A TrkA-selective, Fast Internalizing Nerve Growth Factor-Antibody Complex Induces Trophic but Not Neuritogenic Signals*

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Nerve growth factor (NGF) is a neurotrophin that induces neuritogenic and trophic signals by binding to TrkA and/or p75 receptors. We report a comparative study of the binding, internalization, and biological activity of NGF versus that of NGF in association with an anti-NGF monoclonal antibody (mAb NGF30), directed against the C termini of NGF. NGF-mAb complexes do not bind p75 effectively but bind TrkA with high affinity. After binding, NGF-mAb complexes stimulate internalization faster and to a larger degree than NGF. NGF-mAb-induced activation of TrkA, Shc, and MAPK is transient compared with NGF-induced activation; yet NGF and NGF-mAb afford identical trophic responses. In contrast, NGF induces Sce-1-associated neurotrophic activating protein phosphorylation and neuritogenic differentiation, but NGF-mAb does not. Thus, an absolute separation of trophic and neuritogenic function is seen for NGF-mAbs, suggesting that biological response modifiers of neurotrophins can afford ligands with selected activities.

Nerve growth factor (NGF)† is the prototype member of the neurotrophin family of ligands, which includes brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 (NT-3 and NT-4/5) (1). NGF is important for the survival and differentiation of certain sensory, sympathetic, and cholinergic neurons and many other cell types (2–5). Two NGF receptor proteins have been cloned, termed p75 and TrkA. NGF-responsive cells can express either or both receptor types.

NGF and other neurotrophins bind p75 with low affinity ($K_d \sim 10^{-9}$ M) (6), except NT-3 which binds p75 with high affinity in some cells (7). The p75 receptor belongs to the tumor necrosis factor family, and a pro-apoptotic function is revealed in some cells that express p75 receptor in the absence of TrkA (reviewed in Ref. 8).

TrkA receptors bind NGF with intermediate to high affinity ($K_d = 10^{-10}$ to $10^{-11}$ M) (9–11). TrkA receptors possess an intrinsic tyrosine kinase catalytic activity that mediates two distinct biological outcomes typically associated with NGF: trophic survival and neuritogenic differentiation. The trophic activity of NGF is revealed by its ability to prevent apoptotic cell death (12, 13). The neuritogenic activity of NGF is revealed by its promotion of cellular differentiation and axon elongation (4).

The TrkA receptor mediates all of the trophic and neuritogenic activities of NGF whether or not p75 is expressed (13–15) or bound by the ligand (16, 17). However, co-expression of p75 affects ligand affinity and TrkA efficacy (11, 13) and also affects ligand-induced internalization (18).

NGF-mediated activation of the TrkA tyrosine kinase is the earliest step leading to signal transduction (17, 20). Tyrosine phosphorylation (Tyr(P)) of specific intracellular residues of NGF creates docking sites for adaptor signaling molecules. Phospholipase C-γ, Shc, and PI3-kinase adaptors activate trophic responses via p21ras/MAPK (17, 19–22), although MAPK activation can occur via cAMP-dependent protein kinase A (24). Phospholipase C-γ and SNT activation via TrkA are obligatory for neuritogenesis (20–23).

Cellular commitment to trophic or neuritogenic outcomes may be related to the kinetics of TrkA-mediated activation of MAPK. For example, NGF binding to TrkA causes a relatively slow but sustained activation leading to cellular differentiation (23–26), but epidermal growth factor (EGF) binding to EGF receptors (EGFR) causes a rapid but transient activation of MAPK (ERKs) leading to cellular proliferation (26, 27). Furthermore, signals leading to proliferation or differentiation may be affected by altering the kinetics of internalization (28). TrkA/NGF reportedly internalize in clathrin-coated vesicles or in caveoli, thought to deliver the activated receptor to the cell soma located at some distance from nerve terminals (29, 30).

Here we report interesting signaling properties of a complex between NGF and an anti-mouse NGF monoclonal antibody (mAb NGF30) that binds the C-terminal region of NGF. The NGF-mAb complex binds TrkA with high affinity but does not bind p75 efficiently, suggesting a role in receptor binding to the C-terminal region of NGF. The NGF-mAb complex induces faster internalization of ligand and TrkA than does NGF, and the NGF-mAb complex induces full trophic signals but does not induce any neuritogenic signals. The biological properties of

* This work was supported by Medical Research Council of Canada grants (to H. U. S. and A. C. C.) and by National Institutes of Health Grant NS24380 (to K. E. N.). This is National Research Council publication 57673. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be regarded by the author as public information, and it is not subject to copyright.

