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HSV-1 Vector–Mediated Gene Transfer of the Human Nerve Growth Factor Receptor p75^{hNGFR} Defines High-Affinity NGF Binding

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A series of recombinant herpes simplex virus (HSV-1) vectors have been constructed that encode either the full-length cDNA of the human p75 NGF receptor (p75^{hNGFR}) or truncated forms of the receptor. Infection of cultured fibroblast cells with viral stocks results in abundant expression of all three cDNAs, as detected by affinity cross-linking, immunoblot analysis, and equilibrium binding. Furthermore, viral infection of primary neuronal cultures gives easily detectable p75 expression by immunofluorescence and affinity cross-linking. When p75 was introduced by viral infection into fibroblast cells expressing the *trk* proto-oncogene, a new binding site was created, consistent with high-affinity NGF binding. This site is not created by the coexpression of truncated forms of p75 that lack either the extracellular ligand binding domain or the cytoplasmic domain of the receptor, suggesting that both of these regions of the receptor are required for the formation of the high-affinity NGF binding site. Hence, these HSV-1 vectors give rise to appropriate NGF receptor binding after viral infection. The application of these HSV-1 constructs to primary neuronal culture and *in vivo* models of p75^{NGFR} function is discussed.

[Key words: NGF receptor, herpes simplex virus, primary culture,* trk *oncogene, neurotrophin, receptor binding]

NGF signal transduction results in cell survival and maintenance of discrete neuronal populations, including sensory neurons of neural crest origin and sympathetic neurons (Thoenen and Barde, 1980; Levi-Montalcini, 1987). NGF also regulates neural populations in the brain, most notably cholinergic fibers of the basal forebrain (Gnahn et al., 1983; Hefti, 1986; Mobley et al., 1986). The identification of neurotrophic factors BDNF, NT-3, NT-4, and NT-5 has uncovered a family of related polypeptide differentiation factors with similar biological effects (Leibrock et al., 1989; Ernfors et al., 1990; Hohn et al., 1990; Maisonpierre et al., 1990; Rosenthal et al., 1990; Berkemeier et al., 1991; Hallbook et al., 1991). Furthermore, a primary signaling mechanism for these factors has been defined with the discovery that the p140^{prototrk} receptor tyrosine kinase (*trk*) interacts specifically with NGF, resulting in increased tyrosine phosphorylation of cellular substrates in PC12 cells (Kaplan et al., 1991b; Klein et al., 1991a). Other *trk* family members, including *trk*B and *trk*C, have since been found to interact with multiple neurotrophic ligands (Cordon-Cardo et al., 1991; Klein et al., 1991a,b; Lambelle et al., 1991; Soppet et al., 1991; Squinto et al., 1991).

An important question concerning the action of neurotrophins is how the functional receptor complex is encoded and specified. NGF and the other neurotrophin family members are known to interact with a 75,000 Da transmembrane receptor, p75^{NGFR} (Johnson et al., 1986; Radeke et al., 1987; Ernfors et al., 1990; Rodriquez-Tebar et al., 1990; Squinto et al., 1991). NGF binds to this receptor with low affinity ($K_d = 10^{-9}$ M), but this interaction does not initiate any measurable signal transduction in fibroblast cells expressing p75^{NGFR} (Hempstead et al., 1989). Only in NGF-responsive cells are high-affinity ($K_d = 10^{-11}$ M) and low-affinity binding sites displayed (Sutter et al., 1979; Schechter and Bothwell, 1981; Green and Greene, 1986). High-affinity binding is required for functional responses to NGF both in culture and *in vivo* and can be reconstituted in cultured cells that express p140^{prototrk} (Hempstead et al., 1991; Klein et al., 1991a). Whether this binding requires the concomitant expression of p75^{NGFR} has been the subject of debate (Bothwell, 1991; Chao, 1992).

Both receptors are known to be colocalized in sensory, sympathetic, and central cholinergic neurons during NGF-responsive periods (Buck et al., 1987; Hohaman et al., 1992; Schechterson and Bothwell, 1992), and monoclonal antibodies against p75 have been shown to display inhibitory effects upon neurite regeneration (Chandler et al., 1984) and c-fos induction in PC12 cells (Milbrandt, 1986). Previous equilibrium binding studies have indicated that p75^{NGFR} participates in high-affinity NGF binding (Green and Greene, 1986; Hempstead et al., 1989, 1991; Pleasure et al., 1990; Matsushima and Bogenmann, 1991) and that p140^{prototrk} binds to NGF with low affinity (Kaplan et al., 1991a). Other experiments, however, have reached the opposite conclusion, suggesting that the unique expression of p140^{prototrk} defines a high-affinity receptor for NGF (Klein et al., 1991a). The involvement of p75^{NGFR} in NGF signal transduction was recently discounted with results demonstrating that p140^{prototrk} and the other *trk* receptor tyrosine kinase family members could function independently in fibroblast cells without the coexpression of p75^{NGFR} (Cordon-Cardo et al., 1991; Glass et al., 1991). Moreover, use of anti-p75^{NGFR} antibodies and site-directed mutants of NGF has implied that the biological effects of NGF

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may be mediated through the *trk* receptor alone, without binding to p75^{NGFR} (Weskamp and Reichardt, 1991; Ibãnez et al., 1992).

To address the functional role of the p75^{NGFR} in mediating the differentiative effects of NGF action, we have sought to express p75 cDNAs in defective herpes simplex virus (HSV-1) vectors (Geller and Breakefield, 1988; Geller et al., 1991). Here we report the construction and expression of three novel HSV-1 vectors carrying a full-length human p75 receptor cDNA as well as two mutant receptor clones, each lacking an essential domain of the receptor. Our results confirm that the coexpression of both p75^{NGFR} and p140^{prototrk} is required for the functional reconstitution of a high-affinity binding site for NGF. The expression of *trk* with either the ligand binding domain or the cytoplasmic domain of p75, however, was not sufficient to mediate this high-affinity interaction. Moreover, expression studies in primary culture indicate that the HSV-1 vectors employed in this study are capable of faithfully delivering high levels of receptors into neurons, and therefore these HSV-1 vectors will be useful in addressing questions about the physiological roles that p75^{NGFR} plays in nerve cells.

Materials and Methods

Materials. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Mouse NGF (β-dimer) was obtained from Bioproducts for Science (Indianapolis, IN). Anti-2.5S NGF antiserum, a rabbit anti-mouse polyclonal antibody, was purchased from Collaborative Research (Bedford, MA), and anti-p75 antibody, a polyclonal antibody that was generated against the cytoplasmic domain of the low-affinity NGF receptor, was generously supplied by Dr. S. J. Decker (Parke-Davis, Ann Arbor, MI). Antibody ME20.4 against the human NGF receptor (hNGFR; Ross et al., 1984) was harvested from cultured hybridoma cell line supernatants (from American Type Culture Collection), and rhodamine-conjugated goat anti-mouse (Fab')$_2$ antibody was purchased from Jackson Labs (Bar Harbor, ME). Ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) was purchased from Pierce Chemical Co. (Rockford, IL). ^{125}I-NGF was prepared by the lactoperoxidase method as described previously (Green and Greene, 1986).

Cell culture. PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 5% horse serum (GIBCO, Grand Island, NY). NIH-3T3 cells, which overexpress p140^{prototrk} (3T3-*trk* cells) (Kaplan et al., 1991a), were maintained in DMEM with 10% fetal bovine serum and 0.4 mg/ml of G418 (GIBCO).

Construction of vectors and packaging of HSV-1 virus particles. p75 human NGF receptors pSL and px1 were constructed as described previously (Hempstead et al., 1990). For mutant p5A, a 1.5 kilobase (kb) hNGFR cDNA was digested with both *StuI* and *Sau3A* and religated. The resulting 1.0 kb fragment lacking nucleotides 215–682 was recovered. The pSL, px1, and p5A cDNA inserts were then isolated from carrier sequence by *EcoRI* digestion and subcloned into the recipient HSV-1 vector pHSVPrpUC (Geller and Breakefield, 1988). pHSV-hNGFR, pHSV-x1, and pHSV-5A contain the pSL insert (2.3 kb), the px1 insert (2.3 kb), and the p5A insert (1.0 kb), respectively. The orientation of each insert was verified by restriction enzyme analysis. pHSVlac contains the *lacZ* gene and was constructed as previously described (Geller and Breakefield, 1988).

