Optimal Nerve Growth Factor Trophic Signals Mediated by Synergy of TrkA and p75 Receptor-Specific Ligands

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Nerve growth factor (NGF) receptor-mediated signaling was studied using specific monoclonal antibodies (mAbs) as ligands that discriminate between the receptors TrkA and p75. mAb-induced trophic signals were compared with the signals of the natural ligand NGF. In cells expressing TrkA but no p75 receptors (TrkA⁺ p75⁻), binding of TrkA with mAb 5C3 leads to optimal signals. In cells expressing both TrkA and p75 (TrkA⁺ p75⁺), binding of TrkA with mAb 5C3 leads to significant but suboptimal signals, and optimal trophic signals are obtained by concomitant binding of TrkA and p75 with mAbs 5C3 and MC192. In TrkA⁺ p75⁺ cells, binding of anti-p75 mAb MC192

also enhances the trophic effect of suboptimal concentrations of NGF. In contrast, in cells expressing p75 receptors singly (TrkA⁻ p75⁺), binding with mAb MC192 or NGF causes very limited or no trophic effects. Thus, the data support the hypothesis that unbound p75 may modulate TrkA trophic signals. Importantly, the data also demonstrate for the first time that in multireceptor systems appropriate combinations of anti-receptor mAbs can fully mimic the signals of a polypeptide growth factor.

Key words: NGF; receptor; TrkA; p75; trophic signals; agonist; ligand; mAb

Nerve growth factor (NGF) is a 26 kDa dimeric polypeptide that binds two receptors characterized on the basis of their binding affinity. One NGF receptor is a 140 kDa protein (p140 TrkA) with intrinsic tyrosine kinase enzymatic activity. NGF binds TrkA with intermediate affinity (K_d 10^{-10} – 10^{-11} M) (Hempstead et al., 1991; Kaplan et al., 1991; Klein et al., 1991). Another receptor is a 75 kDa protein (p75) that is bound by NGF and other neurotrophins such as BDNF with lower affinity (K_d $\sim 10^{-9}$ M) (Benedetti et al., 1993).

Coexpression of TrkA and p75 on the cell surface leads to the formation of a limited number of high-affinity NGF binding sites (K_d $\sim 10^{-12}$ M), which are presumably composed of p75-TrkA heteromers (Hempstead et al., 1991; Mahadeo et al., 1994); however, biochemical detection of p75 and TrkA heteromers has not been conclusive.

Although expression of TrkA alone is sufficient for cellular responses (Nebreda et al., 1991; Rovelli et al., 1993), p75 can regulate TrkA-ligand interactions and signal transduction (Hempstead et al., 1989; Verdi et al., 1994; Dobrowsky et al., 1995). Moreover, p75 activates its own signaling pathway (for review, see Chao, 1994; also see Canossa et al., 1996; Carter et al., 1996; Cortazzo et al., 1996). It has been suggested that in certain systems ligand-bound p75 receptors may activate apoptotic sig-

nals, whereas in other systems unbound p75 receptors activate apoptosis.

One problem in elucidating the molecular structure of the functional NGF receptor and in determining the individual role of each receptor and a putative cross-modulation between TrkA and p75 has been the difficulty in obtaining high-affinity ligands that discriminate completely between the receptors. Mutant neurotrophins that bind Trk receptors preferentially over p75 function like wild-type neurotrophins in biological assays (Ibáñez et al., 1992; Barker and Shooter, 1994; Ryden et al., 1995); however, NGF seems to dock onto multiple sites of TrkA, [the IgG-like domain (Perez et al., 1995) and/or the leucine zipper domain (Windisch et al., 1995)]. Ligand binding to multiple TrkA sites may cause signaling and may lead to p75 immobilization and p75-independent signals (Wolf et al., 1995; Ross et al., 1996). This would be consistent with the agonistic effect of anti-TrkA polyclonal antisera, which has multiple binding sites (Clary et al., 1994).

We have previously described a monoclonal antibody (mAb) 5C3 that binds a restricted epitope of TrkA with high affinity and acts as a full agonist (when compared with NGF) on cells that express TrkA but do not express p75 (LeSauter et al., 1996). In the present study, combinations of the TrkA-specific mAb 5C3 and the p75-specific mAb MC192 (Chandler et al., 1984) were used as ligands to analyze NGF receptor in functional and biochemical assays. These mAbs maintain high binding affinity regardless of expression of co-receptors.

