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p75-Nerve Growth Factor as an Antiapoptotic Complex: Independence versus Cooperativity in Protection from Enediyne Chemotherapeutic Agents

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ABSTRACT

Growth factors, including nerve growth factor (NGF), have been hypothesized to play a role in resistance to chemotherapeutic agent-induced apoptosis. Induction by NGF of resistance to apoptosis is primarily thought to be the result of its binding to its high-affinity receptor, TrkA. The low-affinity NGF receptor, p75, has long been thought merely to facilitate NGF binding to TrkA. However, we have previously shown that the binding of NGF to its low-affinity receptor, p75, protects neuroblastoma cells that do not express TrkA against apoptosis induced by enediyne chemotherapeutic agents. In cells that express both receptors, it is not clear what determines which receptor is responsible for the protective effect of NGF. We now show that, in enediyne-treated SH-SY5Y neuroblastoma transfectants with native levels of p75 and a low TrkA/p75 ratio (1/100), the anti-apoptotic effect of NGF requires binding to p75. In contrast, in transfectants with native levels of p75 and a high TrkA/p75 ratio (100/100), NGF treatment prevents enediyne-induced apoptosis by a mechanism independent of p75 binding. Treatment of low TrkA/p75 ratio cells with NGF results in activation and nuclear translocation of NF-κB and tyrosine phosphorylation of TrkA. Analogous treatment of high TrkA/p75 ratio cells results only in phosphorylation of TrkA even though nuclear factor (NF)-κB signaling is not inactive and can be initiated by other ligands. The ratio of TrkA/p75 in cells that express both receptors probably contributes to the determination of which of the two known roles of p75 (i.e., TrkA independent or TrkA facilitatory) are responsible for NGF-mediated protection from enediyne-induced apoptosis.

Several growth factors have recently been found to increase chemotherapeutic resistance of cancer cells (Koutsilieris et al., 1999; Schor, 1999; Sola et al., 1999; Bunn et al., 2000; Powis et al., 2000; Sezer et al., 2001). In the case of tumors of the nervous system, nerve growth factor (NGF), among other neurotrophins, has been implicated in resistance to apoptosis induction (Cortazzo and Schor, 1996; Schor, 1999; Schor and Saragovi, 1999).

The biological activities of NGF result from its binding to one or both of its receptors termed TrkA and p75, respectively. This binding in turn triggers a cascade of cellular signaling events, the precise nature of which is still the subject of considerable investigation and controversy (Bono et al., 1999). TrkA, the high-affinity NGF receptor, is a tyrosine kinase the autophosphorylation of which results in initiation of the mitogen-activated protein kinase (Ulrich et al., 1998), inositol-1,4,5-triphosphate, adapter protein Shc, fibroblast growth factor receptor substrate 2, and other pathways (van der Geer et al., 1996; Meakin et al., 1999). TrkA is primarily but not exclusively expressed on cells of neural lineage. In contrast, the signaling mechanism related to the binding of NGF to its low-affinity receptor, p75, has only recently begun to be defined. The p75 receptor has variously been found to induce apoptosis when not bound to NGF (Rabizadeh et al., 1993; Huang et al., 2000), induce (Kuner and Hertel, 1998; Sedel et al., 1999) or prevent (Rabizadeh et al., 1993; Cortazzo et al., 1996) apoptosis when bound to NGF, and mediate neurotrophin dependence and alter neurotrophin affinity of other neurotrophin receptors when coexpressed with them (Bredesen et al., 1998; Ross et al., 1998). Signaling of NGF through p75 alone is thought to involve the NF-κB/ceramide pathway (Carter et al., 1996; Dobrowsky and Carter, 1998; Brann et al., 1999; Coulson et al., 1999).

It is easy to envision the role of p75 as an independent signal

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ABBREVIATIONS: NGF, nerve growth factor; NCS, neocarzinostatin; 7-AAD, 7-amino-actinomycin D; PBS, phosphate-buffered saline; NF-κB, nuclear factor-κB; PMSF, phenylmethylsulfonyl fluoride; ANOVA, analysis of variance; PLSD, protected least significant difference; TNF, tumor necrosis factor.

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transducer in cells that express only this receptor and not any of the Trk family tyrosine kinase receptors (Cortazzo et al., 1996). Similarly, it is not surprising that the magnitude and duration of NGF signaling through TrkA is dependent on how many p75 receptors participate in the enhancement of TrkA affinity for NGF (Chao et al., 1998; Twiss et al., 1998). This is especially so because, in addition to enhancing TrkA-NGF affinity, p75 is also a functional regulator of TrkA trophic activity (Maliartchouk and Saragovi, 1997; Saragovi et al., 1998). What is unexpected and novel is the p75-mediated protective effect of NGF against enediyne chemotherapeutic agent-induced apoptosis in NGF-independent cells (Cortazzo et al., 1996). This is in sharp distinction to the proapoptotic effects of p75 alone in NGF-dependent cells deprived of NGF (Bredesen et al., 1998) and of NGF through p75 in developing motoneurons in the rat embryonic spinal cord (Sedel et al., 1999). We now demonstrate that the ratio of TrkA/p75 in the cell membrane is one determinant of the relative importance of the two alternative signaling pathways triggered by NGF for its negative effect on enediyne-induced apoptosis.

Materials and Methods

Cells. SY5Y-TrkA (high TrkA/p75) and SY5Y-ET (low TrkA/p75) cells were the kind gift of Dr. Alonzo Ross (University of Massachusetts Medical Center, Worcester, MA). These cells were generated by transfection by electroporation of the TrkA expression vector, pIRVCMV-TrkA, or the corresponding vector lacking the *trkA* insert, respectively, into SH-SY5Y cells. Unlike SY5Y-ET cells, SY5Y-TrkA cells overexpress TrkA. Like native TrkA, the overexpressed product is phosphorylated upon NGF treatment and mediates NGF-induced neurite outgrowth in SY5Y-TrkA cells (Poluha et al., 1995). All cells used in these studies were demonstrated to be mycoplasma-free using a MycoTect Kit (Invitrogen, Carlsbad, CA). SY5Y-TrkA and SY5Y-ET cells were cultured in RPMI 1640 medium supplemented with 1% (v/v) glutamine (200 mM), 10% fetal bovine serum, and 0.4% (v/v) G418 (5 mg/ml) as the selection antibiotic for these transfectants.

Chemicals and Reagents. Neocarzinostatin (NCS), an enediyne antineoplastic agent previously demonstrated to induce apoptosis in SH-SY5Y neuroblastoma cells in culture (Hartsell et al., 1995, 1996), was obtained from Kayaku Pharmaceuticals Ltd. (Tokyo, Japan). NCS was stored in powder form at −20°C; a 47 μM (0.5 mg/ml) working stock solution in 0.015 M sodium acetate buffer, pH 5.0, was stored in the dark at 4°C for up to 2 weeks and diluted with medium immediately before each experiment. NGF was obtained from Roche Applied Science (Indianapolis, IN).

Preparation and characterization of the monoclonal antibody mAbNGF30 were described in our previous publication (Saragovi et al., 1998). This antibody binds to the p75 binding site of NGF, blocking the binding of NGF to p75. Monoclonal antibody 5C3, a TrkA-specific NGF agonist, was prepared as described previously (LeSauteur et al., 1996). Mouse monoclonal anti-TrkA IgG (Ab-1) was obtained from Calbiochem. Rabbit polyclonal anti-p65 IgG, mouse monoclonal anti-phospho-TrkA IgG (E-6) and rabbit anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cy3-conjugated AffiniPure Goat Anti-rabbit IgG(H+L) and Cy2-conjugated AffiniPure Rabbit Anti-mouse IgG(H+L) were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) and used for fluorescent immunostaining of cells. Geneticin Solution (antibiotic G418), 7-amino-actinomycin D (7-AAD), and saponin were purchased from Sigma Chemical Corporation (St. Louis, MO).

