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Mediation of NGF-stimulated Extracellular Matrix Invasion by the Human Melanoma Low-affinity p75 Neurotrophin Receptor: Melanoma p75 Functions Independently of *trkA*

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Although overexpression of the low-affinity p75 neurotrophin receptor ($p75^{NTR}$) is frequently associated with advanced stages of human melanoma progression, the functional significance of this finding is unknown. We examined whether the degree of cell surface expression of $p75^{NTR}$ in human melanoma cell variants determines their extent of invasion stimulated by nerve growth factor (NGF). Treatment of MeWo melanoma cells or a metastatic spontaneous wheat germ agglutinin-resistant variant subline (70W) of MeWo cells with 2.5S NGF resulted in a dose-dependent enhancement of invasion through a reconstituted basement membrane. This effect was most pronounced with the 70W subline that exhibits brain-metastasizing potential in nude mice but was not found with a poorly metastatic MeWo variant subline (3S5). The expression of $p75^{NTR}$ as determined by Northern blotting and immunoprecipitation analysis of ^{125}I-labeled cell surface proteins correlated with NGF-stimulated invasion. The MeWo melanoma sublines used in this study did not express $p140^{proto\text{-}trkA}$ mRNA or any $p140^{proto\text{-}trkA}$ variant transcripts including $p70^{trkA}$ as determined by Northern analysis and RT-PCR analysis. Thus, these melanoma cells would not be expected to form functional p75–p140 heterodimers or p140-p140 homodimers capable of transducing an NGF-generated signal to $p140^{proto\text{-}trkA}$ cytoplasmic substrates. These cells did express authentic $p145^{trkC}$ transcripts. However, NGF did not catalytically activate $p145^{trkC}$ receptors via increased tyrosine phosphorylation as would be expected if $p145^{trkC}$ participated in the signaling established by NGF. Furthermore, a NGF-stimulated purine-analogue-sensitive kinase activity was found to coimmunoprecipitate with $p75^{NTR}$. This $p75^{NTR}$-associated kinase may coordinate initial signaling events evoked by $p75^{NTR}$ ligand interaction. Addition of 2.5S NGF, at concentrations that should saturate cell surface $p75^{NTR}$, to matrix-adherent cultures of human MeWo and 70W but not 3S5 melanoma cells suppressed the expression of 92-kDa type IV collagenase and stimulated the production of 72-kDa type IV collagenase in its fully active 68-kDa form. In the absence of $p140^{proto\text{-}trkA}$, the matrix-dependent effects of NGF on metalloproteinase expression of brain-metastatic 70W melanoma cells suggest a signaling role for the low-affinity melanoma $p75^{NTR}$ receptor and its associated purine-analogue-sensitive kinase in signaling enhanced matrix penetration of NGF-rich stromal microenvironments such as the brain.

INTRODUCTION

The progression of malignant melanoma occurs through a series of clinically defined stages, from benign proliferative lesions without clinical evidence of invasion to lesions with local invasion of surrounding stromal tissue and ultimately to highly aggressive metastatic disease capable of invasion and growth at distant organ site

microenvironments. Interestingly, one of the primary metastatic targets and often a clinical endpoint of melanoma progression is colonization of the brain. At autopsy ~55% of advanced melanoma cases present with brain metastases (Patel *et al.*, 1978). The progressive steps of this process have revealed alterations in in vitro cell growth properties, adhesive characteristics, cell motility, growth factor dependency, and extracellular matrix/basement membrane degradation (Herlyn *et al.*, 1990; Hart *et al.*, 1991; Nicolson, 1991).

Coincident with the progression of malignant melanoma to the metastatic phenotype are modifications in the cell surface display of growth factor receptors and the production of various autocrine growth factors. Comparison of growth factor production between normal human foreskin melanocytes and malignant human melanoma cell lines indicates enhanced production of transforming growth factor-β_2, transforming growth factor-α, basic fibroblast growth factor, keratinocyte growth factor, and the A-chain of platelet-derived growth factor in melanoma cells from advanced lesions (Albino *et al.*, 1991). The functional significance of these alterations in the development of various aggressive properties exhibited by invasive melanoma cells remains largely unknown.

Of particular interest, because of the neural crest origin of melanocytes, is the increased production of the low-affinity receptor neurotrophin ($p75^{NTR}$, previously termed $p75^{NGFR}$) in cultured melanoma cells established at advanced stages of melanoma progression (Herlyn *et al.*, 1985). Histopathological examination of $p75^{NTR}$ production in situ reveals increased synthesis in advanced melanoma stages (Ross *et al.*, 1984). Despite their ability to internalize nerve growth factor (NGF), it is not known whether NGF is capable of modulating the biological behavior of human melanoma cells (Fabricant *et al.*, 1977).

To determine a possible role for $p75^{NTR}$ in regulating invasion-associated properties of melanoma cells we used a series of human melanoma cell variants selected from a parental MeWo line by subjection to spontaneous wheat germ agglutinin (WGA) toxicity (Ishikawa *et al.*, 1988). We focused on two MeWo variant lines, the poorly metastatic subline 3S5 and the highly metastatic subline 70W. The 70W subline displays a highly aggressive pattern of experimental metastasis in nude mice and has the ability to colonize lung, muscle, subcutis, and mesentery. The 70W variant is considered a unique human melanoma cell line because of its capacity to form brain colonies in nude mice. As such, target organ site colonization by the 70W line is similar to the clinical presentation of human melanoma metastasis in advanced disease stages. Here we show that overexpression of $p75^{NTR}$ is associated with enhancement of basement membrane invasion and secretion of matrix-degrading enzymes and that human melanoma $p75^{NTR}$ can function independently of $p140^{proto\text{-}trkA}$.

MATERIALS AND METHODS

Cell Culture

The human melanoma cell line MeWo and the WGA-selected MeWo variants (3S5 and 70W) have been previously described (Ishikawa *et al.*, 1988). The human A875 melanoma cell line was a gift of Dr. George Todaro, Fred Hutchinson Cancer Research Center (Seattle, WA). All melanoma cell lines were cultured in Dulbecco's modified Eagles medium/F12 (DME/F12, 1:1 vol/vol) containing 5% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2-95% air atmosphere in the absence of antibiotics. The SY-5Y human neuroblastoma cells were obtained from Dr. Hideyuki Saya, The University of Texas, M.D. Anderson Cancer Center (Houston, TX) and cultured in DME/F12, 1:1 vol/vol containing 10% FBS. Biological assays of NGF (Collaborative Research, Bedford, MA) activity were performed on PC12 pheochromocytoma cells cultured in RPMI-1640 medium (GIBCO-Bethesda Research Laboratories, Gaithersburg, MD) supplemented with 10% horse serum and 5% FBS. PC12 cells were grown on a gelatin substrate. The bioassays of NGF were performed with 50 ng/ml 2.5S NGF at PC12 cell densities of $2–8 \times 10^5$ per 35-mm tissue culture dish for 72 h. Bioactivity of NGF was determined by a neurite extension assay (Greene and Tischler, 1976). Cells were subcultured using EDTA (1 mM in phosphate-buffered saline) treatment and were grown without addition of antibiotics. All cells were screened for mycoplasma contamination using a ^{3}H-single-stranded DNA detection procedure (Gen-Probe, San Diego, CA) and were found to be mycoplasma-free.

In Vitro Invasion Assays

The invasive potentials of the melanoma cells used in this study were assayed according to the method of Albini *et al.* (1987) with modification. Reconstituted basement membrane Matrigel (Collaborative Research) was diluted 1:30 in cold DME/F12 and applied (~20 μg/100 μl/filter) to the upper surface of a polycarbonate filter insert (8 μm pore size/6.5 mm diameter) in a Transwell chamber (Costar, Cambridge, MA). Cells were seeded into the upper compartment of the Transwell insert at 2×10^4 cells/chamber in serum-free DME/F12 containing 0.1% bovine serum albumin with or without the addition of 2.5S NGF. The bottom compartment contained serum-free human brain microvessel endothelial cell-conditioned medium collected 48 h after shifting the cells to serum-free culture conditions (Hamada *et al.*, 1992). The cells were incubated for 72 h on Matrigel, and the number of cells that penetrated the Matrigel and filter and were found in the lower compartment were counted in triplicate assays. To assist in counting, the cells were fixed in 3% glutaraldehyde and stained with Giemsa.

