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Nerve Growth Factor Stimulates Proliferation and Survival of Human Breast Cancer Cells through Two Distinct Signaling Pathways*

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We show here that the neurotrophin nerve growth factor (NGF), which has been shown to be a mitogen for breast cancer cells, also stimulates cell survival through a distinct signaling pathway. Breast cancer cell lines (MCF-7, T47-D, BT-20, and MDA-MB-231) were found to express both types of NGF receptors: p140^{trkA} and p75^{NTR}. The two other tyrosine kinase receptors for neurotrophins, TrkB and TrkC, were not expressed. The mitogenic effect of NGF on breast cancer cells required the tyrosine kinase activity of p140^{trkA} as well as the mitogen-activated protein kinase (MAPK) cascade, but was independent of p75^{NTR}. In contrast, the anti-apoptotic effect of NGF (studied using the ceramide analogue C2) required p75^{NTR} as well as the activation of the transcription factor NF-kB, but neither p140^{trkA} nor MAPK was necessary. Other neurotrophins (BDNF, NT-3, NT-4/5) also induced cell survival, although not proliferation, emphasizing the importance of p75^{NTR} in NGF-mediated survival. Both the pharmacological NF-κB inhibitor SN50, and cell transfection with IkBm, resulted in a diminution of NGF anti-apoptotic effect. These data show that two distinct signaling pathways are required for NGF activity and confirm the roles played by p75^{NTR} and NF-κB in the activation of the survival pathway in breast cancer cells.

Nerve growth factor (NGF)[1] is the archetypal member of the neurotrophin superfamily, which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5, and NT-6 (1). NGF interacts with two classes of membrane receptor: the TrkA proto-oncogene product p140^{trkA}, which possesses intrinsic tyrosine kinase activity, and a secondary receptor, p75^{NTR}, that belongs to the tumor necrosis factor (TNF) receptor family (2). The stimulation of cell survival and cell differentiation by NGF and other neurotrophins have been described primarily in neuronal cell systems (3). Although the neurotrophic effect through p140^{trkA} is known to involve the MAPK cascade, the role of p75^{NTR} is still controversial; there is evidence that it can both positively and negatively regulate neuronal cell death and differentiation, depending on the cell type examined (4). In some cases, p75^{NTR} is an inducer of apoptosis, even without NGF stimulation (5), whereas in other cases the activation of p75^{NTR} by NGF results in a protection from cell death (6). In addition to its neurotrophic function, other activities of NGF have been described. For example, NGF can modulate gene expression in monocytes (7), it is chemotactic for melanocytes (8), and its inhibition on p75^{NTR} can block the migration of Schwann cells (9). NGF also stimulates the proliferation of chromaffin cells (10), lymphocytes (11), and keratinocytes (12). We have previously shown that NGF is mitogenic for cancerous but not normal human breast cells (13), and these data, as well as others showing a role for NGF in the stimulation of prostatic cancer cells (14–17), implicate NGF in non-neuronal carcinogenesis.

Both cellular proliferation as well as tumor cell survival are crucial for malignant progression. The effect of NGF on the survival of cancer cells through the p75^{NTR} receptor has been shown for neuroblastoma (18) and schwannoma (6). In prostate cancer, p75^{NTR} has been shown to be a mediator of NGF's effects during critical phases of developmental cell death and carcinogenic progression (19). To date only the mitogenic effect of NGF for breast cancer cells has been described (13), with its roles in the control of breast cancer cell survival unknown.

In this study, we have shown that, in addition to its mitogenic effect, NGF is also an anti-apoptotic factor for breast cancer cells. These cells express mRNA for both p140^{trkA} and p75^{NTR} receptors. Our results indicate that the mitogenic effect of NGF requires p140^{trkA} and the MAPK cascade, but not the p75^{NTR} receptor, whereas the promotion of cell survival strictly requires p75^{NTR} as well as NF-κB, but not p140^{trkA} and MAPK. Thus the mitogenic and anti-apoptotic effects of NGF on breast

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[1] The abbreviations used are: NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; NF-κB, nuclear factor-κB; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; PARP, polyADP-ribose polymerase; TNF, tumor necrosis factor; TBP, TATA box binding protein; RT-PCR, reverse transcriptase-polymerase chain reaction; FCS, fetal calf serum; DTT, dithiothreitol; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; GFP, green fluorescence protein; IκBm, dominant-negative IκBα mutant; bp, base pair(s); PD98059, Park Davis 98059.

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cancer cells are mediated through two different signaling pathways.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were purchased from BioWhittaker (France) except insulin, which was obtained from Organon (France). *Recombinant* human nerve growth factor, brain derived growth factor (BDNF), and neurotrophins 3 (NT-3) and 4 (NT-4) were from R & D Systems (UK). K-252a (inhibitor of trk-tyrosine kinase activity) and PD98059 (inhibitor of MAPK cascade) were from Calbiochem (France). The mouse monoclonal anti-NGF receptor ($p75^{NTR}$) antibody was from Euromedex (France) and was previously described for its ability to block the interaction between $p75^{NTR}$ and NGF (20). The anti-lamin B (C-20), goat polyclonal IgG, and the polyclonal anti-$p140^{trkA}$ (trk763) were from Santa Cruz Biotechnology. C2 ceramide analogue (*N*-acetyl-D-sphingosine), Hoechst 33258, and electrophoresis reagents were from Sigma Chemical Co. (France). The SN50 NF-κB inhibitor peptide, the rabbit polyclonal anti-NF-κB p65 antibody, was obtained from TEBU (France). Anti-PARP antibody was from Oncogene Research Products (UK). Primers and probes for TrkA and $p75^{NTR}$, probe for TATA box binding protein (TBP) were from Eurogentec (Belgium). RT-PCR reagents were from Applied Biosystems (France). Lipofectin reagent and Opti-MEM were provided by Life Technologies, Inc. (France). The green fluorescence protein plasmid (EGFPC1) was purchased from CLONTECH, and the dominant-negative IκBα mutant (IκBm) expression vectors (in PCDNA3) containing a Ser to Ala substitution at residues 32 and 36 were obtained from Dr. Jean Feuillard (UPRES EA 1625, Bobigny, France). p65 (rel-A) and c-rel cDNA were cloned at *Eco*RI site in PSVK3 expression plasmid. All vectors were obtained from Dr. Pascale Crépieux (McGill University, Montreal). The SY5Y subclone of SK-N-SH neuroblastoma cell line was a kind gift of Dr. Luc Buée (INSERM, U422, Lille, France). NT-2 (Ntera/D1) human neural precursor cells (Stratagene) are derived from a clone of the NT-2 teratocarcinoma.

