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TrkA Cross-linking Mimics Neuronal Responses to Nerve Growth Factor

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TrkA, a tyrosine kinase receptor, is an essential component of the nerve growth factor (NGF) response pathway. The binding of NGF to the receptor induces receptor autophosphorylation and activation of intracellular signaling pathways, resulting in diverse biological effects. We prepared polyclonal antibodies against the entire extracellular domain of rat trkA produced using a baculovirus expression system. These antibodies specifically recognize rat trkA on antigen blots and in immunoprecipitations. Both IgG and Fab fragments block binding of NGF to trkA expressed by the PC12 cell line. In NGF binding studies using anti-trkA and anti-low-affinity NGF receptor (LNGFR) immunoglobulin (Ig) G, essentially all binding of NGF can be inhibited. The results imply that ≥97% of the NGF binding sites on PC12 cells are accounted for by trkA and the LNGFR. The binding data also argue that all low-affinity NGF binding sites on PC12 cells reflect interactions with the LNGFR, while all high-affinity sites are trkA dependent. A fraction of the high-affinity (or slow) binding sites seem to require both trkA and the LNGFR. Although the monovalent anti-trkA Fab fragments inhibited the biological effects of NGF, such as induction of tyrosine phosphorylation, and survival and neurite outgrowth of sympathetic neurons, the IgG preparation was not effective as an inhibitor. Instead, the IgG fraction by itself was almost as effective as NGF at stimulating receptor activation, cell survival, and neurite outgrowth. Thus, it appears oligomerization of trkA by antibody-induced cross-linking is sufficient to produce the known cellular effects of NGF.

INTRODUCTION

The neurotrophins are a family of small, homodimeric proteins that promote effects on distinct but partially overlapping sets of neurons during development of the nervous system (reviewed in Levi-Montalcini, 1987; Barde, 1989; Eide *et al.*, 1993; Korsching, 1993). Nerve growth factor (NGF), the best characterized member of the neurotrophin family, was originally purified on the basis of its ability to promote survival and process outgrowth of embryonic chick sensory neurons. It is now clear that NGF functions as a target-derived molecule that aids in determining the level of innervation during development by regulating the survival and differentiation of the innervating neuronal population. Apart from its effects on cell death, NGF has been found to influence other facets of neuronal development, for example, regulation of axon branching and of gene expression (Barde, 1989).

The important biological effects of the neurotrophins have stimulated interest in their receptors and signal transduction mechanisms. Most approaches to this problem have focused on the pheochromocytoma cell line PC12, which is transformed into a sympathetic neuron-like cell when exposed to NGF (Greene and Tischler, 1976). Early NGF binding studies using either

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Abbreviations used: CMF-PBS, calcium- and magnesium-free phosphate-buffered saline; HRP, horseradish peroxidase; LNGFR, low-affinity nerve growth factor receptor; NGF, nerve growth factor; REX, anti-rat LNGFR antibody; RTA, anti-rat trkA antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

PC12 cells or sensory neurons indicated that NGF could bind to at least two different sites, a low-affinity or fast-dissociating binding site and a high-affinity or slow-dissociating binding site (Sutter *et al.*, 1979; Schechter and Bothwell, 1981). Subsequently the first identified receptor for NGF, known as the LNGFR, or low-affinity nerve growth factor receptor, was cloned from rat and human (Johnson *et al.*, 1986; Radeke *et al.*, 1987). It is a transmembrane glycoprotein of 75 000 relative molecular weight and is expressed in many neuronal and nonneuronal cell types (Wyatt *et al.*, 1990; Wheeler and Bothwell, 1992). However, LNGFR was unable to bind NGF with a high affinity in transfected fibroblastic cell lines (Radeke *et al.*, 1987), and cross-linking studies (Hosang and Shooter, 1985), biochemical characterization (Meakin and Shooter, 1991; Radeke and Feinstein, 1991), and binding and culture studies with the anti-LNGFR polyclonal antibody REX (Weskamp and Reichardt, 1991) implied that another receptor that could bind NGF was expressed by PC12 cells. That receptor has since been identified as the receptor tyrosine kinase $p140^{trk}$ or trkA (Kaplan *et al.*, 1991a; Klein *et al.*, 1991).

TrkA is expressed in sensory and sympathetic neurons in the periphery, and basal forebrain neurons in the central nervous system, all cell types that show responses to NGF (Martin-Zanca *et al.*, 1990; Holtzman *et al.*, 1992; Schecterson and Bothwell, 1992). Biochemical studies have demonstrated that trkA becomes autophosphorylated in response to NGF (Kaplan *et al.*, 1991b; Klein *et al.*, 1991; Jing *et al.*, 1992) and subsequently activates several signal transduction pathways (Vetter *et al.*, 1991; Loeb *et al.*, 1992; Obermeier *et al.*, 1993). Two closely related receptors have been isolated, called trkB and trkC, which can respond to other members of the neurotrophin family (Klein *et al.*, 1989; Martin-Zanca *et al.*, 1989; Lamballe *et al.*, 1991; Middlemas *et al.*, 1991). However, although the importance of trkA in generating a response to NGF is now better understood, the function of the LNGFR in this process has continued to be debated.

Given that trkA holds a central position in the NGF signal transduction mechanism, a number of models for the role of LNGFR have been put forward (Bothwell, 1991; Meakin and Shooter, 1992). For example, it has been proposed that LNGFR and trkA function together in a heterocomplex for NGF binding and possibly signal transduction (Berg *et al.*, 1991; Hempstead *et al.*, 1991). An alternate view holds that LNGFR might function in a more peripheral way to aid NGF signaling, for example, to modify the ligand specificity of trkA (Benedetti *et al.*, 1993) or perhaps to serve as a loading mechanism to increase the NGF association rate for trkA (Jing *et al.*, 1992; Ibanez *et al.*, 1993). On the basis of the homology that LNGFR has to CD40, the Fas antigen, and the tumor necrosis factor receptor, proteins that can function in regulation of cell death (Itoh *et al.*, 1991), one group has proposed a role for LNGFR in regulating cell death independently of trkA (Rabizadeh *et al.*, 1993). They hypothesize that when LNGFR is bound by ligand (in this case, NGF), it is in an inactive state, but when it is unbound, it can activate certain apoptotic cell death pathways.

An approach that has been utilized to test the function of LNGFR is that of blocking polyclonal antisera. Such an antibody prepared against the entire extracellular domain of LNGFR (Weskamp and Reichardt, 1991) was able to block NGF binding completely to LNGFR expressed in transfected fibroblasts. Its effects were more complex on PC12 cells, however. Although it completely eliminated low-affinity binding of NGF, it also inhibited approximately one-half of the high-affinity binding sites as well. The anti-LNGFR antibody was unable to block biological responses to NGF in either PC12 cells or rat sensory neurons in culture (Weskamp and Reichardt, 1991).

We have extended this antibody approach to include the study of the trkA receptor and its relationship to the LNGFR. Here we describe the production of a polyclonal antibody to the rat trkA receptor and its effects on cellular responses to NGF.

MATERIALS AND METHODS

Antibodies and Neurotrophins

Two rabbit polyclonal antisera recognizing rat trkA were generated by using as antigen synthetic peptides derived from the rat trkA amino acid sequence coupled to keyhole limpet hemagglutinin via m-maleimidobenzoylsulfosuccinimide ester (Pierce Chemical Co., Rockford, IL). One of the antisera (rtrkA.EX2) was raised against the peptide CSVLNETSFIFTQFLESALTNETMRH (amino terminal cysteine + trkA amino acids 322–346) and recognized the extracellular domain of trkA by immunoblot. The other antibody (called rtrkA.cyt) is directed against a peptide corresponding to the carboxy-terminal end of the rat trkA receptor (CARLQALAQAPPSYLDVLG; amino terminal cysteine + trkA amino acids 782–799) and was subsequently affinity purified on a column composed of the same peptide coupled to thiopropyl-Sepharose CL-6B (Pharmacia LKB Biotechnology, Piscataway, NJ). Generation of the third trkA antiserum, denoted RTA, is outlined below. Nonimmune rabbit immunoglobulin (Ig) G used as control was obtained from Organon Teknika Corp. (Malvern, PA). The anti-phosphotyrosine monoclonal antibody (4G10) was purchased from Upstate Biologicals Inc. (Lake Placid, NY). Alkaline phosphatase–conjugated goat anti-mouse antibody was obtained from Promega Corp. (Madison, WI), HRP-coupled streptavidin and rabbit anti-mouse antibody were from Zymed Laboratories, Inc. (South San Francisco, CA) and Organon Teknika Corp., respectively. Fab fragments were prepared from nonimmune rabbit IgG or the RTA IgG using papain-agarose (Sigma Chemical Co., St. Louis, MO) by standard methods (Harlow and Lane, 1988). NGF was purified from male mouse submaxillary gland following the procedure of (Mobley *et al.*, 1976) or was the gift of W. Mobley. ^{125}I-NGF was purchased from Amersham Corp. (Arlington Heights, IL).

cDNA Cloning

A PC12 cDNA library was constructed in the plasmid vector CDM8 using nonpalindromic adaptors (Invitrogen Corp., San Diego, CA) essentially as described (Aruffo and Seed, 1987; Seed, 1987). A probe for the rat trkA cDNA was generated from cDNA derived from the human cell line K562 (Martin-Zanca *et al.*, 1989) using reverse tran-

scriptase coupled with polymerase chain reaction and the primers 5' GGC CGA ATT CGC CCG GCG CAG AGA ACC TGA CTG AGC and 5' GGC CGA ATT CAT GTG CTG TTA GTG TCA GGG ATG GGG, which yields a 1027-bp fragment (coding for amino acids 63–397) derived from the region of the transcript encoding the extracellular domain. Bacterial colonies harboring rat trkA–containing plasmids were isolated through the use of colony hybridization (Sambrook *et al.*, 1989). cDNA inserts were recloned into Bluescript vectors and sequenced using the dideoxynucleotide-termination method (Sequenase; United States Biochemical Co., Cleveland, OH). The sequence obtained agrees with that published previously (Meakin *et al.*, 1992), with the exception of a slightly shorter 5' untranslated region.