Northern Blot Hybridization

Total RNA was isolated from subconfluent cell cultures by extraction with guanidinium thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987). Equal amounts of RNA (17 μg per lane, as determined by 260/280 nm absorbance ratio) were electrophoresed in a 1% agarose gel containing 0.22 M formaldehyde. RNA was capillary blotted onto an Immobilon-N membrane (Millipore, Bedford, MA). The probe used to detect $p75^{NGFR}$ mRNA was a 40-mer single-stranded oligonucleotide in the anti-sense orientation derived from translated sequences near the N-terminus of the human $p75^{NTR}$ gene (Oncogene Science, Manhasset, NY). The probe used to detect *trkA* mRNA was a 40-mer single-stranded synthetic oligonucleotide in the anti-sense orientation derived from translated sequences upstream from the putative transmembrane domain of the *trkA* gene (Oncogene Science). Both oligonucleotides were 5′-end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Radiolabeled oligonucleotides were purified from unincorporated nucleotides using G-25 Quick-spin columns (Boehringer Mannheim, Indianapolis, IN). Approximately 0.6 pmol of 5′-end labeled oligonucleotide probe was added to the hybridization buffer during the hybridization step. Prehybridization and hybrid-

ization were performed in Quick-hyb buffer (Stratagene, La Jolla, CA). Hybridization was performed at 65°C for 6 h. Washes were carried out in 2× SSC/0.1% sodium dodecylsulfate (SDS) at 65°C (1× SSC is 150 mM sodium chloride and 15 mM sodium citrate). A total of four washes were performed (10 min per wash). The hybridized membrane was subjected to autoradiography using Hyperfilm-MP (Amersham, Arlington Heights, IL), and the exposed film was developed using an M35A X-OMAT Film Processor (Kodak, Rochester, NY). Hybridized probe was stripped from the membrane for rehybridization according to the manufacturer's instructions.

Cell Surface Radioiodination and Immunoprecipitation Analysis

We have developed a vectorial cell surface-labeling procedure that does not destroy biological activity in whole cells. Subconfluent cell cultures grown in 100-mm tissue culture dishes were washed twice with FBS-free DME/F12 (1:1 vol/vol) followed by two washes with calcium- and magnesium-free *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES CMFH)-buffered balanced salt solution consisting of 129 mM NaCl, 5 mM KCl, 0.3 mM $Na_2HPO_4 \cdot 7\ H_2O$, 1 mM $NaHCO_3$, 5 mM Glucose, 25 mM HEPES pH 7.4 (CMFH). CMFH containing 2 mM EDTA was used to remove adherent cells, and this was followed by centrifugation at 500 × *g*. Pelleted cells were resuspended and washed twice by centrifugation and resuspension in CMFH without EDTA and counted on a Model ZBI Coulter Counter (Coulter, Hialeah, FL), and 2.0×10^7 cells were resuspended in 5 ml CMFH.

Before cell surface radioiodination, polypropylene microcentrifuge tubes were washed with $CHCl_3$ and coated with 100 μg iodogen (1 mg iodogen/ml $CHCl_3$, Pierce Chemical Company, Rockford, IL) and vacuum dried. Sulfosuccinimidyl-3(4-hydroxyphenyl)proprionate (4 mM Sulfo-SHPP, Pierce) stock was prepared in 100 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS) pH 7.2 (Calbiochem, La Jolla, CA) immediately before iodination, and 40 nm (10 μl) of this solution was combined with 1 nm (2 mCi) of carrier-free Na[^{125}I] (400 mCi/ml, ICN Radiochemicals, Irvine, CA). The mixture was transferred to a MOPS buffer-washed, iodogen-coated vial and mixed for 2 min at 25°C on a Vortex Genie mixer (Scientific Industries, Bohemia, NY). The ^{125}I-Sulfo-SHPP product was transferred to 5 ml CMFH at 0°C, mixed, and combined with 2×10^7 tumor cells in 5 ml CMFH at 0°C. Tumor cells in the reaction mixture were incubated on wet ice for 30 min with occasional resuspension of the cells by inversion of the 13-ml centrifuge tube. The reaction was terminated by the addition of ice-cold FBS-free culture medium. Cells were resuspended in 10 ml ice-cold, FBS-free medium, transferred to a 1.5-ml microcentrifuge tube, and washed twice with 1 ml ice-cold CMFH by centrifugation at 500 × *g* for 5 min in a Beckman Model 12 microcentrifuge (Beckman, Palo Alto, CA). The resulting pellet was solubilized in a buffer consisting of 10 mM (3-[3-chloroamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 10 μM leupeptin, 200 μM phenylmethylsulfonyl fluoride (PMSF), 50 mM NaCl, 100 μM EDTA, and 25 mM HEPES pH 7.8, whose volume was five times the packed cell volume. We performed dilution studies using either sulfosuccinimido-LC-biotin or unlabeled Sulfo-SHPP to determine the appropriate concentration of Sulfo-SHPP to ensure that *N*-hydroxysulfosuccinimidyl-ester that reacted with primary amines on the cell surface would not affect the electrophoretic mobility of cell surface proteins or the adhesive or invasive properties of the cells.

CHAPS detergent cell lysates were precleared by incubation for 60 min with 2 μg affinity-purified rabbit anti-mouse $IgG_{(H+L)}$. To the antibody/lysate mixture 25 μl of recombinant proteinA/G (Oncogene Science) was added, and the slurry was mixed constantly for 60 min at 4°C. Nonspecific immune complexes were recovered by centrifugation. The supernatant was retained for specific immunoprecipitation. The immunoprecipitation reactions were performed in microcentrifuge tubes using 100 μl of radioiodinated-CHAPS-cell lysate in combination with the appropriate antibody: 10 μg of anti-$p75^{NGFR}$ monoclonal antibody (mAb) (Amersham), 10 μg of anti-c-*trk* mAb (Oncogene Science). The reaction mixture was incubated for 60 min at 25°C and mixed occasionally on a Vortex Genie at a setting of 2. The agarose beads were washed twice with solubilization buffer, transferred to a fresh tube, and washed a third time with solubilization buffer. Agarose beads were treated with 25 μl of buffer containing 2% SDS, 30% glycerol, 0.1% bromophenol blue, 14.76 mM tris (hydroxymethyl) aminomethane (Tris), and 6.63 mM HCl pH 8.29, and supernatant samples were electrophoretically resolved on a 6–16% moving boundary velocity gradient-SDS-polyacrylamide gradient gel electrophoresis (PAGGE). Gels were fixed, vacuum dried, and exposed to Kodak XAR 5 film for 72 h with an intensification screen at −70°C and then processed as described for Northern hybridization analysis.

Western Blot Analysis

Immunoprecipitates using Pan-trk and anti-trkC polyclonal antibodies were resolved by high resolution, moving boundary velocity gradient (MBVG)- SDS PAGGE that was developed in conjunction with Dr. Timothy V. Updyke in our labratory. These were purposefully designed to resolve immunoprecipitated samples. This consisted of continuous, linear pH, and buffer strength gradients containing no added SDS (Menter *et al.*, 1992). The gel gradients were designed to improve stacking and resolution of SDS proteins. For polyacrylamide gradient gels, 6–16% T and 5% C_{PDA} were cast in a modified cassette size (15 × 16 × 0.1 cm). The gradients consisted of a stacking leading phase buffer (369 mM Tris base, 166 mM HCl $pH_{25°} = 8.29$) and a resolving leading phase buffer (0.822 M Tris base, 0.068 M HCl $pH_{25} = 9.21$). The anolyte buffer consisted of 20 mM Tris base and 10 mM HCl $pH_{25°} = 8.07$, and the catholyte buffer consisted of 20 mM glycine, 10 mM Tris base, 0.03% SDS $pH_{25°} = 9.18$. The M_r standards consisted of 50 ng each of [^{14}C]-molecular weight standards (GIBCO-Bethesda Research Laboratories). MBVG-SDS-PAGGE was run at 50 V until the dye-fronts entered the continuous gels and were then run at 30 mA/gel until the dye-fronts were 5 mm from the bottom of the gel. High efficiency protein transfers were also developed in our laboratory and consisted of low ionic strength transfer buffers (20 mM Tris HCl, 10 mM glycine) in conjunction with an electrocoupling buffer (390 mM Tris HCl, 130 mM glycine). Nitrocellulose transfer membrane (0.1 μm pore size, Schleicher & Schuell, Keene, NH) and two sheets of blotter paper were presoaked in the electrocoupling buffer at 4°C, assembled into a sandwich with the gel to be transfered, and placed into a GENIE plate electrode transfer unit (Idea Scientific, Corvallis, OR). The transfer was performed at 8 V constant current on ice overnight with a transfer efficiency of >95%. The transfer membrane was blocked using Tween-20 detergent and probed using a 1:2000 dilution of alkaline-phosphatase–conjugated RC20, a recombinant version of anti-phosphotyrosine monoclonal antibody PY20.

Immune-complex Kinase Assays

Immunoprecipitations of CHAPS lysates (10 mM CHAPS, 50 mM NaCl, 25 mM HEPES pH 7.5, 10 μM leupeptin, 200 μM PMSF, 1 mM sodium orthovanadate) were performed on cell equivalents in microcentrofuge tubes using 100 μl of either CHAPS-cell lysate in combination with the appropriate antibody. Lysates were precleared using a nonrelavent antibody such as rabbit anti-mouse IgG followed by protein A/G agarose beads (premixed 1:1 [vol/vol] by packed bead volume and washed twice with solubilization buffer) for 60 min at 4°C. Nonspecific complexes were collected by centrifugation. The pellet was discarded, and the supernatant was used for subsequent antibody reactions with 2 μg of mAb ME20.4. The supernatant was incubated for 60 min at 4°C and mixed occasionally on a Vortex Genie at a setting of 2. After 60 min, the prewashed, packed agarose bead mixture (25 μl) was combined with the specific antibody-CHAPS-cell lysate mixture and incubated for 2 h with gentle agitation. The agarose beads were washed twice with solubilization buffer, transferred to a fresh tube, and washed a third time with solubilization buffer.