Cell Culture—Breast cancer cell lines (MCF-7, T47-D, BT-20, and MDA-MB-231) were obtained from the American Type Culture Collection and routinely grown as monolayer cultures. Cells were maintained in minimal essential medium (Earle's salts) supplemented with 20 mM Hepes, 2 g/liter sodium bicarbonate, 2 mM L-glutamine, 10% fetal calf serum (FCS), 100 units/ml penicillin-streptomycin, 50 μg/ml gentamicin, 1% of non-essential amino acids, and 5 μg/ml insulin.

Detection of Neurotrophin Receptors mRNA Expression —The reverse transcription reaction mixture contained 2 g of purified total RNA (extracted from breast cancer cell lines, NT-2 cells, or SY5Y cells), 1× reverse transcription reaction buffer, 10 mM DTT, 400 mM dNTP each, 2.5 M oligo(dT) 18 primer, 40 units of RNasin, and 200 units of Moloney murine leukemia virus reverse transcriptase were added to 25 μl of total reaction volume. All the reaction mixtures were incubated at 37 °C for 1 h and then inactivated at 95 °C for 5 min. Polymerase chain reaction was performed on cDNAs after RT or corresponding total RNA samples without the RT step for negative controls. The primers used for *trkA* and p75 RT-PCR detection in breast cancer cell lines were as follows: *trkA* sense primer, 5′ (291)-CATCGTGAAGAGTGTCTCCG-3′ (311) and antisense primer, 5′ (392)-GAGAGAGACTCCAGAGCGTT-GAA-3′ (370) or p75 sense primer, 5′ (442)-CCTACGGCTACTACCAG-GATGAG-3′ (462) and antisense primer, 5′ (588)-TGGCCTCGTCG-GAATACG-3′ (571). The primers used for RT-PCR comparative detection of trks in MCF-7 cells were as follows: *trkA* sense primer, 5′ (118)-AGGCGGTCTGGTGACTTCGTTG-3′ (139) and antisense primer, 5′ (1162)-GGCAGCCAGCAGGGTGTAGTTC-3′ (1141) or trkB sense primer, 5′ (134)-CGAGGTTGGAACCTAACAGCATTG-3′ (157) and antisense primer, 5′ (1182)-GTCAGTTGGCGTGGTCCAGTCTTC-3′ (1159) or trkC sense primer, 5′ (219)-CACGGACATCTCAAGGAAGA-GCA-3′ (241) and antisense primer, 5′ (1078)-CTGAGAACTTCACCC-TCCTGGTAG-3′ (1056). Each pair of primers was used in RT-PCR reaction to amplify trks or p75. To PCR tubes were added 5 μl of PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 10 μl of 15 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 1 μl of cDNA or total mRNA (for negative control), 1 μl of 50 mM respective primers, 1 μl of 2.5 units/μl *Taq* DNA polymerase, and water to a total volume of 50 μl. The PCR conditions were as follows: after 95 °C for 3 min for denaturing cDNA, 30 cycles were run at 94 °C for 1 min, 57 °C for 2 min, and 72 °C for 3 min. The PCR tubes were incubated for a further 10 min at 72 °C for the extension of cDNA fragments after the final cycle, and the PCR products were electrophoresed in an agarose gel.

Cell Growth Assay—Experiments were performed as previously described (13). 35-mm diameter dishes were inoculated with 2×10^4 cells/dish in 2 ml of medium containing 10% FCS. After 24 h, cells were washed twice with serum-free medium. Next day, the medium was replaced with 2 ml of serum free medium containing 100 ng/ml NGF or various concentrations of other neurotrophins (BDNF, NT-3, NT-4/5). To study the effect of pharmacological inhibitors or blocking antibodies, various concentrations were added simultaneously with NGF (100 ng/ml). After 2 days of NGF exposure, cells were harvested by trypsinization and counted using an hemocytometer.

Determination of the Percentage of Apoptotic Cell Nuclei—Apoptosis of breast cancer cells was induced by the ceramide analogue C2, which has been described as a pro-apoptotic agent for human breast cancer cells (21, 22). Apoptosis was obtained by treatment with 2 μM C2 for 24 h. To evaluate the anti-apoptotic activity of NGF, various concentrations of this factor were tested; we found that the maximal effect was obtained for 100 ng/ml. Consequently, this concentration was used in all experiments with pharmacological inhibitors or blocking antibody. For determination of apoptotic cell percentage, cells were fixed with cold methanol (−20 °C) for 10 min and washed twice with phosphate-buffered saline (PBS) before staining with 1 μg/ml Hoechst 33258 for 10 min at room temperature in the dark. Cells were then washed with PBS and mounted with coverslips using Glycergel (Dako). The apoptotic cells exhibiting condensed and fragmented nuclei were counted under an Olympus-BH2 fluorescence microscope in randomly selected fields. A minimum of 500–1000 cells was examined for each condition, and results were expressed as a ratio of the total number of cells counted.