The packaging system for HSV-1 viral particles has been previously reported (Spaete and Frenkel, 1982; Geller, 1988). In brief, 1.5×10^5 CV1 cells were plated on 60 mm dishes and transfected with 0.5 ml of a calcium phosphate precipitate containing 1 μg of pHSV-1 vector DNA and 9 μg of salmon sperm DNA. Four hours later the cells were treated with 15% glycerol (Parker and Stark, 1987) and incubated at 37°C for 24 hr. Cells were then infected with 1.5×10^6 plaque-forming units of HSV-1 strain 17 *ts* K (Davison et al., 1984) in 100 μl of medium. HSV-1 *ts* K is a mutant HSV-1 strain that contains a temperature-sensitive mutation in the IE3 gene, resulting in an immediate-early phenotype and no viral replication at the restrictive temperature of 37°C. After 1 hr at room temperature, an additional 5 ml of medium were added to each infected plate. Cultures were then incubated for 72 hr at 31°C and virus subsequently harvested, passaged, and titered (8×10^5 infectious particles/ml) as described previously (Miller and Hyman, 1978).

Northern blot analysis. Cells were grown to 70% confluence on 100 mm tissue culture dishes, infected with 0.4 ml of viral stock at a multiplicity of infection (MOI) of 0.25, and incubated for 6–24 hr at 37°C, and total mRNA was prepared using the RNASol B method (Chomczynski and Nicoletta, 1987). For Northern blot analysis, mRNA (20 μg/sample) was separated by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde, transferred to nitrocellulose filters, and baked *in vacuo* at 80°C for 4 hr (Sambrook et al., 1989). The presence of mRNA species was confirmed by ethidium bromide visualization. Filters were then prehybridized in 50% formamide, 5× SSC, and 5× Denhardt's solution with 500 μg/ml of denatured salmon sperm DNA at 42°C for at least 4 hr. Hybridization with radiolabeled probe was carried out at 42°C in 50% formamide, 5× SSC, and 1× Denhardt's solution with 100 μg of denatured salmon sperm DNA. Randomly primed restriction fragments were generated to specific activity of 5×10^8 cpm/μg. Filters were washed to 0.4× SSC at 68°C and exposed to x-ray film at −70°C with an intensifying screen.

Immunoblotting. For immunoblot analysis, infected cell cultures were prepared as above. Infected fibroblasts were washed in phosphate-buffered saline (PBS; pH 6.4) and rapidly solubilized in 1 ml of solubilization buffer [10 mM Tris-HCl (pH 7.4), 1% SDS, and 0.1 mM each of phenylmethylsulfonyl fluoride (PMSF)/aprotinin/leupeptin]. Cell lysates were incubated on ice for 15 min and then pelleted at top speed in a microfuge at 4°C for 10 min. Supernatants were assayed for protein concentrations with a protein assay kit from Bio-Rad (Richmond, CA). Proteins (200 μg) were immunoprecipitated with anti-p75 antibody, electrophoresed by SDS-PAGE (8%), and electrophoretically transferred to nitrocellulose. Filters were then incubated with anti-p75 antisera followed by ^{125}I-labeled protein A from Amersham (Arlington Heights, IL). Autoradiography was carried out at −70°C with an intensifying screen.

Affinity cross-linking and immunoprecipitation. 3T3-*trk* cells infected at an MOI of 0.25, and uninfected control cells were harvested for affinity cross-linking. NGF receptors were labeled by affinity cross-linking methods previously described (Green and Greene, 1986; Hempstead et al., 1989). For EDAC cross-linking to ^{125}I-NGF, cells were washed in PBS (pH 6.5) and resuspended to a final concentration of 2×10^6 cells/ml in PBS containing 1 mg/ml each of glucose and bovine serum albumin. ^{125}I-NGF was then added to each cell suspension at a final concentration of 25 ng/ml, in the presence or absence of 5 μM unlabeled NGF for 2 hr on a rotator at 4°C. EDAC was next added to a final concentration of 4 mM at room temperature for 30 min. After cross-linking, cells were centrifuged, washed in PBS-lysine (50 mM), andsolubilized in lysis buffer (10 mM Tris, pH 7.6, 66 mM EDTA, 1% NonidetP40, 0.4% deoxycholate, and 0.1 mM each of PMSF/aprotinin/leupeptin) at 4°C for 20 min. Lysates were immunoprecipitated with anti-2.5S NGF antisera at 4°C for 2 hr and washed extensively in RIPA buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM KCl, 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, and 1 mM EDTA). The samples were then resuspended in sample buffer, boiled for 5 min, and subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel. The dried gels were exposed to autoradiographic film at −70°C with an intensifying screen.

Equilibrium binding analysis. Infections were carried out as described above. Equilibrium binding of ^{125}I-NGF to crude cellular membrane preparations was performed as described previously (Hempstead et al., 1989). All measurements were verified in triplicate, and the data were assessed by Scatchard analysis (Scatchard, 1949). Only binding values above 50% specific binding were used in the final analysis. The LIGAND program was used to analyze the data (Munson and Rodbard, 1980).

Primary neuronal cultures and immunohistochemistry. Timed-pregnant Sprague–Dawley rats [embryonic day 16 (E16)] were purchased from Charles River Labs (Wilmington, MA) and embryos removed under sterile conditions. Brain tissue was isolated and the cerebral cortex was carefully dissected away from hippocampus, basal forebrain, and olfactory bulbs. Meninges were also removed. Cortical cells were then manually dissociated and plated on poly-D-lysine–coated dishes. Cultures were enriched for nerve cells by maintaining cultures in serum-free conditions for at least 5 d as described previously (di Porzio et al., 1980).

For immunohistochemistry, primary cortical cells were infected at an MOI of 0.25 for 8 hr at 37°C. Fresh media was then added and the cells were placed at 37°C for an additional 36 hr prior to fixation. Cells were fixed in buffered 4% paraformaldehyde for 1 hr on ice, washed with

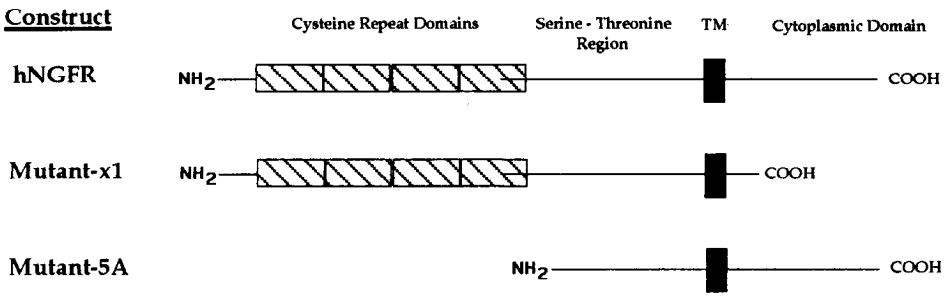

Figure 1. Schematic representation of $p75^{hNGFR}$ and receptor mutants. Constructs were generated as outlined in the Materials and Methods. The *hNGFR* construct represents the full-length cDNA of the human low-affinity NGF receptor, p75. *Mutant-x1* encodes for an intracellular deletion receptor that is truncated 4 amino acids after the transmembrane domain at amino acid position 248. *Mutant-5A* encodes for an extracellular deletion mutation that lacks the NGF binding domain due to a large deletion (Welcher et al., 1991; Yan and Chao, 1991) spanning amino acids 34–191.

PBS, and preincubated in PB buffer (0.1 M PBS, pH 7.4, with 0.1% Triton X-100 and 1 mg/ml BSA) for 1 hr at room temperature. ME20.4 hybridoma supernatants were diluted 1:5 in PB buffer and incubated with cells overnight at 4°C. Cells were thoroughly washed and then incubated in a 1:250 dilution of a rhodamine-conjugated goat anti-mouse secondary antibody (Jackson Labs). Cells were washed extensively and mounted. Control cultures were assayed in the absence of primary antibody.

Results

hNGFR constructs and HSV-1 vectors

The NGF receptor constructs used in this study are shown schematically in Figure 1. The hNGFR construct is a 2.3 kb full-length cDNA of the human NGF receptor that contains the entire coding region of the receptor but lacks most of the 3′-untranslated sequences. The cytoplasmic deletion mutant hNGFR-x1 was generated by the site-directed insertion of a universal Xba-termination linker 4 amino acids beyond the transmembrane domain at amino acid position 248 (Hempstead et al., 1990). The extracellular deletion mutant hNGFR-5A contains a large deletion in the ligand binding domain (Welcher et al., 1991; Yan and Chao, 1991), spanning amino acids 34–191. Each of these receptor constructs was subcloned into the 4.8 kb defective HSV-1 vector (Geller and Breakefield, 1988), which is shown in Figure 2, and then packaged into HSV-1 particles as outlined in the Materials and Methods.

Expression studies in cell culture

To determine if the HSV-1 vectors appropriately express human $p75^{NGFR}$ ($p75^{hNGFR}$) and mutant receptor constructs, 3T3-*trk* cells were infected with 8×10^5 infectious particles/ml of viral stock and analyzed for both mRNA and cell surface protein. These cells were chosen as the recipient cell line because they lack endogenous $p75^{NGFR}$ (Kaplan et al., 1991a; Klein et al., 1991a). Expression of all three receptor constructs in fibroblasts is readily detectable by Northern blot analysis (Fig. 3*A*). In Figure 3*A*, a prominent 2.3 kb message was detected in pHSV-hNGFR and pHSV-x1 lanes (lanes 2 and 3) while in mock-infected 3T3-*trk* cells, no signal was detected (lane 1).