The pellet was resuspended in a reaction buffer consisting of 25 mM HEPES pH 7.5, 4 mM $MgCl_2$, 5 mM $MnCl_2$, and 20 μM (5 μCi) [γ-^{32}P]ATP (3000 Ci/mmol) with or without the appropriate concentration of purine analogue (6-thioguanine). The reaction occurred at 24°C for 15 min and was terminated by the addition of sample loading buffer (2% SDS, 30% glycerol, 0.1% bromophenol blue, 14.76 mM Tris, and 6.63 mM HCl pH 8.29). Terminated reactions were frozen at −70°C and later resolved after heating to 95°C for 4 min by a 6–16% MBVG-SDS-PAGE. Gels were fixed, vacuum dried, and exposed to Kodak XAR-5 autoradiography film.

RT-PCR Analysis

Total RNA was obtained from asynchronous, early passage 70W human melanoma cells, human SY5Y neuroblastoma cells, and rat total brain tissue (prepared as described in Northern Hybridization Analysis section). Total RNA was used as a template for first strand cDNA synthesis using Superscript AMV reverse transcriptase (GIBCO-Bethesda Research Laboratories) and oligo-d(T) as an extension primer. Oligonucleotide primers (GenoSys Biotechnologies, The Woodlands, TX) for polymerase chain reaction PCR) analysis of the various neurotrophin receptors were selected based on their specificity using GCG Sequence Analysis Software (Genetics Computer Group, Madison, WI). Oligonucleotide primer design was further enhanced with respect to thermodynamic considerations and target sequence specificity using the OLIGO-4 Primer/Probe Analysis Software Package (National Biosciences, Plymouth, MN). Primer, template, salt concentration, as well as primer annealing temperature were provided by OLIGO-4. The amplification profile consisted of an initial template denaturation step at 94°C for 2 min followed by 30 cycles of 94°C, 1 min; 57°C, 1 min; 72°C, 1 min. Amplification products were analyzed on a 1% agarose/Tris-Borate-EDTA gel using a 123-base pairs (bp) ladder fragment size standard (Gibco-Bethesda Research Laboratories). The following oligonucleotide primers were used for PCR: human actin forward primer, 5′-ATGGATGATGATATCGAAGCG-3′; human actin reverse primer, 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC-3′; human $trkA_{2024}$ forward primer, 5′-TGGCGGGTCTGCATTTTGTG-3′; human $trkA_{2463}$ reverse primer, 5′-CATCCAGGTAGACAGGAGGT-3′; human $p70^{trk}$ lower strand primer, 5′-CATCCATGATGCTGTGGCGT-3′; rat $trkB_{2658}$ forward primer, 5′-ACCTGGCATCCCAACACTTC-3′; rat $trkB_{3078}$ reverse primer, 5′-CTTCGCCAAGTTCTGAAGGA-3′; rat $trkC_{2059}$ forward primer, 5′-TTGGCCTCCCAGCACTTTGT-3′; rat $trkC_{2507}$ reverse primer, 5′-GCCAAGAATGTCCAGGTAGA-3′; rat $trkC_{644}$ forward primer, 5′-CCCTCTTCCGCATGAACATC-3′; rat $trkC_{1325}$ reverse primer, 5′-CCCAAAAGTGTCTTCCTCTG-3′. All primer sequences were based on published cDNA sequences for the various neurotrophin receptors and were taken directly from the GenBank/EMBL sequence databases using the GCG sequence analysis software package (Genetics Computer Group).

Zymographic Analysis of Secreted Proteinases

Cells were seeded onto the upper surface of Transwell polycarbonate filters that were coated with Matrigel at 2×10^4 cells/filter as described for the in vitro invasion assays (see section above). The cells were seeded without FBS supplementation of the medium. Duplicate wells were seeded with or without 50 ng/ml 2.5 S NGF. After a 72-h incubation, the conditioned medium was concentrated using Centricon-30 microconcentrator units (Amicon, Beverly, MA). Conditioned medium was recovered, and any unattached cells or cellular debris was removed by centrifugation at $3000 \times g$ for 10 min. Conditioned medium was analyzed in a discontinuous 4–7.5% polyacrylamide gel containing 1 mg/ml gelatin (Sigma, St. Louis, MO) as previously described (Nakajima *et al.*, 1989). Gelatinolytic enzymes were detected as cleared bands after Coomassie Blue staining. The zymograms were photographed and digitally analyzed using National Institutes of Health (NIH) Image 1.44 (see Table 1 for details).

RESULTS

NGF Enhances In Vitro Invasive Potential of Melanoma Cells

To assess the extent to which NGF alters the invasive capacity of the MeWo, 70W, and 3S5 variant melanoma cell lines, a reconstituted basement membrane matrix (Matrigel) was used. In this assay, the cells seeded in the top well must adhere to the matrix components. After adhesion, the cells must degrade, then move into the matrix, and eventually through the porous membrane. We used FBS-free 48-h conditioned medium from mouse brain microvessel endothelial cells as a chemoattractant (Hamada *et al.*, 1992) in the bottom compartment of the invasion chamber, and the data were calculated as percentage invasion into the lower chamber. Figure 1, A and B shows the results when MeWo and 70W cell lines, respectively, are treated with various concentrations of 2.5 S NGF for 72 h. We found that NGF treatment resulted in a significant enhance-

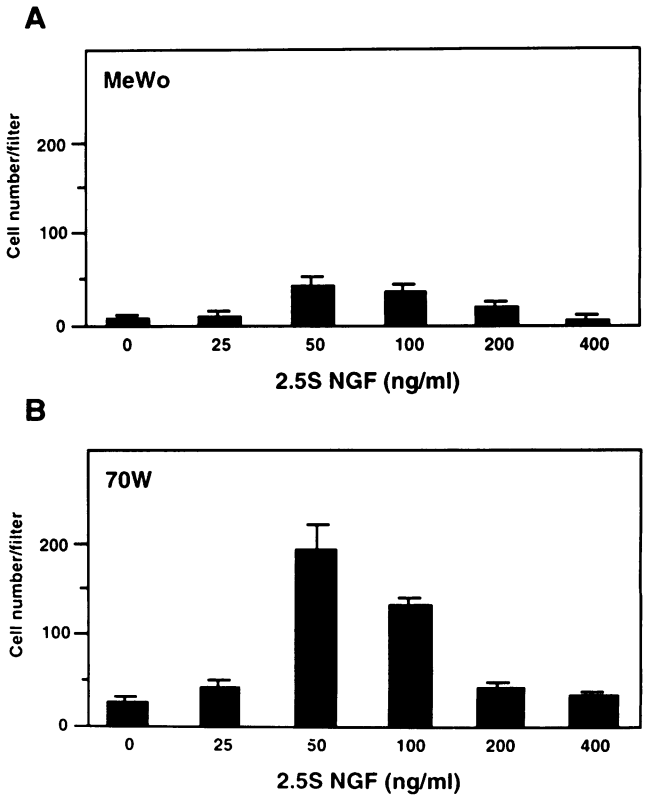

Figure 1. NGF-enhancement of melanoma cell chemoinvasion through a reconstituted basement membrane (Matrigel). (A) MeWo human melanoma cells were examined for their ability to penetrate a reconstituted basement membrane after treatment for 72 h with the indicated concentrations of 2.5 S NGF. (B) 70W variant cells were examined for their in vitro invasive potential as in (A). In experiments A and B, FBS-free conditioned medium obtained from mouse brain microvessel endothelial cells served as the chemoattractant.

ment (at NGF concentrations of 25–50 ng/ml) of MeWo and 70W invasion of Matrigel. The overall extent of NGF-induced chemoinvasion, however, was most pronounced with the 70W variant. We observed a reproducible decline in the extent of Matrigel chemoinvasion at higher concentrations of NGF. The extent of chemoinvasion of the poorly metastatic 3S5 subline and the A875 line under these conditions was negligible, and we did not observe any significant invasion of 3S5 cells after NGF treatment. This may be reflective of other genetic alterations in the A875 and 3S5 sublines. Whether NGF could elicit a chemotactic response was also tested. NGF alone in the lower chamber did not elicit a chemoinvasion response. Additionally, NGF in the absence of extracellular matrix did not enhance chemotaxis toward soluble laminin or conditioned medium from mouse brain microvessel endothelial cells. These results indicate that certain melanoma cells can respond to exogenous NGF by enhancing cell motility and matrix degradative activities necessary for penetration of a reconstituted basement membrane.

p75^{NTR} but Not p140$^{proto\text{-}trkA}$ Steady-state Transcript Level Correlates with NGF-enhanced Chemoinvasion