Statistical Analysis and Software—The statistical analysis of the data gathered from cell and apoptotic nuclei counting was performed using SPSS version 9.0.1 (SPSS inc., Chicago, IL). Analyses of variance were followed by the Tukey's test to determine the significance.

NGF Receptors and PARP Immunoblotting—Subconfluent cell cultures were harvested by scraping in serum-free medium. After centrifugation (1000 × *g*, 5 min), the pellet was treated with lysis buffer (0.3% SDS, 200 mM dithiothreitol) and boiled 5 min. In the case of PARP, the pellet was lysed with urea-rich buffer (62.5 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS), sonicated and incubated at 65 °C for 15 min. The lysates were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane (Immobilon-P, Millipore) by electroblotting (100 V, 75 min), and probed with anti-*trkA*, anti-$p75^{NTR}$ or anti-PARP antibodies at 4 °C overnight. The membranes were then incubated at room temperature for 3 h with biotin-conjugated anti-rabbit (TrkA) or anti-mouse ($p75^{NTR}$ and PARP) immunoglobulin G. After 1 h of incubation with extravidin, the reaction was revealed using the chemiluminescence kit ECL (Amersham Pharmacia Biotech) with Kodak X-Omat AR film.

Detection of $p140^{trkA}$ and MAPK Activation—Proteins were extracted in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.1% SDS, 1% Nonidet P-40, 100 μM sodium orthovanadate) prior to immunoprecipitation. Preclearing was done with protein A-agarose (10 μl/250 μl, 60 min, 4 °C). After centrifugation (10,000 × *g*, 2 min), the supernatant was incubated with monoclonal anti-MAPK (anti-ERK2) antibody (10 μl/250 μl, 60 min, 4 °C). Protein A-agarose (10 μl) was added for 60 min (4 °C) and then pelleted by centrifugation (10,000 × *g*, 2 min). The pellet was then rinsed three times with lysis buffer and boiled for 5 min in Laemmli buffer. After SDS-PAGE and electroblotting, nitrocellulose membranes were blocked with 3% bovine serum albumin. Membranes were then incubated with PY20 anti-phosphotyrosine antibody overnight at 4 °C, rinsed, and incubated with a horseradish peroxidase-conjugated anti-mouse IgG for 3 h at room temperature. Membranes were rinsed overnight at 4 °C before visualization with ECL.

Cell Fractionation and NF-κB Detection—Cell nuclear extracts were prepared as described by Herrmann *et al.* (23). Cells were trypsinized and then pelleted in minimal essential medium containing 10% FCS. After washing with ice-cold PBS, cells were repelleted and resuspended in 400 μl of ice-cold hypotonic buffer (10 mM Hepes, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 3 mM phenylmethylsulfonyl fluoride, and 3 mM DTT). After 10 min on ice, 25 μl of 10% Nonidet P-40 was added and crude nuclei were collected by centrifugation for 5 min. The nuclear pellet was resuspended in high salt buffer (50 mM Hepes, pH 7.4, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% (v/v) glycerol, 3 mM DTT, and 3 mM phenylmethylsulfonyl fluoride). After 30 min on ice with frequent agitation, the insoluble nuclear material was pelleted in a microcentrifuge for 10 min. Crude nuclear protein was collected from the supernatant and snap-frozen in a dry ice/ethanol bath. After thawing and boiling for 5 min in Laemmli buffer, the nuclear extracts were subjected to SDS-PAGE and probed with an anti-NF-κB p65 antibody. A control was established with anti-lamin B antibody.

Transfection of Iκβ, c-rel, and rel-A—Cotransfection experiments were carried out using Lipofectin reagent, as described by the manu-

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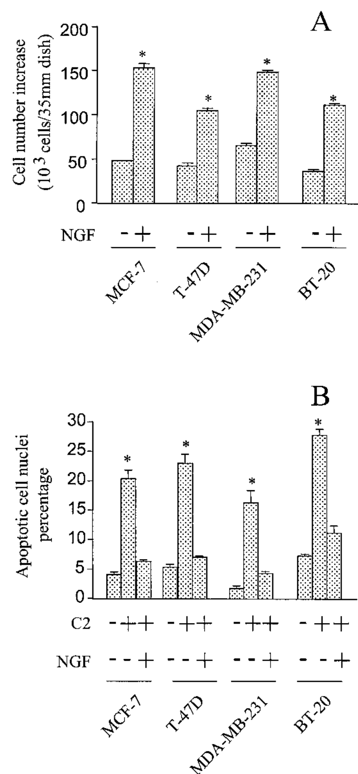

FIG. 1. **Effect of NGF on the growth and survival of breast cancer cells.** *A*, breast cancer cells were serum-deprived in minimum essential medium, and after 24 h the NGF (100 ng/ml) was added. After 48 h, cells were harvested and counted. *B*, cells were serum-deprived in minimum essential medium and treated with 2 μM C2 with or without 100 ng/ml NGF. After 24 h, cells were fixed and the proportion of apoptotic nuclei were determined after Hoechst staining under an Olympus-BH2 fluorescence microscope. For measurement of both cell number and apoptosis, results are expressed as the means ± S.D. of five separate experiments. Significance was determined using the Tukey's test (*, $p < 0.01$).