Cells infected with pHSV-5A also expressed a message of about 2.3 kb in size (Fig. 3*A*, lane 4). Though the cDNA insert of this construct was approximately 1.0 kb, the increased size of this primary transcript was likely due to an alternative polyadenylation event. Unlike the pHSV-hNGFR and pHSV-x1 constructs, which contain an endogenous polyadenylation signal in their cDNA insert, the pHSV-5A construct does not. Therefore, pHSV-5A must rely on the SV40 polyadenylation signal

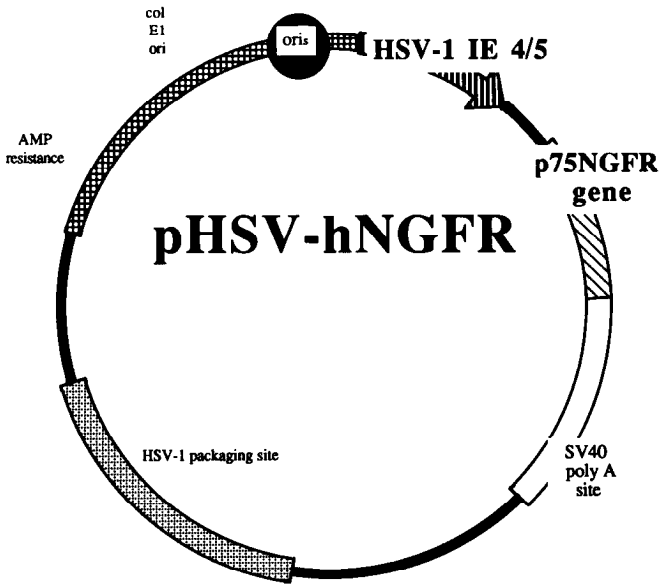

Figure 2. Structure of pHSV-hNGFR. This HSV-1 expression vector is a 4.8 kb plasmid that contains the 2.3 kb hNGFR insert and the following set of genetic elements: (1) sequences from pBR322 that allow for bacterial replication and selection of the plasmid, including the ColE1 origin of DNA replication and the ampicillin-resistance gene; (2) sequences from the HSV-1 genome that allow for packaging of the plasmid into viral particles, including the HSV-1 packaging site and herpes simplex virus DNA replication ori_s (Davison and Wilkie, 1981; McGeoch et al., 1986); and (3) sequences from both HSV-1 and SV40 to compose elements of the transcription unit, including the HSV-1 IE 4/5 promoter (McGeoch et al., 1986) and the SV40 early region polyA site (Hall et al., 1983).

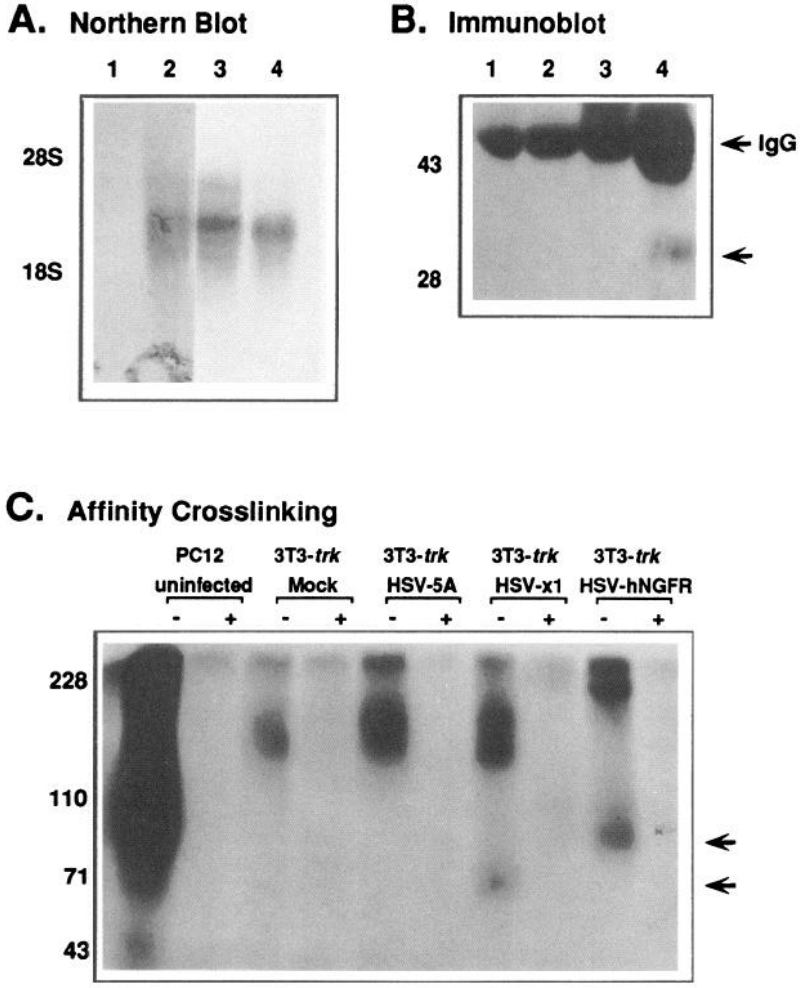

Figure 3. Expression of p75^{hNGFR} and receptor mutants in 3T3-*trk* cells using recombinant HSV-1 vectors. *A*, Northern blot analysis of RNA isolated from 3T3-*trk* cells infected with HSV-1 viral stocks. Cells were infected for 6–24 hr at 37°C, and RNA was isolated 36 hr later as described in Materials and Methods. Northern blots were probed with radiolabeled B9 fragment, a probe specific to p75^{hNGFR} cDNA. Each lane contained 20 μg of total RNA. *Lane 1* is RNA harvested from fibroblasts following mock infection. *Lanes 2–4* contain RNA from fibroblasts infected with 0.4 ml of HSV-hNGFR, HSV-x1, and HSV-5A, respectively. pHSVlac virus, a vector that expresses the *E. coli* β-galactosidase (Geller and Breakefield, 1988), was used for control infection. Positions of 28S and 18S ribosomal markers are indicated. *B*, Immunoblot analysis with anti-p75 antibody of HSV-1–infected 3T3-*trk* cells. Proteins (200 μg) from infected cells were immunoprecipitated and blotted with anti-p75 antibody as described in Materials and Methods. Anti-p75 antibody recognizes the cytoplasmic domain of human p75. *Lane 1* represents mock-infected 3T3-*trk* cells. *Lanes 2–4* are 3T3-*trk* cells infected with HSV-hNGFR, HSV-x1, and HSV-5A, respectively. Molecular mass markers (in kilodaltons) are indicated. An *unlabeled arrow* indicates the position of the HSV-5A protein at about 35 kDa. The broad IgG band (*arrow*) runs at approximately 50 kDa. pHSVlac virus was used for control infection (Geller and Breakefield, 1988). *C*, Affinity cross-linking of NGF receptors in cultured cell lines. NGF receptors were cross-linked to ^{125}I-NGF using EDAC in the presence (+) or absence (−) of excess unlabeled NGF as outlined in Materials and Methods. Samples were loaded as indicated above the lanes. pHSVlac virus was used for control infection (Geller and Breakefield, 1988). Molecular mass markers (in kilodaltons) are indicated on the left. *Arrows* indicate the position of cross-linked p75 at 100 kDa and the cross-linked HSV-x1 protein at approximately 68 kDa.

that is present in the HSV-1 vector about 1.15 kb downstream of the cloning site (Hall et al., 1983).

To verify further that the HSV-1–infected cells appropriately expressed these cDNAs, we assayed both for cell surface protein expression and for ligand binding. Figure 3*B* shows an immunoblot of proteins immunoprecipitated with anti-p75 antibody. The polyclonal anti-p75 antibody was raised against the cytoplasmic domain of p75^{hNGFR} and was used to confirm the expression of the pHSV-5A construct. Cells infected with the HSV-5A virus demonstrated a major 35 kDa protein of predicted size (Fig. 3*B*, lane 4). No signal was detected in this range in mock-infected fibroblasts (Fig. 3*B*, lane 1) or in cells infected with either the full-length receptor HSV-hNGFR or the intracellular deletion mutant HSV-x1 (Fig. 3*B*, lanes 2, 3). The broad IgG band is observed across the top of each lane at approximately 50 kDa.

