Accelerated Publications

Nerve Growth Factor Receptors Are Preaggregated and Immobile on Responsive Cells†

Gita Venkatakrishnan, Christine A. McKinnon, Carmencita G. Pilapil, David E. Wolf,* and Alonzo H. Ross

Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, Massachusetts 01545

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ABSTRACT: It has been hypothesized that signal transduction occurs by ligand-induced receptor clustering and immobilization. For many peptide receptors, cross-linking by anti-receptor antibodies is sufficient for receptor activation. This is not, however, the case for nerve growth factor receptor (NGFR). Using fluorescence microscopy and fluorescence recovery after photobleaching (FRAP), we have analyzed the distribution and diffusibility of NGFR on a series of cell lines. We have found the following: (1) Cells expressing high-affinity responsive NGFR's display clustered NGFR's even in the absence of ligand. In contrast, NGFR's in nonresponsive cell lines are diffusely distributed. (2) Receptors on responsive cell lines are largely nondiffusing while most receptors on nonresponsive cell lines are relatively free to diffuse. (3) NGF does not greatly alter the distribution or diffusion properties of the NGFR on either nonresponsive or responsive cell lines. Thus, NGFR is preclustered and immobile on responsive cells, which suggests that immobilization of NGFR prior to ligand binding is required for signal transduction.

A relationship between aggregation–immobilization and physiological responsiveness has been observed in a number of receptor systems. Acetylcholine receptors are diffusely distributed on nonresponsive myoblasts but become aggregated and immobilized into "hot spots" on myotubes (Axelrod et al., 1976b). EGFR[1] and insulin receptor are reported to be diffusely distributed on responsive cells and become aggregated and immobilized upon ligand binding (Schlessinger et al., 1978). Other investigators (Rees et al., 1984) reported that low-affinity EGFR's are free to diffuse, but high-affinity EGFR's are immobilized. The high-affinity EGFR, but not the low-affinity EGFR, is thought to mediate the biological response even though both sites include the same 170 000-Da glycoprotein (Bellot et al., 1990, Defize et al., 1989). Aggregation of EGFR with some but not all MAbs induces a biological response (Schreiber et al., 1983; Defize et al., 1986). Internalization of the EGFR is not required for modulation of gene transcription and cell growth but may be required for ligand-induced increases in cytosolic calcium (Chen et al., 1989). These results along with those described in this paper for NGFR demonstrate that one cannot hypothesize a generalized model of peptide factor induced receptor aggregation and immobilization. There are at least two classes of receptors, those that are preclustered and immobile and those that only become clustered and immobile upon ligand binding.

NGF is a 26 000-Da polypeptide neurotrophic factor that acts as a survival factor for sympathetic and sensory neurons (Levi-Montalcini & Aloe, 1987). NGF also modulates differentiation of rat pheochromocytoma PC12 cells to sympathetic neuronlike cells and induces a rapid but transient expression of *c-fos* mRNA and protein (Milbrandt, 1986). All of these actions of NGF are mediated by specific cell-surface NGFR's. Molecular cloning of NGFR indicates that the receptor gene is single copy and that the NGFR is a highly conserved protein (Chao et al., 1986; Johnson et al., 1986; Radeke et al., 1987; Large et al., 1989). The human NGFR is a 75 000-Da glycoprotein (gp75) with a hydrophobic signal sequence, a single N-linked glycosylation site, four 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 gp75 is not sufficient for a fully functional NGFR. Additional factors appear to be required. Responsive cell lines express both low- and high-affinity NGFR, but nonresponsive cell lines express only low-affinity NGFR (Green et al., 1986). Expression of recombinant gp75 in nonneuronal cell types resulted in low-affinity nonresponsive NGFR (Chao et al., 1986; Radeke et al., 1987; Reddy et al., 1991). Expression of gp75 in an NGFR-minus variant of PC12 (Hempstead et al., 1989), in human neuroblastoma cell line HTLA230 (Matsushima & Bogenmann, 1990), and in human brain tumor cell line D283 MED (Pleasure et al., 1990) resulted in low- and high-affinity NGFR's that mediated NGF-induced upregulation of *c-fos* oncogene expression. The intracellular domain of gp75 is required for a high-affinity, responsive NGFR (Hempstead et al., 1990), and the high-affinity binding sites may be associated with the cytoskeleton (Schechter & Bothwell, 1981; Vale et al., 1985). The putative second factor may limit the number of high-affinity binding sites and their responsiveness (Reddy et al., 1991). A 135 000-Da cell-surface protein identified by chemical cross-linking with ^{125}I-NGF may be a second subunit of the NGFR or a cross-linked complex of gp75 and the second subunit