Construction of a Baculovirus Strain Expressing a Rat trkA Truncation

Polymerase chain reaction (PCR) was used to generate a version of the rat trkA cDNA that could direct expression of a truncated form of the receptor. The primers used were: 5' CCG AAT TCC ATG GCG CGA GGC CAG CGG CAC GGG CAG CTG G 3' (5' end of cassette) and 5' CCG AAT TCC ATG GCT ATT ATT CGT CCT TCT TCT CCA CTG GGT CTC 3' (3' end of cassette). The resulting DNA construct was flanked by *Eco*RI and *Nco* I restriction sites and contained the DNA sequence between the presumptive start methionine codon and the glutamic acid codon before the transmembrane domain (therefore encoding amino acids 1–416; called rtrkA.trunc). The construct was cloned into the baculovirus transfer vector pVL1393 (Webb and Summers, 1990) using *Eco*RI sites. The resulting plasmid was used to transfer the rtrkA.trunc construct into a linearized baculovirus AcMNPV genome (Invitrogen Corp.), and purified recombinant viruses which express the truncated form of the trkA receptor were isolated using standard methods (Summers and Smith, 1987; Webb and Summers, 1990). Recombinant viruses were identified using PCR and oligonucleotide primers specific for rtrkA.trunc construct and the AcMNPV genome (Invitrogen Corp.)

Purification of rtrkA.trunc Protein and Generation of the RTA Antibody

Large-scale infections with the rtrkA.trunc-expressing baculovirus were performed using the Sf900 cell line (the gift of W. Mobley) grown in SF900 II SFM medium (Gibco/BRL, Life Technologies, Grand Island, NY). The levels of protein throughout expression and purification procedures were measured by antigen blot with the rtrkA.EX2 anti-peptide antibody.

For purification, 1.2 l of Sf900 cells was infected with 160 ml of high-titer viral stock, and 2 d post-infection, the supernatant was harvested and loaded onto a 10-ml lentil lectin-Sepharose column (Pharmacia/LKB Biotechnology). After washing with 500 mM NaCl, 5 mM tris(hydroxymethyl)aminomethane (Tris)-Cl, pH 7.5, protein was eluted with 5 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5 M methyl-α-D-mannopyranoside (Sigma Chemical Corp.). The eluate was dialyzed into 20 mM Tris-Cl, pH 8.0, concentrated by pressure on a YM30 membrane (Amicon, Danvers, MA), and loaded onto a MonoQ column (1 ml; Pharmacia/LKB Biotechnologies). After washing the column with 20 mM Tris-Cl, pH 8.0, the column was developed with a linear gradient to 20 mM Tris-Cl, pH 8.0, 1 M NaCl. The rtrkA.trunc protein eluted in a peak at ~200 mM NaCl and ran on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as an overlapping doublet of ~55 000 molecular weight, with a typical yield of 500 μg. Both of the doublet bands react strongly with the rtrkA.EX2 antibody, indicating that each is derived from the trkA expression vector but differ by posttranslational modification such as glycosylation. The identity of the purified protein was confirmed by the amino acid sequence analysis of two peptides derived from a trypsin digestion of the preparation. The purified protein was used to immunize a rabbit with an initial injection of 175 μg of protein; subsequent boosts were 50 μg each. IgG was prepared from the resulting sera by affinity chromatography on protein-A Sepharose by standard methods (Harlow and Lane, 1988).

Immunoblots and Immunoprecipitations

Lysis buffer contained the following: 150 mM NaCl; 50 mM Tris-Cl, pH 7.6; 1% Triton X-100; 0.1% sodium dodecyl sulfate; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 50 mM NaF; and 10 mM sodium pyrophosphate. When the lysates were to be probed for phosphotyrosine, the lysis buffer contained 0.1 mM sodium orthovanadate in addition. Extracts were prepared by incubating cells with lysis buffer on ice for 15 min; insoluble material was removed with a 10-min, 10 000 rpm centrifugation at 4°C. Extracts were separated on SDS-polyacrylamide gels and transferred to nitrocellulose using standard protocols (Harlow and Lane, 1988). Immunoblots were performed using 80 mM NaCl, 50 mM Tris-Cl, pH 8.0, 0.5% Nonidet P-40, 3% bovine serum albumin, and 1% ovalbumin. The anti-phosphotyrosine antibody 4G10 was used at 1 μg/ml, followed by rabbit anti-mouse coupled to horseradish peroxidase HRP diluted 1:5000. Streptavidin-HRP was also used at 1:5000 dilution; the alkaline phosphatase–coupled secondary antibody was used at 0.1 μg/ml and was developed using the BCIP/NBT method (Harlow and Lane, 1988). HRP conjugates were detected with the electrochemiluminescence protocol developed by Amersham Corp.

TrkA immunoprecipitations were performed by adding 2–5 μg of IgG and 30 μl of protein A-Sepharose per sample. After 2–3 h at 4°C, the Sepharose was washed four times with lysis buffer. Sepharose-bound proteins were eluted in 2× Laemmli SDS sample buffer and loaded on SDS-PAGE for immunoblot analysis.

For biotinylation, COS cell cultures that had been transfected with cDNA constructs 2 d previously were washed two times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (CMF-PBS). CMF-PBS + 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 8.0 and 360 μg/ml sulfosuccinimidobiotin (Pierce Chemical Corp.) was added and the cultures were incubated 1 h at room temperature. The cultures were washed four times with PBS + 5 mM glycine, and lysates were prepared as above. The extracts were precleared with protein A-Sepharose, and then immunoprecipitations were performed as described above.

Binding Assays

Binding assays were performed with PC12 cells and ^{125}I-NGF as previously described (Weskamp and Reichardt, 1991). Briefly, antibodies were preincubated with the cells (5×10^5 cells/ml) at 37 or 4°C for 2 or 4 h before addition of ^{125}I-NGF; cell-associated NGF was determined after a further 60 minutes at 37 or 4°C. Nonspecific binding was determined by addition of 10 μg/ml unlabeled NGF and ranged from 2 to 25% of specific binding. Determinations were made in triplicate. The equilibrium binding data from two experiments were combined for the Scatchard plot, which was analyzed with the program LIGAND (Munson and Rodbard, 1980). The Scatchard data are plotted as femtomoles bound per 10^5 cells (equivalent to a volume of 2×10^{-4} l).

Cell Culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DME-H21) containing 10% fetal calf serum and 5% horse serum on tissue culture plastic. The nnr5 subclone of PC12 and its transfected derivatives were grown in the same medium on a collagen I substrate (Vitrogen 100, Celtrix Pharmaceutical, Inc., Santa Clara, CA). PC12^{nnr5} expressing exogenous rat trkA were generated by infection with a murine retroviral vector in which trkA expression is directed from the cytomegalovirus promotor (Clary and Reichardt, unpublished data). PC12 cells used for outgrowth experiments were treated with NGF in DME-H21 containing 1% horse serum for 8 d before replating in the same medium supplemented with NGF or antibodies.

COS7 cells were grown in DME-H21 containing 10% fetal calf serum. For transfections, 15 μg of the CDM8 vector (Seed, 1987) or the CDM8 vector containing the rat trkA, trkB, or trkC cDNAs was transfected into a 10-cm dish of COS7 cells using the DEAE dextran-chloroquine method (Seed and Aruffo, 1987). After an additional incubation of 2 d, the cells were processed for immunoprecipitation or antigen blotting.

Neonatal rat sympathetic neurons were isolated from superior cervical ganglia of newborn rats as described (Hawrot and Patterson, 1979). The ganglia were treated with 1 mg/ml collagenase/dispase in CMF-PBS (45 min at 37°C; Boehringer Mannheim Biochemicals, Indianapolis, IN) and were washed with the same buffer. The ganglia were triturated to remove the neurons, and the isolated cells were washed several additional times in CMF-PBS. The cells were cultured on a collagen I substrate in a defined medium (Leibowitz L-15/CO_2 medium containing 1× nonessential amino acids, 1 mg/ml bovine serum albumin, and 0.35% methocel, penicillin, streptomycin, glucose, and imidizole as described (Hawrot and Patterson, 1979). In some of the RTA IgG activation experiments, the medium also contained the following: stable vitamin mix, fresh vitamin mix (Hawrot and Patterson, 1979), and the N1 supplement (Bottenstein *et al.*, 1980). Essentially identical results were obtained under either condition. After 24 h, the cultures were fixed with PBS containing 5% sucrose and 2.5% glutaraldehyde before photography and cell counts. Neurons were considered to have survived if they appeared phase-bright and had extended processes greater than two cell diameters in length.

RESULTS

Preparation of an Antibody Against the Extracellular Domain of Rat trkA

To probe the function of the trkA receptor biochemically and biologically, we developed a polyclonal antibody to the extracellular domain of trkA. cDNAs encoding rat trkA were isolated from a PC12 cDNA library constructed in the plasmid vector CDM8 as described in MATERIALS AND METHODS. Ten cDNAs were isolated; sequence analysis revealed that several of these contained the entire rat trkA coding sequence.

A truncated trkA expression cassette containing the entire extracellular domain of rat trkA was generated by PCR and its DNA sequence verified. The cassette was subsequently cloned into a baculovirus transfer vector from which recombinant viruses were isolated through standard protocols. Infections of Sf900 cells with the recombinant virus resulted in the production of an ~55-kDa product that was secreted into the medium. The expression of the truncated trkA protein was monitored by immunoblotting with an anti-trkA peptide antiserum, and the protein was purified as described in MATERIALS AND METHODS. Its identity was confirmed by determining the amino acid sequences of two tryptic peptides derived from it. The truncated trkA protein was subsequently used as an immunogen to raise rabbit polyclonal antisera. This antibody will be referred to here as the RTA antibody.

The ability of the RTA sera to recognize native rat trkA was tested by immunoprecipitation analysis, using lysates of COS cells surface-labeled with biotin after transfection with the CDM8 vector containing rat trkA, rat trkB, rat trkC, or with vector alone as control. After immunoprecipitations were performed with either RTA, an anti-peptide antibody to the trkA cytoplasmic tail (rtrkA.cyt), or control nonimmune rabbit IgGs, the precipitates were analyzed by blot using a streptavidin-HRP conjugate for detection (Figure 1A). RTA was able to precipitate the 140-kDa trkA protein (lane 6), as shown by comparison to the same lysate precipitated with an antibody directed against the cytoplasmic tail of trkA (lane 5). The stronger signal seen with the affinity-purified rtrkA.cyt antibody is due to its higher titer. A doublet of ~100 kDa precipitated with the rtrkA.cyt antibody is not related to trkA, as it is also detected in the vector only transfection (lane 2). The rtrkA.cyt antibody is also able to precipitate the rat trkB and rat trkC proteins (lanes 7 and 9), as the cytoplasmic tails of the three trk receptors have extensive similarity. However, the RTA antibody is unable to recognize either trkB or trkC (lanes 8 and 10).