Flow Cytometric Analysis. Apoptotic cells were quantified by flow cytometry considering 7-AAD staining intensity to be proportional to the DNA content (Lecoeur and Gougeon, 1996). In short, after harvesting, the cells were washed once in PBS and once in PBS/0.05% saponin, followed by addition of 4 μg of 7-AAD in 1 ml of PBS/saponin to the samples. The cells were incubated at room temperature in the dark for 30 min, and DNA histograms were obtained using a CellQuest apparatus and CellQuest software (BD Biosciences, San Jose, CA). Data on 10^4 cells were collected. Electronic gates were set for viable and apoptotic cells with 2N to 4N DNA and subnormal DNA content, respectively, and for exclusion of debris. Percentage of apoptosis was calculated as (number of apoptotic cells / number of total cells) × 100.

Effects of Blocking p75 Binding of NGF on Its Antiapoptotic Activity in SY5Y-ET and SY5Y-TrkA Cells. Sister cultures of SY5Y-ET cells and SY5Y-TrkA cells were treated with a preincubated (15 min) mixture of mAbNGF30 and NGF ([mAbNGF30]: [NGF] = 2:1; final [NGF] = 2 nM) from 24 h before through the completion of the experiment. Twenty-four hours after the addition of the NGF mixture, the cells were treated for 1 h with NCS at two different concentrations (3 and 10 nM). Control conditions for this experiment included cells treated with NGF alone, mAbNGF30 alone, NCS alone, NGF followed by NCS, NGF with mAbNGF30, and mAbNGF30 followed by NCS. Adherent cells were counted as described previously (Hartsell et al., 1995, 1996; Cortazzo et al., 1996), and flow cytometric and fluorescence microscopic assessments of percent apoptosis were performed as described above.

Effect of NGF on NF-κB Activation in SY5Y-ET and SY5Y-TrkA Cells. Cells were treated for 2 h with NGF (2 nM) or an equivalent volume of vehicle. Nuclear extracts were made from these cells as follows: treated cells were washed twice with ice-cold PBS and harvested by scraping into fresh PBS. Harvested cells were washed again with ice-cold PBS, then with 10 mM Tris buffer, pH 7.5, containing 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM PMSF, and 0.1 mM Na_3VO_4, and then suspended in the same buffer made 0.1% in Triton X-100. Cells were vortexed gently on ice, incubated on ice for 10 min, and then centrifuged at 7000 rpm for 5 min at 4°C. The pellet was suspended once again in 20 mM Tris buffer, pH 7.5, containing 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, 0.1 mM Na_3VO_4, 0.1% Triton X-100, and 25% glycerol, and incubated on ice for 20 min. The mixture was centrifuged at 14,500 rpm for 15 min at 4°C, and the pellet was discarded. The supernatant (nuclear extract) was diluted with three volumes of 20 mM Tris buffer, pH 7.5, containing 1.5 mM $MgCl_2$, 25 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM dithiothreitol, 0.1 mM Na_3VO_4, and 20% glycerol. Nuclear extracts were aliquoted (20 μl/vial) and frozen until analysis.

Gel Shift Assays. For gel shift analysis, the ^{32}P-labeled double-stranded NF-κB consensus oligonucleotides [sense, 5′GGGGAGTTGAGGGGACTTTCCCAGGC3′; antisense, 5′GGGGGCCTGGGAAAGTCCCCTCAACT3′ (DNA Synthesis Facility, University of Pittsburgh)] were annealed to the nuclear extracts by PCR thermocycling (85°C, 2 min; 65°C, 15 min; 37°C, 15 min; 22°C, 15 min; 0°C, 15 min). For supershifts, a subsequent incubation was performed with antibodies to the p65 or p50 components of NF-κB (Santa Cruz Biotechnology, Inc.). Samples were run (2.5 μg of protein/lane) on a 4% polyacrylamide gel. The gel was dried and subsequently exposed to X-ray film overnight (4°C) for autoradiographic analysis.

Effects of NGF on Phosphorylation of IκB-α. SY5Y-ET and SY5Y-TrkA cells were treated with NGF (2 nM) for 0 to 6 h. Cells were rinsed with fresh medium and harvested with trypsin. After washing twice, cells were suspended in radioimmunoprecipitation assay buffer containing PMSF, aprotinin, and Na_3VO_4 and was then homogenized. Homogenates were run on a 10% polyacrylamide gel (500 μg protein/lane) and transferred to a Trans-Blot Pure Nitrocellulose Membrane (0.45 μm; Bio-Rad) using a Bio-Rad Trans-Blot apparatus. Staining was performed using antibodies to IκB-α, phospho-IκB-α, and β-actin at 20°C for 2 h, then washed, and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Staining with nonimmune serum in place of primary antibody served as a negative control in these studies. The membrane was finally washed and developed with Western Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology) following the

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manufacturer's instructions. Optical scanning of the membranes was performed using a Optiscan optical scanner (Bio-Rad, Hercules, CA).

Western Blot and Immunohistochemical Demonstration of Phosphorylation of TrkA and Translocation of p65 in SY5Y-ET and SY5Y-TrkA Cells. At the indicated time points after incubation with 40 nM NGF, SY5Y-ET, and SY5Y-TrkA cells were lysed in radioimmunoprecipitation assay buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 4 μg/ml aprotinin, and 1 mM sodium orthovanadate). Subsequently the protein concentrations of the lysates were estimated using the Bio-Rad protein assay (Bio-Rad) with bovine serum albumin as a standard. An aliquot of each lysate containing 150 μg of protein was loaded onto each lane and electrophoresed on a 15% SDS-polyacrylamide gel, followed by blotting on a nitrocellulose membrane (Bio-Rad). After blotting, nonspecific binding was blocked with 5% nonfat dry milk in PBS and the membrane was incubated with either anti-TrkA or anti-phospho-TrkA antibodies (primary antibody) diluted in 5% nonfat dry milk in PBS at 20°C for 2 h. The blot was then developed with Western Blotting Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology), as described above for IκB-α blots. For SY5Y-TrkA cell lysates, the blot was developed for 1 min. For SY5Y-ET cell lysates, known to contain 100-fold less TrkA than SY5Y-TrkA cell lysates (Poluha et al., 1995), the blot was developed for 10 min. In all cases, the same blot was stained for TrkA, stripped using standard methods, and restained for phospho-TrkA using the same development time as for TrkA.

For immunohistochemical staining, SY5Y-TrkA and SY5Y-ET cells were grown on glass coverslips in six-well tissue culture plates. The cells were incubated with NGF and/or NCS, washed three times with PBS, fixed in 95% ethanol for 20 min, and washed three more times with PBS. The cells were then permeabilized by incubation in 2% Triton X-100 for 30 min followed by three PBS washes. After blocking of nonspecific staining (0.5% bovine serum albumin + 2% horse serum in PBS; 10 min), the cells were incubated for 2 h at 37°C with the appropriate primary antibody (anti-p65, anti-TrkA, or anti-*p*-TrkA) and then with a 1:100 dilution of goat anti-rabbit Cy3 immunoconjugate or rabbit anti-mouse Cy2 immunoconjugate followed by three additional washes (10 min each) with PBS. Coverslips were removed from the wells and mounted with Gelvatol [15.3% (w/v) polyvinyl alcohol, 33% (v/v) glycerol] onto glass microscope slides. A Zeiss light microscope equipped for epifluorescent illumination was used for examining these preparations. Nonspecific immunostaining (negative control) was assessed by subjecting sister cultures to this same procedure minus treatment with the primary antibody.

Statistical Methods. For studies involving the comparison of multiple samples, statistical significance was assessed by one-way ANOVA followed by Fisher's protected least significant difference test (PLSD). For studies involving the comparison of paired samples, statistical significance was assessed by Student's (paired) *t* test. In all cases, $p < 0.05$ was considered to be indicative of statistical significance.

Results

The use of *trkA* (SY5Y-TrkA) and mock (SY5Y-ET) transfectants, respectively, of SH-SY5Y human neuroblastoma cells (Poluha et al., 1995) allowed us to examine the effects of manipulation of the TrkA/p75 ratio without alteration of the absolute amount of p75 on the cell surface. SY5Y-ET cells express TrkA and p75 at native levels. SY5Y-TrkA cells express native levels of p75, and TrkA in 100-fold excess over native levels. The TrkA/p75 ratio of these cells is 100/100, whereas that of native SH-SY5Y cells and SY5Y-ET cells is 1/100. The p75 content of both transfectants is equivalent (Poluha et al., 1995).