The data support the hypothesis that NGF-trophic signals are mediated by TrkA and that unbound p75 negatively modulates TrkA trophic function. More importantly, the data show that optimal agonistic ligand mimicry for a multireceptor complex can be achieved by a combination of the natural ligand and an anti-receptor antibody, or by a combination of two antibodies against different receptors. This information will be useful in the design of artificial agonists in multireceptor systems, including neurotrophin receptors.

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MATERIALS AND METHODS

Cell cultures. Rat PC12 pheochromocytomas cells express p75 and TrkA; B104 rat neuroblastoma cells express ~50,000 surface p75 receptors/cell and none of the Trks (TrkA[−] p75⁺); 4-3.6 cells are B104 cells transfected with human trkA cDNA and express equal levels of surface p75 and TrkA (TrkA⁺ p75⁺) (Bogenmann et al., 1995). The C10 cell line is a selected subclone of 4-3.6 expressing ~50,000 surface TrkA receptors but no detectable surface p75 (TrkA⁺ p75[−]). Lack of detectable surface p75 receptors on C10 clones was assessed by FACS analysis (with a sensitivity of <500 receptors/cell). All cell lines were maintained in RPMI media (Life Technologies, Toronto, Ontario) supplemented with 5% fetal bovine serum and antibiotics. Appropriate drug selection was added to 4-3.6 and C10 cells.

Antibodies as NGF receptor ligands. Anti-rat p75 mAb MC192 (IgG1) (Chandler et al., 1984) and anti-human TrkA mAb 5C3 (IgG1) (LeSauter et al., 1996) ascites were purified with Protein G Sepharose (Pharmacia, Baie d'Urfe, Québec), dialyzed against PBS, and stored at −20°C. mAb 5C3 is agonistic and can fully substitute for NGF in E25 cells expressing TrkA but not p75 (LeSauter et al., 1996). Further characterization of mAb 5C3 is published in LeSauter et al. (1996). Purified mAbs were characterized by SDS-PAGE under nonreducing or reducing (100 mM 2-mercaptoethanol) conditions to >95% purity (data not shown).

Binding assays with directly labeled mAbs 5C3 and MC192 demonstrated that each antibody binds to its receptor with relative affinity and saturation profiles regardless of whether the other receptor is expressed and bound. For example, mAb 5C3 binds similarly to TrkA⁺ p75[−] cells or TrkA⁺ p75⁺ cells regardless of whether mAb MC192 is present (data not shown). This is not unusual or unexpected and has been reported for other antibodies binding different subunits of multireceptor systems (Chastagner et al., 1996; Pinkas-Kramarski et al., 1996).

Protection from cell death. Five thousand cells/well in protein-free media (PFHM-II, Life Technologies) containing 0.1% BSA (crystalline fraction V, Sigma, St. Louis, MO) were added to 96-well plates (Falcon, Mississauga, Ontario, Canada). The cultures were untreated or supplemented with serial dilutions of neurotrophins (positive control), test mAbs, or mouse IgG (negative control). The survival profile of the cells was quantitated using the MTT colorimetric assay (Mosmann, 1983) after 48–72 hr. Percentage protection was standardized relative to 1 nM NGF concentrations using the MTT optical density (OD 590 nm) and the following formula: $[(OD_{\text{test}} - OD_{\text{untreated}}) / (OD_{1 \text{ nM NGF}} - OD_{\text{untreated}})] \times 100$. The OD of untreated samples [serum-free medium (SFM) only] was ~10% of 1 nM NGF control.

Some survival experiments were also performed in the presence of various concentrations of the tyrosine kinase inhibitor K252a (kindly provided by Dr. WenHua Zheng, McGill University). The concentrations of K252a used were reported previously (Dobrowsky et al., 1995; Buck and Winter, 1996).

DNA fragmentation and apoptosis. Apoptotic death was confirmed by analysis of DNA fragmentation patterns by extraction of genomic DNA as described (Sambrook et al., 1989). Equal amounts of DNA for each condition were resolved in a 1.5% agarose gel and visualized with ethidium bromide. Note that DNA isolated from apoptotic PC12 cells often does not appear as a typical apoptotic ladder (Xia et al., 1995; Barrett and Georgiou, 1996).