To detect the hNGFR and mutant-x1 proteins, an affinity cross-linking assay was used (Fig. 3*C*). This cross-linking reaction results in a 90–100 kDa labeled species in PC12 cells, human melanoma cells, and rat brain, and has been shown to represent a complex between labeled NGF and the p75 receptor (Grob et al., 1983; Green and Greene, 1986). Infected 3T3-*trk* fibroblast and PC12 cells were incubated with ^{125}I-NGF in suspension for 2 hr, cross-linked with EDAC, and then immuno-

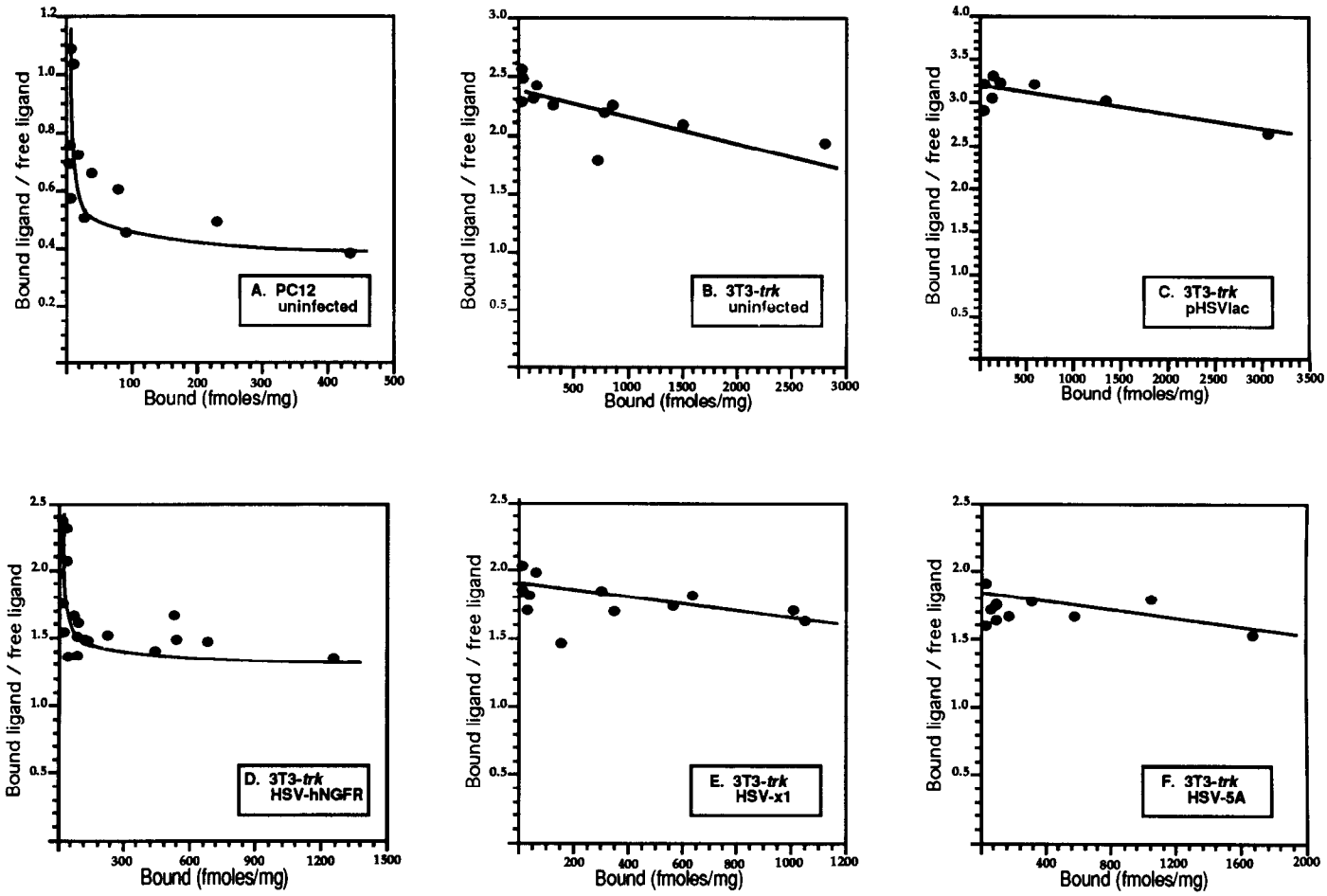

Figure 4. Equilibrium binding and Scatchard plot analysis of NGF receptors in PC12 cells and HSV-1–infected 3T3-*trk* cells. 3T3-*trk* cells were infected with viral stocks for 6–24 hr and crude membrane preparations were prepared 36 hr later as described previously (Hempstead et al., 1989). Binding assays were performed as presented in the Materials and Methods. Binding data were graphed according to Scatchard (1949). *A* is the Scatchard plot of uninfected PC12 cells. *B* is the Scatchard plot of uninfected 3T3-*trk* cells. *C* is the Scatchard plot of mock-infected 3T3-*trk* cells. *D–F* show the binding curves for 3T3-*trk* cells infected with the HSV-hNGFR, HSV-x1, and HSV-5A viruses, respectively. Control infections were performed using pHSVlac (Geller and Breakefield, 1988).

precipitated with anti-NGF antibody (see Materials and Methods). 3T3-*trk* cells infected with HSV-hNGFR viral stock demonstrated the same cross-linked complex as PC12 cells (100 kDa), while cells infected with the mutant construct HSV-x1 show a specific cross-linked species with ^{125}I-NGF that migrated with a slightly faster mobility (68 kDa). Proteins from HSV-5A–infected and mock-infected cells, however, did not cross-link ^{125}I-NGF in the 70–100 kDa range as expected. The specificity of the observed reaction can be seen in adjacent lanes in which an excess of unlabeled NGF (5 μM) is added.

In these same experiments, affinity cross-linking of ^{125}I-NGF to p140^{prototrk} is also detected, producing a species of approximately 160–170 kDa (Fig. 3*C*) in mock, HSV-x1, and HSV-5A lanes. However, it is of interest to note that in 3T3-*trk* cells that have been infected with the full-length receptor HSV-hNGFR, the *trk*–^{125}I-NGF cross-linked complex is no longer observed at 170 kDa. Instead, a new band can be observed, slowly migrating at about 200–240 kDa. This effect was not observed either in mock-infected cells or in cells infected with either of the mutant p75 constructs. A similar phenomenon in several independent *trk*-positive cell lines into which the full-length p75 receptor has been introduced has also been observed (B. L. Hempstead, unpublished observations). The significance of this event is presently unclear and may represent either the downregulation of p140^{prototrk} protein at the cell surface or, more likely, the specific association of the *trk*–^{125}I-NGF cross-linked product with other signaling proteins.

These expression data indicate that the pHSV-hNGFR and the pHSV-x1 constructs are appropriately expressed in infected cells and are capable of binding ^{125}I-NGF. The absence of a specific cross-linked product in cells infected with HSV-5A confirms that this extracellular deletion mutant will not bind NGF even though it is being transcribed and expressed correctly in infected cells (Fig. 3*A,B*).

Equilibrium binding of ^{125}I-NGF

NGF-responsive sensory neurons in culture demonstrate high- and low-affinity ligand binding (Sutter et al., 1979). Furthermore, the presence of high-affinity receptor binding has been shown to be correlated with NGF-responsive periods *in vivo* (Herrup and Shooter, 1975; Godfrey and Shooter, 1986). As a functional assay for the HSV-1–encoded constructs, we set out

Table 1. Summary of Scatchard plot analysis

Cell line	Infection	Temperature	Affinity site 1		Affinity site 2		Site 1: Site 2
			K_d (M)	Sites/mg of protein	K_d (M)	Sites/mg of protein	
PC12	Uninfected	30°C	4.15×10^{-9}	$8.54 \times 10^{+11}$	6.62×10^{-11}	$4.93 \times 10^{+10}$	17:1
3T3-*trk*	Uninfected	30°C	4.23×10^{-9}	$4.53 \times 10^{+12}$	N.A.		
3T3-*trk*	pHSVlac	30°C	5.67×10^{-9}	$6.76 \times 10^{+12}$	N.A.		
3T3-*trk*	HSV-hNGFR	30°C	3.99×10^{-9}	$4.69 \times 10^{+12}$	5.11×10^{-11}	$1.44 \times 10^{+11}$	32:1
3T3-*trk*	HSV-x1	30°C	4.81×10^{-9}	$3.12 \times 10^{+12}$	N.A.		
3T3-*trk*	HSV-5A	30°C	10.46×10^{-9}	$5.53 \times 10^{+12}$	N.A.		

