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41. Cells (2×10^7) or DRGs were treated with NGF, washed, and lysed in buffer containing 1% NP40; the lysates were then immunoprecipitated with antibody 43-4 to $p140^{prototrk}$. Immunoprecipitates were either electrophoresed on 7.5% SDS-polyacrylamide gels and subjected to immunoblot analysis with the phosphotyrosine (Ptyr) monoclonal antibody 4G10 as described (7) or were analyzed in kinase assays (*7, 34*). DRGs were subjected to Dounce homogenization during lysis in buffer containing 1% NP40. *rtrk*-3T3 cells were generated by $CaPO_4$-mediated transfection of a mouse-rat *trk* cDNA hybrid into NIH-3T3 cells. Rat *trk* cDNAs were obtained from an embryonic rat DRG cDNA library. The longest *trk* cDNA obtained (2.4 kb) was missing approximately 150 bp of the coding region as compared to available mouse and human *trk* sequence. The missing bases plus minimal (~50 bp) 5′ flanking noncoding sequences were replaced from mouse first coding exon sequences, and the reconstructed gene was placed downstream of an MSV-LTR (D. Martin-Zanca, E. Sanz, L. F. Parada, manuscript in preparation). *rtrk* 3T3 cells (2×10^7) were treated with 1 mM suramin in Dulbecco's modified Eagle medium (DMEM) for 2 hours or mock-treated. After extensive washing of the cells with DMEM, NGF was added.
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43. ^{125}I-labeled NGF was prepared by lactoperoxidase treatment to specific activities of 2500 to 3500 cpm/fmol. Cross-linking of $p140^{prototrk}$ to ^{125}I-labeled NGF was performed as described (*18*). Cells (2×10^6/ml) were incubated with 0.5 nM ^{125}I-labeled NGF for 2 hours at 4°C. HSAB (50 μM) was added, and the reaction was exposed to long ultraviolet wavelength light (365 nm) for 10 min. After washing in 50 mM lysine in phosphate buffered saline, the cells were lysed in buffer containing 1% NP40, and the lysates were immunoprecipitated and analyzed by 7.5% SDS–polyacrylamide gel electrophoresis as described (7).
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45. We thank M. C. Fishman for the rat DRG cDNA library, J. Maragos, J. Blair, and S. Rabin for technical help, and R. Handley for preparation of the manuscript. Supported in part by NCI contract NO1-CO-74101 with Advanced Biosciences Laboratory (D.R.K., D.M.-Z., and L.F.P.) and by grants from the March of Dimes, Andrew W. Mellon Foundation, and the National Cancer Institute (B.L.H.) and from the National Institutes of Health, the American Cancer Society, Hirschl-Caulier Trust Fund, Parke Davis, and the special group of donors to the Dorothy Rodbell Foundation (M.V.C.).

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Induction by NGF of Meiotic Maturation of *Xenopus* Oocytes Expressing the *trk* Proto-Oncogene Product

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The effect of nerve growth factor (NGF) was assessed in *Xenopus* oocytes expressing the human *trk* proto-oncogene product, $p140^{prototrk}$. Oocytes injected with *trk* messenger RNA expressed polypeptides recognized by antibodies to the *trk* gene product. Exposure of these oocytes to nanomolar amounts of NGF resulted in specific surface binding of ^{125}I-labeled NGF, tyrosine phosphorylation of $p140^{prototrk}$, and meiotic maturation, as determined by germinal vesicle breakdown and maturation promoting factor ($p34^{cdc2}$) kinase activation. Thus the *trk* proto-oncogene product can act as a receptor for NGF in a functionally productive manner.

THE MEMBERS OF THE *TRK* GENE family (*1*) code for receptor-like, tyrosine kinase molecules that are transcribed in a pattern that suggests that they have a specialized role in neural tissue development (*2*). NGF (*3*) induces tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product ($p140^{prototrk}$) in PC12 cells (*4*), suggesting that this protein may transduce NGF signals. In *Xenopus* oocytes, factors that control cell proliferation and regulate the cell cycle (including hormones, growth factors and their receptors, and oncogenes) can easily be studied (*5–7*). We have now expressed the product of the human *trk* proto-oncogene in *Xenopus* oocytes by injecting them with *trk* mRNA synthesized in vitro and analyzing the biochemical and biological effects produced by NGF in these oocytes.