Tyrosine phosphorylation assays. The tyrosine phosphorylation of TrkA was assayed after a 15 min treatment of 4-3.6 cells with the indicated agent(s). Analysis was performed by Western Blot of whole cell lysates with the enhanced chemoluminescence detection system (ECL, Amersham, Oakville, Ontario) as described (LeSauter et al., 1996), using anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY) or affinity-purified polyclonal antisera DF-49 recognizing phosphotyrosine PY490 of TrkA, which forms the Shc recognition/docking site on TrkA (Segal et al., 1996). Quantitation of protein loading was performed with the Bio-Rad Detergent Compatible Protein Assay reagent (Bio-Rad Laboratories, Mississauga, Ontario, Canada), and by Coomassie blue staining of gels. Bands in x-ray films were quantified by densitometry [Scanmaster3+ scanner (Howtec Inc.) and MSCAN software (Scanalytic, CSP Inc., Hudson, NH)]. Band intensities were standardized using the relative OD of NGF treatment in each film as 100%. Statistical analysis of densitometry of three to five gels was performed using paired Student's *t* tests.

Table 1. p75 binding does not protect from apoptotic death in SFM

	Treatment in SFM cultures	PC12 (TrkA ⁺ p75 ⁺)	B104 (TrkA [−] p75 ⁺)	4-3.6 (TrkA ⁺ p75 ⁺)
1	Mouse IgG	0 ± 2.3	0.5 ± 0.7	0 ± 2.2
2	1 nM NGF	100 ± 5.1	−3 ± 2.2	100 ± 4.6
3	100 pM NGF	80 ± 5.5	1 ± 1.9	87.4 ± 4.5
4	10 pM NGF	40 ± 3.4	2 ± 1.7	52.3 ± 4.7
5	1 pM NGF	12 ± 1.1	−0.5 ± 1.2	10.1 ± 5.5
6	2 nM BDNF	Not tested	1 ± 1.3	2.1 ± 1.7
7	200 pM BDNF	Not tested	2.5 ± 1.3	0.9 ± 1.2
8	20 pM BDNF	Not tested	1.6 ± 1.3	3.4 ± 2.2
9	2 pM BDNF	Not tested	0 ± 0.7	0.5 ± 3.7
10	MC192 10 nM	2 ± 1.2	0.7 ± 0.8	1.2 ± 2.3
11	MC192 1 nM	−1 ± 3.4	0.6 ± 2.3	3.7 ± 3.1
12	5% serum	157 ± 0.9	100 ± 7.7	148 ± 7.2

PC12, B104, and 4-3.6 cells were cultured in serum-free media (SFM) supplemented with test or control ligands as indicated. Cell protection was quantitated after 48 hr by measuring OD using the MTT colorimetric assay. Data were standardized relative to optimal NGF treatment (PC12 and 4-3.6 cells). B104 cells do not respond to NGF, thus in this assay they were standardized with respect to 5% serum. A representative experiment is shown (average ± SD; *n* = 4) from more than three independent experiments.

RESULTS

Functional consequences of NGF receptor binding

Cells undergo apoptotic death when cultured in SFM (Table 1). B104 cells expressing p75 but not TrkA were not protected by p75 ligands [neurotrophins NGF and BDNF (lanes 2–9) or by various concentrations of anti-p75 mAb MC192 (lanes 10 and 11)]. Lack of significant p75 ligand-induced protection in SFM was independent of TrkA expression, and apoptotic death occurred in p75⁺ TrkA⁺ PC12 cells (Table 1, lanes 10 and 11) and in p75⁺ TrkA⁺ 4-3.6 cells (Table 1, lanes 6–11). In contrast, NGF binding to TrkA protected cells from apoptotic death in SFM (Table 1, lanes 2–5). NGF-mediated protection of PC12 and 4-3.6 cells was dose dependent and consistently suboptimal at ~1–10 pM (Table 1, lanes 4 and 5). Standard cell culture conditions containing 5% serum (Table 1, lane 12) afford both proliferation and survival. Therefore, higher readings are detected when compared with 1 nM NGF, which in SFM preferentially acts as a survival factor.