The specificity of the RTA antibody was also tested by immunoblot (Figure 1B). COS cell cultures were transfected as above with the rat trk cDNAs, or vector alone, and lysates from all three cultures were probed with the RTA sera. Two prominant bands were detected in the lysate of the trkA-transfected culture, migrating at an approximate molecular mass of 110 and 140 kDa. No cross-reaction to trkB or trkC was detected, although they were abundantly expressed, as determined in Figure 1A and by probing a parallel blot with an antibody against the trk cytoplasmic tail. An additional band is detected sporadically with RTA antibody, migrating with an approximate molecular mass of 180 kDa. This band seems to be a nonspecific reaction of the sera, as it is detected in untransfected COS cells, as well as other cell lines. It has not been detected by immunoprecipitation (Figure 1A), indicating that either it is an intracellular protein and therefore not labeled by the biotinylation reagent or that it is not recognized by the antibody in an undenatured state.

RTA Blocks the Binding of NGF to the trkA Receptor

From the immunoprecipitation analysis it is clear that the RTA antibody can recognize the native trkA receptor protein. We wished to determine if the antibody could disrupt the NGF-binding activity of the receptor and therefore serve as an inhibitor of the receptor's function. This was tested in a series of experiments in which the effects of the antibody preparations were tested in NGF binding assays of the PC12 cell line, known to express both the LNGFR as well as trkA. A whole-cell binding assay containing 200 pM ^{125}I-NGF was employed to measure the dose-dependent inhibition of binding (Figure 2A). The RTA IgG and Fab preparations were able to inhibit the binding of NGF to PC12 cells partially, achieving a maximum inhibition of ~30%. Approximately 125 μg/ml antibody was required to reach this

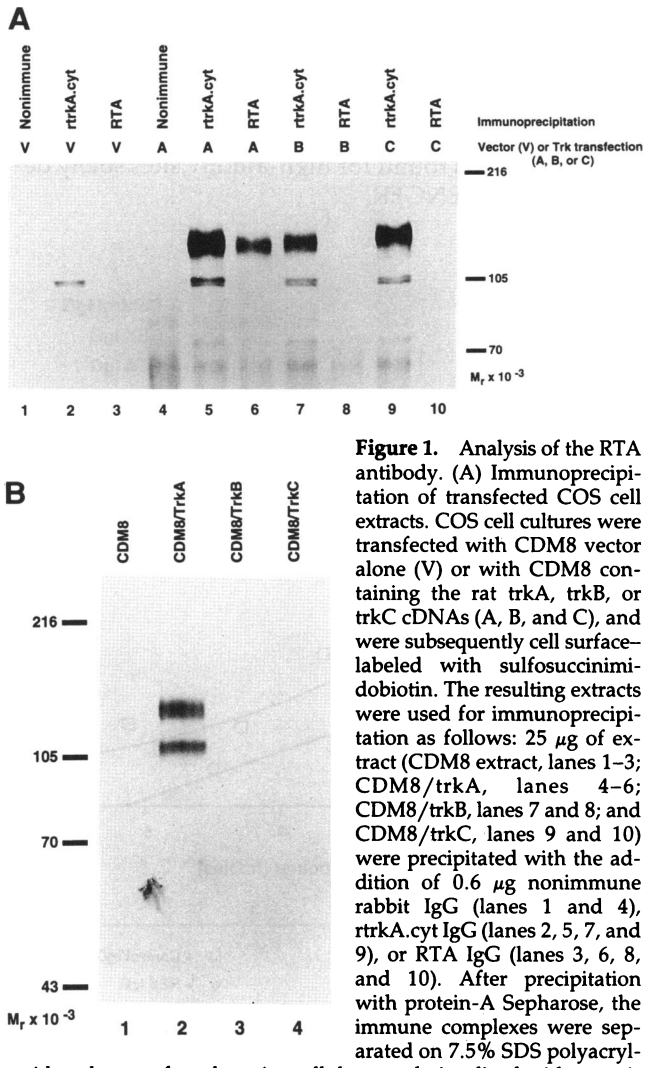

Figure 1. Analysis of the RTA antibody. (A) Immunoprecipitation of transfected COS cell extracts. COS cell cultures were transfected with CDM8 vector alone (V) or with CDM8 containing the rat trkA, trkB, or trkC cDNAs (A, B, and C), and were subsequently cell surface–labeled with sulfosuccinimidobiotin. The resulting extracts were used for immunoprecipitation as follows: 25 μg of extract (CDM8 extract, lanes 1–3; CDM8/trkA, lanes 4–6; CDM8/trkB, lanes 7 and 8; and CDM8/trkC, lanes 9 and 10) were precipitated with the addition of 0.6 μg nonimmune rabbit IgG (lanes 1 and 4), rtrkA.cyt IgG (lanes 2, 5, 7, and 9), or RTA IgG (lanes 3, 6, 8, and 10). After precipitation with protein-A Sepharose, the immune complexes were separated on 7.5% SDS polyacrylamide gels, transferred to nitrocellulose, and visualized with peroxidase-coupled streptavidin and the electrochemiluminescence protocol (Amersham Corp.). (B) Immunoblotting of cell extracts from COS cell cultures. The antigen blot is derived from a 7.5% PAGE of COS cell lysates (5 μg each) transfected with the CDM8 vector (lane 1), CDM8/rat trkA (lane 2), CDM8/rat trkB (lane 3), or CDM8/rat trkC (lane 4). The filter was incubated with 1 μg/ml RTA IgG, followed by an anti-rabbit-HRP secondary antibody. The HRP was visualized using the ECL system (Amersham Corp.). The expression of trkB in lane 3 and trkC in lane 4 was confirmed by blotting a parallel experiment with an anti-trk cytoplasmic tail antibody.

maximal level of inhibition. As IgG preparations directed against extracellular domains of receptor tyrosine kinases have been found to increase the rate of receptor internalization (Gherzi *et al.*, 1987; Ganderton *et al.*, 1989), in principle the inhibition of NGF binding promoted by the RTA IgG preparation could be due to some combination of steric interference of the ligand binding site and receptor internalization. However, the Fab fraction was an equally effective inhibitor of NGF binding, even though previous reports indicate that the binding of Fab fragments does not promote the internalization of receptors expressed on the cell surface (Ganderton *et al.*, 1989). To test directly that the RTA antibody could sterically block the NGF binding site, the antibodies were used to block binding in assays performed at 4°C, a temperature that does not allow antibody- or ligand-induced endocytosis of the receptor. Both the RTA IgG and Fab preparations were able to inhibit the binding of ^{125}I-NGF when the antibody and ligand incubations were performed at 4°C (Figure 3B, 0-min time point), to a level similar to that found in 37°C incubations (Figure 2B). Therefore, interaction of the RTA antibody with trkA can directly block the ligand binding site.

We were able to show that the residual NGF binding in the presence of RTA is due to binding to the low-affinity NGF receptor, by testing saturating concentrations of antibodies to both trkA and the LNGFR (Figure 2B). Here, NGF binding assays on PC12 cells were performed in the presence of RTA IgG or Fab, anti-rat LNGFR IgG, or combinations of the antibodies. The anti-LNGFR antibody (anti-REX; Weskamp and Reichardt, 1991) is a polyclonal antiserum raised against the extracellular domain of the LNGFR and has been shown to inhibit NGF binding to that receptor completely. As shown in Figure 2B, the anti-LNGFR antibody blocked ~80% of NGF binding, whereas the RTA IgG or Fab blocked ~25% of NGF binding. The combinations of antibodies were able to inhibit almost completely the binding of NGF to PC12 cells, indicating that trkA and the LNGFR account for ≥97% of the NGF binding proteins on PC12 cells. Confirmation of RTA specificity was obtained using NGF cross-linking assays (Weskamp and Reichardt, 1991), where preincubation of the cells with the RTA antibody sharply reduced the production of the 160-kDa band, previously identified as the trkA ^{125}I-NGF cross-linked product, without altering the levels of the 100-kDa, LNGFR-derived product (Hosang and Shooter, 1985; Johnson *et al.*, 1986; Kaplan *et al.*, 1991a; Klein *et al.*, 1991).

To analyze the characteristics of the NGF binding sites remaining in the presence of the anti-LNGFR or -trkA antibodies, equilibrium binding assays were performed from 3.5 to 4000 pM NGF. The results are plotted by the Scatchard method in Figure 3A. As is now well known, PC12 cells have NGF binding sites with differing kinetic characteristics, leading to low- and high-affinity binding sites (Schechter and Bothwell, 1981). This is observed in the biphasic graph of NGF binding determined in the presence of control IgG (Figure 3A, □; the binding parameters are presented in Table 1). The number and affinity of the two measured NGF binding sites in the presence of control IgG are similar to those previously reported for PC12 cells (Weskamp and Reichardt, 1991). Preincubation with anti-LNGFR IgG blocks all of the low-affinity sites, and blocks a fraction of the high-affinity sites, as shown previously

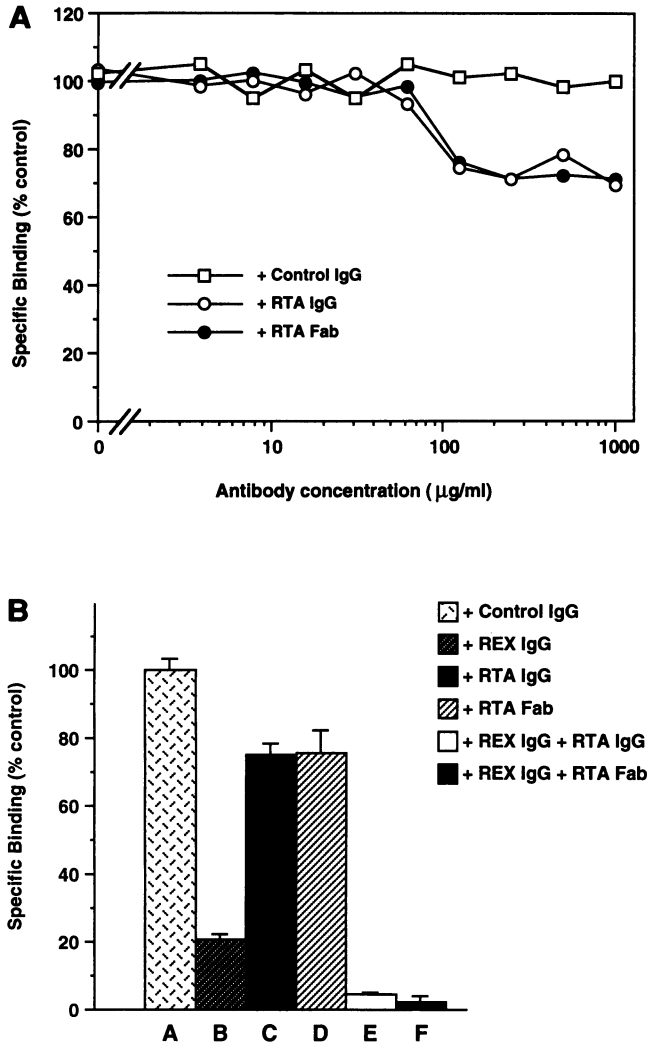

Figure 2. RTA IgG and Fab fragments block NGF binding to PC12 cells. (A) Dose response. PC12 cells were incubated with antibodies at the indicated concentration for 4 h at 37°C, and binding was initiated by the addition of 200 pM ^{125}I-NGF. After a 1-h incubation, the amount of cell-associated NGF was determined. Antibodies tested were control rabbit IgG, RTA IgG, and RTA Fab fragments. Nonspecific binding was determined by addition of 10 μg/ml unlabeled NGF and has been subtracted. Values were determined in triplicate and are shown as percent control (no antibody addition). (B) Additive effects of RTA and anti-LNGFR antibodies. PC12 cells were incubated at 37°C for 4 h with 500 μg/ml of each indicated antibody, followed by the addition of 250 pM ^{125}I-NGF. After a further 60 min at 37°C, the amount of cell-bound NGF was determined. Antibodies tested were as follows: A, control rabbit IgG; B, anti-LNGFR IgG (REX); C, RTA IgG; D, RTA Fab fragments; E, anti-LNGFR IgG + RTA IgG; and F, anti-LNGFR IgG + RTA Fab fragments. Nonspecific binding was determined as in panel A; values are shown as percent control (no antibody addition). The values shown are the average of two experiments of triplicate samples and include calculated SEM.

