The Neurotrophin Receptor, gp75, Forms a Complex with the Receptor Tyrosine Kinase TrkA

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Abstract. The high-affinity NGF receptor is thought to be a complex of two receptors, gp75 and the tyrosine kinase TrkA, but direct biochemical evidence for such an association has been lacking. In this report, we demonstrate the existence of such a gp75–TrkA complex by a copatching technique. Gp75 on the surface of intact cells is patched with an anti-gp75 antibody and fluorescent secondary antibody, the cells are then fixed to prevent further antibody-induced redistributions, and the distribution of TrkA is probed with an anti-TrkA antibody and fluorescent secondary antibody. We utilize a baculovirus–insect cell expression system which allows high level expression of wild-type and mutated NGF receptors. TrkA and gp75 copatch in both the absence and presence of NGF. This association is specific, since gp75 does not copatch with other tyrosine kinase receptors, including TrkB, platelet-derived growth factor receptor-β, and Torso (Tor). To determine which domains of TrkA are required for copatching, we used a series of TrkA-Tor chimeric receptors and show that the extracellular domain of TrkA is sufficient for copatching with gp75. A chimeric receptor with TrkA transmembrane and intracellular domains shows partial copatching with gp75. Deletion of the intracellular domain of gp75 decreases but does not eliminate copatching. A point mutation which inactivates the TrkA kinase has no effect on copatching, indicating that this enzymatic activity is not required for association with gp75. Hence, although interactions between the gp75 and TrkA extracellular domains are sufficient for complex formation, interactions involving other receptor domains also play a role.

NGF is an essential neurotrophic factor required for the development, maintenance, and repair of the nervous system (41). All of the actions of NGF are thought to be mediated by two cell surface receptors, gp75 and TrkA (1, 5). Gp75 is a 75,000-D glycoprotein with a cysteine-rich extracellular domain, a single transmembrane domain and a 155–amino acid cytoplasmic domain (21, 38). It has been suggested that gp75 activates signal transduction by interacting with G proteins (10), cytoplasmic kinases (51), or by initiating sphingomyelin hydrolysis (8). In addition to binding NGF, gp75 binds other members of the neurotrophin family (brain-derived neurotrophic factor (BDNF),[1] neurotrophin-3 (NT-3), and

neurotrophin-4 (NT-4) (39). The second NGF receptor is TrkA, a 140,000-D transmembrane protein with an intracellular tyrosine-specific kinase domain. Binding of NGF activates the TrkA kinase and initiates a signal transduction cascade (23, 26). In addition to TrkA, there are two other members of the Trk family of receptors, TrkB and TrkC. Whereas TrkA preferentially binds NGF, TrkB binds both BDNF and NT-4, and TrkC binds NT-3 (1, 5).

A number of experiments indicate that the two NGF receptors interact and synergize with each other. Addition of NGF to the developing isthmo-optic nucleus inhibits the neurotrophic effects of BDNF and NT-3, presumably by competing for binding to gp75 (52). Transgenic mice which lack gp75 have neurological deficiencies involving death of TrkA-positive sensory neurons (27). Both gp75 and TrkA undergo retrograde transport from the synapse to the cell body (9, 22, 28). In cell culture models, co-expression of gp75 enhances the biological response of cells expressing TrkA (12, 17, 31, 36, 49). Although Jing et al. (20) report that TrkA is sufficient for high-affinity NGF binding, Hempstead et al. (14) find that expression of both gp75 and TrkA is required for high-affinity binding. In addition, co-expression of gp75 with TrkA enhances the rate of

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1. *Abbreviations used in this paper:* BDNF, brain-derived neurotrophic factor; FRAP, fluorescence recovery after photobleaching; NT-3 and -4, neurotrophin-3 and -4; PDGFR-β, PDGF receptor-β; TGF-β, transforming growth factor-β.

NGF binding (29). Antibody to gp75 inhibits responses of PC12 cells to NGF (2, 4). The synergistic action of these receptors has led to the hypothesis that gp75 and TrkA form a heteromolecular complex which is the high-affinity NGF receptor (14). However, thusfar, chemical cross-linking and co-immunoprecipitation have yielded only limited evidence for the complex (19).

Since solubilization of membranes with detergent can disrupt complexes of membrane proteins (25, 34), co-immunoprecipitation of membrane proteins from detergent extracts detects a limited subset of interactions. An alternative approach which overcomes this problem is to study these interactions in intact membranes using appropriate immunological and biophysical techniques. For example, types II and III TGF-β receptors show extensive copatching on the surface of intact cells, but co-immunoprecipitation of these receptors from detergent extracts is slight (18). In another case, the association between the erythrocyte anion transporter and glycophorin A was detected by measurements of rotational diffusion in intact membranes, while the complex failed to be detected by co-immunoprecipitation (33).

Using fluorescence recovery after photobleaching (FRAP), we have measured the lateral diffusion of gp75 in intact cells (46, 47, 54). Gp75 is highly mobile on cells which lack TrkA and are nonresponsive to NGF. In contrast, gp75 is relatively immobilized on TrkA-expressing, NGF-responsive cells (46). Expression of recombinant TrkA in cells lacking endogenous TrkA causes immobilization of gp75, even in the absence of NGF (54). These biophysical studies demonstrate an interaction between gp75 and TrkA supporting, but not proving, the concept of a specific intermolecular complex (14). Furthermore, we were surprised to find that the interaction between gp75 and TrkA was not synonomous with the display of high-affinity NGF binding sites. Gp75(Xba) is a truncated gp75 lacking nearly all of the intracellular domain, and TrkA(K538N) bears a point mutation which inactivates the tyrosine kinase domain. Cells expressing gp75(Xba) + TrkA or gp75 + TrkA(K538N) receptors have little or no capacity for high-affinity binding of NGF (15) (Stephens, R.M., D.R. Kaplan, B.L. Hempstead, personal communication). Based on analysis of diffusion coefficients, an interaction between gp75 and TrkA was detected for cells expressing either gp75(Xba) + TrkA or gp75 + TrkA(K538N).