There are two known receptors for NGF, a low-affinity receptor class (Chao *et al.*, 1986; Radeke *et al.*, 1987) and a high-affinity receptor class (Hempstead *et al.*, 1991; Kaplan *et al.*, 1991; Klein *et al.*, 1991). We considered the possibility that the greater degree of matrix invasion responsiveness exhibited by the 70W variant subline after 2.5 S NGF treatment resulted from increased expression of the low-affinity p75^{NTR} and/or the high affinity c-*trkA* tyrosine kinase proto-oncogene product p140$^{proto\text{-}trkA}$. To examine these possibilities, total RNA was isolated from 3S5, MeWo, and 70W cells and analyzed by Northern blot hybridization analysis using 40-mer oligonucleotides specific for p75^{NTR} and p140$^{proto\text{-}trkA}$ mRNAs. As a control for p75^{NTR} expression, RNA was isolated from A875 human melanoma cells that are known to express only the low-affinity NGF receptor. The A875 melanoma line does not express detectable c-*trkA* mRNA (Kaplan *et al.*, 1991). Likewise, total RNA was isolated from SY5Y human neuroblastoma and PC12 rat pheochromocytoma cells as positive controls for hybridization with the *trkA* oligonucleotide. Hybridization with a p75^{NTR}-specific probe revealed that all the melanoma cell sublines used in this study expressed a single 3.8-kilobase (kb) mRNA encoding the low-affinity receptor (Figure 2A). The level of p75^{NTR} correlated with the differences in NGF-induced invasion responses for the MeWo and 70W lines (70W > MeWo > 3S5). The size of this transcript agrees with the previously determined size for the NGF receptor mRNA from A875 melanoma cells (Johnson *et al.*, 1986). When the p140$^{proto\text{-}trkA}$ oligonucleotide was used as a probe for the same membrane, hybridization was not observed

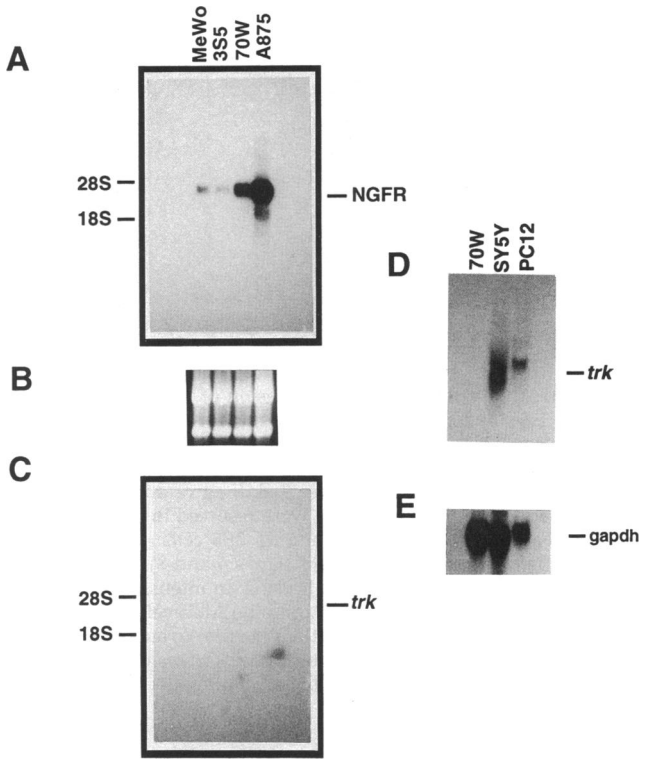

Figure 2. NGF-induced chemoinvasion of melanoma cells correlates with p75^{NGFR} but not with p140$^{proto\text{-}trkA}$ transcript levels. Northern hybridization analysis was performed using total RNA isolated from various human melanoma cells. (A) Hybridization using a single-stranded 40-mer oligonucleotide specific for the low-affinity p75^{NGFR} mRNA. (B) Ethidium bromide staining of RNA to show equal loading of the agarose gel. (C) Hybridization of the same blot used in A with a single-stranded 40-mer oligonucleotide specific for the p140$^{proto\text{-}trkA}$ mRNA. (D) Control hybridization of the trkA-specific 40-mer oligonucleotide. (E) Hybridization to same blot used in D with a full-length human glyceraldehyde-3-phosphate dehydrogenase (gapdh) cDNA to demonstrate RNA loading. Migration positions of the 28S and 18S ribosomal RNAs are indicated.

to RNA from the MeWo melanoma lines (Figure 2C), indicating that the melanoma cells used in this study either do not express p140$^{proto\text{-}trkA}$ or that they produce extremely low levels of p140$^{proto\text{-}trkA}$ mRNA that cannot be detected by Northern blot analysis. The *trkA* 40-mer oligonucleotide hybridized specifically to a transcript expressed by subconfluent SY5Y and PC12 control cells but failed to detect *trkA* mRNA expression by the 70W melanoma cells (Figure 2D).

Expression of Membrane-associated p75^{NTR} Is Elevated in the Highly NGF Responsive 70W Variant

We next examined the expression of p75^{NTR} and p140$^{proto\text{-}trkA}$ by immunoprecipitation analysis of radioiodinated cell surface proteins. Consistent with the Northern blot analysis that demonstrated differential

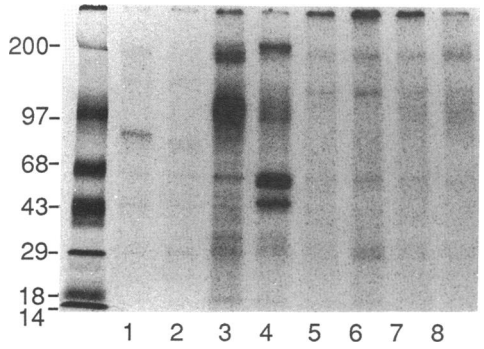

Figure 3. $p75^{NGFR}$ but not $p140^{proto\text{-}trkA}$ cell surface expression correlates with NGF-stimulated chemoinvasion of extracellular matrix. Cell surface iodinated proteins were immunoprecipitated with anti-p75 mAb or an affinity-purified polyclonal antibody generated against the extreme most carboxy-terminal 14 residues of trkA (anti-trk). Lanes 1–4, 10 μg of anti-p75 ME.20; lanes 5–8, 5 μg of anti-trk. Samples (based on equivalent cell numbers) were resolved in a MBVG-SDS-PAGGE gel. MeWo cells (lanes 1 and 5), 3S5 cells (lanes 2 and 6), 70W cells (lanes 3 and 7), A875 cells (lanes 4 and 8). The gels were exposed to Kodak XAR film for 72 h using an intensifying screen at −70°C and then processed. The 70W and the A875 cell lines exhibited higher levels of $p75^{NGFR}$ expression than the MeWo and 3S5 lines. All of the cell lines express small amounts of cell surface iodinatable material recognized by the anti-trk polyclonal antibody.

expression of $p75^{NTR}$, the anti-$p75^{NTR}$ mAb immunospecifically precipitated greater amounts of an appropriate sized, radioiodinated component in the 70W and A875 cells (Figure 3, lanes 3 and 4) than that found in the MeWo or 3S5 cells (Figure 3, lanes 1 and 2). The expression level of $p75^{NTR}$ on the surface of the MeWo line was extremely low but detectable by our analysis. However, despite the presence of the $p75^{NTR}$ transcript in the 3S5 cells, we could not detect $p75^{NTR}$ receptor expressed on the cell surface. This is an indication that the amount of $p75^{NTR}$ expressed on the cell surface by these cells was below the sensitivity of the immunoprecipitation assay, or the $p75^{NTR}$ exists as a cytoplasmic proform of the mature cell surface $p75^{NTR}$. The observed M_r for the protein detected by the anti-$p74^{NTR}$ antibody is ~90 000 for the MeWo line and 97 000 for the 70W line, which is a M_r consistent with other reports on $p75^{NTR}$ (Johnson *et al.*, 1986; Kaplan *et al.*, 1991). These observed differences in $p75^{NTR}$ M_r between the MeWo and 70W receptors may reflect changes resulting from WGA selection of the metastatic variant clones 70W and 3S5. We also observed in the A875 melanoma cells the presence of small M_r forms of $p75^{NTR}$ that were previously described to be the result of proteolytic processing of the low-affinity NGF receptor (Figure 3, lane 4) (Zupan *et al.*, 1989).

Immunoprecipitation of cell surface iodinated components using an affinity-purified polyclonal antibody generated to the C-terminal 14 residues (residues 776–790) of $p140^{prot\text{-}trkA}$ resulted in precipitation of radioiodinated components in the usual M_r range for this protein. However, based on our Northern and Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (Figures 2 and 5), we believe that the M_r 140 000–145 000 immunoprecipitate is not authentic $p140^{proto\text{-}trkA}$ but rather $p145^{trkC}$. The polyclonal antibody used in our immunoprecipitation procedure has been found to recognize $p145^{trkB}$ and $p145^{trkC}$ in addition to $p140^{proto\text{-}trkA}$ (Lamballe *et al.*, 1991). Such cross-reactivity is most likely a reflection of the high degree of C-terminal sequence conservation shared between these molecules. Taken together, the Northern, RT-PCR analysis, and immunoprecipitation results demonstrate a lack of $p140^{proto\text{-}trkA}$ expression and indicate that cell-surface $p145^{trkC}$ is expressed by the malignant melanoma cell lines used in our experiments.