(Figure 3A, ◇; Weskamp and Reichardt, 1991). In contrast, we find that in the presence of the RTA antibody, all of the remaining detectable binding sites are of low affinity (Figure 3A, O). These results indicate that all detected high-affinity binding sites require trkA. However, this analysis also suggests that there may be two types of high-affinity sites, one that is dependent solely on trkA and one that requires both trkA and the LNGFR. No evidence was found for high-affinity sites solely dependent on the LNGFR.

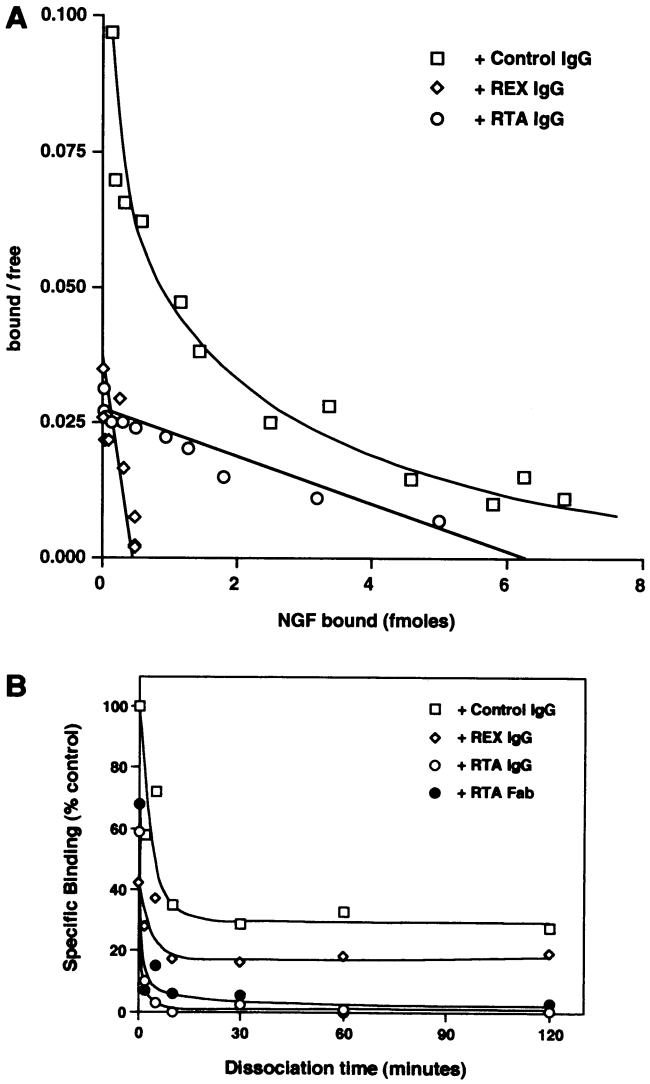

Figure 3. Analysis of anti-LNGFR- and RTA-resistant NGF binding sites. (A) PC12 cells were preincubated with 500 μg/ml control rabbit IgG, anti-LNGFR (REX) IgG, or RTA IgG for 2 h at 37°C. ^{125}I-NGF (3.5 pM to 4 nM) was added, and the amount of cell associated NGF determined after 1 h at 37°C. Data from two similar experiments were pooled and are presented in the form of a Scatchard plot. Nonspecific binding was determined as in Figure 2A; values were determined in triplicate. (B) Binding and dissociation at 4°C. PC12 cells were preincubated at 4°C for 4 h in the presence of the indicated antibody at 500 μg/ml, followed by the addition of 200 pM ^{125}I-NGF. After an additional 60-min incubation at 4°C, triplicate samples were taken. Dissociation was initiated by the addition of unlabeled NGF to 500 nM. At the indicated times, triplicate samples were removed and assayed for bound ^{125}I-NGF.

The Scatchard analysis was performed at 37°C to promote equilibrium binding conditions. However, at that temperature, ligand-induced internalization of the receptors will affect the measured affinities of the binding sites. To examine more closely the apparent heterogeneity of the high-affinity binding sites detected in the Scatchard analysis, we performed a dissociation experiment at 4°C, conditions where receptor internalization does not occur. PC12 cells were preincubated with control IgG, anti-LNGFR IgG (REX), or the RTA IgG or Fab preparations for 4 h at 4°C, followed by a 1-h incubation at 4°C with 200 pM ^{125}I-NGF. Dissociation of the labeled NGF was initiated at time 0 by the addition of 500 nM unlabeled NGF. Two kinetic classes of dissociating NGF are detected in the presence of control rabbit IgG, which correspond to the fast- and slow-dissociating NGF binding sites previously described (Figure 3B, □) (Schechter and Bothwell, 1981). Preincubation of the cells with the anti-LNGFR antibody blocks 60% of the bound NGF at time 0; one-half of the REX-resistant sites show slow dissociation kinetics (◇). However, although preincubation of the cells with either the RTA IgG or Fab antibodies blocks only 30% of the NGF binding sites, all of these sites exhibit fast dissociation kinetics (○, ●). Thus dissociation kinetics measured at 4°C are compatible with the findings of the equilibrium binding experiments. NGF binding sites that are resistant to the RTA antibody have the properties of the LNGFR, displaying fast dissociation kinetics and low-affinity binding. At least one-half of the REX-resistant sites show slow dissociation and likely correspond to the high-affinity sites detected in the equilibrium-binding experiment. Also, the inhibition of NGF binding to the LNGFR decreased substantially the number of slow-dissociating sites formed during the binding experiment, again corresponding to the decrease in high-affinity binding sites detected in the equilibrium binding experiment after REX preincubation (Figure 3A and Table 1).

RTA Fab Fragments Inhibit the Phosphorylation Response to NGF

PC12 cells respond to NGF rapidly, and after 5 min of exposure, a reproducible burst of tyrosine phosphorylation of many cellular proteins occurs (Maher, 1988). This effect can be measured by probing blots of cell lysates with anti-phosphotyrosine antibodies. Such an experiment is shown in Figure 4, where in lane 2 several cellular substrates for NGF-dependent tyrosine phosphorylation are detected after 5 min of NGF treatment (arrows), but not in the absence of NGF (lane 1). We tested the ability of the anti-trkA antibody to block this rapid response by pretreating the PC12 cultures with RTA Fab fragments or control nonimmune Fab fragments before exposure to NGF. As shown in Figure 4, RTA Fab fragments were able to block almost completely the phosphorylation response to NGF (lane 4), whereas control nonimmune Fab fragments did not affect the response (lane 3).

RTA IgG Mimics NGF in Tyrosine Phosphorylation Assays

In contrast to the inhibitory effects of the monovalent RTA Fab fragments, we found that the bivalent RTA IgG promoted phosphorylation of the same cellular proteins as NGF, even in the absence of NGF (Figure 5A). Each of the PC12 cultures were treated for 5 min with NGF or various antibody preparations and changes in cellular tyrosine phosphorylation were determined as described above. Significant changes in phosphorylation were detected in response to NGF and the RTA IgG preparation, and the RTA IgG-induced pattern was indistinguishable from that found in response to NGF. In contrast, both control rabbit IgG and anti-LNGFR IgG were ineffective. Also, Fab fragments of the RTA antibody had no effect.

The changes in protein tyrosine phosphorylation promoted by the RTA antibody preparation could in principle come from other receptor tyrosine kinases. We took advantage of a variant of the PC12 cell line, called PC12^{nnr5}, which lacks functional trkA protein and responses to NGF (Loeb *et al.*, 1991). Neither NGF nor RTA antibody treatment caused detectable changes in protein tyrosine phosphorylation in these cells (Figure 5B, lanes 4–6). However, when rat trkA was ectopically expressed in PC12^{nnr5}, responses to both NGF and the RTA antibody were restored (lanes 7–9). Therefore, the effects of the RTA antibody on protein tyrosine phosphorylation require trkA expression.

Table 1. Effects of anti-LNGFR IgG and RTA IgG on equilibrium binding of ^{125}I-NGF to PC12 cells[a]

Control IgG		Anti-LNGFR IgG		RTA IgG	
K_d (M)	Receptors per cell	K_d (M)	Receptors per cell	K_d (M)	Receptors per cell
3.8×10^{-11}	4 900 ± 700	9.2×10^{-11}	2 800 ± 400	—	—
1.8×10^{-9}	34 000 ± 3 200	—	—	1.1×10^{-9}	28 300 ± 3 000

[a] Binding parameters derived from the Scatchard analysis shown in Figure 3, as determined by the LIGAND program.

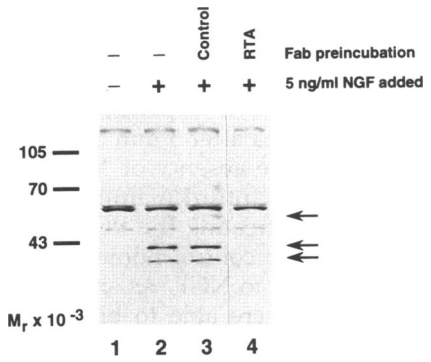

Figure 4. RTA Fab fragments block NGF-dependent cellular tyrosine phosphorylation. PC12 cultures were preincubated with 500 μg/ml nonimmune Fab (control, lane 3), RTA Fab (lane 4), or no antibody (lanes 1 and 2), for 4 h before the addition of 5 ng/ml NGF for 5 min (lanes 2–4). The cleared lysates (10 μg) were fractionated by 10% SDS-PAGE, blotted, and probed for changes in cellular tyrosine phosphorylation using the 4G10 monoclonal, HRP-coupled anti-mouse secondary antibody, and ECL. The positions and relative molecular weights of the marker proteins are shown at the left, and the arrows on the right indicate several prominent proteins that become hyperphosphorylated during the assay.