A cDNA fragment coding for the complete human *trk* proto-oncogene product (*8*) was inserted into vector pGEM2 to generate plasmid pGEMTRK-R1 from which full-length sense *trk* mRNA can be transcribed. An analogous H-ras^{lys12} construct, used as a control in these studies, was generated with a 670-bp EcoR I fragment from plasmid pJCL30 (*9*). These constructs were used to synthesize in vitro the full-length *trk* mRNA (2.7 kb) and *ras* mRNA (0.7 kb) (Fig. 1A), which were then injected into the cytoplasm of full-grown *Xenopus* oocytes. Expression of the protein products, after incubation to allow for translation of the injected mRNA, was assessed by immunoprecipitation of lysates of radiolabeled, injected oocytes with specific antibodies (Fig. 1B). Immunoprecipitation with two different antibodies to either the whole $p70^{trk}$ oncogene protein (*10*) (lanes 1 and 2) or the COOH-terminal region of $p140^{prototrk}$ (*8*) (lanes 3 and 4) revealed significant de novo accumulation of two polypeptide bands of 110 and 140 kD (Fig. 1B, lanes 2 and 4) that corresponded in size to the glycosylated protein products of human *trk* (*8*). The antibodies did not react with these molecules in control oocytes injected with water (lanes 1 and 3).

Expression of $p21^{ras}$ in oocytes was carried out in a similar manner by injecting the

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ras mRNA and allowing translation of the functional protein product. Expression of $p21^{ras(lys12)}$ protein in oocytes injected with *ras* mRNA resulted in rapid induction of germinal vesicle breakdown (GVBD), with an efficiency similar to that caused by progesterone, the physiological inducer of GVBD in oocytes (Table 1). Oocytes injected with water remained morphologically unaltered. In addition, injection of water and subsequent incubation before progesterone treatment did not alter the ability of the injected oocytes to undergo hormone-induced meiotic maturation (Table 1), indicating that the microinjection procedure does not impair the maturation ability of the oocytes.

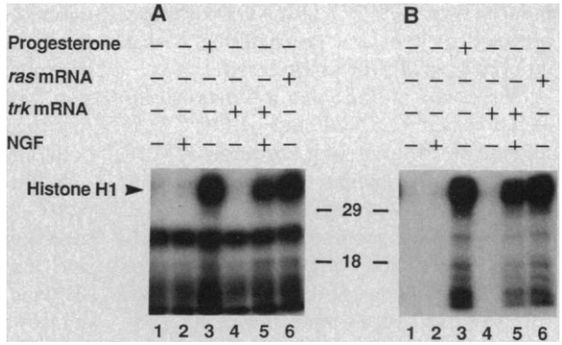

Fig. 2. Activation of MPF kinase complex. Histone H1 kinase assay (*18*) of oocytes from same samples analyzed in Table 1, experiment 3. Similar results were obtained in all other experiments in Table 1. (**A**) Autoradiogram of total reaction mixtures. (**B**) Same gel as in (A) after alkaline treatment (*19*). Position of exogenously added histone H1 is indicated. Lane 1, control, water-injected oocytes; lane 2, NGF, 35 nM; lane 3, progesterone, 15 μM; lane 4, untreated, *trk*-expressing oocytes; lane 5, *trk*-expressing oocytes treated with 35 nM NGF; lane 6, *ras*-expressing oocytes. Gels were exposed at room temperature for 5 hours (A) and 16 hours (B).

In contrast, injection of *trk* mRNA and subsequent expression of $p140^{prototrk}$ did not produce any significant GVBD by itself (Table 1). Addition of low concentrations of NGF (1.7 to 35 nM) to oocytes expressing $p140^{prototrk}$ resulted in meiotic maturation, with the highest levels of GVBD obtained in those experiments where translation of the mRNA proceeded for the longest time before NGF treatment (Table 1). Addition of NGF to uninjected oocytes or to oocytes injected with water alone did not produce any meiotic maturation (Table 1), indicating that NGF-induced GVBD was dependent upon $p140^{prototrk}$ expression.