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[1] Abbreviations: EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; Fl-Fab, fluoresceinated Fab; FRAP, fluorescence recovery after photobleaching; MAb, monoclonal antibody; NGF, nerve growth factor; NGFR, nerve growth factor receptor; % *R*, percent recovery; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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(Hosang & Shooter, 1985; Hempstead et al., 1989). Since recombinant NGFR expressed in the medulloblastoma cell line is high affinity and responsive but does not efficiently internalize ^{125}I-NGF, additional factors may be required for NGF internalization (Pleasure et al., 1990). Recently, Minamoto et al. (1990) reached a similar conclusion for the interleukin 2 receptor.

FRAP has been used to analyze the lateral diffusibility of membrane proteins and lipids (Wolf, 1989) and interactions between membrane proteins and the cytoskeleton (Wade et al., 1989). Fluid dynamic theory predicts that proteins should diffuse only slightly slower than lipids (Saffmann & Delbruck, 1975). However, membrane proteins typically diffuse 1–3 orders of magnitude slower than membrane lipids, with a significant fraction not diffusing at all (Edidin, 1981). For class I major histocompatibility antigens (Edidin & Zuniga, 1984), EGFR (Livneh et al., 1986), vesicular stomatitis virus glycoprotein (Scullion et al., 1987), and NGFR (Venkatakrishnan et al., 1990), deletion of the cytoplasmic domain has no effect on the rate of lateral diffusion. For class I histocompatibility antigens (Wier & Edidin, 1988), mutations that reduce the size of the extracellular domain or eliminate glycosylation enhance diffusion. In contrast, lateral diffusion of class II major histocompatibility antigens was enhanced by deletion of the cytoplasmic domain (Wade et al., 1989). Hence, lateral diffusion of membrane proteins may be limited by both exo- and cytoskeletal interactions.

In the present study, we utilize fluorescent Fab fragments of anti-NGFR MAbs, which do not compete with NGF for binding to NGFR (Venkatakrishnan et al., 1990). We report the cell-surface distribution and lateral diffusion of NGFR for a variety of responsive and nonresponsive cell types. We address two question: (a) Do the diffusion and distribution properties of NGFR differ in cells that are NGF responsive and nonresponsive? (2) Does the addition of NGF to cells affect the rate of diffusion and the % R of the receptors?

Experimental Procedures

Materials. Iscove's modified medium, DMEM, and RPMI 1640 were purchased from Gibco. DME was obtained from the MIT Cell Culture Center. NGF (2.5 S) was from Bioproducts for Science. E-C-L cell attachment matrix, a suspension of entactin, collagen IV, and laminin prepared from Englebreth–Holm–Swarm mouse tumors, was purchased from Upstate Biotechnology. FITC was from Molecular Probes. The fluoresceinated Fab fragment of goat anti-mouse IgG was obtained from Cappel.