To show that the RTA IgG effects reflected activation of the trkA receptor, effects of NGF and various antibodies on trkA phosphotyrosine levels were examined. TrkA is a tyrosine kinase receptor that has been shown previously to be tyrosine phosphorylated and activated upon binding of NGF (Kaplan *et al.*, 1991b; Klein *et al.*, 1991). We treated PC12 cultures as above with NGF or antibody preparations and then immunoprecipitated the trkA receptor from the corresponding lysates. The precipitates were analyzed by blotting with the anti-phosphotyrosine antibody. As shown in Figure 5B, the RTA IgG preparation was able to induce a concentration-dependent increase in trkA tyrosine phosphorylation after 5 min, whereas the RTA Fab preparation and control nonimmune IgG did not.

RTA Fab Inhibits and RTA IgG Stimulates Survival of Neonatal Sympathetic Neurons

To determine the effects of the trkA antibodies on long-term responses to NGF, we utilized a classic in vitro assay for NGF action, the NGF-dependent survival and process outgrowth of rat neonatal sympathetic neurons from the superior cervical ganglion. These cells are dependent on NGF during this period in vivo and in vitro; the withdrawal of NGF leads to a lack of process outgrowth and apoptotic cell death. We isolated neonatal sympathetic neurons and cultured them in the presence of NGF, NGF plus control nonimmune Fab fragments, or NGF plus RTA Fab fragments. As had been seen in the tyrosine phosphorylation assays, the RTA Fab fragments were able to inhibit the NGF responses of these cells, as assayed by survival and neurite outgrowth (Figures 6 and 7).

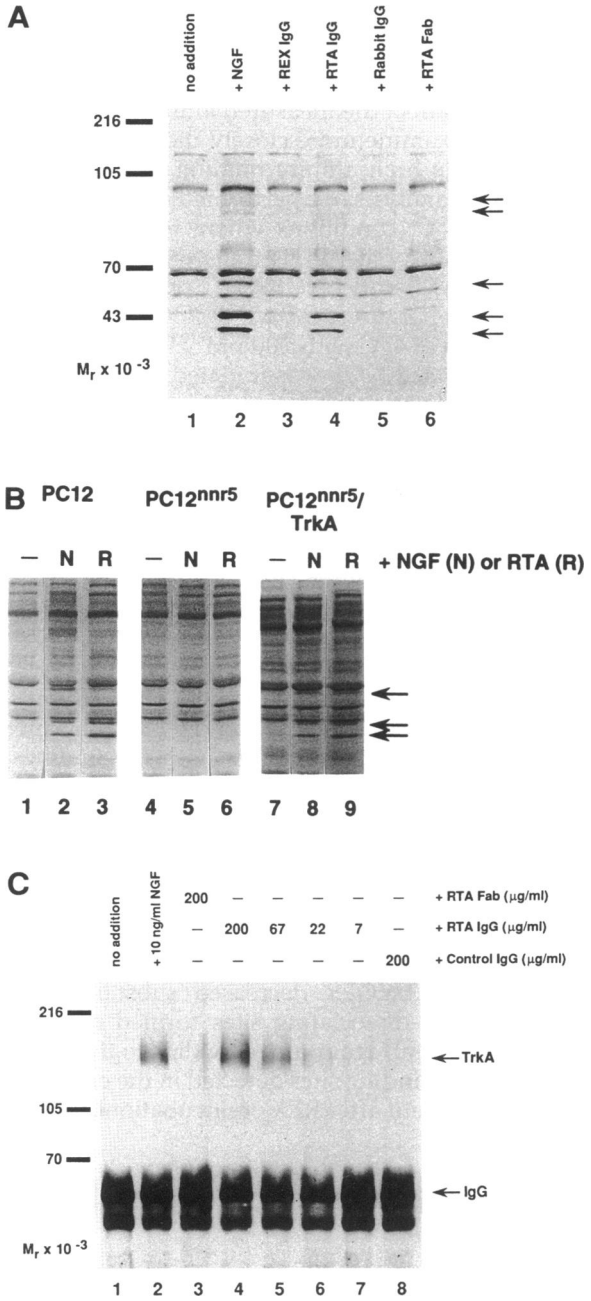

Figure 5. RTA IgG stimulates protein tyrosine phosphorylation in the absence of ligand. (A) PC12 cells were treated for 5 min with growth factors or antibody preparations at 37°C. Lysates were prepared and analyzed for changes in cellular phosphotyrosine as described in Figure 4. The treatments were as follows: no addition (lane 1); 50 ng/ml NGF (lane 2, +NGF); 100 μg/ml anti-LNGFR IgG (lane 3, REX IgG); 100 μg/ml RTA IgG (lane 4); 100 μg/ml nonimmune IgG (lane 5, rabbit IgG); and 67 μg/ml RTA Fab (lane 6). The arrows on the right indicate proteins that show increased tyrosine phosphorylation during the assay. (B) Stimulation of protein tyrosine phosphorylation by RTA requires trkA expression. PC12 cells (lanes 1–3), $PC12^{nnr5}$ (lanes 4–6), or $PC12^{nnr5}$ transfected with trkA (lanes 7–9) were incubated for 5 min with 50 ng/ml NGF (lanes 2, 5, and 8), 50 μg/ml RTA (lanes 3, 6, and 9), or no addition (lanes 1, 4, and 7). Changes in tyrosine phosphorylation were detected by incubating

When the effect of the bivalent RTA IgG preparation was tested quantitatively in the sympathetic neuronal survival assay, the RTA antibody caused a small but reproducible decrease in NGF-dependent viability, whereas the anti-LNGFR and nonimmune IgG preparations had no obvious effects (Figure 8). However, when NGF was omitted from the culture medium, the RTA antibody had a strong survival promoting activity, yielding a maximal survival of ~60% of that obtained with NGF (Figure 8) and promoted extensive process outgrowth (Figure 9). Neither the control IgG nor the anti-LNGFR antibody alone had any in vitro survival activity. In addition, RTA Fab fragments were unable to support sympathetic neuron survival. The effects of RTA IgG were also tested on cultures containing primed PC12 cells (PC12 cells that have been previously exposed to NGF for 7 d, resulting in differentiation). As shown in Figure 10, the RTA IgG showed a clear ability to promote neurite outgrowth of these cells in the absence of NGF; ~60% as many cells extended neurites as responded in the presence of NGF.

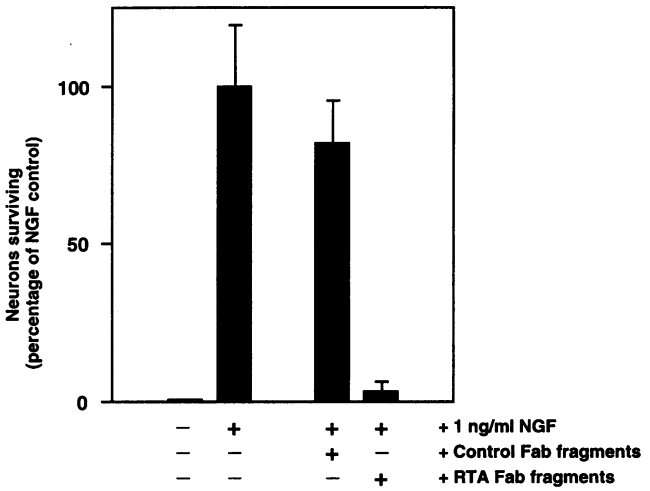

Figure 6. RTA Fab fragments inhibit NGF-dependent survival and outgrowth of sympathetic neurons. Isolated neurons from neonatal rat superior cervical ganglia were cultured in the presence or absence of 1 ng/ml NGF and 500 μg/ml nonimmune or RTA Fab fragments as indicated. After 24 h, the cultures were fixed and the number of process-bearing cells determined. The average and range from duplicate wells are shown.

DISCUSSION

Development of an Antiserum to the Rat trkA Receptor

Generation of antisera to both the LNGFR and trkA receptors allows the expression and function of each to be studied individually. Several monoclonal antibodies and the REX anti-LNGFR polyclonal antibody have been used to characterize in some detail the LNGFR receptor, but an antiserum of equivalent specificity to trkA has not been available. We found that the RTA antibody is highly specific; we detected no cross-reaction with the closely related trkB or trkC receptors on immunoblots or in immunoprecipitations (Figure 1). It also shows a discrete immunohistochemical staining pattern in mouse brain consistent with what is known about trkA gene expression. This pattern is inconsistent with the known expressions patterns of either trkB or trkC (Fariñas, Clary, and Reichardt, unpublished results). The RTA antibody could recognize the rat trkA receptor in its native state, as judged by immunoprecipitation analysis (Figure 1), and is therefore useful in a variety of experiments aimed at obtaining a better understanding of trkA and NGF responsiveness in neurons. We found that this antibody could inhibit NGF binding, as well as neuronal responses to NGF when Fab fragments were employed. In contrast to this, the anti-trkA IgG promoted NGF-like cellular responses even in the absence of NGF, presumably through antibody-dependent receptor cross-linking.

NGF binding experiments demonstrated that monovalent Fab fragments prepared from the RTA antibody can inhibit NGF binding to trkA (Figure 2), and subsequently inhibit downstream cellular NGF responses, such as increases in tyrosine phosphorylation and survival of sympathetic neurons (Figure 4 and 6). In contrast to what has been found with IgG preparations, experiments that have examined the interaction of Fab fragments with the insulin receptor have failed to detect receptor internalization as a result of Fab binding (Ganderton *et al.*, 1989), and we find that the RTA Fabs can inhibit NGF binding at 4°C; therefore, the most likely mechanism for receptor inhibition at 37°C is steric blockage of ligand binding. The use of Fab fragments for trkA receptor blocking will likely be a useful method for confirming or disproving in an in vivo context some of the functions assigned to NGF based on in vitro studies. In fact, this could be a general approach to studying the roles of trk receptors during development in many species. Because it is thought that several of the functions of trk receptors are based on competition for limiting amounts of neurotrophin in the animal (Barde, 1989), blocking of a large number of the recep-

the antigen blot with the 4G10 antibody and an anti-mouse secondary antibody coupled to alkaline phosphatase. The secondary antibody was visualized using the BCIP/NBT method. Several hyperphosphorylated proteins are indicated with the arrows at right. (C) RTA IgG induced trkA autophosphorylation. PC12 cells were treated as described in A with no addition (lane 1); 10 ng/ml NGF (lane 2); 200 μg/ml RTA Fab (lane 3); 200, 67, 22, and 7 μg/ml RTA IgG (lanes 4–7); and 200 μg/ml rabbit IgG (lane 8, control IgG). TrkA receptor was immunoprecipitated from 250 μg of each lysate by the addition of rtrkA.cyt IgG and protein A-Sepharose. The precipitations were separated on a 7.5% SDS gel and tyrosine phosphorylation detected as in Figure 4. The positions of phosphorylated trkA receptor and IgG used in the precipitation are shown at right.