Next, cells expressing p75 and human or rat TrkA receptors were used to test potential synergy of mAb MC192 as a p75 ligand and suboptimal NGF doses (5 pM) as a preferential high-affinity ligand. MAb MC192 alone affords very limited (or insignificant) protection in SFM (Table 1; Table 2, lanes 4–6); 5 pM NGF alone affords suboptimal cell protection ranging from ~30 to 50% (Table 1; Table 2, lane 3).

NGF (5 pM) + mAb MC192 synergized to significantly increase cell protection in SFM (Table 2, lanes 7–9). This protection was dependent on the concentration of mAb MC192 and was maximal at 0.2 μg/ml (1 nM) (Table 2, lane 8). MAb MC192 concentrations ranging from 0.1 nM to 1 μM were tested, but only some concentrations are shown for clarity. At 2 μg/ml (10 nM) or higher concentrations, mAb MC192 afforded limited synergy (Table 2, lane 7), and at 0.02 μg/ml (0.1 nM) or lower concentrations it did not synergize with NGF (Table 2, lane 9). Thus, a bell-shaped dose–response resulted wherein low or high concentrations of mAb do not afford synergy with 5 pM NGF.

Similar tests were performed with 4-3.6 cells (human TrkA⁺ p75⁺) and C10 cells (a sorted subclone of 4-3.6 cells that expresses human TrkA but is p75[−]). 4-3.6 and C10 clones express a

Table 2. Concomitant p75 and TrkA binding protects cells from apoptotic death

	Treatment added to SFM cultures	PC12 (TrkA ⁺ p75 ⁺)	4-3.6 (TrkA ⁺ p75 ⁺)
1	Mouse IgG	0 ± 1.3	0 ± 1.5
2	1 nM NGF	100 ± 6.5	100 ± 4.8
3	5 pM NGF	28 ± 8.4	48 ± 3.5
4	MC192 10 nM	2 ± 1.5	1 ± 4.1
5	MC192 1 nM	6 ± 2.7 ^a	6 ± 3.5 ^a
6	MC192 0.1 nM	1 ± 2.0	1 ± 4.2
7	5 pM NGF + MC192 10 nM	49 ± 3.3	85 ± 6.5
8	5 pM NGF + MC192 1.0 nM	86 ± 7.4	108 ± 5.6
9	5 pM NGF + MC192 0.1 nM	26 ± 5.1	55 ± 4.5

Assays were performed as described in Table 1 legend. mAb MC192 synergizes with suboptimal (5 pM) NGF in protecting PC12 and 4-3.6 cells from apoptotic death in SFM (lanes 7 and 8).

^a The small increase in survival induced by mAb MC192 is statistically significant.

similar number of surface human TrkA receptors. In these cells it is possible to replace NGF with mAb 5C3 as a test ligand for human TrkA (Table 3).

Combinations of mAbs 5C3 and MC192 afforded optimal 4-3.6 cell protection (Table 3, lanes 10–13), which is comparable with that afforded by optimal NGF (Table 3, lane 2). Synergy by combination of mAbs 5C3 and MC192 is demonstrated by significantly higher protection than treatment with either mAb alone (Table 3, lanes 6–9). Interestingly, although binding of TrkA with mAb 5C3 alone affords only ~20–40% protection to 4-3.6 cells, similar treatment of C10 cells affords 65–80% protection in SFM (Table 3, lanes 8 and 9). MAb 5C3 concentrations ranging from 0.01 to 5 µg/ml (0.05–250 nM) were tested, but only some concentrations are shown for clarity.

Consistent with C10 cells lacking surface p75, the combination of mAbs MC192 and 5C3 does not enhance the effect of mAb 5C3 alone (Table 3, lanes 10–13). As expected, C10 cells are less responsive to low doses of NGF than 4-3.6 cells (Table 3, lanes 3–5) because they lack detectable p75. Furthermore, no synergy was observed in C10 cells when mAb MC192 and 5 pM NGF were tested in combination (data not shown).