Cells and Cell Culture. COS (monkey kidney; Gluzman, 1981) cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% FBS. The human melanoma cell line A875 (Fabricant et al., 1977) was grown in DMEM supplemented with 10% FBS. SHEP/NGFR (human neuroblastoma infected with NGFR defective retrovirus; Reddy et al., 1991), MED/NGFR (human medulloblastoma infected with NGFR defective retrovirus; Pleasure et al., 1990), and SHSY5Y (human neuroblastoma; Biedler et al., 1978) cells were grown in RPMI 1640/10% FBS. GICAN cells (human neuroblastoma; Longo et al., 1988) were grown in RPMI 1640/20% FBS. CHP707m cells (human primitive neuroectodermal tumor; Baker et al., 1990) were grown in RPMI 1640/10% FBS supplemented with 1.1 mM *cis*-oxaloacetic acid, 0.45 mM pyruvic acid, and 200 units/L insulin. Rat pheochromocytoma PC12 cells (Greene & Tischler, 1976) and nnr5 cells (nonresponsive variant of PC12; Green et al., 1986) were grown in DMEM/7% horse serum/7% FBS. Glutamine (2 mM) and gentamycin (50 μg/mL) were added to all culture media except for CHP707m, for which 4 mM glutamine was used. Cultures were maintained in a 5% CO_2 humidified atmosphere.

Dorsal root ganglia were dissected from rat embryos (E16) and dissociated for 25 min at 37 °C with 0.25% trypsin. The resulting cells were plated on glass coverslips (5×10^4 cells per 12-mm coverslip) pretreated with E-C-L matrix. The cells were cultured for 2–4 days in DME supplemented with 10% FBS and 100 units/mL penicillin and 100 μg/mL streptomycin.

MAb and Fab Fragments. The preparation of MAb's 192 and NGFR5 (both IgG_1) has been described (Chandler et al., 1984; Marano et al., 1987). IgG was purified from mouse ascites with either a protein A or protein G affinity column. Fab fragments were prepared by brief digestion of the purified IgG with papain as described (Hudson & Hay, 1976). Fab was purified by passage through a DE-52 column or a Mono-Q column, was concentrated with a Centricon 30 filter, and was judged pure by SDS–PAGE. The conjugation of FITC (100 μg) to Fab (400 μg) was carried out at 4 °C in 1 mL of 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. The final fluorescein:Fab molar ratio was 1.1:1.4.

Transfection of COS Cells. Cells were transfected with plasmid DNA (1–2 μg per 35-mm dish) by the calcium phosphate precipitation technique of Wigler et al. (1978). All experiments were carried out for 42–44 h after transfection. Construction of the plasmids pCMVX-7, encoding the full-length human NGFR, and pCMVPvu, expressing a truncated NGFR lacking the cytoplasmic domain, has been described (Reddy et al., 1990).

Immunofluorescence Microscopy and FRAP. Fl-Fab fragments of MAb's (100 μg/mL) NGFR5 (anti-human NGFR) and 192 (anti-rat NGFR) were used to label cells as previously described (Venkatakrishnan et al., 1990). Photomicrographs were taken with a Zeiss Axiovert 35 microscope using a plan neofluor 100×, 1.3 NA objective and a Photometrics 200 CCD liquid-cooled camera. A background image was subtracted from the Nomarski/bright field image to eliminate inhomogeneities in the field, and fluorescence images have been histogram equalized. Hard copies of the images were printed with a Mitsubishi color printer CP100.

All FRAP measurements, as described previously (Venkatakrishnan et al., 1990; Wolf, 1989), were made at room temperature with a Zeiss 63×, 1.4 NA plan Apochromat objective, which gave a 0.9-μm exp(−2) beam radius with the 488-nm line of a Lexel 95-2 argon laser. The monitoring intensity was ≈0.13 μW, and the bleaching intensity was ≈1.3 mW for ≈5 ms. These conditions were chosen so that there would be no detectable bleaching due to the monitoring beam. For most cell types, fluorescence background levels were neglible. Samples were discarded if background exceeded 10%. No significant recoveries of background were observed. Thus, when necessary pre- and postbleach backgrounds were subtracted. Data were fitted to the diffusion theory of Axelrod et al. (1976a) by a modification of the nonlinear least-squares procedure (Bevington, 1969; Wolf & Edidin, 1981).