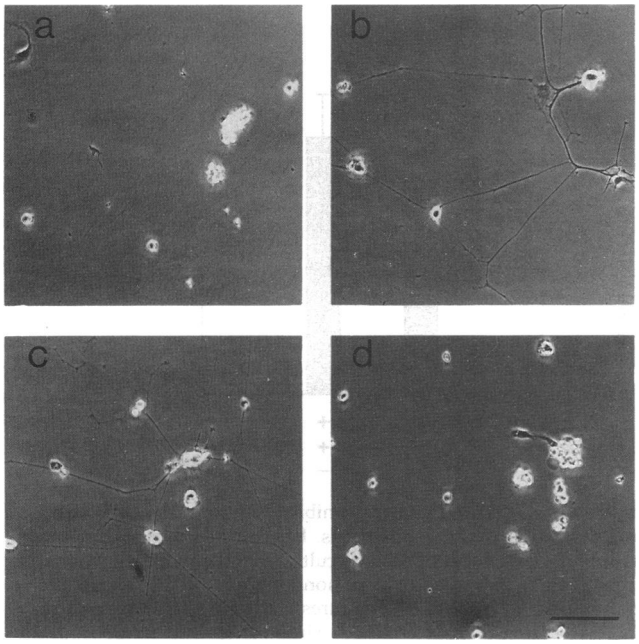

Figure 7. Photomicrographs of sympathetic neurons cultured in the presence of (a) no addition; (b) NGF; (c) NGF + nonimmune Fab; and (d) NGF + RTA Fab. NGF was added at 1 ng/ml, and the Fab fragments were used at 500 μg/ml. After 24 h, the cultures were fixed and photographed. Bar, 100 μm.

tors by Fab could yield dramatic effects during development. We were able to block sympathetic survival only at low concentrations of NGF and high concentrations of Fab, which is likely due to their differences in receptor affinity. However, given that in vivo concentrations of NGF are much lower than those used in culture, it seems possible that this Fab preparation would be an effective means of blocking trkA function in developing rats or mice.

Ligand-independent Activation of trkA by RTA

Although the IgG and Fab preparations of the RTA sera are equivalent in their ability to inhibit NGF binding to trkA, as judged by binding assays using radiolabeled ligand, the IgG fraction has only a very slight inhibitory effect on NGF-dependent survival. In fact, RTA IgG is almost as effective as NGF in activating trkA and downstream signaling pathways (Figures 5 and 8). Ligand-independent receptor activation by anti-receptor antibodies has been demonstrated previously in the receptor tyrosine kinase family. For example, monoclonal antibodies to either the insulin-like growth factor 1 receptor or epidermal growth factor receptor cause increases in receptor kinase activity, and an antibody to the insulin-like growth factor 1 receptor stimulates DNA synthesis in serum-starved NIH 3T3 cells (Spaargaren *et al.*, 1991; Xiong *et al.*, 1992). Both polyclonal and monoclonal antibodies to the insulin receptor could mimic insulin activation in several cell types as judged by 2-deoxyglucose and thymidine uptake, and this process required an active kinase domain (Gherzi *et al.*, 1987; Hawley *et al.*, 1989; Soos *et al.*, 1989). It was shown that bivalent antibodies are required for antibody-dependent activation of the epidermal growth factor receptor, probably because they mimic the ligand in promoting receptor dimerization (Spaargaren *et al.*, 1991). Evidence has been presented that the trkA receptor functions as a homooligomer, most probably a dimer, in signal transduction (Jing *et al.*, 1992), so it seems likely that the bivalent RTA IgG promotes dimerization or oligomerization sufficient to cause transphosphorylation of the receptor and subsequent activation of the NGF signal transduction pathway. The RTA antibody at saturating concentrations exhibits a somewhat lower survival activity than NGF (Figures 8 and 9). This seems to be an intrinsic property of the receptor-antibody interaction. As the presence of NGF does not restore the maximal level of survival, the lack of NGF bound to the LNGFR is not likely to be cause. It is known that antibodies to the insulin receptor cause a greater down-regulation of the receptor at the cell surface than insulin itself (Ganderton *et al.*, 1989); such a decrease of trkA on the surface of sympathetic neurons might lead to a slight decrease in viability. Alternately, some of the antibodies in the IgG pool may bind to the receptor in a fashion that blocks receptor oligomerization and activation.

Activation of trkA by RTA is a useful method for separating the effects of trkA from those of the LNGFR. In fact, this property can be exploited to differentiate the effects of the three receptors of the trk family, as antibodies have the potential to be of different specificity from the neurotrophins themselves. For example, in some systems NT-3 has been shown to activate trkC strongly, to activate trkB moderately, and to activate trkA weakly (Cordon-Cardo *et al.*, 1991; Glass *et al.*, 1991; Lamballe *et al.*, 1991; Soppet *et al.*, 1991; Squinto *et al.*, 1991). Studies designed to analyze the various functions of trkC by using NT-3 as ligand may also be measuring its effects on trkB or trkA. An activating antibody to trkC, however, would produce effects that are attributable solely to trkC.

TrkA Activity but Not LNGFR Activity Is Required for Sympathetic Neuron Survival In Vitro

One purpose of these experiments was to probe the relative contributions of LNGFR and trkA in high-affinity NGF binding and in the NGF-dependent survival of sympathetic neurons. A model of LNGFR function that has been proposed holds that LNGFR and trkA form a high-affinity heterodimer and that the LNGFR participates in the NGF signal transduction pathway (Berg *et al.*, 1991; Hempstead *et al.*, 1991). It now seems clear that activation of trkA is necessary and sufficient

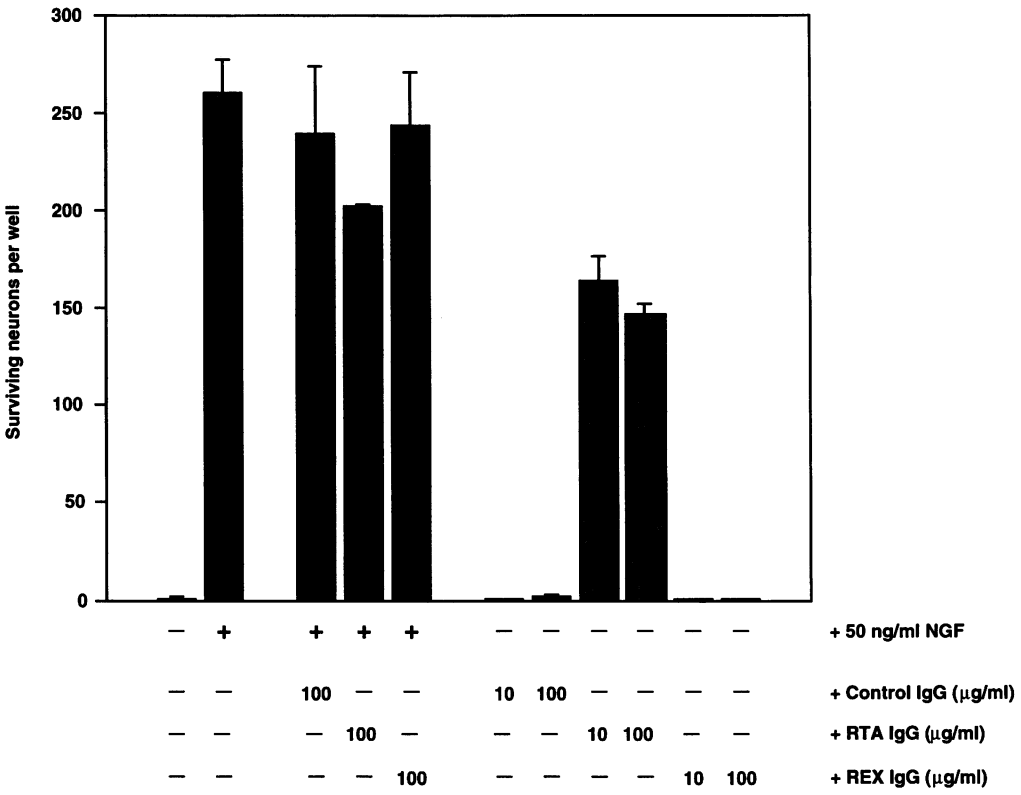

Figure 8. RTA promotes survival of sympathetic neurons. Neonatal rat superior cervical ganglion neurons were cultured in combinations of NGF and IgG preparations as indicated. NGF was used at 50 ng/ml and the IgG preparations at 10 or 100 μg/ml. After 24 h, the cultures were fixed and the number of process-bearing neurons was counted. The data shown are the average and range of duplicate cultures.

to achieve most cellular NGF effects, at least under in vitro culture conditions. This is born out by our finding that in the absence of NGF the RTA IgG facilitates survival and differentiation of both sympathetic neurons and PC12 cells by activating trkA. Several previous observations also support this conclusion. Evidence that trkA is a necessary component of the NGF responsive pathway in PC12 cells comes from studies of the trkA-deficient cell line PC12^{nnr5}, where it was shown that its lack of NGF response could be rectified by the reintroduction of trkA (Loeb *et al.*, 1991). There are also lines of evidence that suggest that the LNGFR does not play a crucial role in NGF signal transduction. The anti-LNGFR polyclonal antibody REX was able to block binding of NGF to LNGFR completely, yet had no obvious effect on the biological activities of NGF in culture (Weskamp and Reichardt, 1991). In agreement with this result, a mutated NGF that had lost its ability to bind

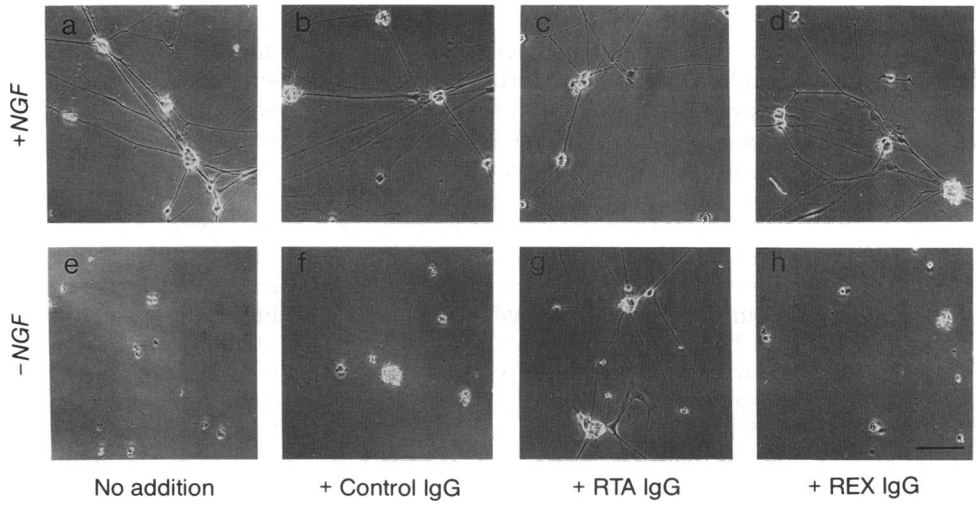

Figure 9. Sympathetic neurons were cultured in the presence of the following: (a) NGF; (b) NGF + normal rabbit IgG (control IgG); (c) NGF + RTA IgG ; (d) NGF + anti-LNGFR IgG (REX IgG); (e) no addition; (f) normal rabbit IgG; (g) RTA IgG; and (h) anti-LNGFR IgG. The antibodies and NGF were added at 100 μg/ml and 50 ng/ml, respectively. Cultures were incubated 24 h before fixation. Bar, 100 μm.