Oocyte meiotic maturation requires the previous activation of the maturation promoting factor (MPF) complex (*5, 11*), which leads irreversibly to GVBD (*5–7*). This active complex contains a serine-threonine histone H1 kinase activity that can be readily assayed in oocyte lysates. We confirmed biochemically the morphological estimation of GVBD in Table 1 by assaying the H1 kinase activity in lysates of the oocytes matured under the various conditions tested (Fig. 2). There was the same amount of kinase activity in oocytes that had been treated with NGF plus *trk* mRNA as in oocytes that had been induced to mature with progesterone or $p21^{ras}$ (Fig. 2). Unlike other endogenous substrates that became phosphorylated in the assays (Fig. 2A), the phosphorylation of exogenous histone H1 was in all cases resistant to alkali treatment (Fig. 2B), presumably due to threonine phosphorylation. Oocytes expressing $p140^{prototrk}$, but not treated, did not show H1 kinase activity. No H1 kinase activity was detected either in control, uninjected oocytes treated with NGF alone, or in oocytes injected with water and treated with NGF. Therefore, it appears that maturation

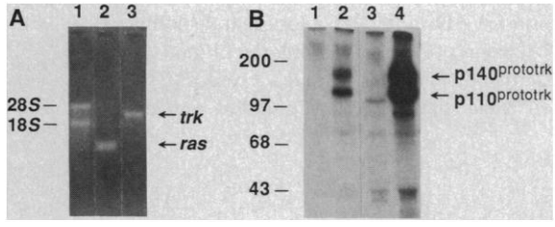

Fig. 1. Expression of $p140^{prtotrk}$ in *Xenopus* oocytes injected with synthetic *trk* proto-oncogene mRNA. (**A**) Ethidium bromide staining of RNA markers (lane 1), ras^{lys12} mRNA (lane 2), *trk* proto-oncogene mRNA (lane 3) synthesized (*17*) in vitro and electrophoresed on 1% agarose gels. (**B**) Immunoprecipitation of *trk* mRNA–injected (lanes 2 and 4) and water-injected (lanes 1 and 3) oocytes with rabbit antiserum 6-3 (lanes 1 and 2) to full length, bacterially expressed $p70^{trk}$ (*10*) or with rabbit antiserum 43-4 (lanes 3 and 4) to a COOH-terminal *trk* proto-oncogene peptide (*8*). The oocytes (five per gel lane) were injected and maintained in ND-96 medium (*16*) supplemented with 1 mCi/ml each of [^{35}S]methionine (1100 Ci/mmol) and [^{35}S]cysteine (900 Ci/mmol) (NEN) for 14 hours at 20°C. Oocyte lysates prepared in "radioimmunoprecipitation" (RIPA) buffer (30 μl per oocyte) were centrifugated and the supernatants were immunoprecipitated (3 hours, 4°C) with antisera 6-3 or 43-4 preadsorbed to protein A–Sepharose were (Pharmacia). The immunoprecipitates were washed four times with RIPA buffer, mixed with SDS-electrophoresis sample buffer, and analyzed by electrophoresis with 10 to 20% SDS-polyacrylamide gradient minigels (Integrated Separation Systems) followed by autoradiography for 18 hours at −70°C.

Table 1. Induction of meiotic maturation in injected oocytes. Oocytes obtained from different frogs for each experiment were injected with ras^{lys12} mRNA, *trk* mRNA, or water (*16*) and maintained in ND-96 medium without KCl. At the indicated times after injection, oocytes were treated with the different agonists for 12 to 15 hours. *Ras* mRNA was injected at the time of agonist treatment of the *trk*- and water-injected oocytes, and GVBD was scored 12 to 15 hours later. Percentage GVBD was estimated by scoring the absence of the nucleus (germinal vesicle) in oocytes fixed with 10% trichloroacetic acid. Number of oocytes matured per total number of oocytes used in each experiment is indicated in parenthesis. Time between injection and agonist treatment: experiment (exp.) 1, 48 hours; exp. 2, 25 hours; exp. 3, 25 hours; exp. 4, 16 hours; n.d., not done.