Results

The cell-surface distribution of NGFR was determined by using the Fl-Fab fragments of either anti-rat NGFR 192 MAb or anti-human NGFR NGFR5 MAb, neither of which competes with NGF for binding to NGFR's (Chandler et al., 1984; Marano et al., 1987). For nonresponsive cell lines A875 and SHEP/NGFR and COS cells transfected with expression

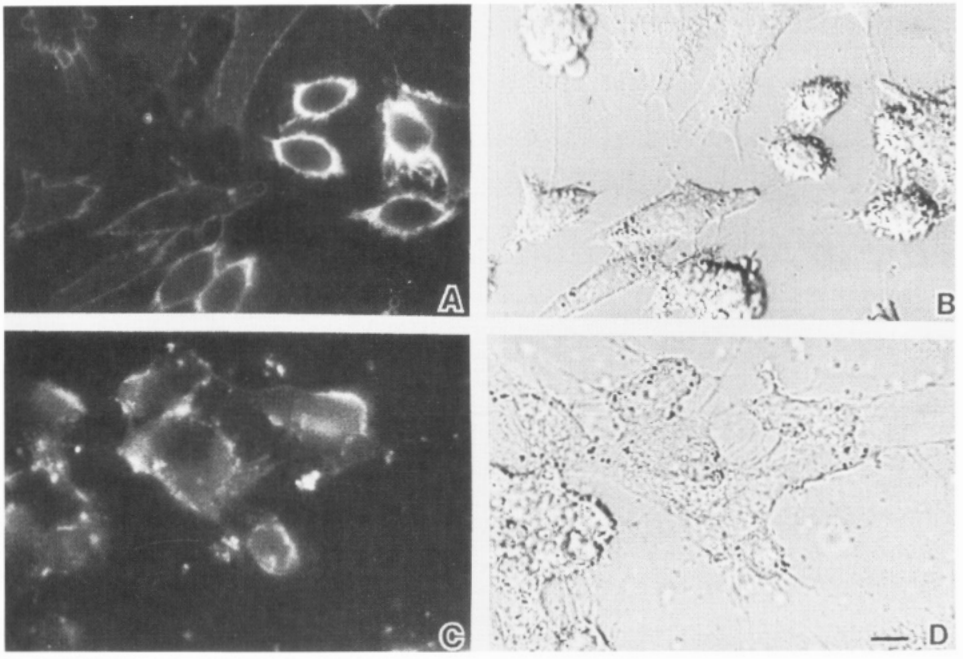

FIGURE 1: Fluorescence photomicrographs showing characteristic distributions of NGFR on adherent cells expressing either only low-affinity NGFR or both low- and high-affinity NGFR. (A) SHEP/NGFR (low-affinity receptors, NGF nonresponsive; Reddy et al., 1991). NGFR was diffusely and homogeneously distributed except for concentrations in the cell periphery that may reflect surface amplification (Wolf & Ziomek, 1983), which also results in the more rounded cells appearing brighter. (B) Accompanying Nomarski image of SHEP/NGFR. (C) SHSY5Y (both low- and high-affinity receptors, NGF responsive; Chen et al., 1990). While there are some diffusely labeled regions over the cell surface, fluorescence is generally clustered. Several high-intensity clustered regions are prominent. Similar staining patterns were observed for the other cell types considered, depending on the class of receptor as described in the text. NGF does not alter the distribution for any of the cell types. (D) Accompanying bright field image of SHSY5Y. Bar = 10 μm.

vectors for full-length NGFR (pCMVX-7) or truncated NGFR (pCMVPvu), the distribution was uniform and homogeneous (Figure 1A,B). For responsive cell lines MED/NGFR, GICAN, PC12 (Venkatakrishnan et al., 1990), CHP707m, and SHSY5Y, the distribution of NGFR was uneven, with some cells showing clustered regions. These clustered regions were most evident for SHSY5Y (Figure 1C,D) and were not altered by prefixation with 1% paraformaldehyde (not shown). The lack of staining of COS cells that do not express NGFR and the complete inhibition of staining by intact nonfluorescent anti-NGFR MAb (20 μg/mL) demonstrated the specificity of the staining. Addition of NGF (100 nM) did not significantly alter the NGFR distribution.