All binding data presented above were analyzed using the LIGAND program (Munson and Rodbard, 1980). N.A. indicates that only one kinetic site was identified. Temperatures refer to the equilibrium binding assay conditions.

to determine if we could reconstitute high-affinity NGF binding in 3T3-*trk* cells, a recipient fibroblast cell line that overexpresses the *trk* proto-oncogene at high levels (>200,000 sites per cell). Since the high-affinity NGF binding complex can be distinguished from low-affinity complex by equilibrium binding, Scatchard analysis was conducted. A membrane binding assay was employed in order to minimize the effects of internalization of NGF. Crude cell membranes were prepared from PC12 cells and HSV-1–infected 3T3-*trk* cells. PC12 cell membranes (Fig. 4*A*) displayed two binding sites for NGF as previously reported (Landreth and Shooter, 1980; Block and Bothwell, 1983). The high-affinity binding site represents only about 5% of the total number of NGF binding sites and demonstrates a K_d of 6.62×10^{-11} M. The low-affinity site has a K_d of 4.15×10^{-9} M.

Multiple binding studies of the 3T3-*trk* recipient cell line demonstrate that this cell line contains predominately low-affinity receptors for NGF. A representative Scatchard plot is shown in Figure 4*B*. A single binding site with a K_d of 4.23×10^{-9} M is observed, consistent with a low-affinity interaction. pHSVlac infections of 3T3-*trk* cells generated a similar binding curve, with a K_d of 5.67×10^{-9} M (Fig. 4*C*). In contrast, when membranes were assayed from 3T3-*trk* cells infected with HSV-hNGFR, a biphasic Scatchard plot was observed, consisting of two discrete binding sites (Fig. 4*D*). Similar to PC12 cells, the reconstituted high-affinity site represents a small percentage of the total number of NGF binding sites and has a K_d of 5.11×10^{-11} M. The low-affinity site in the HSV-hNGFR–infected cells has a K_d of 3.99×10^{-9} M. A summary of these results is shown in Table 1. From these data, we conclude that infection of 3T3-*trk* cells with HSV-hNGFR is capable of creating a new binding site for NGF, and therefore p75^{NGFR} appears to be required in the high-affinity NGF binding complex.

To investigate the nature of the high-affinity binding site more completely, we sought to express and characterize the binding properties of our mutant constructs of p75 in the presence of p140^{prototrk}. In these experiments, 3T3-*trk* cells were infected with either the HSV-x1 or the HSV-5A virus and assayed by Scatchard analysis as above. Representative binding curves are shown in Figure 4, *E* and *F*. In both cases, linear binding curves were generated with a K_d of 4.81×10^{-9} M for HSV-x1 and a K_d of

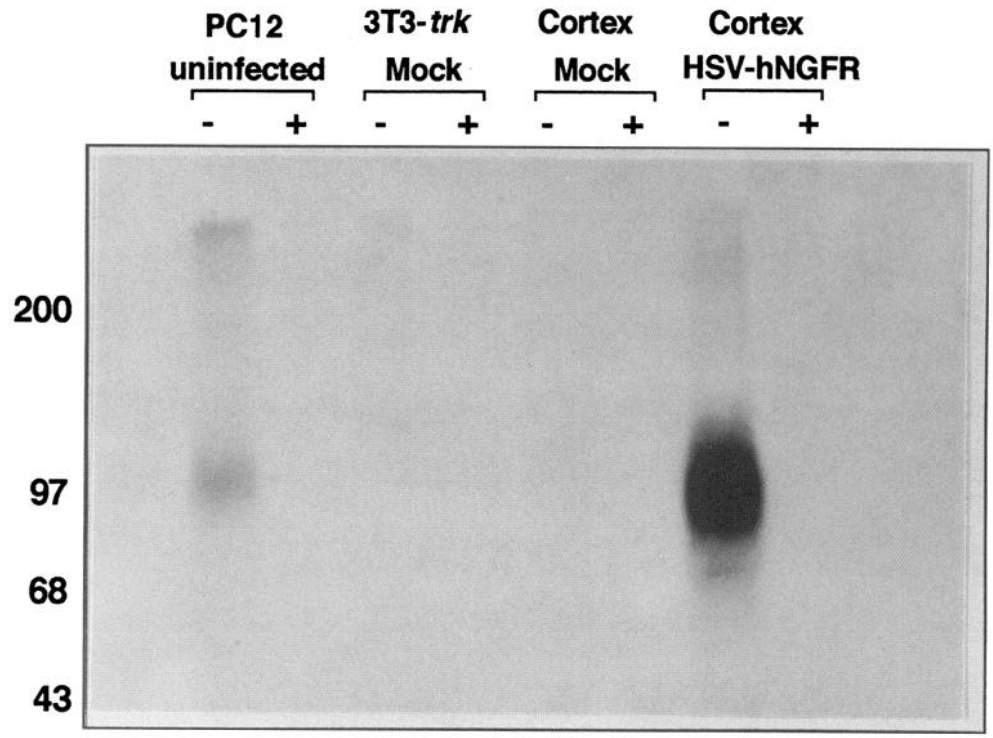

Figure 5. EDAC affinity cross-linking of NGF receptors in primary cortical neurons infected with HSV-hNGFR. Primary cortical cultures were dissected from E16 rat brain and cultured for 5 d in serum-free media as indicated in Materials and Methods. Neuronal cultures were then infected with either pHSVlac virus or HSV-hNGFR virus for 6 hr. Following this incubation period fresh medium was added, and cells were harvested 36 hr later. Uninfected PC12 cells and mock-infected 3T3-*trk* cells were used as controls. NGF receptors were labeled by the cross-linking to ^{125}I-NGF as described in Figure 3*C*. Lanes are loaded as indicated at the top, and all samples contain 2×10^6 cells. Molecular weight standards (in kilodaltons) are shown on the left.

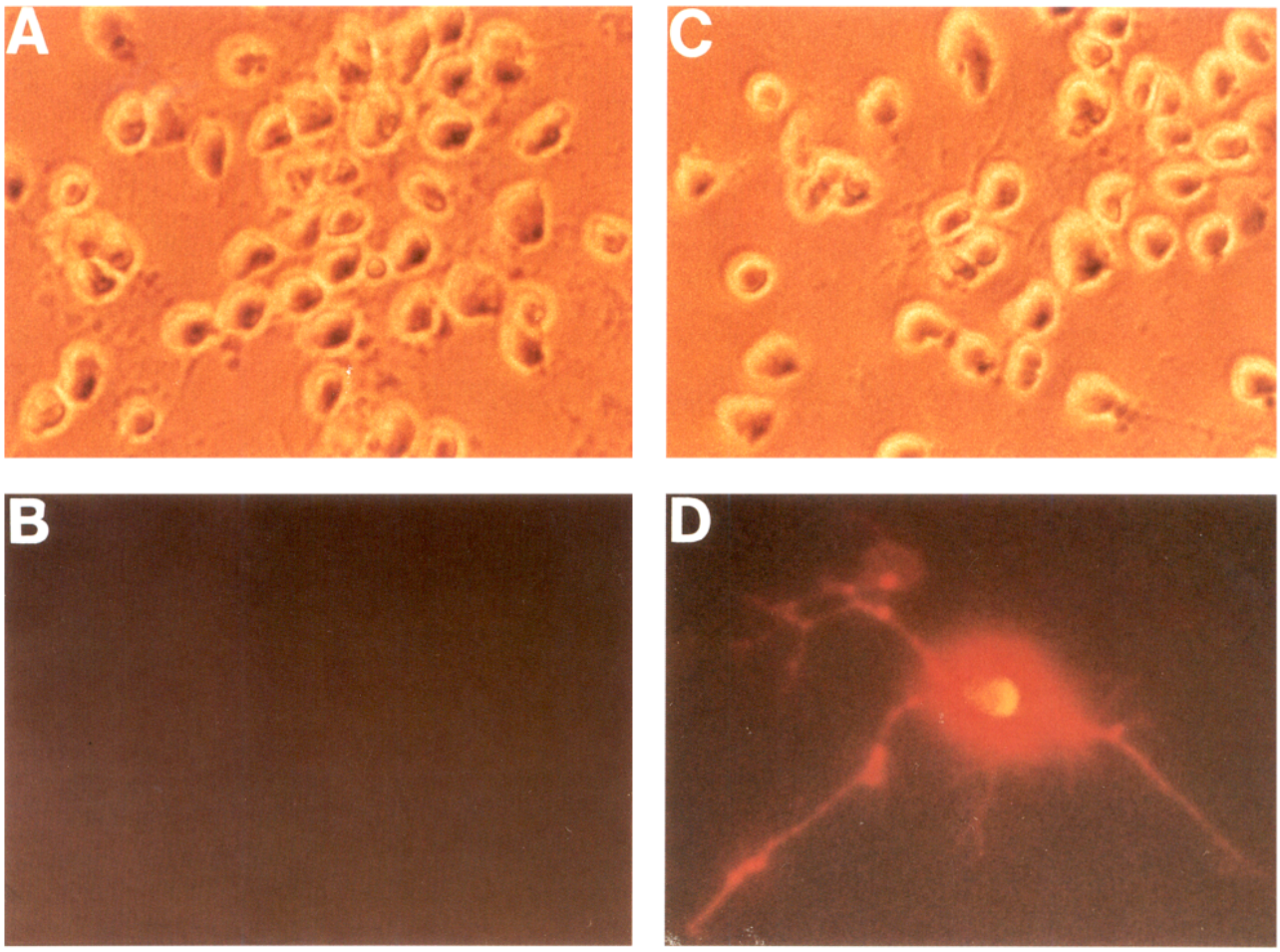

Figure 6. Immunofluorescent localization of hNGFR in primary cortical neurons infected with HSV-hNGFR. Primary cultures of cortical neurons were prepared as described in Figure 5. Following infection, cells were fixed in 4% paraformaldehyde for 1 hr at room temperature, washed extensively with PBS, and incubated overnight at 4°C with ME20.4 antibody. Cells were again thoroughly washed and then stained with a 1:250 dilution of a rhodamine-conjugated goat anti-mouse antibody (Jackson Labs). *D* demonstrates the immunoreactivity of cortical cultures infected with HSV-hNGFR. *C* is the corresponding bright field. *B* demonstrates the immunoreactivity of cultures infected with pHSVlac. *A* is the corresponding bright field. Note that the $p75^{hNGFR}$ is highly expressed both in the nerve cell body as well as in the distal processes.