Injection	Agonist	GVBD (percentage)			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4
ras mRNA	–	100 (10/10)	100 (8/8)	100 (8/8)	100 (8/8)
Water	Progesterone (15 μM)	100 (10/10)	100 (8/8)	100 (8/8)	100 (9/9)
Water	–	0 (0/9)	0 (0/8)	0 (0/6)	0 (0/8)
Water	NGF (35 nM)	0 (0/10)	0 (0/8)	0 (0/6)	0 (0/9)
trk mRNA	–	0 (0/5)	12 (1/8)	0 (0/7)	0 (0/8)
trk mRNA	NGF (35 nM)	100 (6/6)	100 (7/7)	88 (7/8)	40 (4/10)
trk mRNA	NGF (1.7 nM)	n.d.	n.d.	n.d.	20 (2/10)

induced by p140^{prototrk} plus NGF treatment proceeds by the same mechanisms as that induced by p21^{ras} or progesterone.

Our results indicate that a functional interaction between NGF and p140^{prototrk} is responsible for triggering activation of MPF and subsequent meiotic maturation. Thus, p140^{prototrk} may act as a receptor for NGF. To test this, we examined the specific binding of ^{125}I-labeled NGF to the surface of oocytes expressing p140^{prototrk} (Fig. 3A). Incubation of oocytes expressing p140^{prototrk} with increasing concentrations of ^{125}I-labeled NGF resulted in specific binding that was greater than the background binding in control, water-injected oocytes. The radioactivity bound to oocytes that had been injected with water was considered nonspecific because addition of excess (>150-fold) unlabeled NGF to the reaction mixtures did not change the amount of radioactivity incorporated by water-injected oocytes, whereas it reduced the radioactivity bound to *trk*-injected oocytes to the same levels as for water-injected oocytes. Furthermore, the specific binding of ^{125}I-labeled NGF to *trk*-injected oocytes was completely blocked by a neutralizing antibody to NGF (Fig. 3A). Thus, the ^{125}I-labeled NGF specific binding was defined as the difference between radioactivity bound to *trk*-injected and that bound to water-injected oocytes (Fig. 3A, inset). Immunoprecipitation with antibodies to p140^{prototrk} of extracts of oocytes after the binding reaction with ^{125}I-labeled NGF (*12*) showed that there was a direct, physical interaction between NGF and p140^{prototrk}.

A functional, direct interaction between p140^{prototrk} and NGF was indicated by the detection of tyrosine-specific phosphorylation of p140^{prototrk} after NGF binding (Fig. 3B, lane 2). A 5-min exposure to NGF of oocytes expressing p140^{prototrk} allowed the detection in immunoprecipitates of a tyrosine-phosphorylated band that comigrated with the mature p140^{prototrk} product (*8*) expressed in the oocytes injected with *trk* mRNA (Fig. 1B). Expression of p140^{prototrk} alone or treatment of control oocytes with NGF did not produce such phosphorylation (Fig. 3B, lanes 1 and 3).

Our results demonstrate that p140^{prototrk} expressed in oocytes can interact functionally with exogenously added NGF. Binding of NGF to p140^{prototrk} results in rapid tyrosine phosphorylation of this protein and triggers GVBD by activation of the MPF kinase complex. Similar tyrosine phosphorylation and GVBD induction upon ligand binding occurs in oocytes expressing the human epidermal growth factor (EGF) receptor (*7*). It is likely that the NGF-induced tyrosine phosphorylation of p140^{prototrk} constitutes the initiating signal for the observed biological effects.