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‡ The abbreviations used are: NGF, nerve growth factor; ELISA, enzyme-linked immunosorbent assays; wt, wild type; HRP, horseradish peroxidase; BSA, bovine serum albumin; Tyr(P), tyrosine phosphorylation; MAPK, mitogen-activated protein kinase; mAb, monoclonal antibody; Ab, antibody; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FRTC, fluorescein isothiocyanate; PI3, phosphatidylinositol 3-kinase; NT, neurotrophin; SNT, Suc-1-associated neurotrophic activating protein; SFM, serum-free media; MBP, myelin basic protein.

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the NGF-mAb complex as a ligand of TrkA may be due to conformational restrictions in NGF caused by mAb NGF30.

MATERIALS AND METHODS

Cell Lines—PC12 cells are rat pheochromocytomas that express ~40,000–50,000 p75/cell and no Trk (p75+/− TrkA−) (4). The 6-2.4 cells are PC12-transfected with human trkA cDNA and express high levels of receptors/cell of each p75 and TrkA (p75+/+ TrkA−). The nmr5 cells are a subline of PC12 that express intermediate levels of p75 and undetectable TrkA (p75+/− TrkA−). E25 cells are NIH3T3 fibroblasts transfected with human trkA cDNA, and express high levels TrkA/cell (p75− TrkA+/+) (9). B104 cells are rat neuroblastomas that express 40,000–50,000 p75/cell and no Trk (p75+/− TrkA−). The 4-3.6 cells are B104 transfected with human trkA cDNA, and express 40,000–50,000 receptors/cell of each p75 and TrkA (p75+/+ TrkA−). C10 cells are a subline of 4-3.6 cells that express 40,000–50,000 TrkA/cell and no p75 (p75− TrkA+/+) (13). ELA cells are mouse thymomas negative for p75 and TrkA (p75− TrkA−).

Septal Neuronal Cultures, Treatments, and Choline Acetyltransferase Activity—Cell cultures were established from the septal area of 17-day-old rat embryos using established procedures (31) and were treated with control media, NGF alone, or NGF-NGF30 1 day after plating. Choline acetyltransferase activity was evaluated after 4, 6, and 8 days in vitro (32).

Production of Extracellular Domain of TrkA (TrkA-ECD)—Human TrkA-ECD in TNMFH +10% fetal bovine serum (Life Technologies, Inc.) was propagated using serum-free SF9 cells. At 72 h post-infection the supernatant was harvested and purified to >99% purity as described (33).

Antibodies—Rat anti-mouse β-NGF mAb NGF30 (IgG2a) (34), mouse anti-rat p75 mAb MC192 (IgG1), and mouse anti-human TrkA mAb 111 (111) were used. The same binding data were obtained whether sequential binding to TrkA (NGF, washing, mAb NGF30) or pre-associated complexes of NGF-NGF30 were used. Cells were acquired on a FACSscan, and bell-shaped histograms were analyzed using the LYSIS II program.

Receptor Internalization—4-3.6 cells were incubated in media with NGF plus rat IgG control or NGF-NGF30 complexes at 4 °C for 1 h and then allowed to internalize at 37 °C for the times indicated. After washing at 4 °C, cells were analyzed by FACSscan for cell surface TrkA and p75 expression using directly labeled 5C3-FITC and MC192-FITC respectively.

Ligand Binding and Internalization—125I-NGF (73.1 mCi/mg; NEN Life Science Products) binding assays and Scatchard plot analysis were done as described (35). 125I-NGF in BB were pre-mixed with rat IgG (control) or with the indicated molar excess of mAb NGF30 or NGF30 Fab. A 1000-fold molar excess unlabeled NGF was used to assess nonspecific background (always <10% of binding). For 125I-NGF internalization, 4-3.6 cells (3 × 105 cells/ml) were incubated at 4 °C for 1 h with 2 × 106 125I-NGF (alone, with mAb NGF30, NGF30 Fab, or rat IgG control) and then shifted to 37 °C. At different times 3 × 106 cells were washed with citric acid buffer (10 mM sodium citrate, 150 mM NaCl, pH 4.0) to remove surface 125I-NGF. <10% of bound 125I-NGF is resistant to acid wash under conditions that do not allow internalization (NaCl and 4 °C throughout).

Proliferation and Survival Assays—Cells (5,000–10,000 cells/well) were added to 96-well plates (Becton Dickinson, Lincoln Park, NJ) and cultured either in media containing 5% fetal bovine serum or in serum-free media + 0.1% BSA (SFM). Ligands consisted of serial dilutions of rat IgG (control), mAb NGF30 alone, wild type mouse NGF, NGF mutants, or NGF-NGF30 complexes. The proliferative/survival profile of the cells was quantitated using the tetrazolium salt reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) and optical density (OD) readings as described (13). Assays were done ≥5 times, each assay n = 4–10.