10.46×10^{-9} M for HSV-5A. This binding profile is quantitatively similar to that of membranes from control cells (Fig. 4*B,C*). Thus, overexpression of either the ligand binding domain or the cytoplasmic domain of p75 in the presence of high levels of $p140^{prototrk}$ gives rise to only low-affinity binding of ^{125}I-NGF, suggesting that neither of these two domains of p75, when independently expressed, is sufficient for the functional reconstitution of the high-affinity NGF binding site.

Expression of hNGFR in primary neuronal cultures

To demonstrate that HSV-1 vectors could be used efficiently to introduce p75 receptors into neurons, primary cortical cultures were employed. Cultures were maintained in serum-free conditions for at least 5 d. These culture conditions have been shown to enrich for nerve cells in the absence of mitotic inhibitors (di Porzio et al., 1980). Neuronal cultures were infected with HSV-hNGFR virus or pHSVlac virus at 37°C. Following infection, cells were either harvested for affinity cross-linking experiments or fixed in 4% paraformaldehyde for immunohistochemistry.

An equal number of PC12 cells and cortical neurons were incubated with ^{125}I-NGF and chemically cross-linked with EDAC as before. In comparison to endogenous levels of p75 in PC12 cells, the cells infected with HSV-hNGFR were readily detected at high levels (Fig. 5). The addition of excess cold NGF in alternate lanes confirmed the specificity of the cross-linked product. Furthermore, in uninfected cultures, cross-linking was not detected, suggesting that NGF receptors were either absent or at very low levels in E16 cortical neurons.

For immunohistochemistry, cultures were fixed in paraformaldehyde, infected with either pHSVlac or HSV-hNGFR virus, and then stained with ME20.4 antibody, a monoclonal antibody specific for the human p75 receptor (Ross et al., 1984). Representative immunofluorescent photomicrographs of these cortical nerve cells are shown in Figure 6. In Figure 6*D*, a brightly stained nerve cell body with its less intensely stained processes centers the field. The corresponding phase-contrast photomicrograph reveals a field of about 20 cells and is shown in Figure 6*C*. About 20–25% of these surrounding cells are expressing lower levels of human receptor that could be detected above background. Figure 6*A* is the phase-contrast field of cortical cells infected with the pHSVlac virus. The corresponding immuno-

fluorescent field is shown in Figure 6*B*. These results indicate that HSV-mediated expression of p75^{NGFR} can be readily extended to primary nerve cell cultures.

Discussion

A central theme that has emerged from the study of NGF is that a limited supply of neurotrophic factor is available for developing neurons during target innervation (Levi-Montalcini, 1987; Barde, 1989; Oppenheim, 1989; Davies, 1991). Competition for target-derived factors therefore depends upon the precise interaction of neurotrophins for specific receptors. For NGF, two receptors have been identified, p75^{NGFR} and the product of the *trk* proto-oncogene, p140^{prototrk}. The colocalization of both of these receptors in the majority of NGF-responsive cells *in vivo* suggests that the specificity of NGF action must be derived from an interaction of NGF with both receptors. Furthermore, the equilibrium binding constants of NGF binding to each of these receptors suggest that the sensitivity with which responsive cells bind NGF may also depend upon an interaction between both of these receptors.

Measurements of ^{125}I-NGF binding to p75^{NGFR} reveal a single low-affinity class of receptors with a $K_d = 10^{-9}$ M (Chao et al., 1986; Radeke et al., 1987; Rodriquez-Tebar et al., 1990). Similar measurements of p140^{prototrk} indicate that the majority of sites display a low-affinity K_d (Hempstead et al., 1991; Kaplan et al., 1991a; Klein et al., 1991a). A small percentage of higher-affinity binding sites have been observed for the *trk* receptor (Klein et al., 1991a). These results have led to several models of high-affinity NGF binding, one including both p75 and the *trk* receptor and another in which p140^{prototrk} acts independently as a high-affinity receptor site for NGF (Bothwell, 1991; Ross, 1991).

In a further attempt to define the requirements for high-affinity NGF binding, we have expressed p75 cDNAs in defective HSV-1 vectors (Geller and Breakefield, 1988). Here we have reported the construction of three novel HSV-1 vectors that encode either p75^{hNGFR} or mutant forms of the receptor. pHSV-hNGFR contains the full-length human p75 cDNA. The two mutant constructs, pHSV-x1 and pHSV-5A, represent large intracellular and extracellular deletions, respectively. Direct infection of cultured fibroblasts with each of these three HSV-1 vectors resulted in the transcription and correct processing of receptor RNAs, such that high levels of surface-bound receptor protein were detected. These high levels of expression for p75^{NGFR} are required for high-affinity binding.

The binding properties of each virally encoded construct were then assessed by equilibrium binding and Scatchard analysis following direct infection into 3T3-*trk* cells, a fibroblast cell line that stably overexpress the *trk* proto-oncogene (>200,000 sites per cell). HSV-hNGFR–infected cells revealed a distinctive two site Scatchard plot, consistent with both high- affinity ($K_d = 5.11 \times 10^{-11}$ M) and low-affinity ($K_d = 3.99 \times 10^{-9}$ M) NGF binding. This binding curve parallels that of PC12 cells and sensory neurons, which are known to possess both classes of receptors (Schecter and Bothwell, 1981; Green and Greene, 1986). The total number of high-affinity sites observed represents only a small proportion of the total number of NGF binding sites. However, from our studies it is difficult to assess the absolute number of each of these two NGF binding sites on a per cell basis because not every cell was infected with vector. At the MOI used in these experiments, 20–25% of the cells were expressing the transgene as detected by immunofluorescence. Therefore, the percentage of reconstituted high-affinity binding sites per cell may be significantly underestimated. For this reason, the relative number of high- and low-affinity sites for each cell line was reported as sites per milligram of protein (Table 1).

Scatchard analysis of 3T3-*trk* cells infected with either HSV-x1 or HSV-5A has served to characterize further the nature of the high-affinity NGF binding site. When either of these two constructs were expressed to high levels in the presence of p140^{prototrk}, only low-affinity NGF binding was observed, suggesting that an intact p75 receptor is required to form the high-affinity complex. We can also conclude from these experiments that p75^{NGFR} is not merely functioning as a binding protein for NGF. If p75 served to present NGF to the high-affinity receptor, then the HSV-x1 construct, which binds NGF, would have been predicted to reconstitute a high-affinity binding site. The extracellular domain of p75 alone is not sufficient to mediate this high-affinity interaction. The lack of high-affinity binding in HSV-x1–infected cells was not due to insufficient expression of this construct at the cell surface, since a 10-fold increase in the MOI did not change the K_d (data not shown). Moreover, the uninfected parental cell line, 3T3-*trk* fibroblasts, as well as control cells infected with pHSVlac virus displayed only a single low-affinity ($K_d = 10^{-9}$ M) binding site.

From these observations we can propose several hypothetical models to describe the high-affinity receptor complex. Four of these models are shown in Figure 7. Model A demonstrates a p75^{NGFR}–p140^{prototrk} heterodimer. This model assumes that p140^{prototrk} signals as a monomer. Models B–D assume that p140^{prototrk} is activated, like other receptor tyrosine kinases (Ullrich and Schlessinger, 1990), as a homodimer interacting with p75^{NGFR}. Whether this interaction requires a p75^{NGFR} monomer (model B), dimer (model C), or multimer (model D) is presently unclear. In PC12 cells, p75^{NGFR} appears to be in excess of the *trk* receptor. Furthermore, membrane fusion data has suggested that the stoichiometric ratio of p75^{NGFR} to p140^{prototrk} for the reconstitution of high-affinity binding is about 10:1 (Hempstead et al., 1991). A *trk* dimer–p75 multimer (model D) may be consistent with these observations.