Our results also suggest that NGF is a functional ligand for p140^{prototrk}, and thus p140^{prototrk} may act in vivo as a receptor for this multifunctional growth factor (*3*). This work with *Xenopus* oocytes confirms and extends other observations (*14*) showing that p140^{prototrk} is a functional receptor for NGF in PC12 cells. Interestingly, expression in *Xenopus* oocytes of a different NGF receptor, coded by a gene unrelated to *trk*, does not result in meiotic maturation, even upon exposure to NGF (*15*).

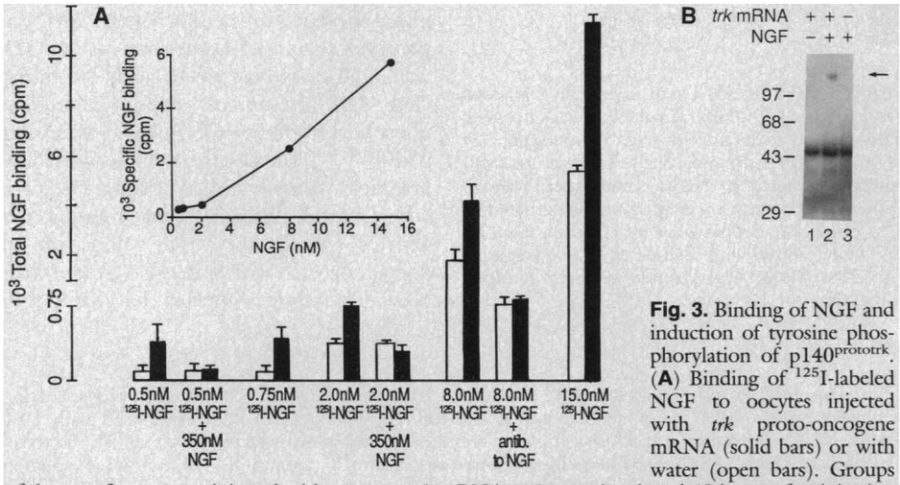

Fig. 3. Binding of NGF and induction of tyrosine phosphorylation of p140^{prototrk}. (**A**) Binding of ^{125}I-labeled NGF to oocytes injected with *trk* proto-oncogene mRNA (solid bars) or with water (open bars). Groups of three to five oocytes injected with water or *trk* mRNA (*16*) were incubated 48 hours after injection with the indicated concentrations of ^{125}I-labeled 2.5*S* murine NGF (1500 Ci/mmol) (Amersham) at 0°C (to avoid internalization) for 3 to 4 hours in ND-96 medium supplemented with bovine serum albumin (1 mg/ml). Where indicated, an excess (350 nM) of unlabeled 2.5*S* murine NGF (Boehringer Mannheim) or a neutralizing antibody to NGF (dilution 1/50) (Sigma) were included in the reaction mixtures. Oocytes were washed five times at 0°C, counted individually, and the average and SD of radioactivity incorporated per oocyte calculated. Similar results were obtained in two separate experiments performed with oocytes from different animals. (**B**) NGF-induced tyrosine phosphorylation of p140^{prototrk} (arrow) expressed in oocytes. Lysates of oocytes (16 per gel lane) injected 48 hours previously with *trk* mRNA (lanes 1 and 2) or water (lane 3) were incubated with 35 nM NGF for 5 min (lanes 2 and 3), followed by extraction with Triton X-100–containing buffer (*13*). p140^{prototrk} immunoprecipitates prepared with antibody 43-4 (*8*) as in Fig. 1 were electrophoresed in 10 to 20% SDS-polyacrylamide gradient minigels and analyzed by Western blotting with antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology Inc.) and ^{125}I-labeled sheep antibody to mouse immunoglobulin G (IgG) (1500 Ci/mmol) (Amersham). The autoradiogram was exposed for 48 hours at −70°C. Bands at the 45-kD marker correspond to cross-reaction between the rabbit 43-4 IgG heavy chain and ^{125}I-labeled antibody to mouse IgG.