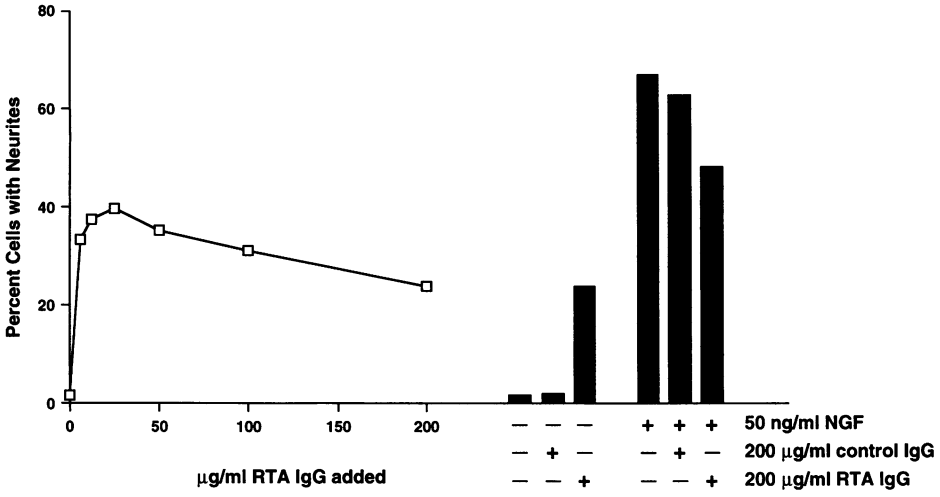

Figure 10. RTA IgG promotes outgrowth from PC12 cells. PC12 cells that had been treated for 8 d in the presence of 50 ng/ml NGF were replated in the presence of either increasing concentrations of RTA IgG or combinations of NGF and control or RTA IgGs as indicated. After an additional 3 d, the cultures were fixed and the percentage of process-bearing cells was determined.

to LNGFR was able nevertheless to support neurite outgrowth from PC12 cells and survival of neonatal sympathetic neurons (Ibanez *et al.*, 1992). A recent study, published after the completion of the present work, examined the activity in PC12 cells of a chimeric receptor consisting of the trkA intracellular kinase domain fused to the tumor necrosis factor receptor extracellular domain and found that treatment of the modified PC12 line with tumor necrosis factor could promote PC12 process outgrowth, presumably without interaction with the LNGFR (Rovelli *et al.*, 1993).

In each of the above examples, it is assumed that the LNGFR is not contributing to the NGF response if the LNGFR is not binding ligand, something that is difficult to prove using those models. Jaenisch and colleagues have addressed this issue by creating a mouse strain in which the gene for the LNGFR has been inactivated (Lee *et al.*, 1992). The resulting mice were viable and fertile. Examination of the nervous system revealed a loss of some sensory innervation and a defect in heat sensitivity, although the sympathetic innervation was largely normal (but see Lee *et al.*, 1994). Examination of the in vitro sensitivity to NGF of the trigeminal sensory neurons and sympathetic neurons has recently been reported; while a three- to fourfold decrease in the NGF dose-response was measured for the sensory neurons, no differences were detected between the LNGFR-deficient and wild-type sympathetic neurons (Davies *et al.*, 1993). Thus for at least some of the targets of NGF, the LNGFR is not required either in an NGF-bound or -unbound state for NGF to achieve its effects.

One of the more interesting models proposed recently has the LNGFR acting in a dominant fashion; when the receptor is bound by ligand, it is inactive, but when unbound, it is free to activate a cellular pathway leading to apoptotic cell death (Rabizadeh *et al.*, 1993). Our evidence argues against this model for sympathetic neurons, as activation of trkA by RTA in the absence of NGF (and therefore when the LNGFR is presumably unbound) could support sympathetic neuron survival, and the combination of RTA and NGF did not yield much more survival than the antibody alone. At a minimum, the activation of the trkA-signaling pathway overrides the putative LNGFR-dependent apoptotic pathway. As the model suggests that the LNGFR works in a dominant fashion to cause apoptosis, it does not easily explain the deficits observed in the LNGFR-deficient mouse. If the dominant model of LNGFR action is correct, the deletion of the LNGFR gene might actually be expected to increase the number of cells in certain populations, rather than eliminate them, and would be unlikely to lead to the observed deficit in sensory innervation.

Is trkA Activity Modified by the LNGFR?

Our analysis of NGF binding to PC12 cells in the presence of RTA and the anti-LNGFR antibodies has implied that most or all of the low-affinity NGF binding sites are LNGFR dependent, that all of the high-affinity NGF binding site are trkA dependent, and that a fraction of the high-affinity sites are dependent on both LNGFR and trkA (Figure 3A). The equilibrium binding data are supported by dissociation data obtained at 4°C, where we found that the RTA antibody blocks all slow-dissociating sites, whereas the anti-LNGFR antibody blocks one-half of the slow-dissociating sites (Figure 3B) (Weskamp and Reichardt, 1991). Although evidence has been presented that trkA receptors can form low- as well as high-affinity binding sites (Kaplan *et al.*, 1991a; Klein *et al.*, 1991), these studies have utilized transfected fibroblastic cell lines that express relatively large numbers of receptors. The expression of trkA in PC12 cells is comparatively low, and we can detect only high-affinity sites that are clearly attributable to trkA (Figure 3 and Weskamp and Reichardt, 1991). A majority of these sites are insensitive to anti-LNGFR antibodies and therefore appear to be solely dependent on trkA. The

high-affinity sites that are inhibited by anti-LNGFR antibodies are not likely to be due to a small number of LNGFR dimers exhibiting a higher affinity for NGF than the monomer LNGFR (Grob and Bothwell, 1983; Chandler *et al.*, 1984) because such sites should not be sensitive to the effects of the RTA antibody. Although there is no biochemical demonstration of a direct association of the LNGFR with trkA, it is possible that the anti-LNGFR/anti-trkA–sensitive high-affinity sites comprise a complex that contains both LNGFR and trkA. Evidence for LNGFR/trkA interaction has been presented previously, also through NGF binding analysis (Hempstead *et al.*, 1991; Battleman *et al.*, 1993).

An alternate explanation for the heterogeneity in the slow-dissociating NGF binding sites is that the LNGFR increases the rate of slow site formation by trkA. However, such a model predicts that the increase in the NGF association rate would lead to an increase in the affinity of trkA for NGF; conversely, in the presence of the anti-LNGFR antibody trkA should display a lower affinity. We found high-affinity binding of NGF in the presence of the anti-LNGFR antibody, but fewer binding sites than in its absence. One way to reconcile this discrepancy is to consider the rate of endocytosis of the trkA/NGF complex at 37°C (Hosang and Shooter, 1987). Within the 60 min we used for the binding reaction, the majority of the trkA bound to NGF was found to become unavailable to a chemical cross-linker and thus probably endocytosed. When endocytosis occurs during measurement of receptor binding affinities, the measured rate constant sets only an upper limit on the actual receptor binding affinity (Wiley and Cunningham, 1981). Endocytosed NGF could therefore appear as high-affinity binding, and so one explanation for the effects of the anti-LNGFR antibody on the Scatchard analysis is that in its presence, trkA is forming slow-dissociating sites less efficiently, thereby decreasing the rate of the internalization of NGF.

Although inhibition of the LNGFR ligand binding site does not prevent the major responses of neurons to NGF (Weskamp and Reichardt, 1991), there may be an additional role for the putative LNGFR- and trkA-dependent binding site; for example, it might as mentioned above increase trkA binding kinetics or change trkA binding specificity (Benedetti *et al.*, 1993). The decrease in slow NGF binding sites formed at 4°C is compatible with a modulation of trkA association kinetics. Also, such a function for the LNGFR would be consistent with known defects in the LNGFR-deficient mouse strain, as it has recently been reported that embryonic trigeminal sensory neurons from the mutant strain show a fourfold decrease in sensitivity to NGF in vitro when compared to wild type (Davies *et al.*, 1993). A drop in NGF sensitivity could conceivably result in the observed decrease in sensory neuron innervation (Lee *et al.*, 1992) as these neurons compete for limiting amounts of NGF during development. However, the interaction between trkA and the LNGFR is probably more complicated than this hypothesis implies. For example, the trigeminal sensory neurons and sympathetic neurons both respond to NGF in vivo and in vitro, and although the lack of the LNGFR in the trigeminal neurons decreases their dose-response to NGF, no differences between LNGFR-deficient and wild-type sympathetic neurons were measured (Davies *et al.*, 1993). Thus it seems that our understanding of the process by which neuronal development is controlled by neurotrophins will be strengthened by a further characterization of the relationships between trkA, the LNGFR, and NGF.

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REFERENCES

Aruffo, A., and Seed, B. (1987). Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. Proc. Natl. Acad. Sci. USA *84*, 8573–8577.

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

Battleman, D.S., Geller, A.I., and Chao, M.V. (1993). HSV-1 vector-mediated gene transfer of the human nerve growth factor receptor p75hNGFR defines high-affinity NGF binding. J. Neurosci. *13*, 941–951.

Benedetti, M., Levi, A., and Chao, M.V. (1993). Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc. Natl. Acad. Sci. USA *90*, 7859–7863.

Berg, M.M., Sternberg, D.W., Hempstead, B.L., and Chao, M.V. (1991). The low-affinity p75 nerve growth factor (NGF) receptor mediates NGF-induced tyrosine phosphorylation. Proc. Natl. Acad. Sci. USA *88*, 7106–7110.