An important next step toward the understanding of the physiological role of p75^{NGFR} will be to assess the function of this receptor in neurons, rather than in heterologous cultured cell lines. HSV-1 vectors offer an effective method to introduce cloned genes in nerve cells (Geller, 1988; Geller and Breakefield, 1988; Chiocca et al., 1990; Geller et al., 1991; Federoff et al., 1992). In fact, recombinant HSV-1 vectors provide the only method for direct gene transfer and stable gene expression in postmitotic cells. Here, we have also demonstrated that the HSV-1 vectors employed in this study can faithfully deliver high levels of NGF receptors into neurons in primary culture with minimal cytotoxicity.

Infection of cortical neurons in culture with HSV-hNGFR (MOI = 0.25) resulted in high levels of receptor expression as detected by both affinity cross-linking and immunofluorescence. In mock-infected cortical neurons, p75^{NGFR} was not detectable, suggesting that endogenous receptor was either expressed at very low levels or not present at this developmental stage in rat cortex. Previous work in feline cortex has demonstrated a transient immunohistochemical staining of p75 in subplate neurons during brain development (Allendoerfer et al., 1990). However, Yan and Johnson (1988) failed to detect p75 expression in the developing rat cortex. Moreover, if endogenous receptor were

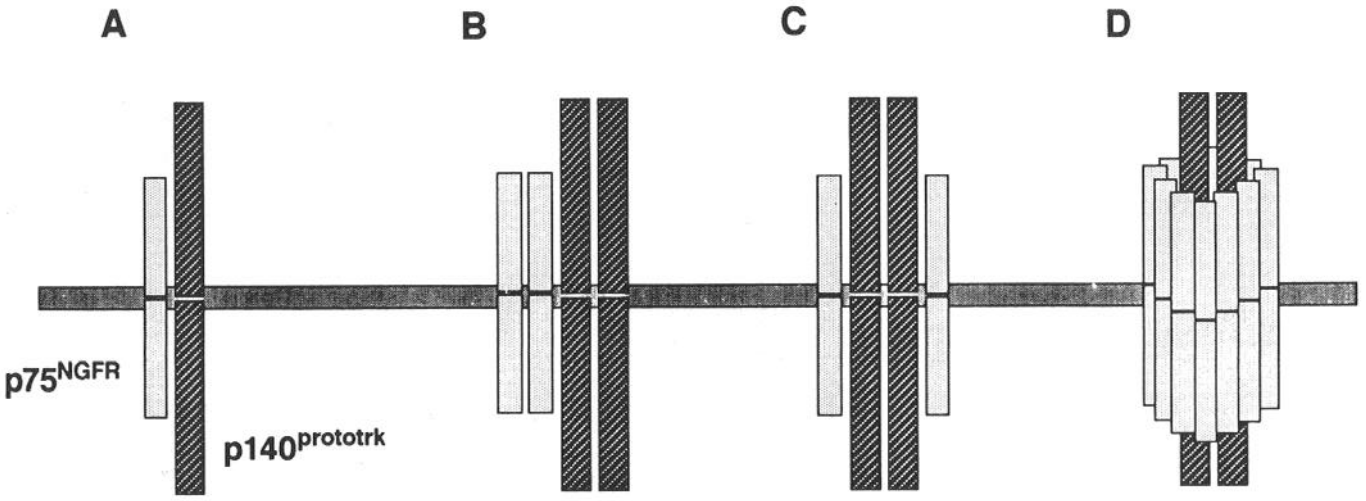

Figure 7. Hypothetical models of the high-affinity NGF receptor complex. Reconstitution of high-affinity NGF binding following infection of 3T3-*trk* cells with HSV-hNGFR suggests several possible models. *Model A* represents a p75–p140 heterodimer. *Models B–D* depict a *trk* homodimeric signaling receptor, interacting with either a p75 monomer (*model B*), dimer (*model C*), or multimer (*model D*). See Discussion.

being expressed at significant levels in these embryonic cells, it would have been detected by affinity cross-linking, an assay that does not discriminate the human from the rat receptor. From these data it would seem that E16 cortex presents a suitable recipient nerve cell population for studies of $p75^{NGFR}$ function in primary culture and *in vivo*.

In summary, we have confirmed by equilibrium binding that $p140^{prototrk}$ binds to NGF with a single, low affinity in uninfected 3T3-*trk* cells; that $p75^{NGFR}$ participates with $p140^{prototrk}$ in the formation of the high-affinity NGF binding complex, following direct infection of 3T3-*trk* cells with HSV-hNGFR; and that neither the extracellular ligand binding domain nor the cytoplasmic region of the p75 receptor when coexpressed with $p140^{prototrk}$ in fibroblasts is sufficient to mediate a high-affinity interaction. We have also shown in this study that our HSV-1 vectors can rapidly deliver high levels of functional p75 receptor in both cultured cell lines and in primary neurons, and further, we have identified E16 cortical neurons as a suitable, p75-negative cell population for future investigations of the physiological significance of high-affinity NGF receptors.

References

Allendoerfer KL, Shelton DL, Shooter EM, Shatz CJ (1990) Nerve growth factor receptor immunoreactivity is transiently associated with the subplate neurons of the mammalian cerebral cortex. Proc Natl Acad Sci USA 87:187–190.

Barde Y-A (1989) Trophic factors and neuronal survival. Neuron 2:1525–1534.

Berkemeier LR, Winslow JW, Kaplan DR, Nikolics K, Goeddel DV, Rosenthal A (1991) Neurotrophin-5: a novel neurotrophic factor that activates *trk* and *trk*B. Neuron 7:857–866.

Block T, Bothwell M (1983) The nerve growth factor receptor on PC12 cells: interconversion between two forms with different binding properties. J Neurochem 40:1645–1663.

Bothwell M (1991) Keeping track of neurotrophin receptors. Cell 65: 915–918.

Buck CR, Martinez HJ, Black IB, Chao MV (1987) Developmentally regulated expression of the nerve growth factor receptor gene in the periphery and the brain. Proc Natl Acad Sci USA 84:3060–3063.

Chandler CE, Parsons LM, Hosang M, Shooter EM (1984) A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. J Biol Chem 259:6882–6889.

Chao MV (1992) Neurotrophin receptors: a window into neuronal differentiation. Neuron 9:583–593.

Chao MV, Bothwell MA, Ross AH, Koprowski H, Lanahan AA, Buck CR, Sehgal A (1986) Gene transfer and molecular cloning of the human NGF receptor. Science 232:518–521.

Chiocca EA, Choi BB, Cai W, DeLuca NA, Schaffer PA, DiFiglia M, Breakefield XO, Martuza RL (1990) Transfer and expression of the *lacZ* gene in rat brain neurons mediated by herpes simplex virus mutants. New Biol 2:739–746.

Chomczynski P, Nicoletta S (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Cordon-Cardo C, Tapley P, Jing S, Nanduri V, O'Rourke E, Lambelle F, Kovary K, Klein R, Jones KR, Reichardt LF, Barbacid M (1991) The *trk* protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. Cell 66:173–183.

Davies AM (1991) Nerve growth factor synthesis and nerve growth factor receptor expression in neural development. Int Rev Cytol 128: 109–138.

Davison AJ, Wilkie NM (1981) Nucleotide sequences of the joint between L and S segments of herpes simplex virus types 1 and 2. J Gen Virol 55:315–331.

Davison MJ, Preston VG, McGeoch DJ (1984) Determination of the sequence alteration in the DNA of the herpes simplex virus type 1 temperature-sensitive mutant *ts* K. J Gen Virol 31:360–369.

di Porzio U, Daquet M-C, Glowinski J, Prochiantz A (1980) Effect of striatal cells on *in vitro* maturation of mesencephalic dopaminergic neurons grown in serum-free conditions. Nature 288:370–373.

Ernfors P, Ibãnez CF, Ebendal T, Olson L, Persson H (1990) Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. Proc Natl Acad Sci USA 87:5454–5458.

Federoff HJ, Geschwind MD, Geller AI, Kessler JA (1992) Expression of nerve growth factor *in vivo* from a defective herpes simplex virus 1 vector prevents effects of axotomy on sympathetic ganglia. Proc Natl Acad Sci USA 89:1636–1640.

Geller AI (1988) A new method to propagate defective HSV-1 vectors. Nucleic Acids Res 16:5690.

Geller AI, Breakefield XO (1988) A defective HSV-1 vector expresses *Escherichia coli* β-galactosidase in cultured peripheral neurons. Science 241:1667–1669.

Geller AI, During MJ, Neve RL (1991) Molecular analysis of neuronal physiology by gene transfer into neurons with herpes simplex virus vectors. Trends Neurosci 14:428–432.