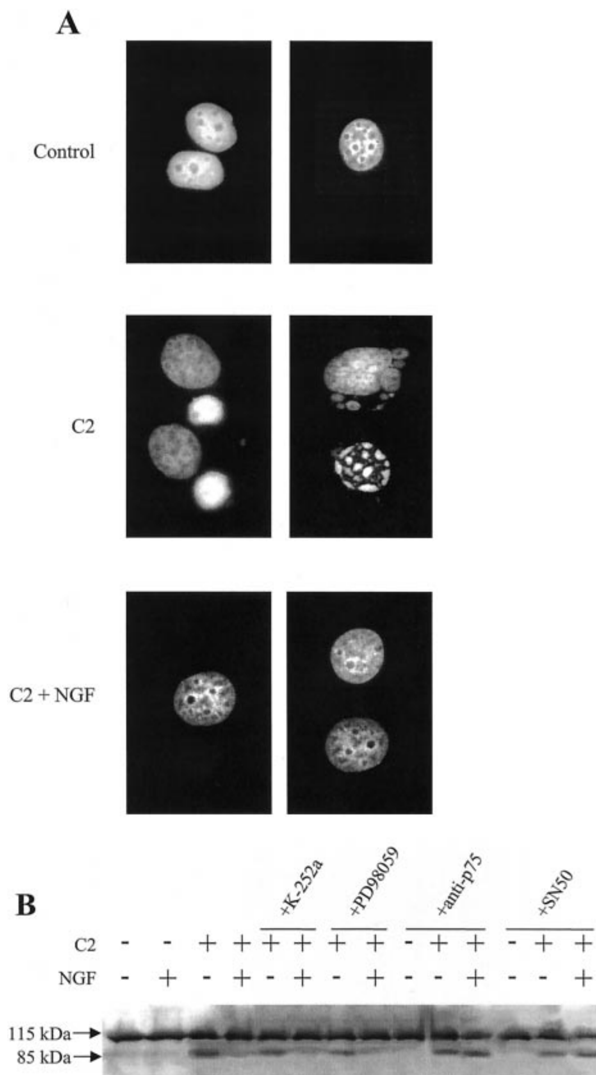

FIG. 2. **Anti-apoptotic effect of NGF.** *A*, Hoechst staining of apoptotic cell nuclei in control, C2 and C2+NGF-treated MCF-7 cells. Cells were serum-deprived in minimum essential medium and treated with C2. NGF was added at 100 ng/ml. After 24 h, cells were fixed and apoptotic nuclei were observed after Hoechst staining. *B*, immunoblot detection of PARP cleavage. C2-induced PARP cleavage was reversed by $p75^{NTR}$ activation mediated by NGF. MCF-7 cells were serum-deprived in minimum essential medium for 24 h and were then treated with 100 ng/ml NGF in the presence or absence of 2 μM C2, 10 nM K-252a, 10 μM PD98059, or 10 μg/ml anti-$p75^{NTR}$-blocking antibody (Euromedex) for another 24-h period. Proteins were detected after SDS-PAGE of cell preparations from MCF-7 breast cancer cells, electroblotting onto nitrocellulose, and immunodetection with anti-PARP antibodies.

facturer. Briefly, MCF-7 cells were incubated for 5 h in 1 ml of Opti-MEM transfection medium containing 8 μl of Lipofectin reagent, 0.8 μg of green fluorescence protein (GFP)-carrying vector and 0.2 μg of empty vector PCDNA3 or 0.2 μg of IκBm. In the case of c-rel or rel-A, cells were cotransfected with 0.8 μg of GFP-carrying GFP and 0.6 μg of $PSVK_3$ (empty plasmid), c-rel, or rel-A. Cells were then grown for 24 h with 10% FCS minimal essential medium and rinsed for 2 h in serum-free medium before incubation in serum-free medium in the presence or absence of 100 ng/ml NGF and/or 2 μM C2 for another 24 h. Cells were then fixed with paraformaldehyde 4% (4 °C) for 30 min, and the percentage of apoptotic cell nuclei in GFP-stained cells was determined as described above.

RESULTS

NGF Mitogenic and Anti-apoptotic Activity for Breast Cancer Cells—The effects of 100 ng/ml NGF on cell proliferation and C2-induced apoptosis were evaluated by cell counting and Hoechst staining, respectively. The results show that NGF induces an increase in cell number for all breast cancer cell lines tested (Fig. 1*A*). We have previously demonstrated that NGF has a direct mitogenic effect on breast cancer cells by recruiting cells in G_0 phase and by shortening the G_1 length (Descamps *et al.*, 1998). In addition, NGF rescued breast cancer cells undergoing C2-induced apoptosis; the maximum survival was observed at 200 ng/ml (Fig. 1*B*). The morphology of cells undergoing this NGF-induced anti-apoptotic rescue was quite distinct (Fig. 2*A*). The induction of apoptosis by C2 was found to involve cleavage of poly(A)DP-ribose polymerase (PARP); this cleavage was reversed by NGF (Fig. 2*B*).

TrkA and $p75^{NTR}$ Expression—RT-PCR was used to show the expression of mRNA for both high and low affinity NGF receptors in MCF-7, T47-D, BT-20, and MDA-MB-231 cells (Fig. 3*A*); the 102-bp band for the TrkA transcript and a 147-bp band for the $p75^{NTR}$ transcript were readily detectable on 1% agarose gels. Moreover, Western blotting demonstrated that both $p140^{trkA}$ and $p75^{NTR}$ were present in all the breast cancer cell lines (Fig. 3*B*). Real-time quantitative RT-PCR indicated that there was no significant change in the levels of TrkA and $p75^{NTR}$ mRNAs in the presence of FCS, NGF, or C2 (data not shown) and that the levels of mRNA for TrkA and $p75^{NTR}$ in breast cancer cells was between 5 and 10 times lower than the level observed in SY5Y neuroblastoma cells (data not shown). This indicates that NGF receptor expression in breast cancer cells is relatively limited. It should be noticed that, although mRNA levels of NGF receptors differ between breast cancer cells and SY5Y, the protein levels apparently do not. However,