NCS Induces Apoptosis in Both SY5Y-ET and SY5Y-TrkA Cells. Previous studies (Hartsell et al., 1995; Hartsell et al., 1996) have demonstrated that NCS induces apoptosis in the parent cell line, SH-SY5Y. Similarly, 48 to 72 h after a 1-h exposure to NCS, both SY5Y-ET and SY5Y-TrkA cells are seen by light microscopy to shrink, round-up, and detach from the culture surface in a time- and NCS concentration-dependent manner (data not shown). Cells of both transfectants also demonstrate apoptotic changes in nuclear configuration seen after fluorescent staining (Fig. 1, A-D).

To quantify cell number after NCS treatment (0–20 nM), cell counts of adherent cells were performed for SY5Y-ET and SY5Y-TrkA transfectants over an experimental period of 7 days after 1 h of NCS treatment. The effects of NCS on adherent cell number were concentration-dependent and became apparent on day 3 in both transfectants. NCS was equipotent for diminution of adherent cell number in the two transfectants ($n = 6$; data not shown).

In addition, we also examined the apoptotic response of SY5Y-ET and SY5Y-TrkA cells to NCS treatment (performed for 1 h on day 0) with 7-AAD staining and flow cytometric analysis on day 3. As demonstrated in Fig. 1E, a concentration-dependent increase in the apoptotic fraction, detected as cells with sub-2N DNA content, was found after NCS treatment. The within-experiment concentration dependence was robust despite some between-experiment variability of the absolute incidence of apoptosis at a given NCS concentration. Flow cytometric analysis also demonstrates the equipotency of NCS for the two transfectants.

Protective Effects of NGF on NCS-Treated SY5Y-ET and SY5Y-TrkA Cells. To determine whether NGF can induce resistance of SY5Y-ET and SY5Y-TrkA cells to NCS-induced apoptosis, flow cytometric analysis was performed in the presence of NGF (2 nM) from 24 h before NCS (3 or 10 nM) treatment through the duration of the experiment. As shown in Fig. 2, incubation with NGF protected both SY5Y-ET and SY5Y-TrkA cells from NCS-induced apoptosis.

MC192, a p75-Specific NGF Ligand, Protects Low TrkA/p75 (SY5Y-ET) but not High TrkA/p75 (SY5Y-TrkA) Cells from NCS-Induced Apoptosis. Previous work has demonstrated the specificity of MC192 for p75 (Chandler et al., 1984; Barker and Shooter, 1994). MC192 has been shown to synergistically enhance the activity of the TrkA-specific ligand, 5C3, in much the same way as NGF binding to p75 enhances NGF signaling through TrkA (Maliartchouk and Saragovi, 1997). We have therefore used MC192 to determine the effects of ligand binding of p75 alone in SY5Y-ET and SY5Y-TrkA cells.

Fluorescent nuclear staining suggested that MC192 decreased the incidence of condensed nuclei in NCS-treated SY5Y-ET cells, although it did not alter the incidence of condensed nuclei in NCS-treated SY5Y-TrkA cells (data not shown). Similarly, whereas MC192 increased the EC_{50} (as defined in Schor et al., 2000) for decrease in adherent cell number after NCS treatment of SY5Y-ET cells from 5 to 20 nM, it had no effect on the EC_{50} of SY5Y-TrkA cells (Fig. 3A). Consistent with the cell counting results, flow cytometric analysis showed a differential effect of MC192 upon SY5Y-ET and SY5Y-TrkA cells (Fig. 3B). MC192 protects SY5Y-ET cells, but not SY5Y-TrkA cells, from apoptosis induction by NCS.

mAbNGF30, an Anti-NGF Antibody That Renders Inactive the p75 Binding Site of NGF, Protects High TrkA/p75 (SY5Y-TrkA) but Not Low TrkA/p75 (SY5Y-ET) Cells from NCS-Induced Apoptosis. We have previously documented the protection of SH-SY5Y cells, the par-

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ent cells of both transfectants, from the effects of NCS on cell number by NGF binding to p75 under circumstances when NGF binding to TrkA was blocked by an engineered, TrkA-specific mutant NGF (Cortazzo et al., 1996). We have now used the monoclonal antibody mAbNGF30 to block the p75 binding site of NGF, thereby converting NGF into a ligand that binds to TrkA but not to p75 (Saragovi et al., 1998). We have tested the effect of this NGF-NGF30 complex on NCS-treated SY5Y-ET and SY5Y-TrkA cells. As is shown in Fig. 4, the NGF-NGF30 complex does not protect SY5Y-ET cells from the effects of NCS. In contrast, NGF-NGF30 does protect SY5Y-TrkA cells in the same system. Control treatment of either transfectant with mAbNGF30, NGF, or NGF-NGF30 alone (i.e., without NCS) had no effect on apoptosis prevalence, and mAbNGF30 did not alter the increased prevalence of apoptosis seen after NCS treatment (data not shown). This further suggests that the role of p75 is different in these two cell lines, despite the fact that both lines express the same amount of p75.

NF-κB Activation Accompanies NGF-Mediated Protection of SY5Y-ET, but Not SY5Y-TrkA Cells from the Effects of NCS. The NF-κB pathway has been proposed to be involved in independent (i.e., non–TrkA-dependent) signaling of NGF through p75. We therefore examined the activation of this pathway by NGF in SY5Y-ET and SY5Y-TrkA cells. The difference in signaling by NGF between the two transfectants was suggested by gel supershift studies of NF-κB activation after incubation of each transfectant with vehicle or NGF. Treatment of SY5Y-ET cells with NGF (2 nM) for 2 h results in more intense staining of the components of NF-κB with a radiolabeled specific oligonucleotide probe (Fig. 5). Both the p65 and p50 components of NF-κB seem to be represented in this staining, as demonstrated by the displacement and altered staining characteristics of each in turn in the lanes containing antibodies to each of these components, respectively. On the other hand, similar treatment of SY5Y-TrkA cells results in no significant change in the intensity of staining of NF-κB with this probe. Note that both cell lines exhibit some baseline nuclear content of NF-κB, as expected for cells cultured in growth factor- (i.e., serum-) containing medium.

Because of the difficulty quantifying gel supershift data and because the NF-κB signaling pathway involves phosphorylation of IκB-α and p65 translocation, we confirmed these results by examining the phospho- and total IκB-α contents (Fig. 6A) and p65 translocation in SY5Y-ET (Fig. 6B) and SY5Y-TrkA (Fig. 6C) cells over time of exposure to NGF. As demonstrated in Fig. 6A, the cellular content of phospho-IκB-α significantly increased over time in SY5Y-ET cells, whereas the content of IκB-α remained constant. Consistent with this result, translocation of p65 protein from the cytoplasm to the nucleus was observed after NGF (2 nM) treatment of SY5Y-ET cells (Fig. 6B). This phenomenon was also observed in the case of treatment with NCS (1 h) in the presence of NGF (Fig. 6D). These results imply that signaling through an independent p75 receptor and the NF-κB pathway could be an important mechanism by which NGF protects SY5Y-ET cells from NCS-induced apoptosis. In sharp contrast, neither phosphorylation of IκB-α nor translocation of p65 protein changed significantly during incubation of SY5Y-TrkA cells with NGF alone (Fig. 6, A and C) or NCS+NGF (Fig. 6E), implying that despite the invariant content of p75 between these two transfected cell lines, in the