In this study, we use copatching, a technique that has been used extensively to study interactions between cell surface proteins (3, 11, 18), to further test and refine our model for NGF receptor structure. In this procedure, addition of an antibody directed against a cell surface antigen followed by addition of a fluorescent secondary antibody results in two-dimensional aggregates or patches on the cell surface. The cells are fixed to prevent further antibody-induced redistribution, and an antibody directed against a second cell surface antigen is used to determine whether that second antigen is included in the patches. To facilitate these studies, we have prepared a TrkA-specific mAb which binds to the extracellular domain. We find that gp75 and TrkA copatch in the absence of added NGF. In addition, copatching was detected for gp75(Xba)–TrkA and gp75–TrkA(K538N). These experiments provide direct evidence for a gp75–TrkA complex.

Materials and Methods

Baculovirus Vectors

The structures of the receptors used in this study are shown in Fig. 1. Recombinant baculovirus vectors for wild type and mutant human gp75 and TrkA and rat TrkB were described previously (44, 54). Recombinant baculovirus vectors for Tor (43) and murine PDGF receptor-β (PDGFR-β) (24) were obtained from D. Morrison (NCI-FCRDC, Frederick, MD).

Trk/Torso chimeras (trk/tor/tor and tor/trk/trk) were constructed by a two-step procedure. First, both human TrkA and Drosophila Torso (a generous gift from F. Sprenger, Max-Planck-Institut für Entwicklungsbiologie, Tubingen, Germany) cDNAs were mutagenized to introduce a unique Mro1 restriction site on the extracellular side of the transmembrane sequence region of both receptors. The Mro1 site in TrkA was generated by mutating amino acids 404 and 405 from lysines to asparagine and proline, respectively. The second step in the chimera construction involved the ligation of respective 5' and 3' halves of the genes to each other. tor/tor/trk was constructed by fusing a region of the intracellular domains of Torso at the BstE2 site at amino acid 454 with a BspH1 site in TrkA at amino acid 463 using Klenow fragment to fill in overhangs generated by restriction enzyme digestion. The two fragments were then fused in frame to form the full-length chimeric receptor. The TrkA extracellular domain construct was generated by PCR to form a molecule with a stop codon in the extracellular domain to create a 407–amino acid TrkA protein. All three constructs were then cloned into the baculovirus expression
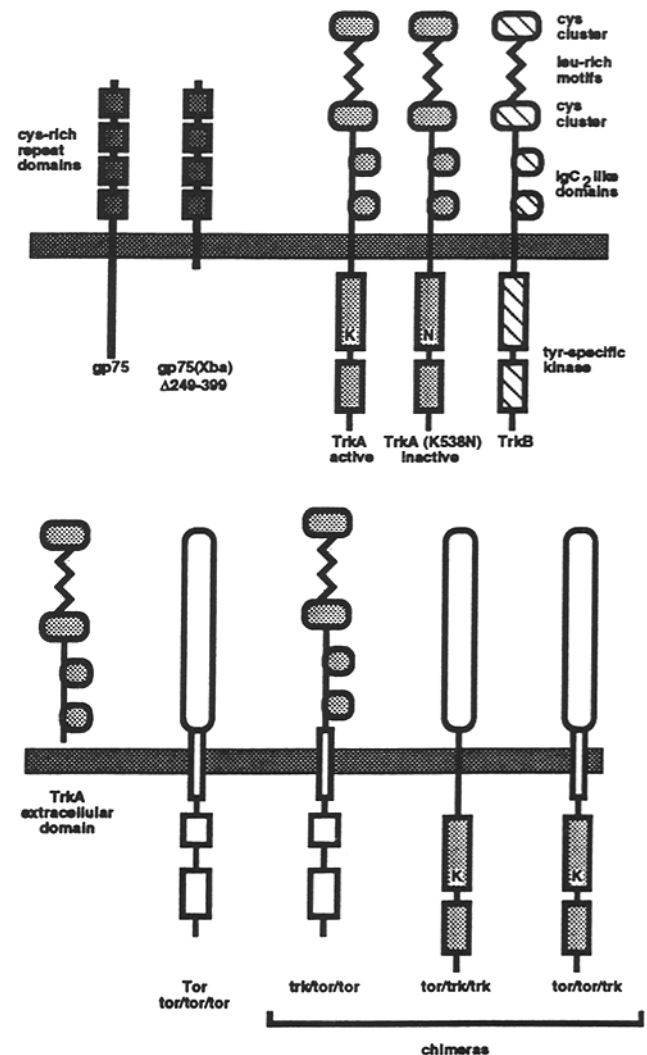

Figure 1. Schematic of neurotrophin receptors: wild type, mutated, and chimeras.

vector pAcc4 as NcoI to EcoR1 fragments. TrkA extracellular domain was secreted from Sf9 cells infected with the recombinant baculovirus for this protein. All three chimeric receptors exhibited ligand-independent tyrosine kinase activity when overexpressed in Sf9 cells. Activity of the trk/tor/tor chimeric receptor could be enhanced by addition of NGF to cells expressing this receptor.

Cell Lines

Sf9 insect cells were maintained in TMN-FH medium from JRH Biosciences (Lenexa, KS) supplemented with 9% heat-inactivated fetal bovine serum and 50 μg/ml of gentamicin at 28–29°C (45). COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 50 μg/ml of gentamicin.

Antibodies

Antibodies against gp75. Two antibodies against gp75 were used in these studies. NGFR5, a mouse mAb directed against human gp75, has been described (30). A rabbit serum R7,8 was prepared against the recombinant extracellular domain of human gp75 (50). Rabbits were immunized with 0.4 mg of extracellular domain in complete Freund's adjuvant followed by two boosts with 0.5 mg of extracellular domain in incomplete Freund's adjuvant. This antiserum specifically detected gp75 by both immunoprecipitation and immunofluorescence labeling of cells. The specificity of this antiserum was similar to that of earlier antisera prepared against extracellular domains of chick and rat gp75 (53). Antibody from R7,8 antiserum was prepared by affinity chromatography with a protein A column.

Antibodies against TrkA. Two anti-TrkA antibodies were used in these studies. Rabbit antiserum 203 is an anti-pan-Trk reagent directed against the Trk COOH terminus (16). The preparation and characterization of anti-TrkA mAb, TA-1, is described below.