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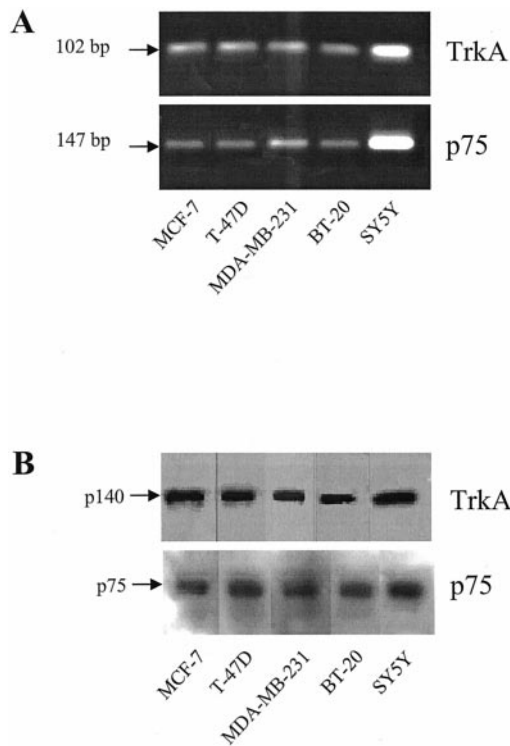

FIG. 3. **TrkA and p75^{NTR} expression in breast cancer cells.** *A*, agarose gel electrophoresis of RT-PCR products evidenced a 102-bp band and a 147-bp band, which are characteristic of TrkA and p75^{NTR}, respectively. Both NGF receptors were found in all cell types tested. *B*, p140^{trkA} and p75^{NTR} were immunodetected after SDS-PAGE of breast cancer cell lines. The neuroblastoma cells SY5Y were used as positive control for the expression of NGF receptors.

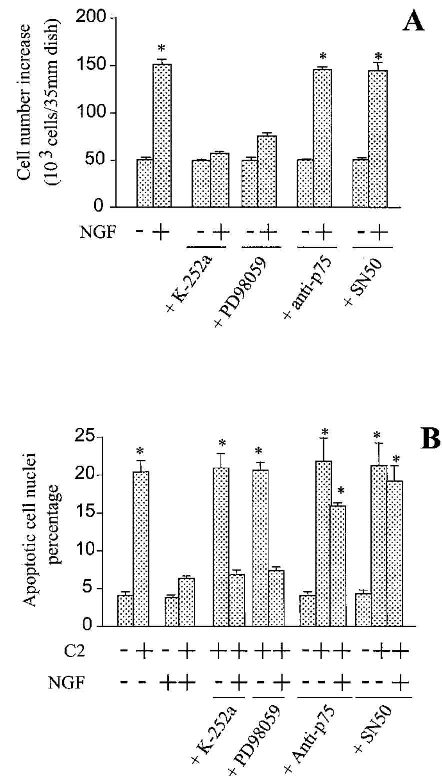

FIG. 4. **Pharmacological modulation of the proliferative and anti-apoptotic effect of NGF.** MCF-7 cells were starved in minimum essential medium, and after 24 h, 100 ng/ml NGF was added with or without inhibitors or antibody. *A*, after 48 h, cells were harvested and counted. *B*, after 24 h, cells were fixed and the proportion of apoptotic nuclei determined after Hoechst staining. The following concentrations were used: 2 μM C2, 10 nM K-252a, 10 μg/ml anti-p75^{NTR}-blocking antibody (Euromedex), 10 μM PD98059, 18 μM SN50. For *A* and *B*, results are expressed as the means + S.D. of five separate experiments. Significance was determined using the Tukey's test (*, $p < 0.01$).

it has been shown before that the level of a given cellular protein cannot be simply deduced from mRNA transcript level (24). One could hypothesize that the stability of mRNA and/or protein for NGF receptors, differs between breast cancer cells and neuroblastoma cells, leading to the observed disproportionality between mRNA and protein levels.

Involvement of p140^{trkA} and p75^{NTR} in Mitogenic and Survival Activities of NGF—We used a combination of specific antibodies and pharmacological inhibitors to study the putative functions of p140^{trkA} and p75^{NTR} in the stimulation of proliferation and cell survival induced by NGF. The Trk tyrosine kinase inhibitor K-252a, and the MEK inhibitor PD98059, both strongly inhibited the growth-stimulatory effect of NGF on MCF-7 cells, but had no effect on its anti-apoptotic effects (Fig. 4). Conversely, neither the anti-p75^{NTR} blocking antibody nor the NF-κB inhibitor SN50 affected NGF-stimulated proliferation, although both strongly reduced the anti-apoptotic effects (Fig. 4). The tyrosine kinase activity of p140^{trkA} was inhibited by K-252a but not by the anti-p75^{NTR} or PD98059 (Fig. 5). On the other hand, the activity of the MAPKs was inhibited by K-252a and PD98059 but not by the anti-p75^{NTR} (Fig. 5). It should be noted that the SN50 peptidic inhibitor of NF-κB, similarly to the anti-p75^{NTR}, inhibited the anti-apoptotic effect of NGF but neither its proliferative effect nor its activation of p140^{trkA} and MAPKs. The effect of other neurotrophins on MCF-7 cell growth and survival was also evaluated (Fig. 6*A*). In contrast to NGF, no proliferative effect was provided by BDNF, NT-3, or NT-4/5. However, all neurotrophins tested exhibited a rescue effect on C2-treated cells that was not altered in the presence of the trk inhibitor K-252a (Fig. 6*B*). These data suggest that trk receptors are not involved in NGF survival activity. Moreover, the participation of trkB and trkC in these events can be ruled out, because they are not expressed in these breast cancer cells (Fig. 6*C*).