To assess whether trophic signals leading to cell survival in SFM were mediated via a tyrosine kinase activity, the K252a inhibitor was used (Table 4). As expected, K252a inhibited trophic survival induced by 1 nM NGF. K252a also inhibited trophic survival induced by optimal concentrations of mAb 5C3 or by optimal combinations of mAbs 5C3 + MC192. Inhibition by K252a was dose dependent. The highest concentration of K252a tested (500 nM) was not toxic to 4-3.6 cells (data not shown), and this dose has been used previously (Dobrowsky et al., 1995; Buck and Winter, 1996).

Analysis of the degradation pattern of genomic DNA confirmed the apoptotic nature of cell death in SFM for 4-3.6 and PC12 cells (Fig. 1) and for B-104 cells (data not shown). The absence or presence of DNA degradation correlated conclusively with protection or lack of protection from death for all treatments and for all cell lines (Tables 1–3).

In 4-3.6 cells, no DNA degradation is seen after culture with 5% serum or with mAbs 5C3 + MC192, although a small amount of DNA degradation is seen for 4-3.6 cells treated with mAb 5C3 (Fig. 1A). In contrast, extensive apoptotic DNA degradation is seen when 4-3.6 cells are cultured with SFM or mAb MC192 alone (Fig. 1A).

Table 3. Concomitant ligand binding of p75 and TrkA synergizes in trophic signals

	Treatment added to SFM cultures	4-3.6 (TrkA ⁺ p75 ⁺)	C10 (TrkA ⁺ p75 [−])
1	Mouse IgG	0 ± 3.4	0 ± 1.7
2	1 nM NGF	100 ± 5.3	100 ± 3.7
3	100 pM NGF	89 ± 6.6	35 ± 3.1
4	10 pM NGF	52 ± 3.6	7 ± 3.1
5	1 pM NGF	4 ± 4.4	0 ± 2.4
6	5 nM MC192	16 ± 5.7 ^a	1 ± 1.2
7	0.5 nM MC192	8 ± 4.0	0 ± 1.8
8	5 nM 5C3	42 ± 3.0	79 ± 5.2
9	0.5 nM 5C3	20 ± 5.5	64 ± 5.3
10	5 nM 5C3 + 5 nM MC192	78 ± 2.7	73 ± 3.8 ^c
11	5 nM 5C3 + 0.5 nM MC192	118 ± 3.1 ^b	59 ± 4.9 ^c
12	0.5 nM 5C3 + 5 nM MC192	65 ± 6.8	62 ± 1.6 ^c
13	0.5 nM 5C3 + 0.5 nM MC192	96 ± 2.3	64 ± 1.4 ^c

Assays were performed as described in Table 1 legend. Binding of p75 and TrkA with mAbs MC192 and 5C3, respectively (lanes 9–12), synergize in protecting 4-3.6 cells from apoptotic death, whereas binding of TrkA with mAb 5C3 alone (lanes 8 and 9) affords suboptimal protection. In contrast, C10 cells are better protected by binding TrkA with mAb 5C3 alone (lanes 8 and 9).

^a The small increase in survival induced by mAb MC192 is statistically significant.

^b The survival higher than 100% is statistically significant from 1 nM NGF.

^c Not statistically significant from each other.

In PC12 cells, no DNA degradation is seen after culture with 5% serum or with 5 pM NGF + 10 nM mAb MC192. PC12 cells treated with 5 pM NGF alone do have limited DNA degradation (Fig. 1B), as expected, because this concentration of NGF affords suboptimal survival. PC12 cells cultured with SFM or mAb MC192 alone show extensive DNA degradation (Fig. 1B).

TrkA tyrosine phosphorylation

To further analyze the signaling mechanism of the antibody-based ligand combinations, TrkA tyrosine phosphorylation (PY) was studied. This was performed by Western blot analysis of whole cell lysates with antibodies against phosphotyrosine (α-PY) or with antibodies that bind phosphotyrosinylated TrkA within the Shc recognition/docking site [phosphotyrosine 490 of TrkA (α-PY490, DF-49 antibody)].