Western Blots—Blots were performed as described (13). Briefly, cells were detergent-solubilized, and protein concentrations were determined. Lysates were immunoprecipitated (Shc with anti-Shc antisera (gift of Dr. J. J. Bergeron); TrkA with anti-TrkA antisera, and SNT with mouse IgG (FITC-G-α-M) secondary antibody. As cellular controls not expressing NGF receptors were used, and for background staining control mAb NGF30 + FITC-G-α-Rat was used without NGF. In direct binding assays FITC-labeled mAb NGF30 (NGF30-FITC) was used. The same binding data were obtained whether sequential binding to TrkA (NGF, washing, mAb NGF30) or pre-associated complexes of NGF-NGF30 were used. Cells were acquired on a FACSscan, and bell-shaped histograms were analyzed using the LYSIS II program.

RESULTS

mAb NGF30 Binds Near Amino Acid Arg-118 of Murine NGF—A panel of mouse NGF mutants was used as targets in ELISAs to map the epitope recognized by mAb NGF30. Ab NGF30 bound equally well to wild type mouse NGF and to mouse recombinant mutants H75A, H84Q, and Δ9-13 (Table 1, rows 1–4). Mouse NGF double mutant H75A/H84Q was bound but at significantly lower levels (~80% of wild type NGF; Table 1, row 5). mAb NGF30 binds wild type rat NGF (data not shown) but does not bind mouse mutant NGF R118A, recombinant human NGF, or recombinant human NT-3 (Table 1,
NGFmAb Complex with Altered Binding and Signaling

**TABLE I**

| Test antigen | Binding ± S.E. % |
|--------------|------------------|
| 1. Wt mouse NGF | 100.0 ± 1.1 |
| 2. Mouse H75A | 100.0 ± 3.4 |
| 3. Mouse H84A | 94.4 ± 4.4 |
| 4. Mouse Δ9-13 | 106.5 ± 1.9 |
| 5. Mouse H75A/H84Q | 80.4 ± 5.0 |
| 6. Mouse R118A | 14.5 ± 9.9 |
| 7. Human rec NGF | 1.9 ± 6.8 |
| 8. Human rec NT-3 | 2.2 ± 2.7 |

Binding of mAb NGF30 to purified wild type (wt) or recombinant (rec) neurotrophins was studied by ELISAs and standardized to wild type mouse NGF after background subtraction (<5%). n = 4 ± S.E. Data are representative of >3 independent assays.

NGF amino acids 119 and 120 are not relevant for mAb NGF30 binding because wild type NGF1–118 is bound as well as recombinant mouse NGF3–120.

Lack of binding by mAb NGF30 to mutant R118A suggests that this residue is directly bound by the mAb. Conformational changes in the mutant are unlikely to account for the data because the mAb works in denaturing Western blots (34). However, reduced binding to mouse H75A/H84Q double mutant suggests conformational hindrance upon the C terminus.

**Stoichiometry of mAb NGF30-NGF Interactions—**

We tested whether bivalent mAb NGF30 interacts with both protomers of dimeric NGF. A sandwich ELISA was used wherein immobilized mAb NGF30 was used to capture NGF, and NGF30-biotin/avidin-HRP was used to reveal binding sites not occupied by the capture mAb. If both NGF epitopes are occupied with the capture mAb, no subsequent binding of mAb NGF30-biotin would occur. This sandwich ELISA was used previously to study anti-NGF mAb 27/21 which binds one epitope of an NGF protomer and leaves the epitope of the other NGF protomer exposed and available for binding (38).

mAb NGF30 with captured NGF did not allow substantial binding of NGF30-biotin (Table II, row 1), compared with background binding in the absence of NGF (Table II, row 2), or to nonspecific binding to BSA (Table II, row 4). In contrast, immobilized NGF was efficiently bound by mAb NGF30-biotin (Table II, row 3). Similar data were obtained when 180-fold molar excess of NGF was used in an attempt to drive NGF30 binding two NGF dimer molecules (data not shown). These results indicate that mAb NGF30 excludes the binding of a second mAb NGF30 and suggest that the stoichiometry of one antibody dimer and one NGF dimer is preferred thermodynamically. In contrast, control mAb 27/21 was effective as capture in sandwich ELISAs (Table II, row 5). mAb 27/21-HRP bound to NGF-mAb 27/21 complexes as efficiently as it bound immobilized NGF (Table II, row 6). mAb NGF30 did not bind NGF-mAb 27/21 complexes (not shown), suggesting that the mAbs may block each other’s binding.