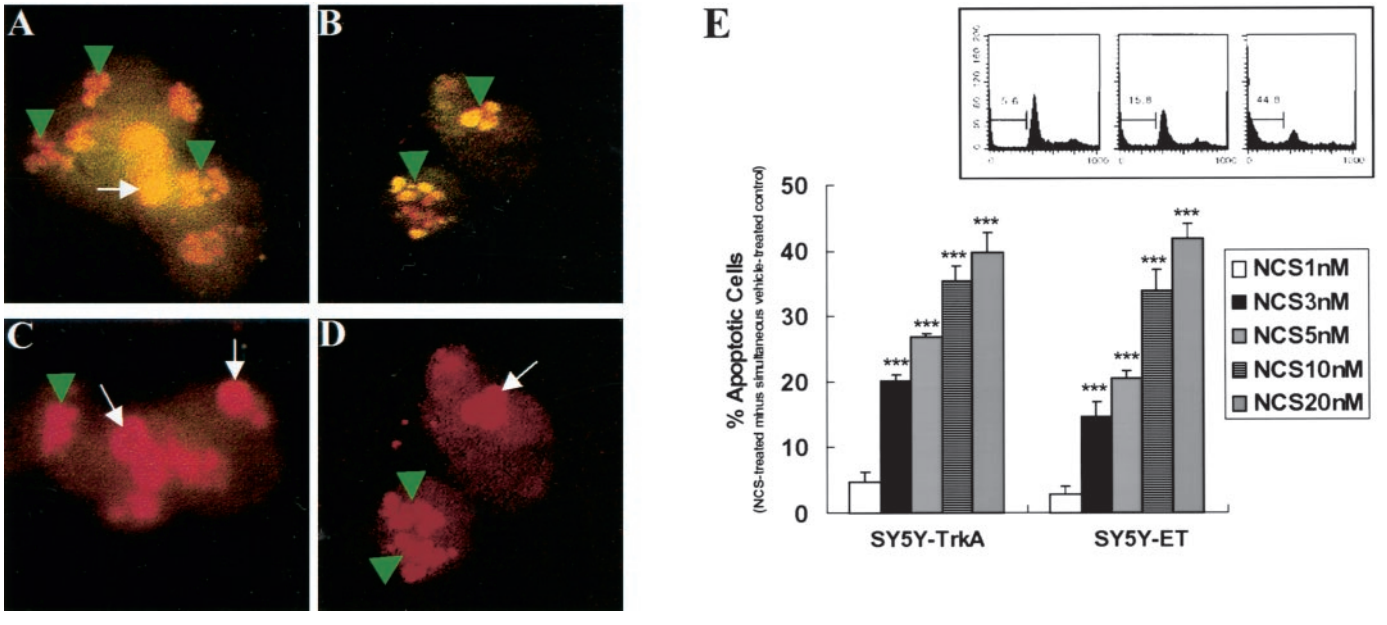

Fig. 1. Nuclear fluorescent staining of SY5Y-ET (A, B) and SY5Y-TrkA (C, D) stained with acridine orange and ethidium bromide to demonstrate apoptosis induced by NCS treatment. Nonadherent cells were collected, stained, and photographed on day 3 after a 1-h NCS treatment. A and C, [NCS], 3 nM; B and D, [NCS], 10 nM. Apoptotic cells are distinguished by condensed (white arrows) and fragmented (green arrowheads) chromatin. (magnification, 1000×) E, NCS (0, 1, 3, 5, 10, and 20 nM; 1 h) increases cell death in a dose-dependent manner in both SY5Y-ET and SY5Y-TrkA cells. Both transfectants were treated with NCS. Pooled adherent and nonadherent cells were harvested, stained with 7-AAD, and analyzed by flow cytometry. Electronic gates were set for viable and apoptotic cells with 2N to 4N DNA and subnormal DNA content, respectively. Percent apoptosis observed in a simultaneous vehicle-treated sister culture was subtracted from the respective value obtained in the presence of NCS. Shown is the cumulative result of five experiments. Error bars on this and all subsequent bar graphs represent the S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with vehicle-treated cells [one-way ANOVA followed by Fisher's PLSD test]. Inset, representative histogram (SY5Y-ET cells).

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SY5Y-TrkA line, NGF does not signal through an independent, NF-κB-linked p75 receptor.

Independent Signaling through p75 in SY5Y-ET Cells Is Not the Result of Lack of a Critical Threshold TrkA Content in These Cells; TrkA Signaling Is Competent in SY5Y-ET Cells. From the results we describe above, it is not possible to distinguish between alteration of TrkA/p75 ratio and expression of a critical threshold number of TrkA receptors as the reason for differential signal transduction. It is theoretically possible that signaling through the TrkA tyrosine phosphorylation pathway requires a critical number of TrkA receptors and that only independent p75-mediated signaling was important in protection against apoptosis of SY5Y-ET cells because no TrkA-mediated signaling of NGF occurred. We therefore examined NGF-induced TrkA phosphorylation in SY5Y-ET and SY5Y-TrkA cells treated with NGF at a concentration (40 nM) sufficient to saturate both TrkA and p75. In so doing, we sought to test the hypothesis that, even with low TrkA content, SY5Y-ET cells are capable of phosphorylating TrkA.

Fig. 7A confirms the differential expression of TrkA in the two transfectants. Figures 7, B and C, and 8 show that even when p75 is saturated with NGF and TrkA is present only at native levels, NGF-mediated TrkA phosphorylation takes place in both transfectants, and that the relatively low TrkA expression of SY5Y-ET cells does not preclude such phosphorylation.

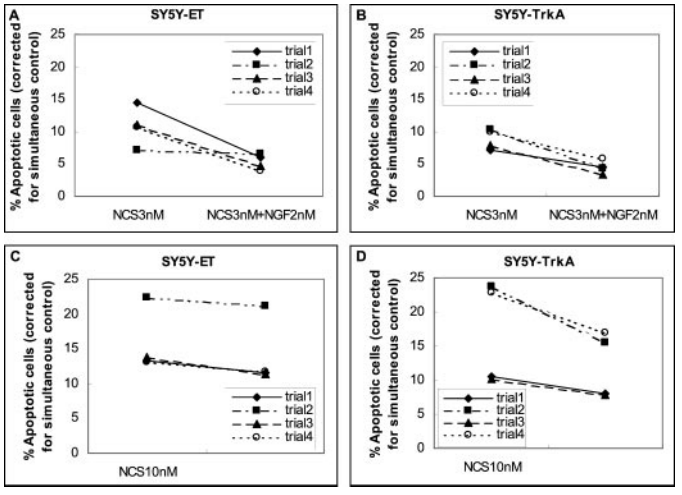

Fig. 2. Protective effects of NGF (2 nM) on NCS (3 nM, 10 nM; 1 h) -induced apoptosis in SY5Y-ET (A, C) and SY5Y-TrkA (B, D) cells. Sister cultures were pretreated with NGF for 24 h, then exposed to NCS for 1 h in the presence of NGF. NGF was maintained in the culture medium throughout the duration of the experiment. On day 3 after NCS treatment, the cells were harvested, stained with 7-AAD, and analyzed by flow cytometry. Percentage of apoptosis observed in a simultaneous vehicle-treated control was subtracted from the respective NCS- or NCS+NGF-treated sister culture. Each line represents one independent trial (paired NCS- and NCS+NGF-treated samples) of four performed.

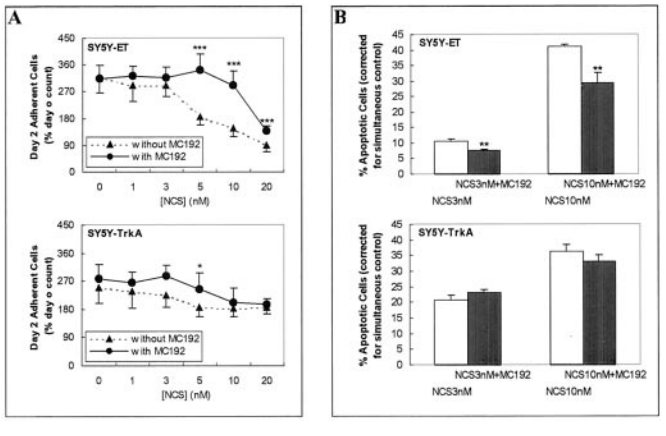

Fig. 3. Effects of MC192 on NCS-induced SY5Y transfectant cell death. A, both SY5Y-ET and SY5Y-TrkA cells were pretreated with 1 nM MC192 for 24 h, then exposed to NCS (1, 3, 5, 10, and 20 nM) for 1 h in the presence of MC192. Where indicated, MC192 was maintained in the culture medium throughout the duration of the experiment. Cell counts are performed at 2 d after NCS treatment. *, $p < 0.05$; ***, $p < 0.001$ (one-way ANOVA followed by PLSD) compared with corresponding group without MC192. B, both transfectants were treated as described in A. Flow cytometry was performed on day 3 after NCS treatment as described for Fig. 2. Percentage of apoptosis observed in a simultaneous vehicle-treated control was subtracted from the respective value obtained in the NCS- or NCS+MC192-treated sister culture. **, $p < 0.05$ compared with NCS treatment alone (Student's t test)