The lateral diffusion of NGFR was analyzed by FRAP (Venkatakrishnan et al., 1990; Wolf, 1989). The D for NGFR expressed on nonresponsive cell lines ranged between 0.53×10^{-9} and 2.4×10^{-9} cm^2/s but for NGFR on responsive cell lines was between 4.2×10^{-9} and 6.5×10^{-9} cm^2/s (Figure 2A). Analysis of these data by the Wilcoxon rank–sum test for independent samples (Bradley, 1968) demonstrated a statistically significant ($P < 0.025$) difference between the nonresponsive and responsive cell lines. The data for CHP707m are shown but were not included in the statistical analysis. CHP707m apparently has only low-affinity NGFR but very weakly upregulates *c-fos* oncogene expression in response to NGF and, hence, does not clearly fall into either the low-affinity, nonresponsive or the high-affinity, responsive categories. The % R for nonresponsive cells was between 43% and 69% but for responsive cells was between 11% and 24% (Figure 2B), which is a statistically significant difference ($P < 0.025$). There was no effect of NGF at a saturating concentration on the diffusion properties of NGFR for any of the cell lines tested. Controls on fixed cells demonstrating that these Fab fragments accurately probe lateral diffusion rather than surface "hopping" were described in a previous paper (Venkatakrishnan et al., 1990).

For SHSY5Y cells, diffuse and clustered areas were photobleached and analyzed. The clustered areas were about 2–3-fold more intense than the diffuse areas. For the diffuse areas, the D and % R were $(5.5 \pm 0.8) \times 10^{-9}$ cm^2/s and 27 ± 3 (25 measurements, average ± standard error of mean), but for the clustered areas the D and % R were $(12 \pm 2) \times 10^{-9}$ cm^2/s and 15 ± 3 (13 measurements). The differences for both D and % R were statistically significant by Student's t test ($P < 0.005$). Thus, immobilization was greater in the clustered areas.

For some cell cultures, it was necessary to enhance the fluorescence signal by using both Fl-anti-NGFR-Fab and Fl-anti-mouse IgG-Fab. With this method, the diffusion parameters for PC12 cells were $D = (1.7 \pm 0.3) \times 10^{-9}$ cm^2/s and % $R = 23 \pm 3$ (25 measurements). The slightly lower D measured with the double Fab may have resulted from the larger bulk of the Fab-Fab-NGFR complex (Wier & Edidin, 1988). The cell line nnr5 is an nonresponsive variant of PC12 that has no high-affinity NGFR and approximately 23% as many total NGFR as PC12 (Green et al., 1986). FRAP analysis of nnr5 revealed that NGFR diffused with a higher % R (45 ± 5) and a lower D [$(0.51 \pm 0.07) \times 10^{-9}$ cm^2/s, 29 measurements] than those on the parent PC12 cells (Student's t test; $P < 0.0005$ for both D and % R), consistent with the differences between responsive and nonresponsive cells seen in Figure 2. Dorsal root ganglia neurons (E16) that require NGF for survival (Sutter et al., 1979) were also analyzed by the double Fab method. The D and % R were $(2.4 \pm 0.6) \times 10^{-9}$ cm^2/s and 19 ± 3 (14 measurements) for NGFR in the cell bodies. In contrast to the NGF-responsive cell lines, the distribution of NGFR on cell bodies was homogeneous and did not display clustered regions. In future studies, we will provide a detailed description of the distribution and diffusion properties of NGFR on neuronal cell bodies and axons. In preliminary studies, NGFR diffusion was measured for a