**NGF and NGF-NGF30 Mediate Identical Trophic Survival—**

NGF can rescue TrkA-expressing cells from death in a serum deprivation model of apoptosis. Ligand-induced survival of various cell lines cultured in serum free media (SFM) was studied by the MTT method (Table III). Cells expressing TrkA and p75 (PC12, 4-3.6, and 6-2.4 cells) were optimally protected from apoptotic death in SFM by 2 nM mouse NGF (Table III, row 1), as well as by NGF-NGF30 (Table III, rows 6, 7, and 8). This was also true for cells that express TrkA in the absence of p75 (C10 and E25 cells). In contrast, cells that express p75 in the absence of TrkB (B104 and mm5) were not protected from apoptotic death in SFM by NGF or by NGF-NGF30 at different molar ratios nor was there accelerated death induced by these ligands (data not shown).

**TABLE II**

| Immobilized | Soluble | Primary | OD |
|-------------|---------|---------|----|
| 1. mAb NGF30 | NGF | NGF30-bio | 56 ± 3 |
| 2. mAb NGF30 | NGF30-bio | 24 ± 3 |
| 3. NGF | NGF30-bio | 218 ± 4 |
| 4. BSA | NGF30-bio | 23 ± 3 |
| 5. mAb 27/21 | NGF | 27/21-HRP | 275 ± 9 |
| 6. NGF | NGF30-bio | 336 ± 9 |

Stoichiometry of mAb NGF30-NGF interactions were studied by sandwich ELISA, using immobilized mAb NGF30 for capture of NGF and mAb NGF30-biotin (bio) for reading. The colorimetric assay was generated with avidin-HRP. Immobilized NGF is the positive control, and immobilized BSA is the negative control. n = 4 ± S.E. Data are representative of three independent assays.

There were no differences in the trophic protection afforded by mouse NGF in the presence or absence of mAb NGF30, even at high molar excess of mAb (2 nM NGF + 64 nM NGF30) (Table III, row 5). Monovalent Fabs of mAb NGF30 had no effect on NGF-mediated trophic protection, even when added at 40-fold molar excess (data not shown).

Various controls demonstrated specificity. First, 64 nM rat IgG did not alter the optimal protective effect of 2 nM mouse NGF (Table III, rows 1 versus 4). Second, a combination of 2 nM human NGF + 64 nM NGF30 afforded the same survival as 2 nM human NGF (Table III, rows 9 versus 10). This result is consistent with lack of mAb NGF30 binding to human NGF (see Table I). Third, 64 nM rat IgG or 64 nM NGF30 mAb alone had no effects on any cell line in SFM (Table III, rows 2 and 3).

**NGF-NGF30 Does Not Induce Neurotogenic Differentiation—**

PC12 or 6-2.4 cells cultured in serum-containing media differentiate in response to mouse NGF, projecting neuritic processes that can be measured morphometrically. Culture of PC12 cells with 2 nM NGF30 differentiated most cells, compared with no NGF added (Fig. 1A). Whereas cells did differentiate in culture with 2 nM NGF + 24 nM monovalent NGF30 Fab (Fig. 1D), cultures with 2 nM NGF + 4 nM mAb NGF30 did not differentiate (Fig. 1C).

Various controls demonstrated the specificity of the neurogenic block. First, co-treatment with 2 nM NGF + 24 nM rat IgG did not affect differentiation (Fig. 1B) compared with NGF alone (data not shown). Second, neuritogenesis in response to 2 nM human NGF (Fig. 1E) was not affected by 24 nM mAb NGF30 (Fig. 1F). This result is consistent with mAb NGF30 not binding human NGF (Table I). Third, cultures in serum-containing media with 24 nM mAb NGF30, 24 nM mAb NGF30 Fabs, 24 nM rat IgG or higher concentration of these agents, either alone or in the presence of NGF, did not reveal any toxicity (data not shown). Fourth, all of these results were repeated using 6-2.4 cells (data not shown).

It is improbable that bivalent NGF30 mAb acts simply by hindering or blocking NGF action, because it inhibits neurotogenic signals without affecting trophic signals (Table III), and monovalent NGF30 Fabs do not affect any of the activities of NGF.