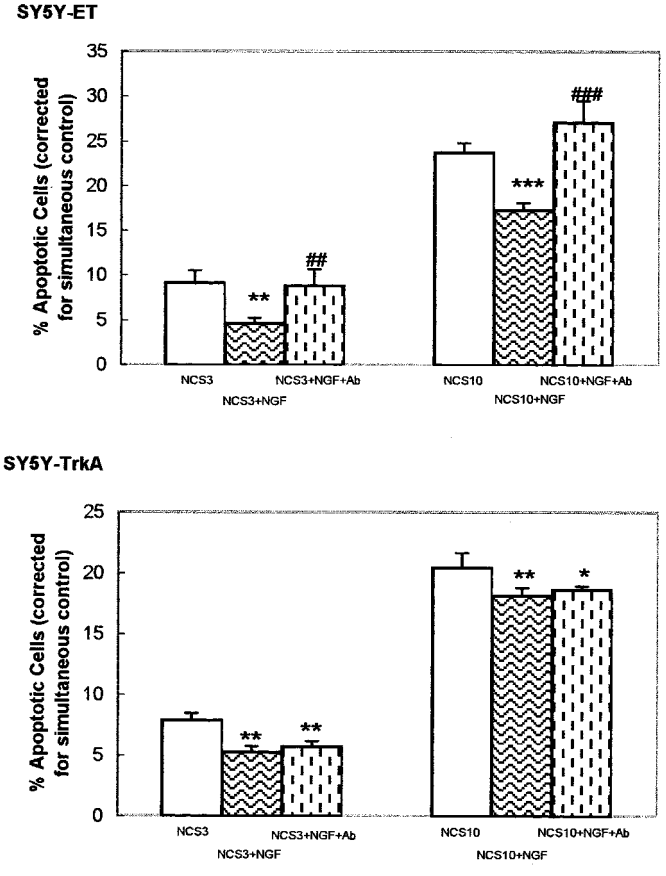

Fig. 4. Effects of blocking p75 binding of NGF on its anti-apoptotic activity in SY5Y-ET and SY5Y-TrkA cells. Sister cultures of SY5Y-ET and SY5Y-TrkA cells were treated with all possible combinations of vehicle, NCS (3 and 10 nM, 1 h, 37°C), NGF (final concentration, 2 nM) present from 24 h before NCS addition through the termination of the experiment, and mAbNGF30 (final concentration, 4 nM), as we have described previously for other antibodies (Cortazzo et al., 1996). Flow cytometry was performed as described for Fig. 2. NGF alone, mAbNGF30 alone, and NGF + mAbNGF30 had no effect on flow cytometric profiles. Similarly, mAbNGF30 alone had no effect on change in flow cytometric profile induced by NCS (data not shown). For both transfectants, NCS+NGF treatment values differ significantly from NCS alone values [*, $p < 0.05$; **, $p < 0.01$ (one-way ANOVA followed by PLSD)]. For SY5Y-ET cells, NGF+NCS+Ab treatment values differ significantly from NCS+NGF treatment values (##, $p < 0.01$; ###, $p < 0.001$), but not from NCS alone values ($p > 0.05$). For SY5Y-TrkA cells, NGF+NCS+Ab treatment values do not differ significantly from NCS+NGF treatment values ($p > 0.05$).

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Absence of Independent Signaling through p75 in SY5Y-TrkA Cells Is Not the Result of Inability of NGF to Induce Activation of NF-κB through p75; p75 Signaling Is Competent in SY5Y-TrkA Cells. NGF, a ligand of both TrkA and p75, does not induce activation of NF-κB in SY5Y-TrkA cells. This could mean either that p75 does not function independently when the TrkA/p75 ratio is 100/100 and both p75 and TrkA are bound to NGF, or that p75 is incapable of independent signaling under any circumstances in this transfectant. To distinguish between these possibilities, we examined SY5Y-TrkA and SY5Y-ET cells for activation of NF-κB after treatment with MC192, a ligand that binds to p75 but not to TrkA (Chandler et al., 1984; Barker and Shooter, 1994). As shown in Fig. 9, in both SY5Y-ET and SY5Y-TrkA cells, binding of MC192 by p75 results in a time-dependent increase in cellular levels of phospho-IκB-α, whereas levels of actin and IκB-α remain constant. Binding of MC192 to p75 therefore activates NF-κB in both transfectants. This is not the case for NGF, which suggests that the inability of NGF to activate NF-κB in SY5Y-TrkA cells is related to its binding to both TrkA and p75 and not to an innately incompetent pathway through p75 to NF-κB activation in these cells.

In SY5Y-ET cells, p75 Enhances TrkA-Mediated Signaling of NGF, Whereas in SY5Y-TrkA Cells, p75 Does Not Synergize with TrkA. We also tested the hypothesis that in SY5Y-TrkA cells, but not SY5Y-ET cells, binding of NGF to p75 serves to enhance phosphorylation of TrkA resulting from exposure to a single concentration of NGF. This

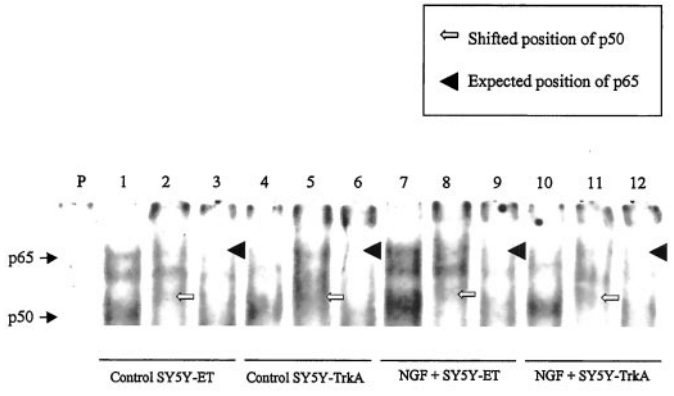

Fig. 5. Effect of NGF on NF-κB activation in SY5Y-ET and SY5Y-TrkA cells. Gel supershift assays were performed as detailed under *Materials and Methods* using oligonucleotides that bind specifically to NF-κB. Nuclear preparations from all experimental conditions in both transfectants were simultaneously run on a single gel. Lanes 1 to 6, cells under native conditions; lanes 7 to 12: cells treated for 2 h with NGF (2 nM). Results are shown from one representative experiment of two performed. P, probe alone; 1, SY5Y-ET nuclear prep. + probe; 2, SY5Y-ET nuclear prep. + anti-p50 antibody + probe; 3, SY5Y-ET nuclear prep. + anti-p65 antibody + probe; 4, SY5Y-TrkA nuclear prep. + probe; 5, SY5Y-TrkA nuclear prep. + anti-p50 antibody + probe; 6, SY5Y-TrkA nuclear prep. + anti-p65 antibody + probe; 7, (SY5Y-ET + NGF) nuclear prep. + probe; 8, (SY5Y-ET + NGF) nuclear prep. + anti-p50 antibody + probe; 9, (SY5Y-ET + NGF) nuclear prep. + anti-p65 antibody + probe; 10, (SY5Y-TrkA + NGF) nuclear prep. + probe; 11, (SY5Y-TrkA + NGF) nuclear prep. + anti-p50 antibody + probe; 12, (SY5Y-TrkA + NGF) nuclear prep. + anti-p65 antibody + probe. The open arrows indicate the shifted position of p50 in lanes including an antibody to this protein. The closed arrowheads indicate the disappearance of the band for p65 in lanes including an antibody to this protein. Binding of specific antibody to p65 has previously been shown to alter binding to the oligonucleotide probe sufficiently to diminish radiographic detection of this protein (Carter et al., 1996).