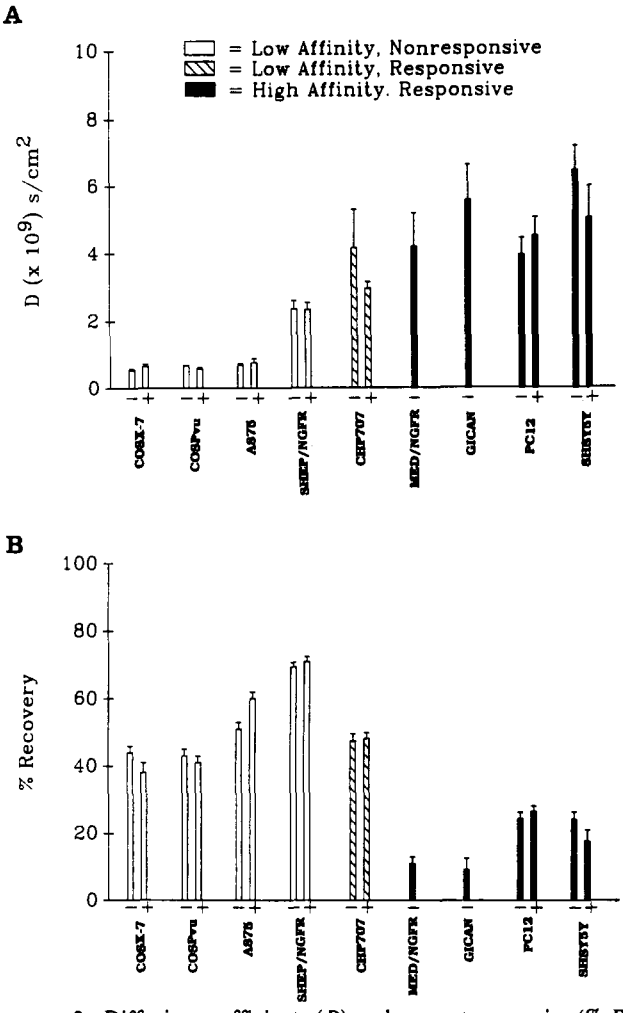

FIGURE 2: Diffusion coefficients (D) and percent recoveries (% R) for NGFR. Open bars denote low-affinity nonresponsive cells, and filled bars denote high-affinity responsive cells. CHP707m, a low-affinity, weakly responsive cell line, is shown as a striped bar. Error bars indicate ±1 standard error of the mean; ± indicates the absence or presence of 100 nM NGF. Low-affinity NGFR cells generally show high % R ranging from 41% to 74%, while high-affinity NGFR cells generally show low % R ranging from 9% to 27%. NGFR's on responsive cells diffuse in the range of (3–7) × 10^{-9} cm^2/s, while NGFR's on the nonresponsive cells diffuse in the range of (0.6–2) × 10^{-9} cm^2/s. NGF (100 nM) did not significantly affect either D or % R for any of the cell types tested. Data for the COS cells and A875 have been published previously (Venkatakrishnan et al., 1990).

limited set of dorsal root ganglia glial cells, which are a mixture of Schwann and satellite cells and are thought to be nonresponsive to NGF (Yasuda et al., 1987; Lindsay et al., 1990). The D and % R were (0.55 ± 0.28) × 10^{-9} cm^2/s and 49 ± 7 (four measurments). Future studies will be made on pure populations of Schwann cells. These results provide further evidence that NGFR is immobile on responsive cells.

Discussion

Using a series of tissue culture cell lines, we have found that NGFR's expressed on responsive cells have a lower % R than NGFR's expressed on nonresponsive cells. The diffusion coefficients of NGFR on responsive cells are greater than those of NGFR on nonresponsive cells. Additionally, on many responsive cell lines NGFR's are heterogeneously distributed, while on nonresponsive cells NGFR's are homogeneously distributed. The NGFR's in the clustered regions of responsive cells were more highly immobilized than in the diffuse regions of the same cells. Addition of NGF at a saturating concentration had no effect on either diffusion or distribution of NGFR on any cell type considered. Thus, we conclude that NGFR is immobilized and preclustered on responsive cells.