NF-κB Involvement in the Anti-apoptotic Effect of NGF—The inhibitory effect of SN50 on the NGF anti-apoptotic activity indicated the potential involvement of NF-κB in the signaling leading to the protective activity of this growth factor. To further investigate this phenomenon, we studied the effect of NGF on the nuclear translocation of NF-κB, as well as the consequence of transfection by IkBm (an inhibitor of NF-κB) or by c-rel and rel-A (constitutively active subunits of NF-κB) on the NGF-mediated anti-apoptotic activity in MCF-7 cells. Western blotting revealed no change in the nuclear levels of NF-κB (p65) during apoptosis induced by C2 (Fig. 7). In contrast, the addition of NGF on C2-treated cells induced a translocation of NF-κB from cytoplasm to nucleus. Computerized quantification revealed a doubling p65 band intensity normalized to the total intensity of the lane (data not shown). Moreover, this NF-κB nuclear translocation was inhibited by the presence of p75^{NTR}-blocking antibody or SN50, but was not affected by K-252a and PD98059. Interestingly, in the absence of C2-induced apoptosis NGF was not able to induce the nuclear translocation of NF-κB, confirming previous observations that p75^{NTR}-mediated NF-κB activation requires cell stress (25). Transfection of MCF-7 cells with IkBm, an inhibitor of NF-κB, reversed the anti-apoptotic effect of NGF (Fig. 8*A*). As a control, we transfected MCF-7 cells with an empty vector; no effect was observed. In addition, transfection with activators of the NF-κB pathway, c-rel or rel-A (Fig. 8*B*), resulted in an inhibition of C2-induced apoptosis of MCF-7 cells, even in absence of NGF,

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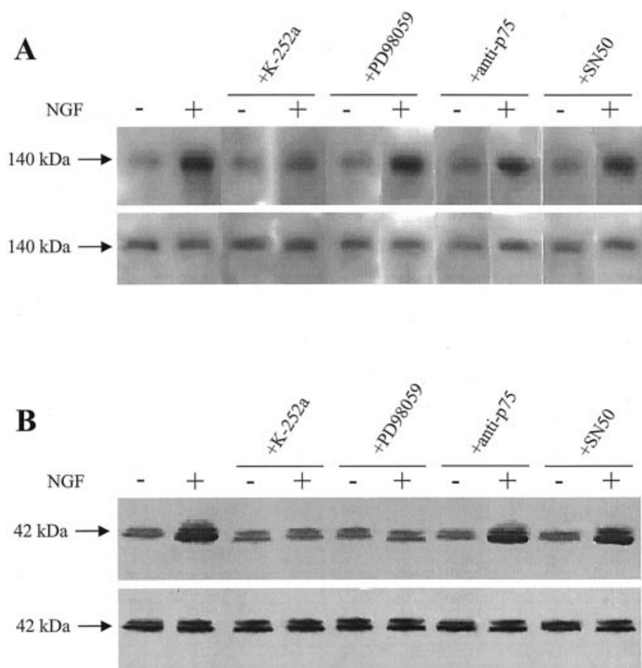
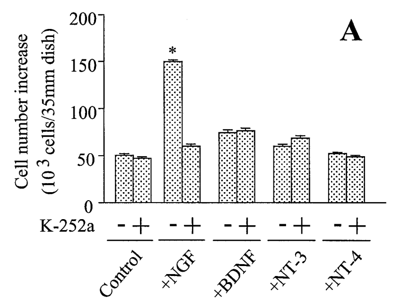


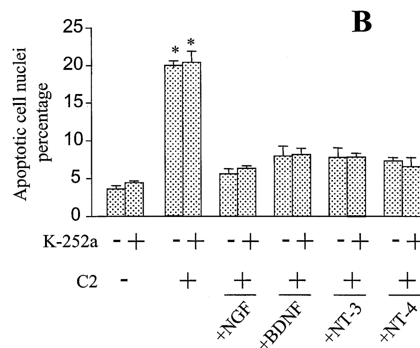
FIG. 5. **p140^{trkA} and MAPK activation.** MCF-7 cells were treated with 100 ng/ml NGF in the presence or absence of 10 nM K-252a, 10 μg/ml anti-p75^{NTR}-blocking antibody, or 10 μM PD98059. p140^{trkA} (*A*) and MAPK activation (*B*) were determined after immunoprecipitation using polyclonal anti-TrkA and monoclonal anti-ERK2 antibodies, respectively. After SDS-PAGE and electroblotting, nitrocellulose membranes were counterprobed with the PY20 anti-phosphotyrosine antibody. For detection of TrkA (*A*) and MAPK (*B*) activation, the *lower panel* shows reprobing of the blots with the immunoprecipitating antibody.

confirming the involvement of NF-κB family members in human breast cancer cell survival.

DISCUSSION

This study shows that, in addition to its mitogenic activity, NGF is anti-apoptotic for breast cancer cells, and that these two biological effects are differentially mediated by the p140^{trkA} and p75^{NTR} receptors, respectively. The growth of breast cancer results from a balance between cell proliferation and apoptosis, both of which can be modulated by various regulatory peptides. For example, epidermal growth factor, fibroblast growth factors, and insulin-like growth factor-1 can all stimulate the proliferation and survival of breast cancer cells (26). On the other hand, agents such as transforming growth factor-β or tumor necrosis factor-α can inhibit growth and induce apoptosis in these cells (27). Recently we have shown that NGF, which was primarily described for its neurotrophic properties, is a strong mitogen for cancerous but not for normal human breast epithelial cells, suggesting a crucial function for this factor in the initiation and progression of human breast tumors (13). In the present study, we have shown that the breast cancer cells express transcripts for both TrkA and p75^{NTR} receptors. In contrast, no expression of TrkB and TrkC was found in any of the breast cancer cells tested, in accordance with the fact that BDNF, NT-3, or NT-4/5 have no mitogenic effect for these cells. The presence of NGF receptors has been detected previously in breast cancer cells (28), and low levels of NGF receptor expression have recently been reported in other breast cancer cell lines (29), leading to the hypothesis of a recruitment and cooperation between p140^{trkA} and p185^{Her-2} for the induction of mitogenesis by NGF. Our results indicate a stimulation of p140^{trkA} tyrosine kinase activity and of the MAPK cascade by NGF, and the use of the pharmacological inhibitors K-252a and PD98059 demonstrate the requirement for these signals in NGF-induced MCF-7 cell proliferation. The induction of MAPK activity required p140^{trkA} activation, but p75^{NTR} did not appear to be involved, because p75^{NTR}-blocking antibodies did not have any effect on NGF-induced MAPK