**NGF-NGF30 Does Not Increase Choline Acetyltransferase Activity—**

To study the block to NGF-induced cellular differentiation further, rat embryonic septal cultures were tested. Septal cultures increased choline acetyltransferase activity ~2.1-fold in response to NGF. Co-culture of NGF with a 2-fold molar excess of mAb NGF30 reduced choline acetyltransferase activity to base line. No differences in cell numbers or health were seen in NGF versus NGF-NGF30-treated cultures (data not shown). These data extend earlier findings of lack of differentiation by septal neurons in response to NGF-NGF30 (34).

mAb NGF30 Blocks NGF Binding to p75 but Not to TrkA—

Binding of NGF-NGF30 to cells expressing NGF receptors was...
The indicated cells were cultured in serum-free media in the presence of the indicated ligands to assess cell survival. Rat IgG was used as a control for mAb NGF30, and human NGF was used to exclude mAb NGF30 toxicity and to control specificity. Data shown are % OD ± S.E. with respect to 2 nM mouse NGF (row 1), m = 4 or n = 8. Data are compiled from >5 independent experiments for each cell line.

### Table IV

| Cells | Preformed NGF + mAb NGF30 | Sequential NGF ↔ NGF30-FITC | NGF30-FITC only |
|-------|---------------------------|-----------------------------|-----------------|
| 1. 4–3.6 (TrkA+++ p75+++) | 73 | 72 | 10 |
| 2. C10 (TrkA+++ p75+) | 110 | 108 | 8 |
| 3. B104 (TrkA p75+++) | 10 | 10 | 8 |
| 4. EL4 (TrkA p75+) | 12 | 11 | 10 |

Fig. 1. mAb NGF30 prevents NGF-induced neuritogenesis. PC12 cells were cultured for 72 h with no NGF (A), with 2 nM mouse NGF (B–D), or 2 nM human NGF (E and F). Abs were co-cultured as follows: 24 nM rat IgG control (B), 4 nM NGF30 mAb (C), 24 nM NGF30 Fab (D), or 24 nM NGF30 mAb (F). Pictures are representative from >4 independent assays. Similar data were obtained with 6-2.4 cells (data not shown).

NGF30-FITC to cell surfaces was detected in the absence of NGF, and there was no binding to TrkA− p75+ B104 cells (Table IV, row 3). No specific binding of NGF30-FITC to cell surfaces was detected in the absence of NGF, and there was no binding to TrkA− p75+ EL4 cells (Table IV, row 4). The same data were obtained in assays using mAb and mAb binding sequentially or using preformed NGF-NGF30 complexes as ligands.

The data suggest that mAb NGF30 blocks or hinders the p75 binding domain of NGF. The data further indicate that mAb NGF30 complexes bind to TrkA independently of p75 expression and that NGF docked onto TrkA can still be bound by mAb NGF30. In contrast, NGF docked onto p75 cannot be bound by mAb NGF30 or the mAb causes NGF to dissociate from p75. Thus, as to NGF binding there is a reciprocal block between mAb NGF30 and p75 but not between mAb NGF30 and TrkA.

This notion was strengthened by Scatchard analysis that demonstrated ablation of 125I-NGF binding to TrkA− p75+++ B104 cells in the presence of mAb NGF30 (Fig. 2). In independent assays 125I-NGF-p75 interactions exhibited a Kd ~25 nM, and ~35,000 NGF molecules/cell were detected. Complexes of 125I-NGF-NGF30 binding exhibited decreased binding, and ~2,000 NGF molecules/cell were detected. Interestingly, the affinity of these p75 receptors for NGF was Kd ~4 ps. Competition with a 1000-fold excess of unlabeled NGF ablated all 125I-NGF binding to B104 cells (Fig. 2). In contrast, Scatchard plot analysis of C10 cells (TrkA+++ p75++) revealed that mAb NGF30 does not hinder TrkA125I-NGF binding (data not shown).

The interactions of NGF-NGF30 with TrkA were further studied in ELISAs testing binding to immobilized extracellular domain of TrkA (TrkA-ECD) (Table V). NGF-NGF30 bound TrkA-ECD specifically (Table V, rows L–5). Only at very high molar ratios of mAb NGF30 to NGF (64:2) there was lower binding to TrkA-ECD (Table V, row 6), suggesting that some interference occurs at this ratio. The presence of immobilized TrkA-ECD on plates was controlled with anti-TrkA mAb 5C3 (Table V, row 11). Comparable data were obtained by adding NGF and mAb NGF30 sequentially (Table V) or by adding...
NGF::NGF30 complexes preformed in solution (data not shown).

In control assays mAb NGF30 did not bind TrkA-ECD in the absence of mouse NGF (Table V, row 10). mAb NGF30 did not bind TrkA-ECD in the presence of human NGF (Table V, row 7), as expected from earlier results (Table I). Human NGF bound TrkA-ECD and competed for the binding of NGF::NGF30 (Table V, row 8), but efficient competition occurred at 25-fold molar excess of human NGF. The affinity of NGF for TrkA-ECD is 2 nM (data not shown), and Ref. 33). The affinity of NGF:NGF30 for TrkA-ECD was estimated to be also 2 nM.