Glass DJ, Nye SH, Hantzopoulos P, Macchi MJ, Squinto SP, Goldfarb M, Yancopoulos GD (1991) *trk*B mediates BDNF/NT-3 dependent survival and proliferation in fibroblasts lacking the low affinity NGF receptor. Cell 66:405–413.

Gnahn H, Hefti F, Heumann R, Scwab ME, Thoenen H (1983) NGF-mediated increase in choline acetyltransferase (Chat) in neonatal rat forebrain: evidence for a physiological role of NGF in the brain? Brain Res 285:45–52.

Godfrey EW, Shooter EM (1986) Nerve growth factor receptors on chick embryo sympathetic ganglion cells: binding characteristics and development. J Neurosci 6:2543–2550.

Green SH, Greene LA (1986) A single M_r approximately 103,000 ^{125}I-β-nerve growth factor-affinity labeled species represents both the low and the high affinity forms of the nerve growth factor receptor. J Biol Chem 261:15316–15326.

Grob P, Berlot CH, Bothwell MA (1983) Affinity labeling and partial purification of nerve growth factor receptors from rat pheochromocytoma and human melanoma cells. Proc Natl Acad Sci USA 80: 6819–6823.

Hall CV, Jacob GM, Ringold F, Lee J (1983) Expression and regulation of *Escherichia coli lacZ* gene fusions in mammalian cells. Mol Appl Genet 2:101–109.

Hallbook F, Ibãnez C, Persson H (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in the *Xenopus* ovary. Neuron 6:845–858.

Hefti F (1986) Nerve growth factor (NGF) promotes survival of septal cholinergic neurons after fimbrial transection. J Neurosci 6:2155–2162.

Hempstead BL, Schleifer LS, Chao MV (1989) Expression of functional nerve growth factor receptors after gene transfer. Science 243: 373–375.

Hempstead BL, Patil N, Thiel B, Chao MV (1990) Deletion of cytoplasmic sequences of the nerve growth factor receptor leads to loss of high affinity ligand binding. J Biol Chem 17:9595–9598.

Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High-affinity NGF binding requires coexpression of the *trk* proto-oncogene and the low-affinity NGF receptor. Nature 350:678–683.

Herrup K, Shooter EM (1975) Properties of the beta-nerve growth factor receptor in development. J Cell Biol 67:118–125.

Hohn A, Leibrock J, Bailey K, Barde Y-A (1990) Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic family. Nature 344:339–341.

Holtzman DM, Li Y, Parada LF, Kinsman S, Chen C-K, Valletta JS, Zhou J, Long JB, Mobley WC (1992) p140^{trk} mRNA marks NGF-responsive forebrain neurons: evidence that *trk* gene expression is induced by NGF. Neuron 9:465–478.

Ibãnez CF, Ebendal T, Barbany G, Murray RJ, Blundell TL, Persson H (1992) Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the trk gene product. Cell 69:329–341.

Johnson D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao MV (1986) Expression and structure of the human NGF receptor. Cell 47:545–554.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991a) The *trk* proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252:554–557.

Kaplan DR, Martin-Zanca D, Parada LF (1991b) Tyrosine phosphorylation and tyrosine kinase activity of *trk* proto-oncogene product induced by NGF. Nature 350:158–160.

Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991a) The *trk* proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189–197.

Klein R, Nanduri V, Jing S, Lambelle F, Tapley P, Bryant S, Cordon-Cardo C, Jones KR, Reichardt LF, Barbacid M (1991b) The *trk*B tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell 66:395–403.

Lambelle F, Klein R, Barbacid M (1991) *trk*C, a new member of the *trk* family of tyrosine kinases, is a receptor for neurotrophin-3. Cell 66:967–979.

Landreth GE, Shooter EM (1980) Nerve growth factor receptors on PC12 cells: ligand-induced conversion from low- to high-affinity states. Proc Natl Acad Sci USA 77:4751–4755.

Leibrock J, Lottspeich AH, Hofer M, Hengerer B, Masiakowski P, Theonen H, Barde Y-A (1989) Molecular cloning and expression of brain-derived neurotrophic factor. Nature 341:149–152.

Levi-Montalcini R (1987) The nerve growth factor 35 years later. Science 237:1154–1162.

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247:1446–1451.

Matsushima H, Bogenmann E (1991) Terminal differentiation in neuroblastoma cells transfected with the NGF receptor gene when treated with NGF. Prog Clin Biol Res 366:227–233.

McGeoch DJ, Dolan A, Donald S, Brauer DHK (1986) Complete DNA sequence of the short repeat region in the genome of herpes simplex type 1. Nucleic Acids Res 14:1727–1745.

Milbrandt J (1986) Nerve growth factor rapidly induces c-fos mRNA in PC12 rat pheochromocytoma cells. Proc Natl Acad Sci USA 83: 4789–4793.

Miller RH, Hyman RW (1978) Palindrome and palindrome-like sequences of herpes simplex virus DNA. Virology 87:34–41.

Mobley WC, Ruttkowski JL, Tennekoon GI, Gemski J, Buchanan K, Johnston MV (1986) Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. Mol Brain Res 1:53–62.

Munson P, Rodbard D (1980) LIGAND: a versatile computerized approach for characterization of ligand binding systems. Anal Biochem 107:220–239.

Oppenheim RW (1989) The neurotrophic theory and naturally occurring motoneuron death. Trends Neurosci 12:252–255.

Parker BA, Stark GR (1987) Regulation of simian virus 40 transcription: analysis of the RNA species present early in infections by virus or viral DNA. J Virol 31:360–369.

Pleasure S, Reddy UR, Venkatakrishnan G, Roy AK, Chen J, Ross AH, Trojanowski JQ, Pleasure DE, Lee V (1990) Introduction of nerve growth factor (NGF) receptors into a medulloblastoma cell line results in expression of high- and low-affinity NGF receptors but not NGF-mediated differentiation. Proc Natl Acad Sci USA 87:8496–8500.

Radeke MJ, Misko TP, Hsu C, Herzenberg LA, Shooter EM (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature 325:593–597.

Rodriquez-Tebar A, Dechant G, Barde Y-A (1990) Binding of brain derived neurotrophic factor to the nerve growth factor receptor. Neuron 4:487–492.

Rosenthal A, Goeddel DV, Nguyen T, Lewis M, Shih A, Laramee GR, Nikolics K, Winslow JW (1990) Primary structure and biological activity of a novel human neurotrophic factor. Neuron 4:767–773.

Ross AH (1991) Identification of tyrosine kinase *Trk* as a nerve growth factor receptor. Cell Regul 2:685–690.

Ross AH, Grob P, Bothwell M, Elder DE, Ernst CS, Marano N, Ghrist BFD, Slemp CC, Herlyn M, Atkinson B, Koprowski H (1984) Characterization of nerve growth factor receptor in neural crest tumors using monoclonal antibodies. Proc Natl Acad Sci USA 81:6681–6685.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Scatchard G (1949) The attraction of proteins for small molecules and ions. Ann NY Acad Sci 52:660.

Schechter AL, Bothwell MA (1981) Nerve growth factor receptors on PC12 cells: evidence for two receptor classes with different cytoskeletal associations. Cell 24:867–874.

Schechterson LC, Bothwell MA (1992) Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron 9:867–874.

Soppet D, Escandon E, Maragos J, Middlemas DS, Reid SW, Blair J, Burton LE, Stanton BS, Kaplan DR, Hunter T, Nikolics K (1991) The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the *trk*B tyrosine kinase receptor. Cell 65:895–903.

Spaete RR, Frenkel N (1982) The herpes simplex virus amplicon: a new eukaryotic defective-virus cloning-amplifying vector. Cell 30: 295–304.

Squinto SP, Stitt TN, Aldrich TH, Davis S, Bianco SM, Radziewski C, Glass DJ, Masiakowski P, Furth ME, Valenzuala DM, DiStephano PS, Yancopoulos GD (1991) *trk*B encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not for nerve growth factor. Cell 65:885–893.

Sutter A, Riopelle RJ, Harris-Warrick RM, Shooter EM (1979) Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chicken embryo sensory ganglia cells. J Biol Chem 254:5972–5982.

Thoenen H, Barde Y-A (1980) Physiology of nerve growth factor. Physiol Rev 60:1284–1334.

Ullrich A, Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. Cell 61:203–212.

Welcher AA, Bitler CM, Radeke MJ, Shooter EM (1991) Nerve growth factor binding domain of the nerve growth factor receptor. Proc Natl Acad Sci USA 88:159–163.

Weskamp G, Reichardt LF (1991) Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. Neuron 6:649–663.

Yan H, Chao MV (1991) Disruption of cysteine-rich repeats of the p75 nerve growth factor receptor leads to loss of ligand binding. J Biol Chem 266:12099–12104.

Yan Q, Johnson EM (1988) An immunohistochemical study of the nerve growth factor receptor in developing rats. J Neurosci 8:3481–3498.