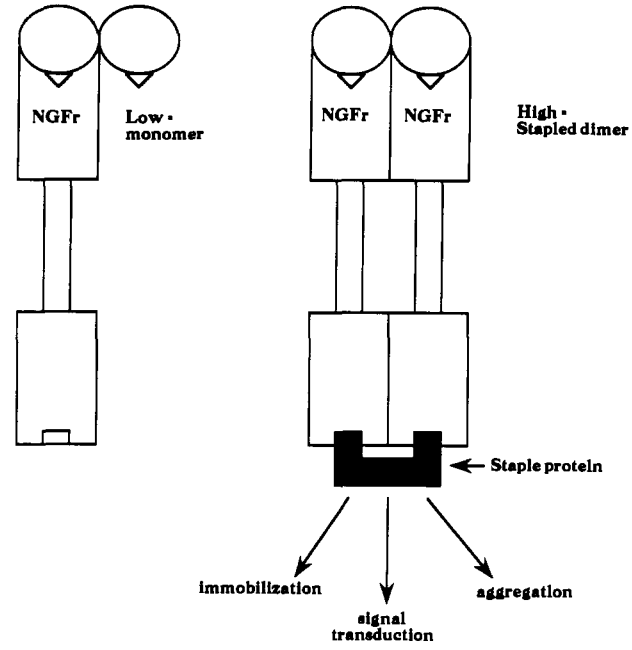

FIGURE 3: Proposed model to account for high-affinity-responsive and low-affinity-nonresponsive forms of NGFR. The model proposes that the high-affinity site is a homo- or heterodimer that is stabilized by an ancillary staple protein. NGF alone in the absence of the staple protein cannot form stable NGFR dimers. This staple protein may also be necessary for NGFR immobilization and signal transduction.

In further support of this conclusion, we have compared the diffusion of NGFR on responsive PC12 cells with the nonresponsive variant nnr5. As predicted, NGFR's on PC12 cells exhibit diffusion properties typical of responsive cell types, while NGFR's on nnr5 cells exhibit diffusion properties typical of nonresponsive cells. Additionally, we have compared the diffusion properties of NGFR on responsive dorsal root ganglia neurons and nonresponsive dorsal root ganglia glial cells and again found that NGFR is largely immobile on responsive cells.

As discussed above, responsiveness is correlated with a decreased mobile fraction of NGFR and an increased diffusion coefficient for that fraction. This opposition of mobile fraction and diffusion coefficient is not ubiquitous in FRAP studies. Duband et al. (1988), for instance, in studying the diffusion of the fibronectin receptor integrin on locomoting and nonmotile embryonic neural cells found a dramatic difference in mobile fractions but the same diffusion coefficient. These different results can potentially be explained in terms of a theory of lateral mobility developed by Elson and Reidler (1979). These authors predicted that interactions of a cell-surface molecule with some stationary membrane component can affect either only % R or both % R and D. The critical parameter in their theory is the lifetime of the membrane complex compared to the characteristic time of the FRAP measurement (10 s to 5 min). If the lifetime is long in comparison to the characteristic time, the complex will result in a reduced % R but will not affect D. If the lifetime is short, both % R and D will be reduced. In the context of this model, one would predict that, in contrast to integrin, NGFR forms a relatively short-lived unstable complex with the immobilizing factor in nonresponsive cells, but in responsive cells, this interaction becomes stabilized and long-lived in comparison to the characteristic time.

In Figure 3 we show a working model that could explain many of the properties of NGFR. This model is a modification

of that previously proposed by Buxser et al. (1985). In this model, the low-affinity receptor is an NGFR monomer, and the high-affinity receptor is an NGFR dimer. Our observations that NGF does not alter NGFR diffusibility indicate that NGFR does not cross-link NGFR monomers, suggesting that dimerization is stabilized by a secondary factor, the "staple" protein that interacts with the cytoplasmic domain of gp75. This domain is required for high-affinity binding of NGF (Hempstead et al., 1990). In this model, the staple protein is also required for interactions that result in clustering, immobilization, and signal transduction. A variation of this model would be that the staple protein promotes the interaction of an NGFR monomer not with another NGFR monomer but with some other membrane component.