**Signal Transduction Studies—Early signaling events induced upon NGF binding include TrkA tyrosine phosphorylation (Tyr(P)), the Tyr(P) of adapter molecules Shc and SNT, and internalization of the ligand-receptor complex. To assess the mechanism by which the mAb splits the trophic and neurotrophic activities of NGF, TrkA-mediated signals induced by NGF versus NGF::NGF30 at 1:2 molar ratio were compared.**

**NGF::NGF30 Induces TrkA Tyrosine Phosphorylation with Altered Kinetics—TrkA Tyr(P) was studied on 4-3.6 cells after a time course of ligand stimulation, by TrkA immunoprecipitation and Western blotting with anti-Tyr(P) mAb 4G10.** NGF and NGF::NGF30 induced comparable TrkA Tyr(P) after 3 min (Fig. 3, lanes 1 and 2) or 15 min (Fig. 3, lanes 4 and 5). In contrast, after 45 min NGF-induced sustained TrkA Tyr(P) (Fig. 3, lane 8), whereas TrkA Tyr(P) induced by NGF::NGF30 was transient and almost disappeared (Fig. 3, lane 7).

No TrkA Tyr(P) was detected at any time in untreated control cells (Fig. 3, lanes 3, 6, and 9). Blots were subsequently stripped and reprobed with anti-TrkA antisera to ensure that equal amounts of p140 TrkA protein were present (data not shown). This control also demonstrated that the p110-phosphorylated band is indeed TrkA as reported previously (39).

**mAb NGF30 Prevents NGF-induced SNT Activation—**The Tyr(P) of SNT was assessed after a 5-min ligand stimulation of PC12 cells. Studies were done by affinity isolation of SNT with Suc-1-agarose beads and Western blotting with anti-Tyr(P) mAb 4G10. Treatment with NGF resulted in the expected SNT Tyr(P) (Fig. 4, lane 1) compared with untreated control cells (Fig. 4, lane 3). These results are consistent with absence of differentiation when cultured with NGF::NGF30 (Fig. 1).

**mAb NGF30 Reduces but Does Not Abolish the Tyr(P) of Shc—**The Tyr(P) of Shc was assessed after a 12-min ligand stimulation of PC12 cells (Fig. 4B). Studies were done by immunoprecipitation with anti-Shc antibodies and Western blotting with anti-Tyr(P) mAb 4G10. Treatment with NGF resulted in the expected SNT Tyr(P) (Fig. 4A, lane 2). Treatment with NGF::NGF30 did not induce SNT Tyr(P) (Fig. 4A, lane 3) compared with untreated control cells (Fig. 4A, lane 1). These results are consistent with trophic survival of cells cultured with NGF or NGF::NGF30 in SFM (Table III) and suggest that only partial Shc activation is required for full trophic support.

The bands indicated with dashed arrows (Fig. 4B) are proteins co-precipitated with Shc which are tyrosine-phosphorylated specifically in response to ligand treatment. Based on their molecular mass the proteins may be TrkA (140 kDa), P13-kinase (85 kDa), and either TrkA or P13-kinase (110 kDa). We have not characterized these proteins.

**Kinetics of Ligand-dependent Activation of MAPK—**The kinetics of MAPK activation were gauged after a time course of treatment of 4-3.6 cells with NGF or NGF::NGF30 (Fig. 5).
Analysis was done by immunoprecipitation of MAPK and study of its activity in \textit{in vitro} kinase assays using myelin basic protein (MBP) as an exogenous substrate.

The kinetics and efficacy of MAPK activation by NGF or NGF-NGF30 were different. For both ligands the $t_{1/2}$ of MAPK activation was $\approx 3$ min, and optimal MAPK activation was induced by 15 min. However, NGF-NGF30 activated only $\approx 80\%$ of MAPK compared with NGF. After 45 min NGF signals sustained 100\% MAPK activation, whereas NGF-NGF30 signals caused transient MAPK activation which decreased to $\approx 30\%$.

\textit{mAb NGF30 Enhances Internalization of NGF and TrkA}—We hypothesized that changes in activation kinetics might be due to differential internalization. Thus, ligand internalization (Fig. 6A) and NGF receptor internalization (Fig. 6B) were tested in two distinct assays.