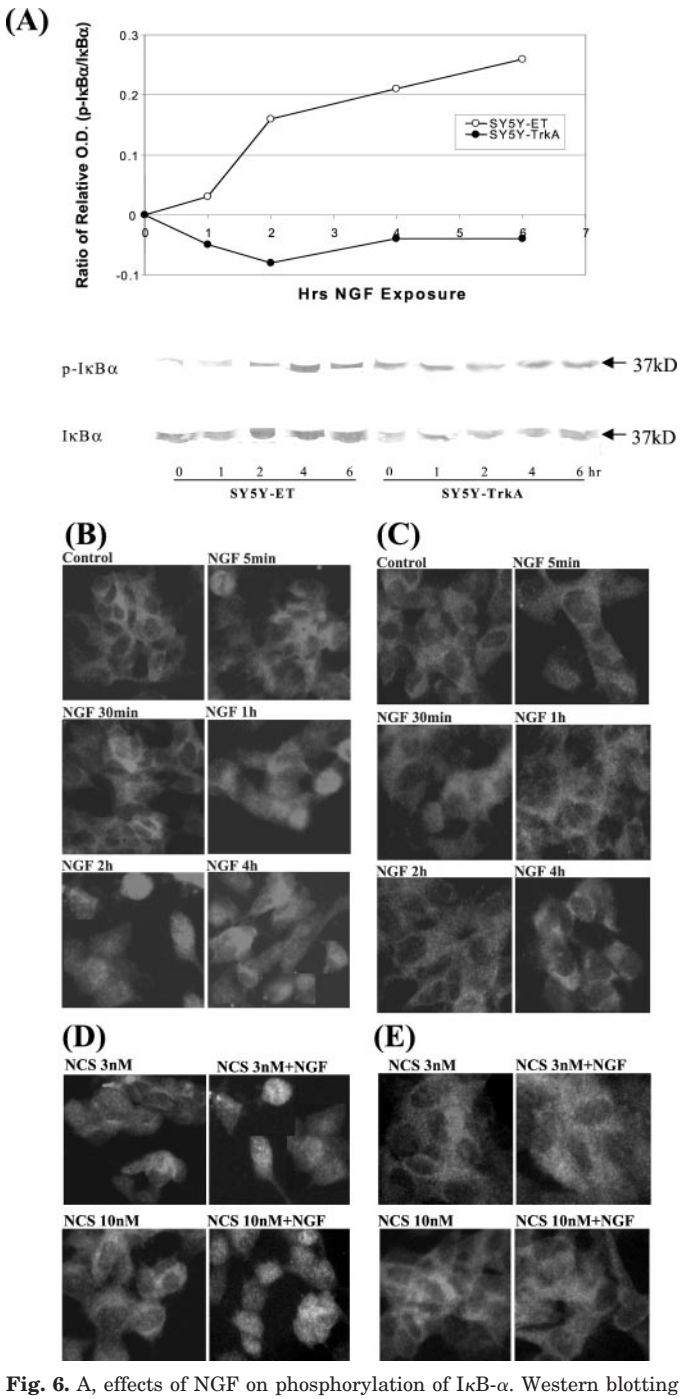

Fig. 6. A, effects of NGF on phosphorylation of IκB-α. Western blotting was performed to examine SY5Y-ET and SY5Y-TrkA cells for phosphorylation of IκB-α after NGF exposure (2 nM; 0–6 h). Blots were stripped and then stained for IκB-α and β-actin as IκB-α expression and loading controls, respectively. Plots of optical density were generated using a Bio-Rad Optiscan optical scanner and normalized to IκB-α expression. Intensity of the band for β-actin did not change significantly from lane to lane (data not shown). Results are shown from one representative experiment of three performed. B to E, expression and translocation of p65 protein after NGF (2 nM) treatment in the absence (B and C) and presence (D and E) of NCS were performed in SY5Y-ET cells (B and D) and SY5Y-TrkA cells (C and E). Sister cultures of SY5Y-ET and SY5Y-TrkA cells grown on glass coverslips were incubated for varying lengths of time with NGF (B and C) or preincubated with NGF for 24 h followed by 1 h of NCS (3 and 10 nM) treatment in the presence of NGF and another 24 h of incubation with NGF alone (D and E). The cells were washed, fixed, blocked, and stained with anti-p65 polyclonal antibody. Subsequent washing, staining with a fluorescent secondary antibody, and microscopy were performed as detailed under *Materials and Methods*.

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was suggested by our finding that the ratio of the optical densities of the band for phospho-TrkA to that for TrkA peaks at 1.25-fold above control levels after 30-min incubation with NGF in SY5Y-ET cells, whereas the analogous ratio peaks at 3.75-fold of control levels after 5-min incubation with NGF and remains close to this level for at least 2 h in SY5Y-TrkA cells (data not shown). This is consistent with synergistic enhancement of TrkA activity by p75 in cells with a high TrkA/p75 ratio, as has been seen previously in other cell systems (Maliartchouk and Saragovi, 1997; Ross et al., 1998; Rabizadeh et al., 1999).

In confirmation of this observation, the p75 ligand MC192 (2 nM) is itself without effect on SY5Y-TrkA cells but affords synergistic enhancement of the antiapoptotic effect of the TrkA ligand 5C3 on these cells. Conversely, 5C3 (5 nM) alone is without effect on SY5Y-ET cells, and coincubation with 5C3 and MC192 does not alter the antiapoptotic effect of MC192 on these cells (Fig. 10). These results underscore the differential signaling capacity of NGF receptors in the two transfectants, and demonstrate the different roles played by p75 in SY5Y-TrkA and SY5Y-ET cells.

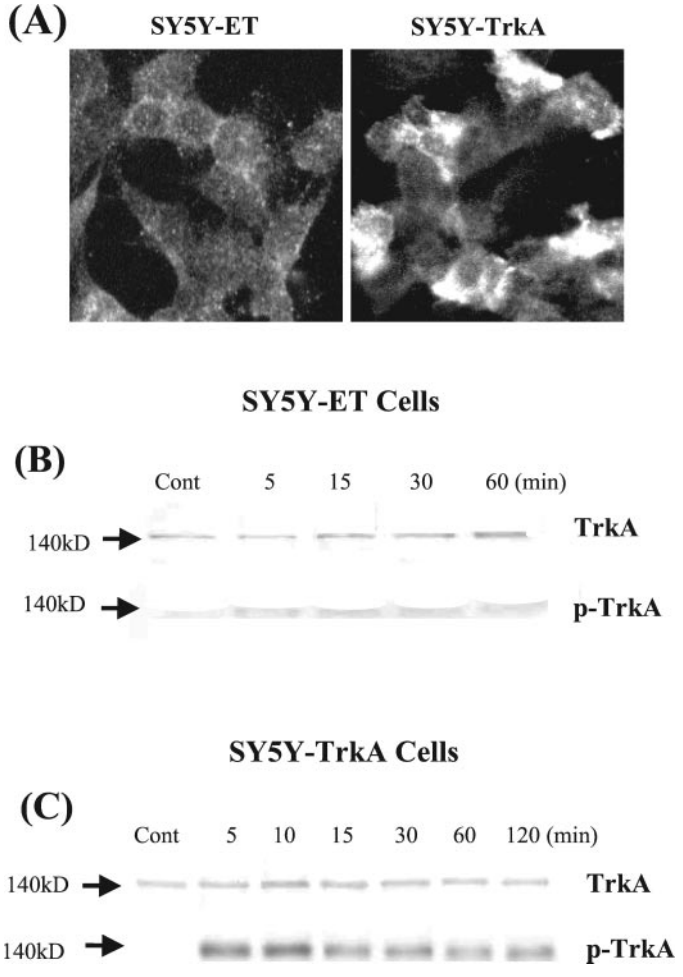

Fig. 7. A, differential TrkA receptor protein expression in SY5Y-ET and SY5Y-TrkA cells. Immunofluorescent staining for TrkA was performed on the native transfectants. B and C, effects of NGF on phosphorylation of TrkA are shown for SY5Y-ET (B) and SY5Y-TrkA (C) cells. Sister cultures of SY5Y-ET and SY5Y-TrkA cells were incubated for varying lengths of time with NGF (40 nM). Results for $t = 0$ (control) are denoted as "Cont". The TrkA and phospho-TrkA (*p*-TrkA) contents of lysates of these cells were determined by Western blotting as described under *Materials and Methods*. In the case of each transfectant, the same blot was stained with an antibody to TrkA, stripped, and subsequently stained with an antibody to *p*-TrkA. The notation to the left of each blot indicates the running position of a 140 kD TrkA standard on the same gel. A representative set of results for one experiment of four performed with SY5Y-TrkA cells and three performed with SY5Y-ET cells is shown.