Other Antibodies. Antiserum anti-TrkBout is an anti-peptide antibody specific for TrkB (residues 76–96). The anti-Torso antibody is a polyclonal rabbit antiserum directed against the extracellular domain of *Drosophila* receptor tyrosine kinase, Tor (42), and was provided by F. Sprenger and D. Morrison. Antiserum directed against a peptide (residues 1013–1025) from human PDGFR-β was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Ascites fluid for anti-hemagglutinin monoclonal antibody 12CA5 was purchased from Babco (Richmond, CA). Fluorescein- and rhodamine-labeled anti-IgG secondary goat antibodies were obtained from Fisher Biotech (Springfield, NJ) or Jackson ImmunoResearch Laboratories (West Grove, PA).

Preparation of Anti-TrkA mAb

COS-7 cells were transfected with the expression vector, pCMV-*htrk*, encoding the human TrkA receptor (14). At 60 h after transfection, the TrkA-expressing COS-7 cells (3×10^6 per mouse) were suspended in Dulbecco's PBS and were used to immunize 6-wk-old female BALB/c mice (Charles River, Wilmington, MA). Five mice were used per protocol. The mice were injected subcutaneously in the lower leg (protocol 1; 50 μl per mouse), in the tail vein (protocol 2; 150 μl) or intraperitoneally (protocol 3; 500 μl). They were boosted every third day for 2 wk, using the same routes of injection. The mice were bled, and the resulting antisera were screened by immunofluorescence, as described below. Two mice from protocol 1 and one mouse from protocol 2 were found to express anti-TrkA antibodies and were injected by the same routes with 2×10^6 TrkA-expressing COS-7 cells every 14 d for 6 wk.

Cells from the spleen of a protocol 2 mouse and from the popliteal and inguinal lymph nodes of a protocol 1 mouse were fused with the nonsecreting mouse myeloma variant P3x63Ag-8.653 (2.5×10^7 cells). The fusion was carried out with 50% (wt/vol) polyethylene glycol in serum-free Dulbecco's modified Eagle's medium, as described (13). The resulting fused cells were seeded on 96-well plates in medium preconditioned with macrophage feeder cells.

Antisera and hybridoma culture supernatants were screened by immunofluorescence. Sf9 insect cells were infected with a baculovirus encoding TrkA or, as a negative control, with baculoviruses encoding gp75 or TrkB. The cells were fixed with 1% paraformaldehyde, blocked with 1 mg/ml of BSA, and stained with antisera (diluted 1:50 to 1:1,350) or with undiluted hybridoma culture supernatants. Immunoglobulin isotypes were determined using a kit from Sigma Chemical Co. (St. Louis, MO).

Radioiodination and Immunoprecipitation of Trk Extracellular Domains

The reaction was carried out in a 6 × 50 mm glass tube containing 5 μg of iodogen (Sigma Chemical Co., St. Louis, MO) dried from chloroform (40). 2 μg of the extracellular domain for TrkA, TrkB, or TrkC (35) (gifts from A. Welcher) were incubated for 15 min at 4°C with Na^{125}I (200 μCi) in 50 μl of 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.5. The protein was separated from free label on a PD10 Sephadex G-25 column (Pharmacia Inc., Piscataway, NJ). The specific activities of the iodinated proteins ranged from 2×10^7 to 6×10^7 cpm/μg.

40 μl of anti-mouse IgG agarose beads (Sigma Chemical Co.) were suspended for 1 h at 4°C in 500 μl of 0.5% (vol/vol) NP-40, 140 mM NaCl, 10 mM Trizma, 10 mM NaF, 5 mM Na$_2$EDTA, and 100 Kallikrein IU/ml of aprotinin supplemented with either 10 μl of TA-1 ascites fluid or 10 μl of 12CA5 ascites fluid. The beads were washed twice with the same buffer. Trk extracellular domains (100,000 cpm) were incubated for 2 h at 4°C with the beads. The beads were washed three times and boiled in SDS sample buffer with 2-mercaptoethanol. The samples were analyzed by SDS-PAGE with a 10% gel followed by autoradiography for 8–22 h.

Baculovirus Expression in Sf9 Cells

To express a single NGF receptor, recombinant baculovirus was added to Sf9 cells (2×10^6 cells in a 25-cm² flask) at a multiplicity of infection (m.o.i.) of 1 for the gp75 virus or a m.o.i. of 4 for TrkA. For coexpression experiments, TrkA baculovirus was added to Sf9 cells at a m.o.i. of 16 followed by immediate addition of gp75 baculovirus at m.o.i. of 1. Infections with the Tor or PDGFR-β baculoviruses were done in the same manner as TrkA. Experiments were carried out 60-h postinfection.

TrkA extracellular domain was produced in the baculovirus Sf9 system using procedures described previously (50). The extracellular domain was purified by TA-1 immunoaffinity chromatography and by ion-exchange chromatography on a MONO-Q column (Pharmacia, Piscataway, NJ).

Copatching Assay

Cells were incubated for 30 min at room temperature with an antibody directed against one of the recombinant receptor proteins expressed by the Sf9 cells. The cells were pelleted (100 g for 4 min) and washed twice with 200 μl of Sf9 medium. The cells were suspended in Sf9 medium with a fluorescein- or rhodamine-labeled anti-IgG antibody. The cells were pelleted, washed twice and fixed for 10 min at 4°C shielded from light, using a fresh solution of 1% glutaraldehyde in Dulbecco's PBS. The cells were washed twice and incubated for 30 min at room temperature with an antibody directed against the other recombinant receptor protein. The cells were washed twice and incubated with a fluorescently labeled secondary antibody and washed again.

For the copatching experiments, the primary antibodies were used at the following dilutions or concentrations: R7,8 antibody, 0.18 mg/ml; NGFR5 antibody, 0.01 mg/ml; TA-1 ascites, 1:100; 203 antiserum, 1:400; anti-Tor antiserum, 1:50; anti-PDGFR-β antiserum, 1:50. Secondary fluorescein- and rhodamine-labeled antibodies were used at 40–75 μg/ml.