An important question concerning this model is how we could detect the immobilization if only high-affinity binding sites are immobilized. For instance, in PC12 cells only about 5% of the NGFR's are high affinity (Green et al., 1986), and immobilization of such a small fraction of molecules would probably not be detected by FRAP. One explanation is that immobilization is a prerequisite for high-affinity binding sites, but not all immobilized NGFR's are high affinity. An immobilized low-affinity NGFR monomer might arise by interaction of a staple protein with a single NGFR. A second possibility is that there are not really two distinct classes of NGFR on responsive cells, as proposed by Buxser et al. (1990). These authors found that the nonlinear Scatchard plots observed on responsive cells result from deficiencies in the Scatchard method of analysis.

To determine the relative roles of immobilization and internalization in NGF responsiveness, we have analyzed the MED/NGFR cell line (Pleasure et al., 1990), which apparently has both low- and high-affinity NGF binding sites, is responsive to NGF (upregulation of *fos* oncogene expression), but has little or no capacity for internalization of NGF. These results suggest that NGFR immobilization rather than NGF internalization is the critical parameter for initial responses to NGF.

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Articles

Purification and Characterization of an Oxidase Activating Factor of 63 Kilodaltons from Bovine Neutrophils†

Marie-Claire Pilloud-Dagher* and Pierre V. Vignais
Laboratoire de Biochimie/LBIO, Dêpartement de Biologie Molêculaire et Structurale, Centre d'Etudes Nuclêaires, 85X, 38041 Grenoble Cedex, France

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ABSTRACT: A 63-kDa protein, which behaves as an oxidase activating factor in bovine neutrophils, has been purified to electrophoretic homogeneity. The protein was isolated from the cytosol of resting bovine neutrophils after several steps, including ammonium sulfate precipitation and chromatography on AcA44, DE-52 cellulose, Mono Q, and Superose 12 in the presence of dithiothreitol. The oxidase activating potency of the protein was assayed with a cell-free system consisting of neutrophil membranes, GTPγS, arachidonic acid, and a complementary cytosolic fraction. The purification factor was 200 and the yield 3%. During the course of gel filtration on calibrated Superose 12, the 63-kDa protein eluted as a dimer. Its isoelectric point was 6.4 ± 0.1. Antibodies raised in rabbits against the 63-kDa protein reacted with a protein of similar size in human neutrophils and in HL60 promyelocytic cells induced to differentiate into granulocytes. No immune reaction was observed in cytosol from undifferentiated HL60 cells, in extracts from bovine skeletal muscle, liver, and brain, or in cytosol prepared from neutrophils derived from a patient with an autosomal cytochrome *b* positive form of chronic granulomatous disease lacking the 67-kDa oxidase activating factor. Immunoblotting with the 63-kDa bovine protein antiserum demonstrated that activation of bovine neutrophil oxidase by phorbol myristate acetate induced the translocation of the 63-kDa protein from cytosol to the membrane.

Resting neutrophils do not generate significant amounts of the superoxide anion $O_2^{\cdot -}$. However, when they are challenged by appropriate soluble or particulate stimuli, their $O_2^{\cdot -}$ production is markedly enhanced. This abrupt enhancement in $O_2^{\cdot -}$ generation, known as the respiratory burst, together with degranulation, contributes to the microbicidal function of neutrophils during phagocytosis. The respiratory burst is catalyzed by an activated plasma membrane-bound NADPH oxidase, which is believed to consist of an NADPH-dependent flavoprotein and a low-potential *b*-type cytochrome [for reviews, see Rossi (1986), Bellavite (1988), and Segal (1990)]. The molecular approach to the mechanism of oxidase activation has largely benefited from the use of a cell-free system

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