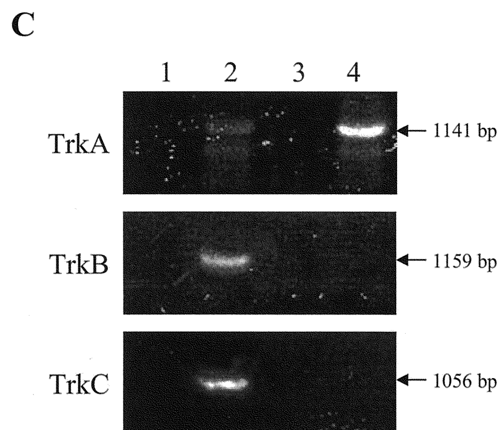


FIG. 6. **Effect of different neurotrophins on MCF-7 cells growth and survival.** MCF-7 cells were serum-deprived in minimum essential medium, and after 24 h the neurotrophins (100 ng/ml NGF, 50 ng/ml BDNF, 50 ng/ml NT-3, 100 ng/ml NT-4/5) were added. *A*, after 48 h, cells were harvested and counted. In contrast with NGF, neither BDNF, NT-3, nor NT-4/5 displayed significant bioactivity (for concentrations up to 400 ng/ml). *B*, MCF-7 cells were serum-deprived in minimum essential medium and treated with 2 μM C2, with or without neurotrophins (100 ng/ml NGF, 50 ng/ml BDNF, 50 ng/ml NT-3, 100 ng/ml NT-4/5). After 24 h, cells were fixed and apoptotic nuclei percentage was determined after Hoechst staining under an Olympus-BH2 fluorescence microscope. For measurement of both cell number and apoptosis, results are expressed as the means ± S.D. of five separate experiments. Significance was determined using the Tukey's test (*, $p < 0.01$). *C*, TrkB and TrkC mRNA expression in MCF-7 cells. Agarose gel electrophoresis of RT-PCR products reveals TrkA expression, but no TrkB or TrkC expression in MCF-7 breast cancer cells. Human NT2 cells were used as positive control for the expression of TrkB and TrkC. *Lane 1*, NT2-negative control without RT step; *lane 2*, NT2-positive control; *lane 3*, MCF-7 cells-negative control without RT step; *lane 4*, MCF-7 cells.

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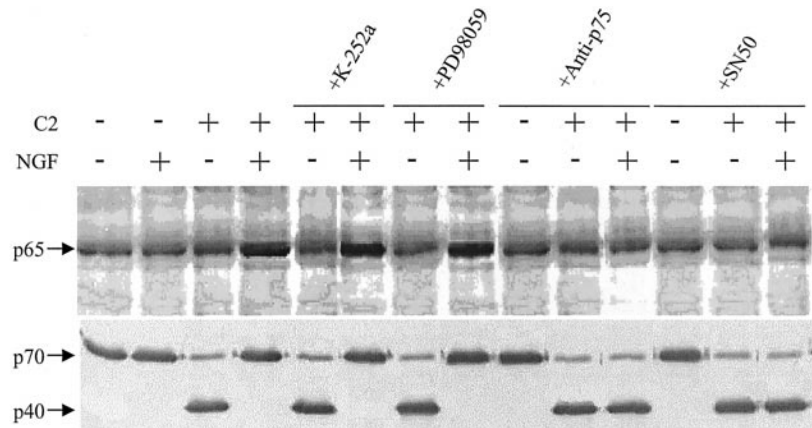

FIG. 7. **Activation of NF-κB during NGF anti-apoptotic effect.** MCF-7 cells were treated with 100 ng/ml NGF in the presence or absence of 10 nM K-252a, 10 μM PD98059, or 10 μg/ml anti-p75[NTR] blocking antibody (Euromedex). Proteins were detected after SDS-PAGE of nuclear extract preparations and immunoblotting with rabbit anti-NF-κB p65. The *lower panel* shows immunoblotting with anti-lamin B.

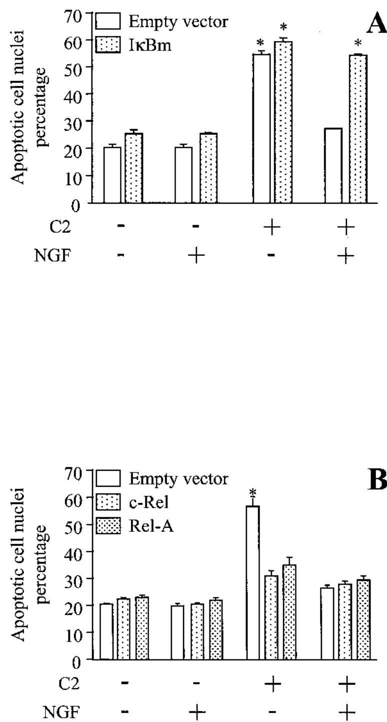

FIG. 8. **Modulation of NGF anti-apoptotic effect by IκBm, c-rel, and rel-A transfection.** MCF-7 cells were co-transfected with EGFPC1 and either IκBm (*A*) or c-rel or rel-A (*B*) using the Lipofectin reagent. Controls were performed with both PCDNA3 and EGFPC1 (for IκBm experiments) and both PSVK$_3$ and EGFPC$_1$ (for c-rel and rel-A experiments). After 24 h, cells were serum-deprived in minimum essential medium and either C2 or neurotrophins added for another 24-h period. Cells were then fixed and the percentage of apoptotic nuclei in transfected cells (with the expression of GFP as a transfection control) determined after Hoechst staining. Results are expressed as the means ± S.D. of six separate experiments. Significance was determined using the Tukey's test (*, $p < 0.01$).