Copatching was screened on a Zeiss Axiovert microscope using an oil immersion 100× planapochromat objective (NA = 1.4) under 100 W Hg illumination. Fluorescein and rhodamine were visualized using Carl Zeiss 909 and 914 filter cubes, respectively. Cells for which >50% of the patches exhibited both fluorescein and rhodamine fluorescence were scored as positive. Although the choice of 50% is arbitrary, the scoring is not greatly affected by this parameter. Most cells are either nearly completely copatched or not copatched at all. Hence, for most cells, setting the cut-off at 25, or 50, or 75% would not change the basic results. In each experiment, ~100 cells were assessed for copatching.

Confocal Microscopy

Photomicrographs were obtained using a Bio-Rad Laboratories (Hercules, CA) MRC 1000 scanhead on a Nikon 200 Diavert microscope using a 60× planapochromatic oil immersion objective (NA = 1.4). The confocal was operated in the two color mode using the T1-Triple Dichroic and TA2-D560 DRLP filter blocks. All images were taken at a zoom setting of 3.2 using slit widths of 2.4–3.0 mm, Kahlman averaging over 20 frames. Images were contrast stretched and then local contrast enhanced. Collected images were colorized using Universal Imaging Metamorph (Westchester, PA) software, and fluorescein and rhodamine images were

merged together. In these combined images, areas of copatching are yellow. The individual and merged images were assembled into montages with CorelDraw (Ontario, Canada) and ultimately printed with a Kodak 8600 dye sublimation color printer.

Results

Preparation and Characterization of Anti-TrkA mAb

To perform these experiments, it was necessary to produce an antibody specific to the extracellular domain of TrkA. Mice were immunized with TrkA-expressing COS-7 cells as described in the Materials and Methods. An IgG$_3$-producing hybridoma, TA-1, was selected and found to immunostain TrkA-expressing Sf9 cells, but not TrkB- or gp75-expressing Sf9 cells (Fig. 2, C and D, not shown for TrkB). In contrast, the anti-gp75 rabbit antiserum R7,8 stained gp75-expressing cells and not TrkA-expressing cells (Fig. 2, A and B). TA-1 also stained TrkA-expressing COS-7 cells (not shown) and cholinergic neurons in rat brain sections (28). TA-1 immunoprecipitated the TrkA extracellular domain but not the TrkB or TrkC extracellular domains (Fig. 3). The epitope for TA-1 appears to be conformation dependent and distinct from the NGF-binding site, since this mAb did not stain TrkA in Western blotting analyses and did not block the effects of NGF on PC12 cells (not shown). To our knowledge, this is the first TrkA-specific mAb to be reported, although there is a TrkA-specific rabbit antiserum (6).

Copatching Studies

In this paper, we exploit patching by antibodies R7,8 and TA-1 (Fig. 2) to demonstrate a heteromolecular complex. The fundamental paradigm of these copatching experiments is to label and patch gp75 on the cell surface, to fix the cells to prevent further redistributions, and then to stain TrkA by immunofluorescence in a second color.

It was first necessary to determine fixation conditions which effectively eliminate antibody-induced patching. Noncross-linking fixatives such as paraformaldehyde do

not immobilize surface proteins and do not prevent redistributions (32). Sf9 cells coexpressing gp75 and TrkA were fixed with 0.1–1.0% glutaraldehyde as well as mixtures of glutaraldehyde and paraformaldehyde. They were then labeled sequentially with R7,8 antibody, rhodamine goat anti–rabbit IgG, TA-1 antibody, and fluorescein goat anti–mouse IgG. Pressure from the coverslip slightly flattened the cells, thereby, presenting a large flat surface suitable for observation with the confocal microscope. We ultimately selected fixation of cells with 0.75% glutaraldehyde in Dulbecco's PBS for 10 min in the dark at 4°C because this was the lowest concentration of glutaraldehyde which consistently prevented redistribution of antigens (Fig. 4, A–C). Use of glutaraldehyde resulted in some autofluorescence, particularly in the cytoplasm. However, because autofluorescence was weaker at the surface of the cell and immunostaining of the baculovirus-infected Sf9 cells was strong, scoring of copatching was not hindered.

Fig. 4 (D–F) show a typical copatching experiment. Sf9 cells expressing both gp75 and TrkA were incubated with R7,8 anti-gp75 antibody followed by rhodamine anti–rabbit IgG, fixed, and then labeled with anti-TrkA mAb TA-1 followed by fluorescein anti–mouse IgG. Using the confocal microscope, we observed extensive co-localization of TrkA and gp75 (Fig. 4, D–F). In the merged images (Fig. 4, C, F, and I), areas of copatching are yellow.

Several possible artifacts were considered. The cross-linking experiment was repeated, using a fluorescein secondary antibody for gp75 and rhodamine secondary antibody for TrkA. The results were essentially identical to those shown in Fig. 4 (D–F), demonstrating that filter bleedthrough was not a problem. Also, since omission of either primary antibody resulted in neglible staining with the corresponding secondary antibody, there was no cross-reactivity of the secondary antibodies. For cells expressing gp75 but not TrkA, gp75 patches were detected, but no TrkA staining was detected. For cells expressing TrkA but not gp75, gp75 staining was not detected and TrkA staining was uniform. Copatches were additionally observed using anti-gp75 mAb NGFR5 and a different anti-Trk antiserum, 203. Copatches also were observed if TrkA was patched with mAb TA-1, the cells were fixed and gp75 was detected with R7,8 antibody. Hence, the immunostaining in these experiments was specific, and the copatching occurred using several different anti-NGF receptor antibodies and labeling protocols.

To quantitate these results, we scored the Sf9 cells for copatching (Fig. 5, see Materials and Methods for details).