Matrix-dependent Regulation of Melanoma Matrix Metalloproteinase Expression by NGF

NGF stimulation of PC12 pheochromocytoma cell neurite extension has been shown to be accompanied by a dramatic transcriptional induction of the matrix metalloproteinase transin (Machida *et al.*, 1989). To determine if the NGF-induced extracellular matrix invasion could be in part because of enhanced secretion of matrix-degrading enzymes, we performed substrate gel zymography with conditioned medium of the melanoma cell lines. Briefly, medium conditioned for 72 h was collected from 3S5, MeWo, or 70W melanoma cells that were cultured on extracellular matrix. The concentration of NGF used for this experiment (50 ng/ml) was selected on the basis of its maximal stimulation of matrix invasion. Conditioned medium was adjusted to reflect equal cell numbers, concentrated, and then analyzed on a polyacrylamide gel embedded with a gelatin as substrate

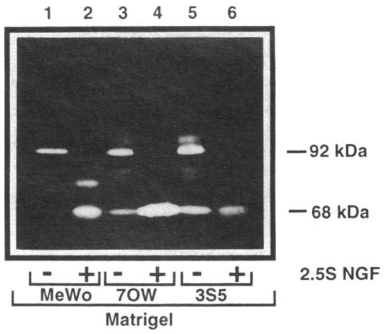

Figure 4. NGF-induced expression of gelatinolytic matrix metalloproteinases. Conditioned medium from MeWo, 70W, or 3S5 human melanoma cells stimulated (+) with 50 ng/ml 2.5 S NGF or untreated (−) was collected and concentrated. The presence of metalloproteinases in the conditioned medium was analyzed by electrophoresis in a gelatin-embedded substrate gel. Renaturation and activation of the gelatin degradative enzymes was performed as described in MATERIALS AND METHODS. All cells were plated on reconstituted basement membrane Matrigel. Molecular weights ($\times 10^{-3}$) of secreted gelatinases are indicated on the right ordinate.

Table 1. Digital analysis of gelatin zymogram

Cell line/ treatment	68-kDa gelatinolytic area in cm^2	Integrated density of 68-kDa gelatinolytic area
MeWo − NGF	0.051	0.002
MeWo + NGF	0.188	0.012
70W − NGF	0.100	0.008
70W + NGF	0.334	0.016
3S5 − NGF	0.148	0.011
3S5 + NGF	0.110	0.008

Analysis was performed by digitizing a zymogram photograph using Deskscan II software on a Macintosh IIci (Apple Computer, Cupertino, CA) and an Hewlett Packard Scanjet IIc scanner (Palo Alto, CA). The digitized image was then analyzed using NIH Image 1.44 (written by Wayne Rasband, National Institutes of Health, Research Services Branch NIMH). It is interesting to note that NGF treatment of 70W cells causes a threefold increase in size that is accompanied by a twofold increase in the integrated density of the 68-kDa gelatinolytic area. NGF treatment of MeWo cells causes a similar increase in the size of the 68-kDa gelatinolytic area with a sixfold increase in the density. The 68-kDa gelatinolytic area of the 3S5 cells by comparison to 70W and MeWo cells appears to not be regulated by NGF.

(Figure 4). We found that NGF treatment under the culture conditions employed essentially abolished the low level secretion of a 92-kDa gelatinase (type IV collagenase). We also observed in the MeWo and 70W lines that NGF induced the expression of a fully active 68-kDa gelatinase (probably derived from the 72-kDa type IV collagenase, see Table 1). The induced secretion of 72-kDa gelatinase (type IV collagenase) was most pronounced in the 70W variant cells consistent with the chemoinvasion results using this subline (Table 1). That these are classic matrix metalloproteinases was demonstrated by addition of EDTA during the incubation period of enzyme activation to inhibit gelatin degradation. Treatment with NGF did not significantly alter the expression of the 72-kDa enzyme in 3S5 cells (see Table 1). Parallel zymography experiments performed with cells cultured on plastic plates without Matrigel did not show enhancement of matrix metalloproteinase secretion in response to NGF treatment. Thus, the NGF induction of melanoma gelatinase production appears to be dependent on extracellular matrix interactions.

Melanoma Cells Express the NT-3 Receptor $p145^{trkC}$ but NGF Fails to Catalytically Activate Melanoma $p145^{trkC}$

As discussed above, the absence of $p140^{proto\text{-}trkA}$ expression by our melanoma cells was demonstrated by Northern blot hybridization analysis using a specific coding strand 40-mer oligonucleotide region immediately upstream of the transmembrane domain of human *trkA* mRNA. Examination of the literature revealed several variants of $p140^{proto\text{-}trkA}$ cloned from human tumors or isolated as transforming recombinants after transfection of NIH 3T3 fibroblasts with the $p140^{proto\text{-}trkA}$ proto-oncogene cDNA (Martin-Zanca *et al.*, 1986; Kozma *et al.*, 1988; Oskam *et al.*, 1988). These investigators have documented several mechanisms of malignant activation of $p140^{proto\text{-}trkA}$, including point mutation, internal deletion, recombination with unknown cellular sequences, and kinase duplication. Interestingly, several of these $p140^{proto\text{-}trkA}$ recombinants encode cytoplasmic molecules that are not membrane inserted. Such is also the case for the oncogenic transforming allele of *trkA* designated $p70^{trk}$ (Mitra *et al.*, 1987).

With this in mind, we sought to identify whether 70W malignant melanoma cells might express some unique recombination variant of the TRK tyrosine kinase family of receptors or their respective proto-oncogenic forms. Oligonucleotide amplimers were designed to specifically amplify the C-terminal half of the kinase domain for each member of the TRK family (*trkA, trkB, trkC*) and oncogeneic $p70^{trk}$ by RT-PCR analysis. We were not able to specifically amplify 70W melanoma cDNA template using primer pairs for *trkA, trkB,* or $p70^{trk}$. The *trkA-, trkB-,* and *trkC*-specific primer pairs performed well with control cDNA templates from total rat brain cDNA and SY5Y human neuroblastoma cells. No amplification product was obtained using the $p70^{trk}$-specific primer pair with any template. Using human 70W melanoma cDNA as target template, however, we were able to specifically amplify a 708-bp product corresponding to the C-terminal half of external ligand-binding domain

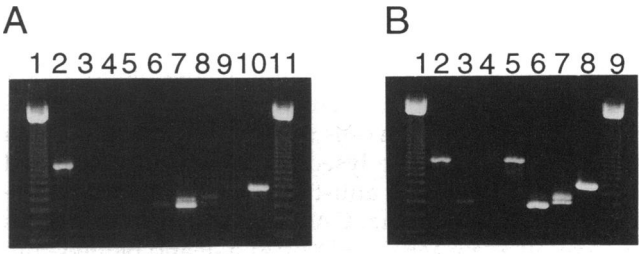

Figure 5. RT-PCR analysis of 70W human malignant melanoma neurotrophin receptor transcript expression. (A) cDNA template was generated from total RNA isolated from exponential growth phase cultures of 70W cells. (B) cDNA was generated from exponential growth phase cultures of SY5Y human neuroblastoma (lanes 2–4) or rat total brain tissue (lanes 5–8). PCR was performed as described in MATERIALS AND METHODS. Amplified products were resolved on a 1% agarose/TBE gel using a 123-bp ladder as a fragment size standard (lanes 1 and 10 left, lanes 1 and 9 right). The lane assignments for each primer pair were as follows: (left) lane 2, human actin control primers; lane 3, trkA 2024–2467; lane 4, p70trk; lane 5, trkB 2568–3078; lane 6, trkC 2059–2507; lane 7, trkC 2059–2507 reamplification of 1:1000 initial PCR reaction dilution; lane 8, trkC 2059–2507 reamplification at 1:10 000 initial PCR reaction dilution; lane 9, trkC 644–1325; and lane 10, trkC 644–1325 reamplification. (Right) Lane 2, actin control primers; lane 3, trkA 2024–2467; lane 4, p70trk; lane 5, actin control primers; lane 6, trkB 2658–3078; lane 7, trkC 2059–2507; lane 8, trkC 644–1325. See MATERIALS AND METHODS for oligonucleotide primer designations.

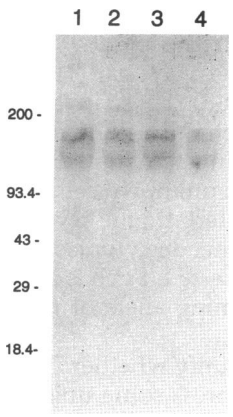

Figure 6. Human 70W malignant melanoma p145trkC is not catalytically activated by NGF. Serum-free cultures of 70W melanoma cells were stimulated with NGF (50 ng/ml) for 5 min (lanes 2–4) or untreated (lane 1) and lysed. Precleared cell lysate was immunoprecipitated with affinity-purified anti-trkC(798) polyclonal antibody (lane 1 and 3), anti-trk(790) (lane 2), or Pan-trk 443 (lane 4).

of $p145^{trkC}$ and a 468-bp product spanning the C-terminal most half of the cytoplasmic tyrosine kinase domain of $p145^{trkC}$ (Figure 5, lanes 6–10). Interestingly, various amounts of cDNA template in the PCR reaction altered the product profile using the primer pair designed to amplify the C-terminal half of the $p145^{trkC}$ kinase domain. Figure 5 (lanes 7 and 8), shows a larger PCR product obtained with the $trkC_{2059}$-$trkC_{2507}$ primer pair. Confirmatory sequence analysis of both PCR products obtained using the trkC-specific tyrosine kinase domain specific primers reveal that only the smaller fragment corresponds to authentic trkC. The larger fragment was generated by a dual priming event involving the $trkC_{2059}$ primer. Thus the larger fragment does not represent one of the alternatively spliced versions of *trkC* mRNA that encode variants of *trkC* that containing small in-frame insertions in the kinase domain (Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993).