First, internalization of $^{125}$I-NGF was assayed by counting ligand that become resistant to acid wash. The internalization rate of $^{125}$I-NGF was increased by mAb NGF30 (Fig. 6A). The $t_{1/2}$ for NGF internalization was $\approx 3$ min, and the $t_{1/2}$ for internalization of untreated NGF was $\approx 6$ min. Monovalent mAb NGF30 Fab or control rat IgG did not affect ligand internalization rates. In addition, an increase in the absolute number of $^{125}$I-NGF internalized was induced by mAb NGF30. After 20 min $\approx 12,000$ cpm of $^{125}$I-NGF were internalized versus $\approx 30,000$ cpm of $^{125}$I-NGF-NGF30 complexes.

Second, the density of receptors on the cell surface were measured by a quantitative FACScan assay after ligand treatment of 4-3.6 cells. Ligand-induced loss of cell-surface receptors is interpreted as receptor internalization (Fig. 6B). NGF induced the internalization of TrkA receptors with a $t_{1/2} \approx 8$ min. A complex of NGF-NGF30 induced faster TrkA internalization, with a $t_{1/2} \approx 4$ min. Furthermore, NGF induced the internaliza-
tion of only ~20% of the total cell-surface TrkA receptors, whereas NGF-NGF30 doublet doubled receptor internalization to ~41% of the surface TrkA. The p75 receptors did not internalize substantially in response to either ligand (Fig. 6B).

**DISCUSSION**

We report on the first split of neurotrophic and neuritogenic activities of NGF. The split is caused by association of NGF to bivalent anti-NGF mAb NGF30. In contrast, there are no detectable differences in the biological function of NGF and NGF-monovalent NGF30 Fab complexes; both ligands induce trophic and neuritogenic effects.

To analyze the mechanisms leading to ligand-induced bioactivity, we compared receptor binding, internalization, and signal transduction (see Table VI for summary). There are three factors that could account for the different bioactivity of NGF-NGF30 as follows: (i) altered receptor binding, (ii) conformational effects of bivalent mAb NGF30 upon NGF, and (iii) altered ligand internalization.

Arguably, NGF-NGF30 complexes do not necessarily remain bound in solution, and low amounts of NGF free of mAb NGF30 may be found. However, a differential trophic and neuritogenic response to low concentrations of NGF has not been observed (data not shown, also see Ref. 13).

**Receptor Binding Studies—**NGF, NGF-NGF30, and NGF-NGF30 Fab complexes bind to TrkA receptors selectively and with comparable affinity. In contrast, while NGF binds p75 with low affinity ($K_d \approx 10^{-9}$ M), neither NGF-NGF30 nor NGF-monovalent NGF30 Fabs bind p75 efficiently.

Since mAb NGF30 and NGF30 Fabs do not block NGF-TrkA interactions, the data suggest that the C-terminal region of NGF may not be required for binding and activating TrkA. This notion is consistent with other studies (16, 36, 40–44). However, our conclusion is partly at odds with reports suggesting that the C terminus of NGF is critical for TrkA binding (41, 45) because trophic support is unaffected by mAb NGF30, but our conclusion is partly consistent with those reports inasmuch as neuritogenesis is ablated by mAb NGF30.

Conversely, since mAb NGF30 and NGF30 Fabs block NGF-p75 interactions, the data indicate that the C terminus of NGF (or adjacent domains) are critical for p75 binding. This is consistent with previous observations (16, 46, 47). Also, important NGF-p75 interactions are mediated by NGF amino acids 30–35 within the $\alpha$-helix $\beta$-turn (39–41); and these residues in one NGF protomer seem to pack closely with the C terminus of the other protomer within the NGF dimer (42, 43).

**Conformational Effects of mAb NGF30 upon NGF and TrkA—**One mechanism by which bivalent mAb NGF30 may affect NGF biology differently than monovalent NGF30 Fabs may be related to the packing or the tertiary structure of NGF.

Predicted distances for the NGF dimer would allow bivalent mAb NGF30 to bind both C termini simultaneously (1 bivalent mAb binding 1 NGF dimer). Indeed, our data strongly suggest that mAb NGF30 binds both NGF protomers.

NGF is a “flexible” molecule, with some unstructured domains that do not resolve crystallographically (42). Bivalent mAb NGF30 could “freeze” the C termini of NGF in a restricted conformation, but monovalent NGF30 Fabs lack the hinge region and do not freeze NGF. We hypothesize that flexible NGF, flexible NGF-NGF30 Fabs, and “frozen” NGF-NGF30 bind TrkA in different ways that result in different biological outcomes. It is postulated that there are two key regions for ligand-binding sites on TrkA, TrkA “hot spots” (48, 49); arguably frozen NGF-NGF30 and flexible NGF could interact differently with each receptor hot spot. Thus, NGF trophic and neuritogenic signals may be intrinsic to the flexible structure of the ligand (50).