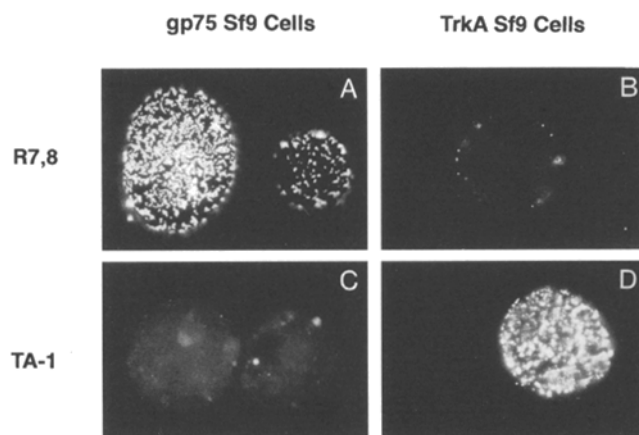gp75 Sf9 Cells   TrkA Sf9 Cells

Figure 2. Specificities of R7,8 and TA-1 antibodies. Sf9 cells infected with a baculovirus encoding gp75 (A and C) or encoding TrkA (B and D) were immunostained with anti-gp75 antibody R7,8 (0.18 mg/ml) (A and B) or anti-TrkA mAb TA-1 (ascites 1:100) (C and D). As shown in A, gp75-expressing cells stain positively with R7,8, and TrkA-expressing cells stain with TA-1.
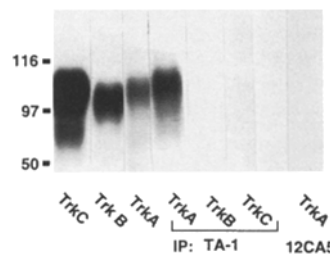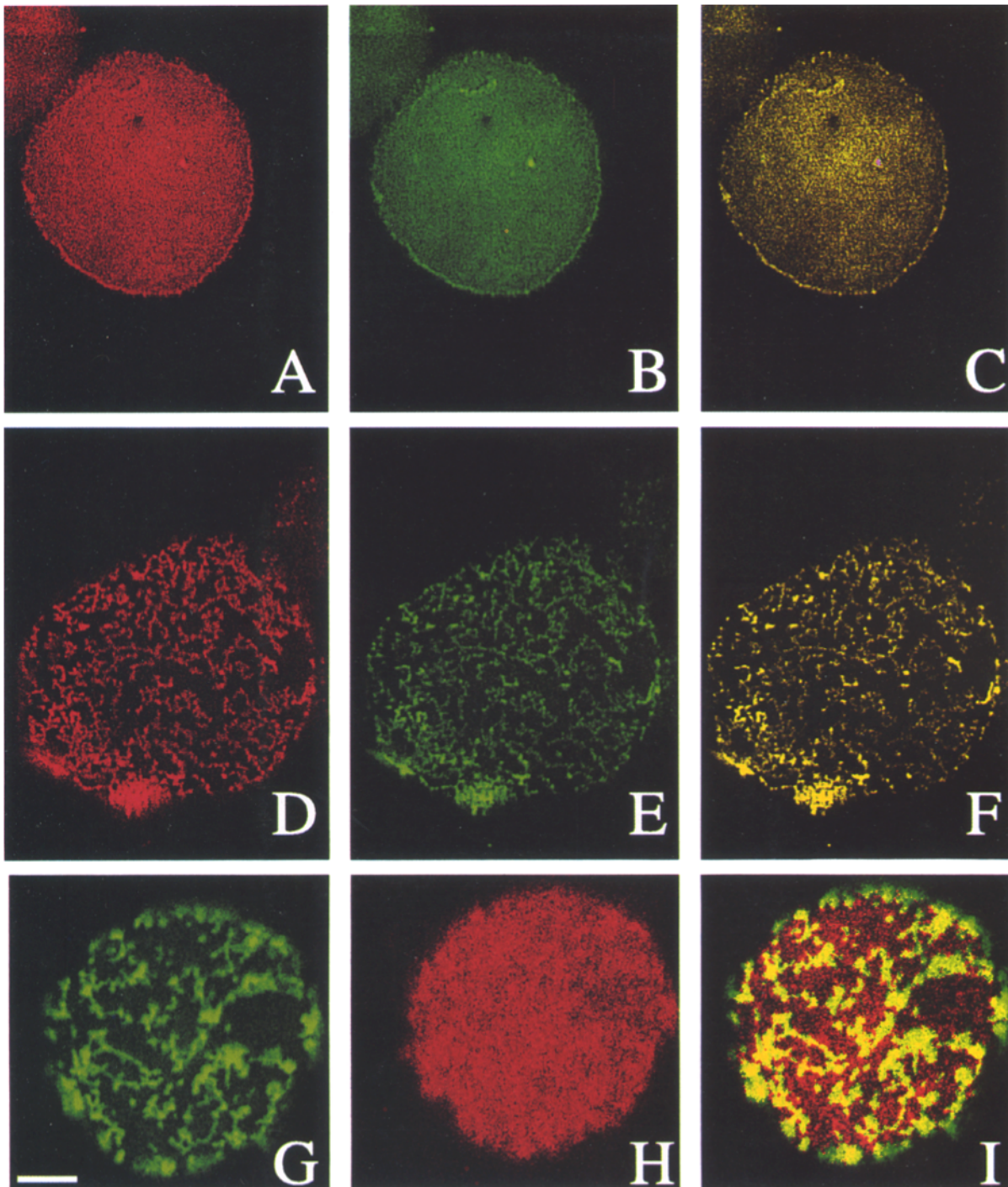
Figure 4. Confocal photomicrographs showing copatching of TrkA, but not Tor, with gp75. *A–C* show the lack of patching when cells were first fixed with 0.75% glutaraldehyde and subsequently labeled for gp75 (*A*) and TrkA (*B*). The sum of images *A* and *B* is shown in *C*, as described in Materials and Methods. This result demonstrates the effectiveness of 0.75% glutaraldehyde in preventing antibody-induced patching. (*D–F*) show copatching of TrkA (*E*) with gp75 (*D*) and the combined image (*F*). gp75 was labeled and patched with R7,8 (0.18 mg/ml) followed by rhodamine goat anti–rabbit IgG. The cells were then fixed with 0.75% glutaraldehyde, and the TrkA was labeled with TA-1 (ascites 1:100) followed by fluorescein goat anti–mouse IgG. A very high degree of copatching (*yellow* in *F*) is observed. *G–I* show the lack of copatching for gp75 (*G*) and Tor (*H*). Cells were treated as in *D–F* except that gp75 was patched and labeled with NGFR5 (0.01 mg/ml) followed by fluorescein goat anti–mouse IgG. Following fixation of the cells, Tor was labeled with anti–Tor rabbit serum (1:50) followed by rhodamine goat anti–rabbit IgG. The combined image (*I*) shows both red and green areas indicating a lack of copatching. Bar, 5 μm.
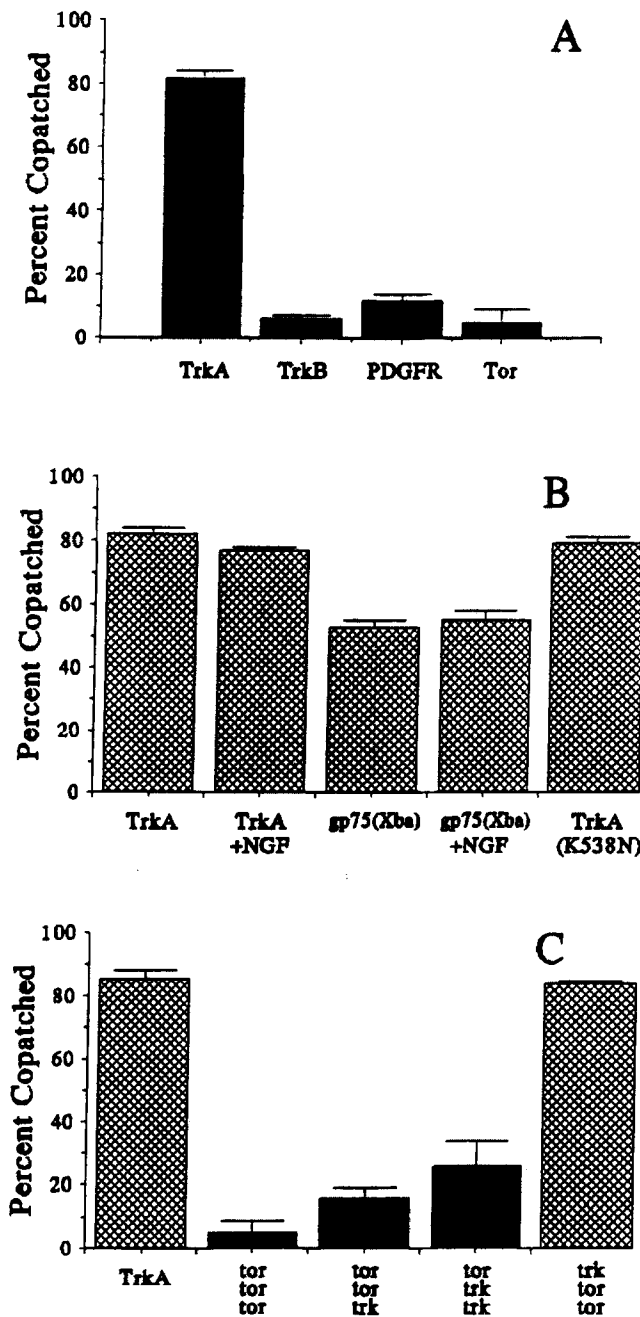
Figure 5. Quantitative assay of copatching. (A) Gp75 copatches with TrkA but not with other receptor tyrosine kinases. (B) Co-patching between modified forms of gp75 and TrkA. TrkA co-patches with wild-type gp75 and a truncated receptor, gp75(Xba). TrkA(K538N) which bears an inactive kinase domain copatches with gp75. Addition of NGF does not enhance copatching. (C) Copatching of gp75 with TrkA-Tor chimeric receptors. The extracellular, transmembrane and intracellular domains of TrkA play a role in formation of the gp75-TrkA complex, but the extracellular domain of TrkA apparently has the largest role. Details of these experiments are given in the text. Gp75 was patched using either mAb NGFR5 (0.01 mg/ml) (solid bars) or rabbit antibody R7,8 (0.18 mg/ml) (cross-hatched bars). The rabbit antibody was visualized with a rhodamine anti-rabbit IgG and the mAb with a fluorescein anti-mouse IgG. The Trk or other tyrosine kinase receptor was then labeled with the other species (rabbit/mouse) of primary antibody and the other color (rhodamine/fluorescein) secondary antibody. Data are given as mean ± SEM.

