 15 μg per 75-cm^2 flask) by the calcium phosphate precipitation technique of Wigler *et al.* (1978). All experiments were carried out 42–44 h after transfection. Construction of the plasmids pCMYX-7, encoding the full-length human NGF-R, and pCMVPvu, expressing NGF-R lacking the cytoplasmic domain, have been described (Reddy *et al.,* 1990).

Immunofluorescence microscopy and FRAP

Unfixed cells on coverslips were washed twice with Hank's buffered saline solution (HBSS) and HBSS + 0.5% bovine serum albumin and incubated with 0.1 mg/ml FITC-Fab for 30 min at 4°C. The fluorescently labeled cells were extensively washed with HBSS, and, immediately before viewing, varying concentrations of unlabeled NGF were added. Cells were observed with the use of a Zeiss (Thornwood, NY) Axioplan microscope equipped with fluorescence optics and a 100× 1.3 NA plan neofluor objective lens. Lateral diffusion of the receptors was measured by the technique of fluorescence photobleaching and recovery, which has been extensively reviewed (Edidin, 1981; Peters, 1981); the details of the specific instrument used in the present study were described by Wolf (1989). All FRAP measurements were made at room temperature with the use of a Zeiss 63× 1.4 NA plan Apochromat objective, which gave a 0.9 μm exp(−2) beam radius using the 488-nm line of a Lexel (Palo Alto, CA) 95-2 argon laser. The monitoring intensity was ≈0.13 μW, and the bleach was ≈1.3 mW for ≈5 ms. These conditions were chosen so that there would be no detectable bleaching due to the monitoring beam.

Data were fitted to the diffusion theory of Axelrod *et al.* (1976) by the method of nonlinear least squares by the use of a modification of the procedures of Bevington (1969). These procedures are described in detail by Wolf (1989).

Labeling with lipid probe

Lipid diffusibility was studied with the use of the fluorescent lipid analogue 1,1,1′,1′-tetramethyl-3,3′-dihexadecyl-indocarbocyanine perchlorate (C_{16}dil; Molecular Probes). Cells were labeled with 5 μg/ml C_{16}dil in HBSS containing 0.5% (v/v) ethanol and for 8 min at RT and then rinsed three times with HBSS.

Analysis of NGF and IgG binding

NGF (2.5S, Bioproducts for Science, Indianapolis, IN) and purified IgG were iodinated by the lactoperoxidase method of Sutter *et al.* (1979). Binding of ^{125}I-NGF and ^{125}I-IgG to intact A875 cells was assayed according to Vale and Shooter (1985). Nonspecific binding was determined in the presence of excess NGF (500 nM) and subtracted from all data. Internalization of ^{125}I-NGF was measured by the method of Bernd and Greene (1984).

Northern blot analysis

Total cellular RNA was isolated by the method of Chomczynski and Sacchi (1987) and analyzed by electrophoresis on a 1.2% agarose/formaldehyde gel. The RNA was transferred on to a Gene Screen Plus membrane (New England Nuclear Research Products, Boston, MA) and hybridized with random primer-labeled probe for *fos* oncogene (1-kb *Pst* I fragment from p*fos*-1 [Curran *et al.,* 1982]).

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