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12. After the ^{125}I-labeled NGF binding reaction, the *trk* mRNA-injected and water-injected oocytes were extracted with Triton X-100–containing buffer (*13*), and the extracts were immunoprecipitated with antibody 43-4 to p140^{prototrk} (*8*). ^{125}I-labeled NGF was detected only in the immunoprecipitates of *trk* mRNA-injected oocytes. In control immunoprecipitates prepared with protein A alone from extracts of *trk*-injected or water-injected oocytes, no ^{125}I-labeled NGF was detected.
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16. Full-grown oocytes were manually dissected from unstimulated *Xenopus laevis* females and maintained overnight in ND-96 medium (5 mM Hepes, 96 mM NaCl, 1 mM $MgCl_2$, 2 mM KCl, 1.8 mM $CaCl_2$, pH 7.6, supplemented with 10 μg/ml each of penicillin and streptomycin sulphate) at 20°C. The oocytes were then injected into the cytoplasm with 30 nl of either *trk* or *ras* mRNA (30 ng) or water, with a computer-assisted (Atto) microinjection system.
17. A 2.7-kb Eco RI cDNA fragment containing the entire coding region of the *trk* proto-oncogene (*8*) was subcloned into pGEM2 (Promega Biotech). The resulting plasmid, pGEMTRK-R1, was linearized by digestion with Hind III and transcribed in vitro with SP6 RNA polymerase, as recommended by the supplier (Promega), in the presence of the CAP analog m7G(5′)ppp(5′)G (New England Biolabs). The product of the reaction was treated with

ribonuclease-free deoxyribonuclease RQ1, extracted with phenol-chloroform, precipitated with ethanol, and dissolved in diethylpyrocarbonate-treated water to a concentration of ~1 mg/ml. For *ras* mRNA synthesis, a 0.7-kb Eco RI fragment from plasmid pJCL30 (*9*) containing the H-*ras*^{lys12} gene was subcloned into pGEM2. The resulting construct was linearized with Bam HI and transcribed in vitro with the same conditions as for *trk*.

18. Groups of two oocytes were collected before fixation with trichloroacetic acid and homogenized in buffer (20 μl per oocyte) containing 20 mM Hepes, pH 7.4, 100 mM B-glycerophosphate, 10 mM EGTA, 15 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, aprotinin (10 μg/ml), and leupeptin (10 μg/ml). Clarified extracts (10 μl) were incubated in a final volume of 15 μl with 100 μM adenosine triphosphate (ATP), 4 μCi of [γ-^{32}P]-ATP (3000 Ci/mmol) (Amersham), and 3 μg of histone H1 (Sigma) for 15 min at room temperature. The reaction was terminated by adding SDS-electrophoresis sample buffer and boiling for 3 min. Phosphorylations were visualized by autoradiography after electrophoresis on 10 to 20% polyacrylamide gradient minigels.
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Chimeric NGF-EGF Receptors Define Domains Responsible for Neuronal Differentiation

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To determine the domains of the low-affinity nerve growth factor (NGF) receptor required for appropriate signal transduction, a series of hybrid receptors were constructed that consisted of the extracellular ligand-binding domain of the human epidermal growth factor (EGF) receptor (EGFR) fused to the transmembrane and cytoplasmic domains of the human low-affinity NGF receptor (NGFR). Transfection of these chimeric receptors into rat pheochromocytoma PC12 cells resulted in appropriate cell surface expression. Biological activity mediated by the EGF-NGF chimeric receptor was assayed by the induction of neurite outgrowth in response to EGF in stably transfected cells. Furthermore, the chimeric receptor mediated nuclear signaling, as evidenced by the specific induction of transin messenger RNA, an NGF-responsive gene. Neurite outgrowth was not observed with chimeric receptors that contained the transmembrane domain from the EGFR, suggesting that the membrane-spanning region and cytoplasmic domain of the low-affinity NGFR are necessary for signal transduction.

THE BIOLOGICAL ACTIVITIES OF NGF, like those of EGF, are mediated by cell-surface receptor proteins that span the plasma membrane (*1, 2*). Receptors for NGF exist in a high- and low-affinity form. However, it is unclear whether the low-affinity form is capable of participating in signal transduction. To investigate the function of the low-affinity NGFR represented by the p75^{NGFR} protein (*2*), and the structural requirements necessary to elicit biological responses by NGF, we constructed chimeric receptors that consisted of various domains from the human EGFRs and NGFRs and tested their ability to carry out signal transduction.