Averaging eighteen experiments of the type shown in Fig. 4 (D-F) (R7,8 and TA-1 antibodies), we found that 85 ± 3% (n = 18) of the cells were copatched. Similar results (82 ± 2% of cells, n = 6) were obtained using mAb NGFR5 for gp75 and rabbit antibody 203 against TrkA. Several other receptor kinases were co-expressed with gp75 and found to give very low levels of copatching: TrkB (6 ± 1%, n = 6), PDGFR-β (12 ± 2%, n = 5), and Drosophila protein Tor (5 ± 4%, n = 5). All of these values are significantly less than that for TrkA (P ≤ 0.0005 by the t test). Fig. 4 (G-I) shows a cell expressing gp75 and Tor. The R7,8 anti-gp75 antibody resulted in patching of gp75, but there is little or no copatching of Tor.

Results of our previous study (54) using FRAP demonstrated that a mutant form of gp75 which lacks its cytoplasmic domain, gp75(Xba), interacts with wild-type TrkA and similarly that a kinase-deficient TrkA mutant TrkA(K538N) interacts with wild-type gp75. These findings were tested using the copatching assay (Fig. 5 B). The gp75(Xba) receptor copatched with TrkA, albeit at a reduced level (53 ± 2% of cells, n = 6, P ≤ 0.01) but still significantly greater than the negative controls (P ≤ 0.0005). TrkA (K538N) was as effective as wild-type TrkA in copatching with gp75 (79 ± 2%, n = 4). Hence, the copatching results confirm the interactions suggested by the the earlier FRAP experiments.