Discussion

Neurotrophins have been implicated in positive and negative modulation of the susceptibility of neural tumors to chemotherapeutic agents (Cortazzo et al., 1996; Kim et al., 1999; Schor, 1999). NGF, the first neurotrophin described, has been found to

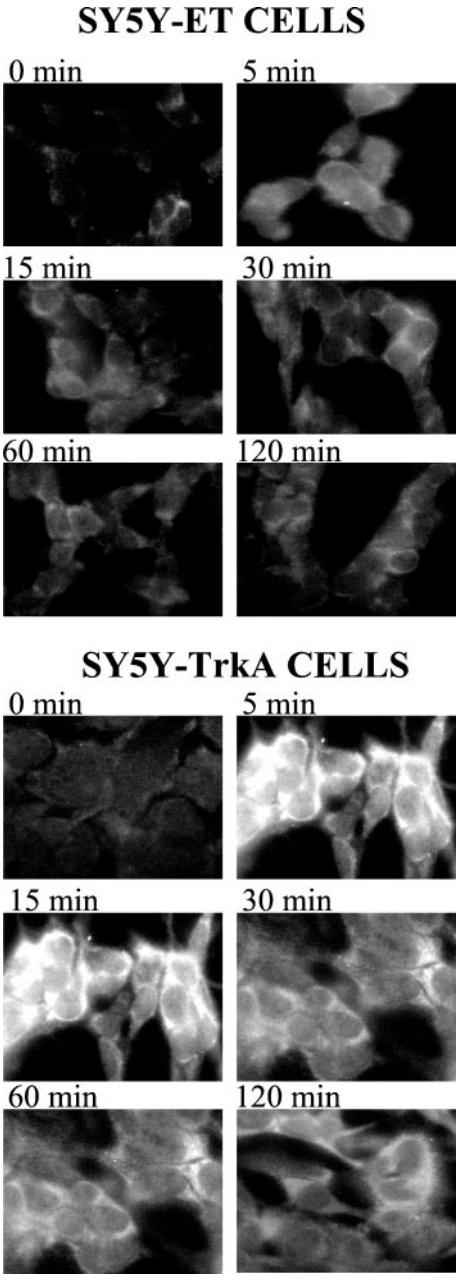

Fig. 8. Immunofluorescent staining for phospho-TrkA in SY5Y-ET and SY5Y-TrkA cells treated with NGF (40 nM). Sister cultures of SY5Y-ET and SY5Y-TrkA cells grown on glass coverslips were incubated for varying lengths of time with NGF and then stained with an antibody to phospho-TrkA. Staining with a fluorescent secondary antibody and subsequent microscopy were performed as detailed under *Materials and Methods*. Panels labeled "0 min" were treated with vehicle only and stained immediately thereafter.

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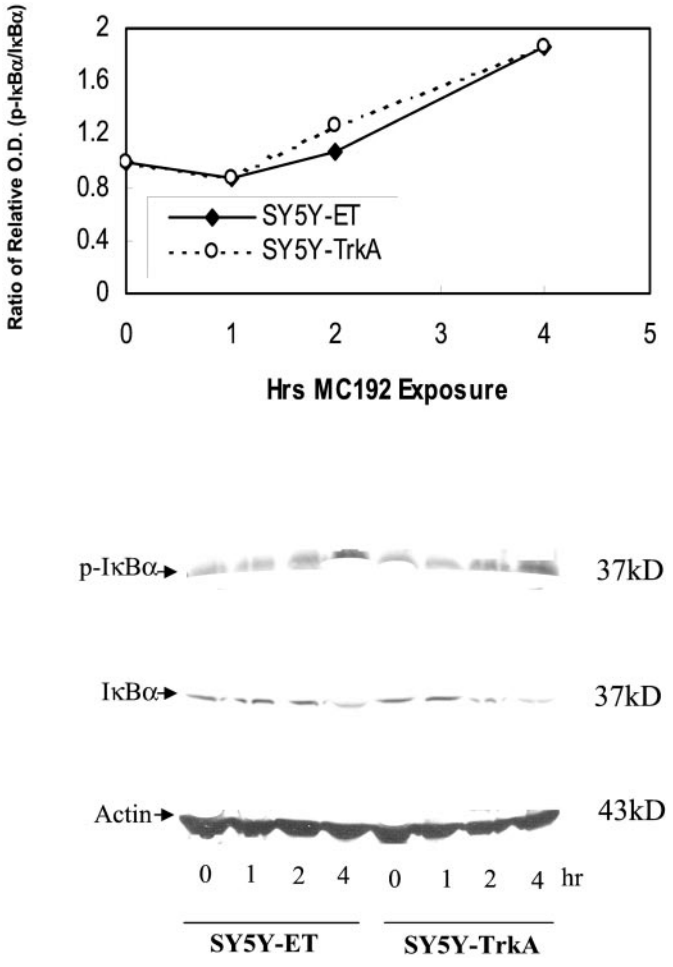

Fig. 9. Effects of monoclonal antibody MC192 on cellular content of phosphorylated IκB-α. Western blotting was performed to examine SY5Y-ET and SY5Y-TrkA cells for phosphorylated IκB-α after MC192 exposure (2 nM; 0–4 h). Plots of optical density were generated using a Bio-Rad Optiscan optical scanner. Each blot was stripped and stained for β-actin and IκB-α as a loading control and control for IκB-α expression, respectively. Results shown are from one representative experiment of two performed.

variably induce or prevent apoptosis of normal and neoplastic neural cells, depending upon the method by which apoptosis is induced and which of the two known NGF receptors (p75 or TrkA) is involved in mediating the response to NGF (Bredesen and Rabizadeh, 1997; Bredesen et al., 1998).

In addition, previous studies have demonstrated the multiple roles of the p75 receptor (Frade and Barde, 1998) and have suggested that its predominant role varies from cell type to cell type (Cortazzo et al., 1996). What determines the function of p75 in different cell types or in the same cell at different times in its development remaines unclear (Fundin et al., 1997). Several studies have indicated that p75 binding to TrkA, TrkB, or TrkC enhances the affinity of the respective Trk for its corresponding neurotrophin (Hempstead et al., 1991; Bredesen and Rabizadeh, 1997; Ryden et al., 1997; Ross et al., 1998; Brennan et al., 1999). Recently, it has been reported that a complex consisting of TRAF6 and atypical protein kinase C-interacting protein (p62) could serve as a bridge between p75 and TrkA signaling (Wooten et al., 2001). This interaction is almost certainly initiated by the physical proximity of the two receptors to one another (Huber and Chao, 1995; Gargano et al., 1997; Bibel et al., 1999). Some authors have hypothesized that the direct interaction of p75 with TrkA requires that the TrkA/p75 ratio be no smaller than 1/10 (Verdi et al., 1994; Greene and Kaplan, 1995). However, most of the studies performed to test this hypothesis compare the response to NGF of different cell lines that undoubtedly differ in characteristics other than just the TrkA/p75 ratio. Furthermore, none of these studies approach the question of what, if anything, p75 does in cells where a physical or functional TrkA-p75 interaction is not apparent.