We sought to examine whether $p145^{trkC}$ could participate in mediating NGF signaling in the highly NGF-responsive 70W human malignant melanoma cells. Immunoprecipitates from cells treated with NGF for 5 min and control cells were lysed and immunoprecipitated using affinity-purified anti-trkC(798) (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitates were resolved by MBVG-SDS-PAGGE and blotted onto a polyvinylidene difluoride membrane. The membrane was probed using a recombinant version of anti-phosphotrysine mAb PY20 (RC20) that was alkaline phosphatase conjugated. We detected a M_r 145 000–150 000 tyrosine-phosphorylated protein representing trkC (Figure 6). This tyrosine-phosphorylated protein was also immunoprecipitated by anti-trk(790) and antiserum 443 both which recognize all members of the Trk family of receptors. However, we found that NGF did not enhance the catalytic activation of $p145^{trkC}$ via tyrosine phosphorylation.

The Melanoma $p75^{NTR}$ Is Associated with an NGF-stimulated Purine-analogue-sensitive Protein Kinase

The lack of a known intrinsic catalytic activity for the low-affinity $p75^{NTR}$ prompted us to examine whether $p75^{NTR}$ in human melanoma cells could be associated with a cytoplasmic kinase(s). Recent experiments indicate that $p75^{NTR}$ expressed by rat PC12 pheochromocytoma cells associates with a kinase sensitive to inhibition by various purine analogues (Volonte *et al.*, 1993). Moreover, in certain conditionally immortalized neuronal cells, $p75^{NTR}$ overexpression in the absence of NGF ligand was found to impose an apoptotic death program (Rabizadeh *et al.*, 1993). Apoptosis of these $p75^{NTR}$-expressing cells was found to occur independently of $p140^{proto\text{-}trkA}$ expression.

As mentioned above, because the low-affinity $p75^{NTR}$ itself does not contain any intrinsic protein kinase activity, we sought to identify $p75^{NTR}$-associated proteins capable of mediating NGF-induced protein phosphorylation resulting from their interaction with $p75^{NTR}$. Consequently, cells were solubilized using mild nonionic detergent lysis conditions to better preserve native structures and complexes of interactive proteins. The 70W human malignant melanoma subline was cultured for different times with or without 2.5S NGF. CHAPS-soluble material that was extensively precleared using secondary rabbit anti-mouse IgG and $p75^{NTR}$ was immunoprecipitated using the anti-$p75^{NTR}$ ME20.4 mAb. Immunoprecipitates were extensively washed, and the immune-complex-kinase assays (ICKAs) were performed under conditions (5 mM Mn^{2+}, 4 mM Mg^{2+} pH 7.4) similar to those described for detection of a $p75^{NTR}$-associated NGF-stimulated kinase activity in rat pheochromocytoma cells (Volonte *et al.*, 1993).

After only 5 min of NGF treatment (50 ng/ml), we were able to detect a consistent NGF-stimulated phosphorylation of, most notably, two small molecular weight proteins migrating as a doublet at approximately 10 and 11 kDa (Figure 7). Whether these represent the hypo- and hyperphosphorylated isoforms of the same protein remains to be addressed. The kinase activity coimmunoprecipitated with $p75^{NTR}$ was found to be inhibited by 6-thioguanine. As such, this kinase most likely is the serine/threonine kinase protein kinase N described by Volonte *et al.* (1993).

Figure 7. NGF-stimulated phosphorylation of 10- and 11-kDa substrates for the human 70W malignant melanoma p75-associated purine-analogue-sensitive kinase. ICKAs were performed using 70W human malignant melanoma cells treated with NGF (5 min, lanes 3 and 4 and 24 h, lanes 5 and 6) and untreated controls (lanes 1 and 2). Immunoprecipitation was performed using anti-human $p75^{NGFR}$ mAb clone ME20-4. Lanes 2, 4, and 6 represent ICKAs performed with 6-thioguanine (500 μM) in the ICKA buffer. Immune precipitates were incubated with 10 μM [γ-^{32}P]ATP for 15 min in kinase buffer at 24°C, and phosphoproteins were analyzed on a 6–16% MBVG-SDS-PAGGE. Comparable results were obtained using anti-p75NGFR mAb 8211. Control experiment for nonspecific IgG binding proteins were performed using nonrelevant rabbit anti-mouse IgG plus protein A/G agarose and did not show kinase activity.

DISCUSSION

Metastasis formation by malignant tumor cells at secondary organ site microenvironments is the result of a sequential multistep process (Nicolson, 1988, 1991). The successful metastatic cell must be able to respond to local concentrations of hormones and organ-specific growth factors to grow at new sites (Nicolson, 1993). In addition, during the process of metastasis, tumor cells encounter several physical barriers, such as basement membranes and surrounding tissue stromal matrices, that are composed of highly ordered networks of collagens, proteoglycans, and glycoproteins. To invade these barriers malignant cells must be able to bind to extracellular matrix components and elaborate a variety of extracellular matrix-degradative enzymes. Malignant cells must also be able to respond to locally produced motility factors, and malignant cells can acquire enhanced motility properties by synthesis of autocrine motility factors (Nicolson, 1988, 1991).

We have found that extracellular matrix-adherent human MeWo melanoma cells can respond to physiological concentrations of NGF. At the concentrations used in our studies, NGF should saturate the low-affinity $p75^{NTR}$ receptors on melanoma cells, and this may stimulate the chemoinvasion of extracellular matrix by the MeWo and 70W lines. The degree of matrix invasion stimulation correlated with the level of $p75^{NTR}$ expression and with the induced secretion of the activated form of the 72-kDa type IV collagenase. When these cells were plated on plastic in the presence of FBS and then switched to FBS-free growth conditions, they did not alter their gelatinolytic profile after NGF treatment. Thus, the regulation of gelatinase expression by NGF was found to be dependent on a Matrigel component(s) and/or a component in the mouse brain endothelial cell-conditioned medium used as a chemoattractant. Recent experiments have shown that the extracellular matrix protein vitronectin can mediate NGF-dependent outgrowth in PC12 pheochromocytoma cells (Grabham *et al.*, 1992). It will be of interest to investigate whether Matrigel or brain endothelial cell-conditioned medium is responsible for promoting this NGF-induced effect.

Maximal NGF stimulation of extracellular matrix penetration was achieved at concentrations of NGF (50 ng/ml) sufficient to saturate all of the 70W and MeWo low-affinity $p75^{NTR}$ receptors with ligand (Marchetti *et al.*, 1993). Interestingly, at the higher concentrations of NGF (>100 ng/ml), we observed a reproducible decline in the extent to which these two cell lines are responsive to NGF-induced chemoinvasion. This phenomenon could reflect the creation of a negative feedback system by NGF at higher concentrations that alters the program(s) by which these cells respond to exogenous NGF. Such a bimodal response has been observed for the proliferative response of various connective tissue cells to transforming growth factor-β and has been associated with the creation of a complex autocrine loop involving platelet-derived growth factor-AA (Battegay *et al.*, 1990).

Recent studies have established that NGF exerts its biological effects by interaction with two receptor classes. One receptor for NGF (designated the low-affinity $p75^{NTR}$) may function as a common binding subunit for the various members of the neurotrophin family (Bothwell, 1991). The low-affinity form of NGF receptor, $p75^{NTR}$, is expressed during normal embryogenesis in a variety of tissues not known to respond to NGF or other neurotrophins, including the testis, muscle anlage and somites, skin mesenchyme, and kidney (Bothwell, 1990; Heuer *et al.*, 1990; Persson *et al.*, 1990; Wyatt *et al.*, 1990; Sariola *et al.*, 1991). The expression of $p75^{NTR}$ in these nonresponsive tissues raises the possibility that this receptor may participate in some more general yet unknown embryonic role (Bothwell, 1990). Previous experiments have demonstrated that $p75^{NTR}$ displays low-affinity NGF binding and is, by itself, unable to mediate a functional biological response upon interaction with NGF (Green *et al.*, 1986; Hempstead *et al.*, 1991). However, other experiments indicate that $p75^{NTR}$ expression is essential to form high-affinity binding sites for NGF presumably via the formation of $p75^{NTR}$–$p140^{proto\text{-}trkA}$ complexes and to elicit tyrosine phosphorylation of $p140^{proto\text{-}trkA}$ (Berg *et al.*, 1991). Despite a lack of intrinsic tyrosine kinase activity associated with $p75^{NTR}$, these studies suggest that $p75^{NTR}$ plays a role in NGF-stimulated tyrosine phosphorylation. Indeed, a purine-analogue-sensitive protein kinase activity that is responsive to NGF has been found that specifically associates with $p75^{NTR}$ in PC12 cells and should be considered a potential component of the NGF-mediated signaling pathway in these cells (Volonte *et al.*, 1993). Furthermore, mice that are homozygous for a mutation in the $p75^{NTR}$ gene have been created (Lee *et al.*, 1992). Targeted disruption of the $p75^{NTR}$ gene in these mice resulted in a complex phenotype with deficits in cutaneous innervation and heat sensitivity. It is possible that other defects were generated in these null-allele mice but were unnoticed because of their subtle nature. The diminished innervation documented for the $p75^{NTR}$ null allele mice also suggests a role for the $p75^{NTR}$ in coordinating neurotrophin-generated signals essential for an invasive process in vivo.