Additionally, we tested by copatching (Fig. 5 B) whether gp75-TrkA interactions are enhanced by the presence of NGF. Sf9 cells expressing gp75 and TrkA were incubated with 100 nM of NGF for 15 min. Then the copatching assay was carried out, including NGF with the R7,8 anti-gp75 antibody and the rhodamine anti-rabbit IgG antibody. Copatching in the presence of NGF (79 ± 2% of cells, n = 5) was similar to the NGF-minus result. It was possible that NGF did not enhance gp75-TrkA copatching because the level of copatching was already maximal. Hence, we tested whether NGF enhances copatching of gp75(Xba)-TrkA and found 55 ± 5% (n = 3) of cells copatched. The plus-NGF and the minus-NGF results for copatching are not statistically different, demonstrating that NGF does not significantly influence this interaction.

To determine which domains of TrkA are required for copatching with gp75, we studied a series of chimeric receptors derived from TrkA and Tor (Fig. 5 C). We chose Tor for these studies because Tor and TrkA have the same overall structure (extracellular NH2 terminus, single transmembrane domain and intracellular kinase domain), but the sequences of the two receptors are not closely homologous. As discussed above, the wild-type Tor (tor/tor/tor which has Tor extracellular, transmembrane and intracellular domains) exhibited copatching for 5 ± 4% (n = 5) of the cells. Substitution of the intracellular TrkA domain for the Tor intracellular domain (tor/tor/trk) led to 16 ± 3% (n = 6) of cells copatched, only slightly higher than tor/tor/tor (P ≤ 0.05) and significantly lower than wild-type trk/trk/trk (P ≤ 0.0005). For the tor/trk/trk chimera, 26 ± 8% (n = 10) of cells were copatched, again higher than tor/tor/tor (P ≤ 0.025) and significantly lower than trk/trk/trk (P ≤ 0.0005). In contrast, the trk/tor/tor chimera formed copatches with gp75 for 84 ± 1% (n = 4) of the cells which is statistically equivalent to trk/trk/trk. Thus, although the intracellular and transmembrane domains of TrkA can in-

duce a complex with gp75, the extracellular domain of TrkA appears to dominate this interaction.

To further examine the role of the TrkA extracellular domain in copatching with gp75, we considered whether soluble TrkA–extracellular domain would compete with membrane-bound TrkA for binding to gp75. We prepared a soluble TrkA-extracellular domain using the baculovirus insect cell system (see Materials and Methods). Fig. 6 *D* shows that by SDS-PAGE the 64,000-D extracellular domain is >90% pure. The molecular weight of this TrkA extracellular domain which was prepared in insect cells (see Materials and Methods) was less than that for the extracellular domain prepared in CHO cells (Fig. 3). This difference is presumably due to decreased glycosylation in the insect cells.

Recombinant TrkA-extracellular domain was included with the anti-gp75 antibody R7,8 and the rhodamine anti–rabbit IgG secondary antibody. The cells were then fixed and stained with anti-TrkA mAb TA-1 and the fluorescein anti–mouse IgG secondary antibody (Fig. 6, *A–C*). Since gp75-expressing Sf9 cells incubated with TrkA extracellular domain did not stain with anti-TrkA mAb (not shown),

the staining in the copatching experiment was specific for the membrane-bound TrkA and not for TrkA extracellular domain that might be nonspecifically adhering to the cells. The presence of the TrkA-extracellular domain reduced the level of gp75-TrkA copatching to $51 \pm 9\%$ ($n = 5$) ($P \leq 0.005$) (Fig. 6 *E*). Therefore, the gp75–TrkA complex detected by copatching results from a saturable interaction at the cell surface.

Discussion

In this study of NGF receptor complexes, we use a copatching technique which in many regards is the two-dimensional analogue of immunoprecipitation. In particular, associations with other cellular components are expected to remain intact under the experimental conditions for copatching. Using monoclonal and polyclonal anti-NGF receptor antibodies, we detected copatching and, therefore, intermolecular complexes of gp75 and TrkA on the surface of Sf9 insect cells. The use of the baculovirus insect cell system greatly aided these studies by allowing rapid, high level expression of these receptors. Analysis of other
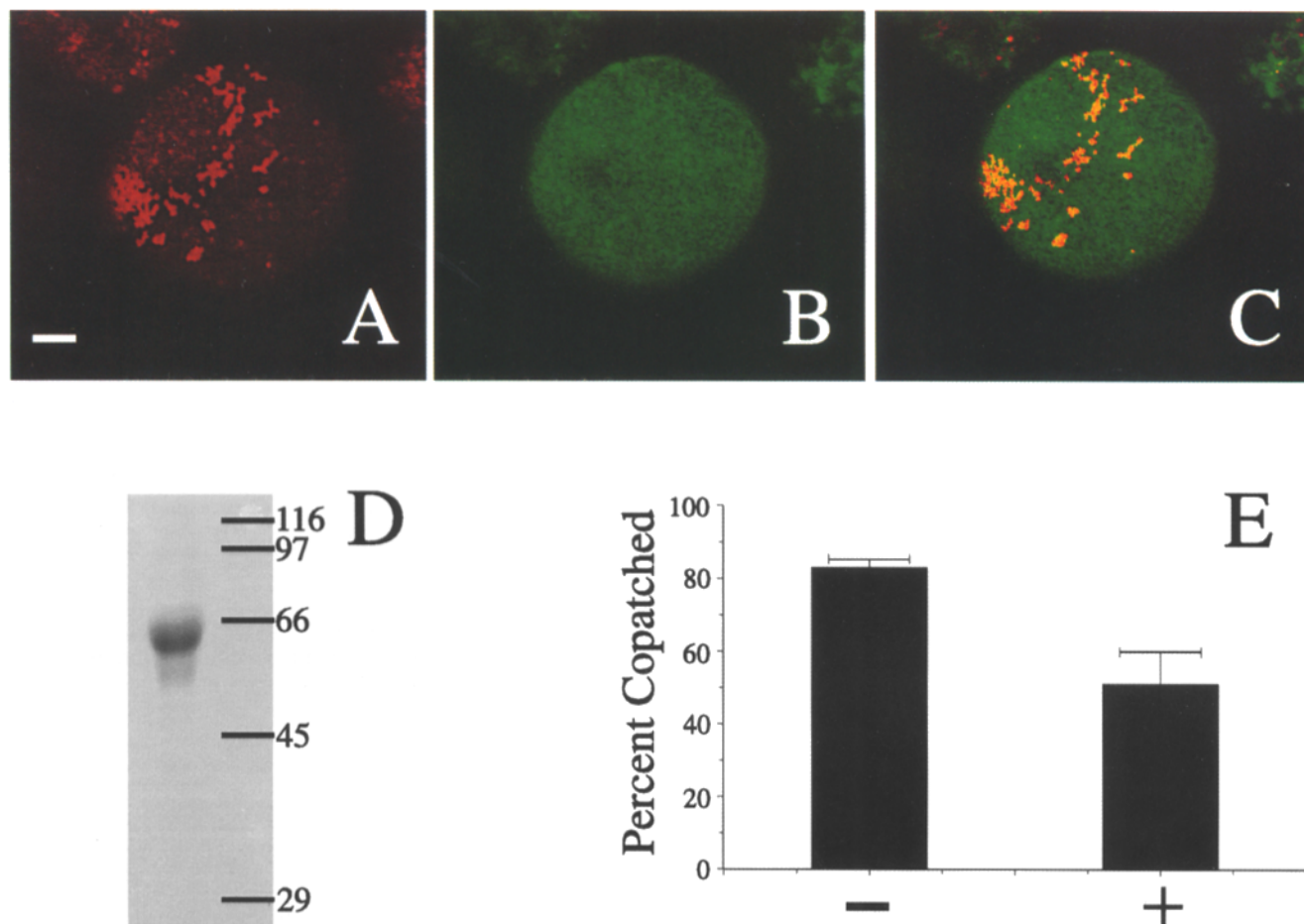