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

Bottenstein, J.E., Skaper, S.D., Varon, S.S., and Sato, G.H. (1980). Selective survival of neurons from chick embryo sensory ganglionic dissociates utilizing serum-free supplemented medium. Exp. Cell Res. *125*, 183–190.

Chandler, C.E., Parsons, L.M., Hosang, M., and Shooter, E.M. (1984). A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. J. Biol. Chem. *259*, 6882–6889.

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

Davies, A.M., Lee, K.-F., and Jaenisch, R. (1993). p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. Neuron *11*, 565–574.

Eide, F.F., Lowenstein, D.H., and Reichardt, L.F. (1993). Neurotrophins and their receptors—current concepts and implications for neurologic disease. Exp. Neurol. *121*, 200–214.

Ganderton, R.H., Whittaker, J., and Siddle, K. (1989). Turnover of insulin receptors in cells transfected with human insulin receptor cDNA. Biochem. Soc. Trans. *17*, 197–198.

Gherzi, R., Russell, D.S., Taylor, S.I., and Rosen, O.M. (1987). Reevaluation of the evidence that an antibody to the insulin receptor is insulinmimetic without activating the protein tyrosine kinase activity of the receptor. J. Biol. Chem. *262*, 16900–16905.

Glass, D.J., Nye, S.H., Hantzopoulos, P., Macchi, M.J., Squinto, S.P., Goldfarb, M., and Yancopoulos, G.D. (1991). TrkB mediates BDNF/NT-3-dependent survival and proliferation in fibroblasts lacking the low affinity NGF receptor. Cell *66*, 405–413.

Greene, L.A., and Tischler, A. (1976). Establishment of a nonadrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc. Natl. Acad. Sci. USA *73*, 2424–2428.

Grob, P.M., and Bothwell, M.A. (1983). Modification of nerve growth factor receptor properties by wheat germ agglutinin. J. Biol. Chem. *258*, 14136–14143.

Harlow, E., and Lane, D.P. (1988). Antibodies: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hawley, D.M., Maddux, B.A., Patel, R.G., Wong, K.-Y., Mamula, P.W., Firestone, G.L., Brunetti, A., Verspohl, E., and Goldfine, I.D. (1989). Insulin receptor monoclonal antibodies that mimic insulin action without activating tyrosine kinase. J. Biol. Chem. *264*, 2438–2444.

Hawrot, E., and Patterson, P.H. (1979). Long-term culture of dissociated sympathetic neurons. Methods Enzymol. *53*, 574–584.

Hempstead, B.L., Martin, Z.D., Kaplan, D.R., Parada, L.F., and Chao, M.V. (1991). High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor [see comments]. Nature *350*, 678–683.

Holtzman, D.M., Li, Y., Parada, L.F., Kinsman, S., Chen, C.K., Valletta, J.S., Zhou, J., Long, J.B., and Mobley, W.C. (1992). p140trk mRNA marks NGF-responsive forebrain neurons: evidence that trk gene expression is induced by NGF. Neuron *9*, 465–478.

Hosang, M., and Shooter, E.M. (1985). Molecular characteristics of nerve growth factor receptors on PC12 cells. J. Biol. Chem. *260*, 655–662.

Hosang, M., and Shooter, E.M. (1987). The internalization of nerve growth factor by high-affinity receptors on pheochromocytoma PC12 cells. EMBO J. *6*, 1197–1202.

Ibanez, C.F., Ebendal, T., Barbany, G., Murray-Rust, J., Blundell, T.L., and 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.

Ibanez, C.F., Ilag, L.L., Murray-Rust, J., and Persson, H. (1993). An extended surface of binding to Trk tyrosine kinase receptors in NGF and BDNF allows the engineering of a multifunctional pan-neurotrophin. EMBO J. *12*, 2281–2293.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S.-I., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell *66*, 233–243.

Jing, S., Tapley, P., and Barbacid, M. (1992). Nerve growth factor mediates signal transduction through trk homodimer receptors. Neuron *9*, 1067–1079.

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

Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M.V., and Parada, L.F. (1991a). The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science *252*, 554–558.

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

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

Klein, R., Parada, L.F., Coulier, F., and Barbacid, M. (1989). trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. EMBO J. *8*, 3701–3709.

Korsching, S. (1993). The neurotrophic factor concept: a reexamination. J. Neurosci. *13*, 2739–2748.

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

Lee, K.-F., Bachman, K., Landis, S., and Jaenisch, R. (1994). Dependence on p75 for innervation of some sympathetic targets. Science *263*, 1447–1449.

Lee, K.F., Li, E., Huber, L.J., Landis, S.C., Sharpe, A.H., Chao, M.V., and Jaenisch, R. (1992). Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell *69*, 737–749.

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

Loeb, D.M., Maragos, J., Martin, Z.D., Chao, M.V., Parada, L.F., and Greene, L.A. (1991). The trk proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. Cell *66*, 961–966.

Loeb, D.M., Tsao, H., Cobb, M.H., and Greene, L.A. (1992). NGF and other growth factors induce an association between ERK1 and the NGF receptor, gp140prototrk. Neuron *9*, 1053–1065.

Maher, P.A. (1988). Nerve growth factor induces protein-tyrosine phosphorylation. Proc. Natl. Acad. Sci. USA *85*, 6788–6791.

Martin-Zanca, D., Barbacid, M., and Parada, L.F. (1990). Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. Genes Dev *4*, 683–694.

Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T., and Barbacid, M. (1989). Molecular and biochemical characterization of the human trk proto-oncogene. Mol. Cell. Biol. *9*, 24–33.

Meakin, S.O., and Shooter, E.M. (1991). Molecular characterizations on the high-affinity nerve growth factor receptor. Neuron *6*, 153–163.

Meakin, S.O., and Shooter, E.M. (1992). The nerve growth factor family of receptors. Trends Neurosci. *15*, 323–331.

Meakin, S.O., Suter, U., Drinkwater, C.C., Welcher, A.A., and Shooter, E.M. (1992). The rat trk protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. Proc. Natl. Acad. Sci. USA *89*, 2374–2378.

Middlemas, D.S., Lindberg, R.A., and Hunter, T. (1991). trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol. Cell. Biol. *11*, 143–153.

Mobley, W.C., Schenker, A., and Shooter, E.M. (1976). Characterization and isolation of proteolytically modified nerve growth factor. Biochemistry *15*, 5543–5552.

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

Obermeier, A., Halfter, H., Wiesmullere, K.-H., Jung, G., Schlessinger, J., and Ullrich, A. (1993). Tyrosine 785 is a major determinant of Trk-substrate interaction. EMBO J. *12*, 933–941.

Rabizadeh, S., Oh, J., Zhong, L., Yang, J., Bitler, C.M., Butcher, L.L., and Bredesen, D.E. (1993). Induction of apoptosis by the low-affinity NGF receptor. Science *261*, 345–348.

Radeke, M.J., and Feinstein, S.C. (1991). Analytical purification of the slow, high affinity NGF receptor: identification of a novel 135 kd polypeptide. Neuron *7*, 141–150.

Radeke, M.J., Misko, T.P., Hsu, C., Herzenberg, L.A., and Shooter, E.M. (1987). Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature *325*, 593–597.

Rovelli, G., Heller, R.A., Canossa, M., and Shooter, E.M. (1993). Chimeric tumor necrosis factor-TrkA receptors reveal that ligand-dependent activation of the trkA tyrosine kinase is sufficient for differentiation and survival of PC12 cells. Proc. Natl. Acad. Sci. USA *90*, 8717–8721.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schechter, A.L., and Bothwell, M.A. (1981). Nerve growth factor receptors on PC12 cells: evidence for two receptor classes with differing cytoskeletal association. Cell *24*, 867–874.

Schecterson, L.C., and Bothwell, M. (1992). Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron *9*, 449–463.

Seed, B. (1987). An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. Nature *329*, 840–842.

Seed, B., and Aruffo, A. (1987). Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. Proc. Natl. Acad. Sci. USA *84*, 3365–3369.

Soos, M.A., O'Brien, R.M., Brindle, N.P.J., Stigter, J.M., Okamoto, A.K., Whittaker, J., and Siddle, K. (1989). Monoclonal antibodies to the insulin receptor mimic metabolic effects of insulin but do not stimulate receptor autophosphorylation in transfected NIH 3T3 fibroblasts. Proc. Natl. Acad. Sci. USA *86*, 5217–5221.

Soppet, D., Escandon, E., Maragos, J., Middlemas, D.S., Reid, S.W., Blair, J., Burton, L.E., Stanton, B.R., Kaplan, D.R., and Hunter, T. (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell *65*, 895–903.

Spaargaren, M., Defize, L.H.K., Boonstra, J., and de Laat, S.W. (1991). Antibody-induced dimerization activates the epidermal growth factor receptor tyrosine kinase. J. Biol. Chem. *266*, 1733–1739.

Squinto, S.P., Stitt, T.N., Aldrich, T.H., Davis, S., Bianco, S.M., Radziejewski, C., Glass, D.J., Masiakowski, P., Furth, M.E., and Valenzuela, D.M. (1991). trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell *65*, 885–893.

Summers, M.D., and Smith, G.E. (1987). A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, College Station, TX: Texas Agricultural Station.

Sutter, A., Riopelle, R.J., Harris-Warrick, R.M., and Shooter, E. (1979). Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia cells. J. Biol. Chem. *254*, 5972–5982.

Vetter, M.L., Martin, Z.D., Parada, L.F., Bishop, J.M., and Kaplan, D.R. (1991). Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-gamma 1 by a kinase activity associated with the product of the trk protooncogene. Proc. Natl. Acad. Sci. USA *88*, 5650–5654.

Webb, N.R., and Summers, M.D. (1990). Expression of proteins using recombinant baculoviruses. Technique *2*, 173–188.

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

Wheeler, E.F., and Bothwell, M. (1992). Spatiotemporal patterns of expression of NGF and the low-affinity NGF receptor in rat embryos suggest functional roles in tissue morphogenesis and myogenesis. J. Neurosci. *12*, 930–945.

Wiley, H.S., and Cunningham, D.D. (1981). A steady state model for analyzing the cellular binding, internalization and degradation of polypeptide ligands. Cell *25*, 433–440.

Wyatt, S., Shooter, E.M., and Davies, A.M. (1990). Expression of the NGF receptor gene in sensory neurons and their cutaneous targets prior to and during innervation. Neuron *4*, 421–427.

Xiong, L., Kasuya, J., Li, S.-L., Kato, J., and Fujita-Yamaguchi, Y. (1992). Growth-stimulatory monoclonal antibodies against human insulin-like growth factor I receptor. Proc. Natl. Acad. Sci. USA *89*, 5356–5360.