The signaling pathways known to be employed by NGF are complex and involve the transiently induced expression of a number of primary response genes that encode transcription factors, such as c-*fos*, c-*jun*, NGFI-B, and *krox*24 (Batistatou *et al.*, 1992). Other NGF-induced changes include activation of protein kinase N and ornithine decarboxylase activity (Volonte and Greene, 1990, 1992). Previous studies have demonstrated that Ha-*ras* can coordinate the NGF-mediated, phosphorylation-dependent activation of several key growth and differentiation molecules, including MAP

kinase, c-*raf*-1, and p90 ribosomal S6 kinase (Wood *et al.*, 1992). These experiments were performed with PC12 pheochromocytoma cells that contain functional $p140^{proto\text{-}trkA}$. Not surprisingly, the contribution of the low-affinity $p75^{NTR}$ receptor to these signals is uncertain.

Two current models for signal generation by NGF have been proposed. Although controversial, one model proposes that 75^{NTR} is essential for the formation of high-affinity NGF-binding sites via $p75^{NTR}$–$p140^{proto\text{-}trkA}$ heterodimerization (Hempstead *et al.*, 1991). Indeed, experiments indicate that $p75^{NTR}$ can enhance NGF-induced tyrosine phosphorylation via the modulation of $p140^{proto\text{-}trkA}$ activity (Berg *et al.*, 1991). Secondly, chimeric NGF-epidermal growth factor (EGF) receptors where the extracellular ligand-binding domain of the human EGF is fused to the transmembrane and cytoplasmic domain of the human $p75^{NTR}$ are capable of eliciting neurite outgrowth in EGF-stimulated PC12 rat pheochromocytoma cells transfected with the hybrid construction (Yan *et al.*, 1991). A second model suggests that $p75^{NTR}$ functions strictly as a procurement receptor for neurotrophins and that $p140^{proto\text{-}trkA}$ can function independently of $p75^{NTR}$. The latter model is supported by experiments that indicate autonomous overexpression of $p140^{proto\text{-}trkA}$ in *Xenopus* oocytes is sufficient to evoke meiotic maturation after NGF treatment (Nebreda *et al.*, 1991).

We have found that NGF-stimulated signals capable of enhancing extracellular matrix penetration of human melanoma cells can be generated independently of $p140^{proto\text{-}trkA}$ expression, because the MeWo cells only express the low-affinity $p75^{NTR}$ receptor class. This finding is significant because it indicates that NGF signal transduction may occur in the absence of $p140^{proto\text{-}trkA}$ function in certain cellular contexts. Supportive evidence for an independent role for $p75^{NTR}$ in certain neuronal cells has been advanced recently. These experiments demonstrate that conditionally immortalized neuronal cells overexpressing $p75^{NTR}$ in the absence of NGF ligand, but not expressing $p140^{proto\text{-}trkA}$, will proceed through an apoptotic death program (Rabizadeh *et al.*, 1993). Exogenous application of NGF can apparently rescue the apoptotic phenotype. Taken together, these data argue for a more complex role for the low-affinity $p75^{NTR}$. Consequently, we propose that $p75^{NTR}$ is more than just a procurement or presentation molecule as has been suggested; rather, $p75^{NTR}$, in the appropriate cellular context, may directly evoke complex biological behavior independent of the other neurotrophin receptors.

The lack of any intrinsic kinase activity attributable to $p75^{NTR}$ and the absence of $p140^{proto\text{-}trkA}$ expression by MeWo melanoma cells prompted us to examine whether these cells could be expressing another member of the TRK family (*trkB* or *trkC*) or a variant of *trkA*. This presumes that $p75^{NTR}$ signaling could occur via interaction with such a surrogate molecule. Analysis of TRK family mRNA expression in human MeWo melanoma cells by RT-PCR indicated that these cells do not express transcripts for *trkA*, the oncogenic variant of *trkA* ($p70^{trkA}$) or *trkB*. However, these melanoma cells do express authentic $p145^{trkC}$ mRNA. Three alternatively processed transcripts with in-frame kinase domain insertions as well as a truncated version lacking a kinase domain have been described for the $p145^{trkC}$ (Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993). We did not observe expression of any of the described kinase insertion isoforms in human melanoma cells. Both the $p145^{trkCki14}$ and $p145^{trkCki39}$ isoforms also display NT-3-induced autophosphorylation in transfected NIH 3T3 fibroblasts and PC12 cells. However, these alternative forms of $p145^{trkC}$ were not found to mediate a proliferative or differentiative response (Tsoulfas *et al.*, 1993; Valenzuela *et al.*, 1993). The failure of the kinase insert isoforms to transduce an apparent biologically relevant signal suggests a role for these variants in serving as neurotrophin-binding molecules. It also possible that the isoforms could interact with $p145^{trkC}$ via heterodimer formation in a manner capable of altering or modulating $p145^{trk}$ substrate interactions. *TrkC* has been cloned from rat and porcine cDNA libraries and dispalys a relatively neuronal-specific expression pattern in adult tissues. Cross-linking experiments in NIH 3T3 fibroblasts indicate that $p145^{trkC}$ is the authentic receptor for NT-3 and that $p145^{trkC}$ fails to bind structurally related neurotrophins NGF, BDNF, xNT-4, or hNT-5 (Lamballe *et al.*, 1991; Tsoulfas *et al.*, 1993). Because these experiments were performed in fibroblasts lacking $p75^{NTR}$, the contribution, if any, of $p75^{NTR}$ to the formation of a NGF-binding complex with $p145^{trkC}$ was not addressed. Consequently, the expression of $p145^{trkC}$ by melanoma cells raises the possibility that the NGF-stimulated matrix invasion that we have described could be signaled through $p75^{NTR}$ cooperation with $p145^{trkC}$ and for NT-3–generated signaling events important for the pathogenesis of human melanoma cells.

If, however, $p145^{trkC}$ was participating in the NGF response described here, application of NGF would be expected to augment catalytic activation of $p145^{trkC}$ via tyrosine phosphorylation. Anti-trkC immunoprecipitates from NGF-stimulated 70W melanoma cells did not display enhanced tyrosine phosphorylation. Interestingly, $p145^{trkC}$ in these cells appears to be phosphorylated on tyrosine residues even when the cells are extensively washed and cultured using serum-free conditions. Catalytic activation of $p145^{trkC}$ in these cells could be envisioned as the result of 1) autocrine production of NT-3, 2) the interaction of NT-3 or an NT-3-related serum component in a high-affinity interaction with the $p145^{trkC}$ receptor resulting in constitutive signaling, or 3) a structural alteration that promotes 70W $p145^{trkC}$ phosphorylation independent of ligand interaction(s).

Initial experiments to determine whether melanoma $p75^{NTR}$ can coordinate, or at least participate, in a signaling event(s) upon NGF ligand interaction indicate that similar to the rat PC12 $p75^{NTR}$ the human 70W melanoma $p75^{NTR}$ can associate with a purine-analogue-sensitive kinase (PASK). Analysis of anti-p75NGFR immunoprecipitates from NGF-stimulated cells using a sensitive immun-complex-kinase assay indicates that NGF-mediated signaling via $p75^{NTR}$-PASK complexes involves primarily phosphorylation of two small molecular weight proteins (10 and 11 kDa). Perhaps the 11-kDa species represents the hyperphosphorylated version of the 10-kDa species. Identification of these and other $p75^{NGFR}$-PASK substrates is currently being performed.

Our results demonstrate that in melanoma cells devoid of $p140^{proto\text{-}trkA}$ expression, NGF can still elicit profound changes in biological behavior presumably through $p75^{NTR}$. A potential contributory role for $p145^{trkC}$ in supporting $p75^{NTR}$ function is currently being pursued. We are also examining whether melanoma cells are capable of responding to autocrine-derived or paracrine-derived NT-3 in terms of $p145^{trkC}$ receptor activation and ligand-induced phosphorylation. Our data are the first to provide evidence of a functional link between the elevated expression of $p75^{NTR}$ observed in the progression melanoma cells from primary tumor to a highly aggressive malignant tumor and metastases. Experiments are in progress to determine the nature of melanoma $p75^{NTR}$ or $p75^{NTR}$-associated signaling components and their downstream targets.