The present study demonstrates that NGF decreases NCS-induced apoptosis in both SY5Y-ET and SY5Y-TrkA cells (Fig. 2). This neuroprotective effect of NGF is mediated by p75 in SY5Y-ET cells and by TrkA in SY5Y-TrkA cells. This conclusion is based on the following observations. First, monoclonal antibody MC192, a p75-specific NGF ligand, selectively protects SY5Y-ET cells, but not SY5Y-TrkA cells, from NCS-induced apoptosis (Fig. 3). Second, mAbNGF30, which occupies and inactivates the p75 binding site of NGF, selectively abolishes the protective effects of NGF on NCS-treated SY5Y-ET cells but not NCS-treated SY5Y-TrkA cells (Fig. 4). Third, although p75–NF-κB signaling is intact in both transfectants (Fig. 9), NGF induces such signaling only in SY5Y-ET cells (Figs. 5 and 6, A, B, and D), and not in SY5Y-TrkA cells (Figs. 5 and 6, A, C, and E). Thus, although

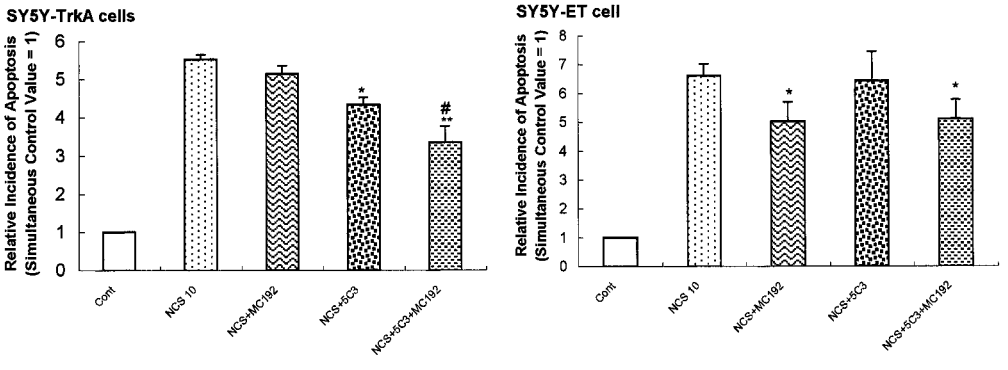

Fig. 10. The synergistic protective effects of 5C3 and MC192 on NCS-induced cell death in SY5Y-TrkA but not SY5Y-ET cells. Sister cultures were pretreated with 5C3 (5 nM) and/or MC192 (2 nM) for 24 h, then exposed to 10 nM NCS for 1 h in the presence of 5C3 and/or MC192. MC192 and 5C3 were left in the medium for the duration of the experiment. Results are expressed as relative incidence of apoptosis with apoptosis incidence in a simultaneous control sample set at 1. This represents the fold-increase in apoptosis after treatment relative to control conditions. *, $p < 0.05$; **, $p < 0.01$ compared with NCS alone; #, $p < 0.05$ protective effect of both ligands in coincubation compared with either ligand alone (one-way ANOVA followed by PLSD)

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the absolute p75 content is the same in both transfectants, the role of p75 signaling in the neuroprotective effects of NGF differs between them. The present studies are consistent with the study by Twiss et al. (1998), who reported that not only the presence of p75 but also the p75/TrkA ratio determines cellular responsiveness to NGF. Although there is ample evidence that changes in ligand concentration or availability that lead to alterations in receptor occupancy rate can alter the predominant signal transduction pathway activated by that ligand (Shimizu and Gurdon, 1999), no prior studies have shown directly that heterologous receptor ratio determines signaling by a single common ligand.

Many recent studies have demonstrated the existence of an independent function of p75 (Carter et al., 1996; Cortazzo et al., 1996; Brann et al., 1999; Coulson et al., 1999; Yamashita et al., 1999; Huang et al., 2000). The exact independent function of p75 seems to depend on whether NGF is bound to it (Rabizadeh et al., 1993; Huang et al., 2000). As a "naked" receptor, p75 seems in some cells to function as a mediator of apoptosis. In contrast, the binding of NGF to p75 prevents apoptosis induction in these lines (Rabizadeh et al., 1993; Cortazzo et al., 1996); p75 has therefore been said to induce "neurotrophin dependence" in cells (Bredesen et al., 1998). A number of studies in other cell types have demonstrated the apoptosis-inducing effect of NGF binding to p75 (Kuner and Hertel, 1998; Sedel et al., 1999). The present experiments differ from these studies in that we use an antimitotic, DNA-cleaving agent in the presence of serum, rather than serum deprivation or NGF exposure itself, to induce cell death. Apoptosis in this model is not itself p75-dependent, as evidenced by the absence of effect of NGF, MC192, or 5C3 alone (i.e., in the absence of NCS) on SY5Y-ET or SY5Y-TrkA cells. Binding of NGF to p75 protects these cells from apoptosis induced by the enediyne chemotherapeutic agent, NCS. This protection may be reflective of potential resistance to chemotherapy, and could therefore presage the existence of residual tumor after treatment. Because these neoplastic cells are mitotically active, even small numbers of chemoresistant cells could be biologically and medically significant.

From the signal transduction standpoint, TrkA is the starting point for a tyrosine kinase pathway that clearly mediates the differentiative and trophic functions of NGF. Many of the intermediate steps in the pathway from TrkA binding of NGF to the induction of neurite outgrowth have been identified (Cordon-Cardo et al., 1991; Kaplan et al., 1991; Kremer et al., 1991; Ohmichi et al., 1991; Saltiel and Ohmichi, 1993). More recently, an antiapoptosis function and pathway for TrkA have been described as well (Pincelli et al., 1997; Garcia Valenzuela and Sharma, 1998).

Other studies have suggested that NF-κB activation is associated with cell survival in some systems (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996). The signal transduction pathways responsible for this association are only recently being elucidated. NF-κB is composed of two subunits (p65 and p50) and exists in a complex with an inhibitory protein, termed IκB, in resting cells (Nagata, 1997). It has been suggested that ligand-induced trimerization of tumor necrosis factor (TNF) results in the recruitment of the death domain adaptor protein TRADD, which in turn recruits and interacts with receptor interaction protein (Ashkenazi and Dixit, 1998). Overexpression of receptor interaction protein activates NF-κB–inducing kinase, which in turn activates IκB kinase complex (IKK). Upon phosphorylation by IKK, IκB becomes ubiquitinated and degraded by proteosome complexes (Zandi et al., 1997). Once free of this inhibitor, NF-κB dimerizes and becomes an active transcription factor that translocates to the nucleus and activates transcription of NF-κB–responsive genes (Ashkenazi and Dixit, 1998). The low-affinity NGF receptor, p75, is a member of the TNF receptor family, and there is evidence to support the notion that activation and translocation of NF-κB are steps in the signaling cascade initiated by NGF binding to p75 (Carter et al., 1996).

In the present study, we demonstrated that NGF induces phosphorylation of IκB-α in SY5Y-ET cells, but not in SY5Y-TrkA cells (Fig. 6A). Furthermore, NGF induces the translocation of p65 from the cytoplasm to the nucleus of SY5Y-ET, but not SY5Y-TrkA, cells (Fig. 6, B-E). These findings, taken together with the effects of selective TrkA or p75 agonists in this system, support the notion that NGF protects against NCS-induced apoptosis through the p75-NF-κB signaling pathway in SY5Y-ET cells, and through a TrkA tyrosine phosphorylation pathway in SY5Y-TrkA cells.

These findings suggest that in cell lines and at stages of in vivo development in which p75 is in overabundance relative to TrkA, p75 serves an independent function, whereas a TrkA/p75 ratio closer to 100/100 implies enhancement of the TrkA-mediated functions of NGF by p75. Given the very wide tissue expression profile of p75 (Wheeler et al., 1998; Guate et al., 1999; Lara et al., 2000), these relationships are likely to have implications in both neural and non-neural tissues and pathological states (Labouyrie et al., 1997; Brann et al., 1999; Hamanoue et al., 1999; Hannila and Kawaja, 1999; Guate et al., 1999; Lara et al., 2000; Sortino et al., 2000). Whether NGF receptors can serve as a prototype for other homo- and heterodimeric receptors remains to be seen, particularly with regard to the mechanism by which receptor pairing is determined. Furthermore, additional studies will determine the generalizability to other chemotherapeutic agents of the effects of NGF on susceptibility to enediyne-induced apoptosis.

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