activation and cell proliferation. In contrast, p75[NTR]-blocking antibodies exhibited an inhibition of NGF-induced survival, attesting to the functionality of these blocking antibodies. Thus, the mitogenic activity of NGF requires the p140^{trkA} and MAPK cascade independently of the p75[NTR] receptor. This signaling pathway for the NGF-proliferative effect appears to be similar to that which is described for the neurotrophic activity of this factor. For example, in PC-12 pheochromocytoma cells, disruption of p75[NTR] does not result in an inhibition of the NGF differentiative activity, which is mediated by the p140^{trkA}/MAPK pathway (30). Interestingly, it has also been shown in PC-12 cells that NGF induces survival and differentiation through two distinct signaling pathways, because the activation of the MAPK cascade is required for the differentiative but not the protective activity of NGF (31). These data emphasize the similarities between the mitogenic and neurotrophic signaling pathways of NGF.

The function of NGF as a survival factor has been extensively described for neurons in both *in vitro* and *in vivo* (32). However, the intracellular signaling involved in the anti-apoptotic activity of NGF in neurons remains controversial. The p140^{trkA}/MAPK cascade is generally described as protective for neuronal cell death, although there has been a recent report of a novel apoptotic pathway mediated by p140^{trkA}/MAPK in medulloblastoma cells (33). Unlike the p140^{trkA} receptors, the definition of the precise physiological role of p75[NTR] has proven difficult (4). The p75[NTR] receptor belongs to the TNF-receptor family, including among others, types I and II of the TNF receptor, the Fas antigen, and CD40 (34). The common cellular responses to activation of this family of receptors are the activation of gene transcription via nuclear factor-kB (NF-κB) and the regulation of cell survival/apoptosis. In some cases apoptosis was shown to develop following NGF binding to p75[NTR], although in other cases it appeared to occur in the absence of ligand (spontaneous apoptosis) and was reversed by NGF (35). The C2 reagent used here is known to induce apoptosis in breast cancer cells such as MCF-7 (21, 22). Morphological analysis after Hoechst staining and the inhibition of PARP cleavage demonstrated that NGF rescues breast cancer cells from C2-induced cell death. Interestingly, K-252a and PD98059 did not affect the anti-apoptotic activity of NGF, indicating that p140^{trkA} tyrosine kinase and MAPK activities are not necessary for the protective effect. Previous reports have noted that NGF is able to elicit its biological effects through p75[NTR] receptors and independently of p140^{trkA} in neurons (36, 37) and Schwann cells (38). In our experiments, a specific role for p75[NTR] in the cell survival effect was first suggested by the fact that other neurotrophins (interacting with p75[NTR] and not with p140^{trkA}) are also able to protect cells from death while having no impact on cell proliferation. The crucial role of p75[NTR] was further demonstrated by the use of p75[NTR]-blocking antibodies, which completely reversed the protective effect of NGF from C2-induced apoptosis. Moreover, BDNF, NT-3, and NT-4/5, all of which can bind P75[NTR], can also stimulate breast cancer cell survival. Because TrkB and TrkC are not expressed in breast cancer cells, these data emphasize the role played by p75[NTR] in the anti-apoptotic effect of NGF. Activation of p75[NTR] specifically induces NF-κB independent of p140^{trkA} in several cell types, including Schwann cells (38). To explore the involvement of NF-κB in the NGF survival effect, we first tested SN50, which inhibits the nuclear translocation of this transcription factor (39). We found that it blocked the anti-apoptotic effect of NGF without affecting the p140^{trkA}/MAPK cascade or cellular proliferation. The involvement of

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NF-κB was further demonstrated by transfection with a mutated form of IkBa, which blocked NF-κB translocation to the nucleus. MCF-7 cells transfected by mutated IkBa were not rescued from C2-induced apoptosis by NGF, confirming the involvement of NF-κB in the anti-apoptotic activity mediated by p75^NTR^. Similar observations have been made in PC12 cells in which the blocking of p75^NTR^-mediated activation of NF-κB resulted in an enhancement of apoptosis (40). In addition, transfections by c-rel or rel-A, which are constitutively activated forms of NF-κB, had a protective effect on MCF-7 cells treated by C2 in absence of NGF stimulation. c-rel and rel-A belong to the NF-κB family of transcription factors. The protection from apoptosis observed after transfection with this factor emphasizes the role played by NF-κB molecules in the control of breast cancer cell survival.

In conclusion, our results demonstrate that NGF is an anti-apoptotic factor for human breast cancer cells and that the signaling pathway leading to this survival activity is distinct from the signaling pathway, which leads to mitogenic stimulation. Although $p140^{trkA}$ and the MAPKs mediate the mitogenic activity of NGF, its anti-apoptotic activity required $p75^{NTR}$ and NF-κB alone. NGF is present in the mammary gland (41, 42) as well as its transcripts,[2] and our present finding therefore emphasizes that NGF is a crucial regulator of mammary tumor growth. The inhibition of breast cancer progression through the targeting $p140^{trkA}$ and $p75^{NTR}$ should be considered as a potential perspective for the treatment of this pathology.

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