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REFERENCES

Albini, A., Iwamoto, Y., Kleinman, H.K., Martin, G.R., Aaronson, S.A., Kozlowski, J.M., and McEwan, R.N. (1987). A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res. *47*, 3239–3245.

Albino, A.P., Davis, B.M., and Nanus, D.M. (1991). Induction of growth factor RNA expression in human malignant melanoma: markers of transformation. Cancer Res. *51*, 4815–4820.

Batistatou, A., Volonte, C., and Greene, L.A. (1992). Nerve growth factor employs multiple pathways to induce primary response genes in PC12 cells. Mol. Biol. Cell *3*, 363–371.

Battegay, E.J., Raines, E.W., Selfert, R.A., Bowen-Pope, D.F., and Ross, R. (1990). TGF-β induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell *63*, 515–524.

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

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

Bothwell, M. (1990). Tissue localization of nerve growth factor and nerve growth factor receptors. Curr. Top. Microbiol. Immunol. *165*, 55–70.

Chao, M.V., Bothwell, M.A., Ross, A.H., Koprowski, H., Lanahan, A.A., Buck, C.R., and Sehgal, A. (1986). Gene transfer and molecular cloning of the human NGF receptor. Science *232*, 518–521.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

Fabricant, R.N., DeLarco, J.E., and Todaro, G.L. (1977). Nerve growth factor receptors on human melanoma cells in culture. Proc. Natl. Acad. Sci. USA *74*, 565–569.

Grabham, P.W., Gallimore, P.H., and Grand, R.J.A. (1992). Vitronectin is the major serum protein essential for NGF-mediated neurite outgrowth from PC12 cells. Exp. Cell Res. *202*, 334–337.

Green, S.H., Rydel, R.E., Connolly, J.L., and Greene, L.A. (1986). PC12 cell mutants that possess low- but not high-affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor. J. Cell Biol. *102*, 830–843.

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

Hamada, J., Cavanaugh, P.G., Lotan, O., and Nicolson, G.L. (1992). Separable growth and migration factors for large-cell lymphoma cells secreted by microvascular endothelial cells derived from target organs for metastasis. Br. J. Cancer *66*, 349–354.

Hart, I.R., Birch, M., and Marshall, J.F. (1991). Cell adhesion receptor expression during melanoma progression and metastasis. Cancer Metastasis Rev. *10*, 115–128.

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

Herlyn, M., Kath, R., Williams, N., Valyi-Nagy, I., and Rodeck, V. (1990). Growth regulatory factors for normal, premalignant and malignant human cells. Adv. Cancer Res. *54*, 213–234.

Herlyn, M., Thurin, J., Balaban, G., Bennicelli, J.L., Herlyn, D., Elder, D.E., Bondi, E., Guerry, D., Nowell, P., Clark, W.H., and Kaprowski, H. (1985). Characteristics of cultured human melanocytes isolated from different stages of tumor progression. Cancer Res. *45*, 5670–5676.

Heuer, J.G., Fatemie, N.S., Wheeler, E.F., and Bothwell, M. (1990). Structure and developmental expression of the chicken NGF receptor. Dev. Biol. *137*, 287–304.

Ishikawa, M., Dennis, J.W., Man, S., and Kerbel, R.S. (1988). Isolation and characterization of spontaneous wheat germ agglutinin-resistant human melanoma mutants displaying remarkably different metastatic profiles in nude mice. Cancer Res. *48*, 665–670.

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

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

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

Kozma, S.C., Redmond, S.M.S., Xiao-Chang, F., Saurer, S.M., Groner, B., and Hynes, N.E. (1988). Activation of the receptor kinase domain of the trk oncogene by recombination with two different cellular sequences. EMBO J. *7*, 147–154.

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

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

Machida, C.M., Rodland, K.D., Matrisian, L., Magun, B.E., and Ciment, G. (1989). NGF induction of the gene encoding the protease transin accompanies neuronal differentiation in PC12 cells. Neuron *2*, 1587–1596.

Marchetti, D., Nakajima, M., and Nicolson, G.L. (1993). Nerve growth factor effects on mouse and human melanoma cell invasion and heparanase production. Int. J. Cancer (*in press*).

Martin-Zanca, D., Hughes, S.H., and Barbacid, M. (1986). A human oncogene formed by fusion of a truncated tropomyosin and protein tyrosine kinase sequences. Nature *319*, 743–748.

Menter, D.G., Patton, J.T., Updyke, T.V., Kerbel, R.S., Maamer, M., McIntire, L.V., and Nicolson, G.L. (1992). Transglutaminase stabilizes melanoma adhesion under laminar flow. Cell Biophysics *18*, 123–143.

Mitra, G., Martin-Zanca, D., and Barbacid, M. (1987). Identification and biochemical characterization of $p70^{trk}$, product of the human TRK oncogene. Proc. Natl. Acad. Sci. USA *84*, 6707–6711.

Nakajima, M., Lotan, D., Baig, M.M., Carralero, R.M., Wood, W.R., Hendrix, M.J., and Lotan, R. (1989). Inhibition by retinoic acid of type IV collagenolysis and invasion through reconstituted basement membrane by metastatic rat mammary adenocarcinoma cells. Cancer Res. *49*, 1698–1706.

Nebreda, A.R., Martin-Zanca, D., Kaplan, D.R., Parada, L.F., and Santos, E. (1991). Induction by NGF of meiotic maturation of *Xenopus* oocytes expressing the trk proto-oncogene product. Science *252*, 558–561.

Nicolson, G.L. (1988). Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites. Biochim. Biophys. Acta *948*, 175–224.

Nicolson, G.L. (1991). Molecular mechanisms of cancer metastasis: tumor and host properties and the role of oncogenes and suppressor genes. Curr. Opin. Oncol. *3*, 75–92.

Nicolson, G.L. (1993). Cancer progression and growth: relationship of paracrine and autocrine growth mechanisms to organ preference of metastasis. Exp. Cell Res. *204*, 171–180.

Oskam, R., Coulier, F., Ernst, M., Martin-Zanca, D., and Barbacid, M. (1988). Frequent generation of oncogenes by in vitro recombination of TRK protooncogene sequences. Proc. Natl. Acad. Sci. USA *85*, 2964–2968.

Patel, J.K., Didolkar, M.S., Pickren, J.W., and Moore, R.H. (1978). Metastatic pattern of malignant melanoma. A study of 216 autopsy cases. Am. J. Surg. *135*, 807–810.

Persson, H., Ayer, L.L.C., Soder, O., Villar, M.J., Metsis, M., Olson, L., Ritzen, M., and Hokfelt, T. (1990). Expression of beta-nerve growth factor receptor mRNA in Sertoli cells downregulated by testosterone. Science *247*, 704–707.

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

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

Ross, A.H., Grob, P., Bothwell, M., Elder, D.E., Ernst, C.S., Marano, N., Ghrist, B.F., Slemp, C.C., Herlyn, M., Atkinson, B., and Kaprowski, H. (1984). Characterization of nerve growth factor receptor in neural crest tumors using monoclonal antibodies. Proc. Natl. Acad. Sci. USA *81*, 6681–6685.

Sariola, H., Saarma, M., Sainio, K., Arumae, U., Palgi, J., Vaahtokari, A., Thesleff, I., and Karavanov, A. (1991). Dependence of kidney morphogenesis on the expression of nerve growth factor receptor. Science *254*, 571–573.

Tsoulfas, P., Soppet, D., Escandon, E., Tessarollo, L., Mendoza-Ramirez, J.-L., Rosenthal, A., Nikolics, K., and Parada, L.F. (1993). The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. Neuron *10*, 975–990.

Valenzuela, D.M., Maisonpierre, P.C., Glass, D.J., Rojas, E., Nunez, L., Kong, Y., Gies, D.R., Stitt, T.N., Ip, N.Y., and Yancopoulos, G.D. (1993). Alternative forms of trkC with different functional capabilities. Neuron *10*, 963–974.

Volonte, C., and Greene, L.A. (1990). Induction of ornithine decarboxylase by nerve growth factor in PC12 cells: dissection by purine analogs. J. Biol. Chem. *265*, 11050–11055.

Volonte, C., and Greene, L.A. (1992). 6-Methylmercaptopurine riboside is a potent and selective inhibitor of nerve growth factor-activated protein kinase N. J. Neurochem. *58*, 700–708.

Volonte, C., Ross, A.H., and Greene, L.A. (1993). Association of a purine-analog-sensitive protein kinase activity with p75 nerve growth factor receptors. Mol. Biol. Cell *4*, 71–78.

Wood, K.W., Sarnecki, C., Roberts, T.M., and Blenis, J. (1992). ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell *68*, 1041–1050.

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

Yan, H., Schlessinger, J., and Chao, M.V. (1991). Chimeric NGF-EGF receptors define domains responsible for neuronal differentiation. Science *252*, 561–563.

Zupan, A.A., Osborne, P.A., Smith, C.E., Siegal, N.R., Leimgruber, R.M., and Johnson, E.M. (1989). Identification, purification, and characterization of truncated forms of the human nerve growth factor receptor. J. Biol. Chem. *264*, 11714–11720.