The cDNA sequences (*3*) that coded for the ligand-binding domain of the human EGFR (amino acid residues −24 to 541) were fused with the sequences that encoded the transmembrane and cytoplasmic sequences (amino acids 188 to 399) of the human p75^{NGFR} (*2*), creating EN10 cDNA (Fig. 1). We also constructed two other chimeric receptor cDNAs with a similar strategy (Fig. 1, EN30 and EN31). EN30 resulted from a ligation between the extracellular domain of the EGFR (amino acids −24 to 619) with p75^{NGFR} transmembrane and cytoplasmic sequences (amino acids 204 to 399), and EN31 consisted of EGFR ligand-binding and transmembrane domains (amino acids −24 to 647) fused to only the cytoplasmic domain (amino acids 250 to 399) of the NGFR. Each hybrid cDNA was introduced into either the pMV7 (*4*) or pCMV5 (*5*) mammalian expression vectors.

Rat PC12 cells were transfected with each plasmid, and stable transfectants were selected by resistance to G418. Expression of the chimeric proteins was assessed by rosetting analysis (*2*) with the R1 monoclonal antibody, which recognizes the human EGFR (*6*). Positive clones were selected and used for immunoprecipitation and cross-linking analyses. Transfected cells were labeled with ^{35}S-labeled Cys and Met, and receptors were immunoprecipitated with the R1 antibody. In cells that expressed the EN10 and EN30 cDNAs, the chimeric receptors were compared to the native EGFR in A431 cells (Fig. 2A). The chimeric receptors migrated at an apparent molecular size of 140 kD, as would be expected for a receptor that contained the comparatively shorter NGFR cytoplasmic domain. Endogenous EGFRs were not detected, as the R1 antibody was specific for human EGFRs (*6*).

Treatment of PC12 cells with NGF led to morphological differentiation into a sympathetic cell phenotype (*7*). No changes in morphology were observed with cells treated with EGF (Fig. 3). We tested the morphological response to NGF and EGF in transfected cells that contained chimeric receptors. All clones responded to NGF at concentrations necessary to observe biological responses (1 to 10 ng/ml) (*8*). In clonal cell lines that contained the EN10 and EN30 chimeric receptors, EGF induced cell flattening within hours and extensive neurite outgrowth after 3 days of treatment with EGF. From 40 to 75% of the EN10- and EN30-containing cell lines showed a phase bright neuronal-like morphology in response to EGF, and the majority of processes were greater than one cell diameter and approached several hundred micrometers. The response of these cells to treatment with concentrations of EGF between 0.5 to 5 ng/ml was qualitatively similar to their

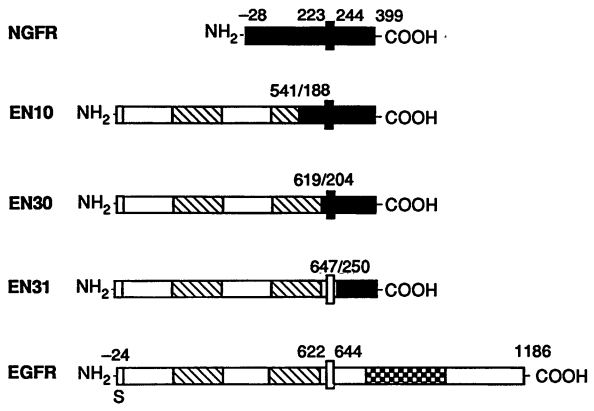

Fig. 1. Schematic representation of EGF and NGF chimeric receptors. The sequences are aligned at their transmembrane domains, shown by the vertical bar. The reading frame was verified by DNA sequencing. Filled region, NGFR; open regions, EGFR; S, signal peptide; striped regions, cysteine rich; checkerboard region, tyrosine kinase domain.

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