Our results are analogous to studies using TrkA receptor mutants activated by wild type NGF. TrkA mutants of the juxtamembrane extracellular domain (21, 51, 52), the juxtamembrane intracellular domain (21, 52), or intracellular tyrosines (e.g. Y785F/Y490F) (17, 19) result in receptors that do not mediate neuritogenesis. However, our study is unique in that our results were obtained with wild type TrkA receptors.

**Does Altered p75 Binding Account for NGFNGF30 Biology?—**Our data and published literature argue against the notion that altered NGF-p75 interactions can cause a split in the trophic and neuritogenic functions of NGF. First, monovalent NGF30 Fabs inhibit NGF-p75 interactions without affecting neuritogenesis. Second, trophic and neuritogenic signals are mediated via TrkA exclusively (14–17). Third, NGF mutants that do not bind p75 do induce differentiation (40, 44).

Thus, p75 blocking does not *per se* explain changes to NGF function. However, it is possible that lack of NGF-p75 interactions result in faster internalization events because p75 hinders ligand internalization (18). Consequently, blocking NGF-p75 interactions could indirectly cause changes to NGF function (see below).

One unexpected and interesting observation is that when the low affinity NGF-p75 interactions are ablated with mAb NGF30, a small number of remaining high affinity p75-binding sites ($K_d \approx 10^{-12}$ M) are unmasked. These putative high affinity p75-binding sites for NGF may be allosteric (53) or may be analogous to high affinity p75-binding sites for NT-3 (7). However, their significance is unclear at this time.

**Signal Transduction—**NGF affords sustained activities that can lead to growth or to differentiation, whereas EGF affords transient activities that lead only to growth (23). NGF and NGF-monovalent NGF30 Fabs induce neuritogenesis by sustained activation of MAPK and SNT phosphorylation (20–22, 26), but NGF-NGF30 did not afford these signals.

NGF-NGF30 induces more transient TrkA phosphorylation, lower Snh phosphorylation, and more transient MAPK activation. Partial Snh/MAPK activation by NGF can induce full trophic signals, but it is not sufficient for neuritogenic signals. The data suggest that neuritogenic NGF signals are more sensitive to Snh/MAPK than TrkA trophic signals or that neuritogenic signals can not be compensated by phospholipase C-δ, PI3-kinase, or ERKs (17–24, 26, 27). Hence, the differences in NGF and NGF-NGF30 signal transduction via TrkA may be analogous to NT-4 and brain-derived neurotrophic factor signal transduction via TrkB, where NT-4 is more dependent on Snh (54).

**Ligand Internalization—**Faster and more extensive internalization of TrkA and of NGF-NGF30 were detected, and it is possible that internalization rates may account for the split of trophic and neuritogenic functions. Receptor internalization is an essential step for growth factor function (23, 28) and can either down-regulate signaling or bring the activated receptor in contact with specific substrates (27–30).

One explanation for faster internalization is that NGF-NGF30...
NGFmAb Complex with Altered Binding and Signaling does not interact with p75. However, our data argue against this possibility because NGF-monovalent NGF30 Faba does not interact with p75 either, yet they afford internalization rates comparable to NGF.

Three lines of evidence suggest that rapidly internalizing ligands may be trophic, whereas slowly internalizing ligands may be neuritogenic. First, ligand-bound EGFR is more rapidly internalized than TrkA and only leads to trophic growth (27); but a slowly internalizing EGFR mutant causes differentiation in PC12 cells (55). Second, regulation of protein phosphorylation with staurosporine or K252a affords sustained EGF-in-
ternalized than TrkA and only leads to trophic growth (27); may be neuritogenic. First, ligand-bound EGFR is more rapidly internalization with staurosporine or K252a affords sustained EGF-induced phosphorylation of EGFR and neuritogenic differentiation in PC12 cells (23, 56). Third, defects in NGF-TrkA transport or internalization are thought to result in neurite retraction and neurodegeneration (57).

Our data are consistent with the evidence above and suggest that rapidly internalizing TrkA ligands are trophic, whereas slowly internalizing TrkA ligands are neuritogenic. Hence, it seems that NGF-NGF30 activates TrkA signal transduction more like the manner in which EGF activates signal transduction via EGF receptors.

It may be desirable to develop novel fast-internalizing TrkA ligands or to use biological modifiers of NGF such as mAb NGF30. These agents could be useful in neuropathies to maintain neurites without inducing de novo sprouting which could be non-functional or pain-causing.

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