Figure 6. Soluble TrkA extracellular domain inhibits gp75-TrkA copatching. Copatching was carried out as described in the text in the presence or absence of 0.1 mg/ml of TrkA extracellular domain. Immunostaining for gp75 with rabbit antibody R7,8 (0.18 mg/ml) followed by rhodamine anti–rabbit IgG (*A*), TrkA with mAb TA-1 (ascites 1:100) followed by fluorescein anti–mouse IgG (*B*) and the combined images (*C*) are shown for a cell which did not copatch. (*D*) Analysis of the TrkA extracellular domain (3 μg/lane) by SDS–polyacrylamide gel electrophoresis and Coomassie blue staining showing the purity of the recombinant protein. (*E*) Quantitation of copatching showing mean ± SEM. Bar, 5 μm.

receptors as well as mutated NGF receptors demonstrated that this interaction is specific. The simplest model to explain our earlier FRAP data (54) and the current experiments is a heterocomplex of gp75 and TrkA as proposed by Hempstead et al. (14). The characteristics of this complex are that (1) it is not dependent on the presence of NGF, (2) the extracellular domains drive complex formation, (3) the complex is not dependent upon the existence of high-affinity NGF binding sites (15) (Stephens, R.M., D.R. Kaplan, and B.L. Hempstead, personal communication).

The copatching is likely to reflect a specific interaction between gp75 and TrkA, since PDGFR-β, Tor and TrkB do not copatch with gp75. Since TrkA and TrkB are closely homologous, the experiments with gp75 and TrkB are particularly cogent. Additionally, the inhibition of copatching by soluble TrkA extracellular domain demonstrates a specific, saturable interaction on the cell surface.

Since use of the baculovirus insect cell system leads to elevated levels of receptor expression, a reasonable question is whether we can extrapolate these conclusions to neuronal cells with lower levels of receptor expression. For instance, Ventura et al. (48) reported that TGF-β receptors I and II normally form a complex only in the presence of TGF-β. These receptors overexpressed in insect cells associate in the absence of TGF-β. However, our previous results using FRAP indicate that the interaction between gp75 and TrkA in the absence of NGF is not related to the higher levels of expression. Interaction between gp75 and TrkA consistent with an intermolecular complex is readily detected in PC12 pheochromocytoma cells and sensory neurons, even in absence of NGF (46, 54). We have so far been unable to extend these copatching studies to PC12 cells. The lower levels of NGF receptors expressed in these cells render it difficult to distinguish specific labeling from the glutaraldehyde autofluorescence. In addition, the TrkA receptor was difficult to patch with anti-TrkA antibody, perhaps due to additional interactions of TrkA in the PC12 membrane. Therefore, the Sf9 cells provide a model well suited for the copatching technique, and the conclusions of the current study are likely to be applicable to neuronal cell types.

In this study, we did not observe copatching between gp75 and TrkB. However, using FRAP, we previously observed a slight but statistically significant interaction (54). Furthermore, Hantzopoulos et al. (12) observed a functional synergy between gp75 and TrkB receptors expressed on fibroblasts. Hence, these two receptors may form a weakly bound and, perhaps, shortlived complex. The functional synergy between gp75 and TrkB (12) might result from this complex, or gp75 and TrkB might act as independent receptors emitting synergistic signals (8, 37).

These data lead to an apparent, possibly informative contradiction. The studies with chimeric receptors demonstrate that the NGF receptor extracellular domains are sufficient for the interaction between gp75 and TrkA. However, mutations of the gp75 and TrkA intracellular domains affect NGF binding (15) (Stephens, R.M., D.R. Kaplan, and B.L. Hempstead, personal communication). If these mutations do not affect formation of a gp75–TrkA complex, then why do they alter NGF binding affinity?

We suggest two possible models to resolve this contradiction. The first is based on allosteric enzymes. For this

type of regulation, a compound binds to the protein at a distance from the active site. The compound induces a conformational change, frequently involving a small shift in the angle between subunits (7). Although the change in structure is small, the effect on activity can be quite large. Such allosteric regulation might be relevant to the affinity of NGF binding. While gp75 and TrkA can form a complex via their extracellular domains, the conformation of the complex and, therefore, its affinity for NGF might be affected at a distance by the intracellular domains. Mutations of the intracellular domains may induce a slight rotation of the extracellular domains, thereby, modulating binding affinity for NGF. In the second model, we propose a third subunit which interacts with gp75 and TrkA through the intracellular domains and is required for high-affinity NGF binding. Since our earlier FRAP study (54) demonstrated a correlation between high-affinity NGF binding and immobilization of gp75, interactions with this third subunit also might result in immobilization of the gp75–TrkA complex.

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