Initial experiments were designed to resolve the concentration of mAb 5C3 that affords optimal PY of TrkA (Table 5). A 15 min treatment with mAb 5C3 at 1 µg/ml (5 nM) induced optimal TrkA PY and TrkA PY490 in C10 (TrkA⁺ p75[−]) and 4-3.6 cells (TrkA⁺ p75⁺). This was consistent with previous survival data

Table 4. K252a inhibits NGF receptor-mediated trophic signals

K252a (nM)	% Cell survival in SFM supplemented with		
	NGF	5C3	5C3 + 192
0	100 ± 9	50 ± 3	112 ± 4
50	60 ± 4	32 ± 3	67 ± 5
500	32 ± 4	13 ± 2	43 ± 2

Assays were performed as described in Table 1 legend. 4-3.6 cell survival in SFM was achieved by incubation with the indicated ligands. Optimal ligand concentrations were used as per Table 3 (1 nM NGF, 5 nM 5C3 mAb, and 5 nM 5C3 + 0.5 nM MC192 mAbs). Cells were challenged with various concentrations of K252a, and % survival was calculated using 1 nM NGF as 100% standard. K252a inhibits both NGF and mAb-mediated survival in a dose-dependent manner and to a similar relative degree.

(A) 4-3.6 cells **(B) PC12 cells**

Figure 1. Changes in apoptotic DNA degradation. Genomic DNA was extracted from (A) 4-3.6 or (B) PC12 cells cultured as indicated for 48 hr in SFM. Equal amounts from each sample were resolved on a 1.5% agarose gel and visualized with ethidium bromide. Standard molecular markers (*M*) are shown. A typical apoptotic DNA ladder is seen for 4-3.6 cells, but PC12 DNA is more smeared and difficult to isolate as a ladder (Xia et al., 1995; Barrett and Georgiou, 1996). Antibody concentrations were selected from optimal survival assays (e.g., Table 3), namely 5 nM mAb 5C3 and 0.5 nM mAb MC192. NGF (5 pM) was suboptimal in survival assays, and some DNA laddering is expected (B). DNA laddering is ablated when NGF is combined with 0.5 nM mAb MC192.

(e.g., Table 3); however, 5 nM mAb 5C3 was less efficient at phosphorylating TrkA when compared with 1 nM NGF (Table 5, lane 5). This result is also consistent with previous survival data.

As expected, TrkA phosphorylation in response to low NGF concentrations (Table 5, lanes 2–4) was decreased in C10 cells compared with 4-3.6 cells, because C10 cells do not express p75 receptors. In contrast, TrkA phosphorylation in response to mAb 5C3 was always stronger in C10 cells compared with 4-3.6 cells (Table 5, lane 8).

Using the optimal NGF and mAb 5C3 concentrations above, we studied TrkA PY after treatment of cells with various combinations of the ligands (Fig. 2). A 15 min treatment of 4-3.6 cells (TrkA⁺ p75⁺) with both 5C3 and MC192 mAbs (Fig. 2*A,B*, lane 5) induced TrkA PY comparable with that induced by optimal NGF doses (Fig. 2*A,B*, lane 2). mAb 5C3 alone (Fig. 2*A,B*, lane 3) caused significant changes in TrkA PY; however, mAb 5C3-induced TrkA PY is lower than that induced by NGF or by combinations of mAbs 5C3 and MC192. Treatment with mAb MC192 alone did not cause significant changes in TrkA PY.

Other cellular proteins of sizes ranging from 40 to 125 kDa are also tyrosine-phosphorylated in response to these ligands. Interestingly, the effect on these unidentified substrates is ligand specific. For example, NGF, mAb 5C3, or 5C3 + MC192 (but not MC192 alone) causes the PY of a ~120 kDa phosphoprotein (Fig. 2*A*, *thick dashed arrow*), whereas only NGF or mAb 5C3 causes the PY of a ~110 kDa phosphoprotein (Fig. 2*A*, *short thin arrow*). All treatments cause the PY of a ~40 kDa phosphoprotein (Fig.

Table 5. TrkA tyrosine phosphorylation in response to mAb 5C3

		C10 Cells		4-3.6 Cells	
	Cells	PY total	PY490	PY total	PY490
1	No ligand	11	1	4	1
2	NGF 1 pM	12	1	7	5
3	NGF 10 pM	12	1	44	33
4	NGF 100 pM	36	45	93	61
5	NGF 1 nM	100	100	100	100
6	5C3 0.05 nM	10	1	5	1
7	5C3 0.5 nM	40	40	32	21
8	5C3 5 nM	91	71	45	43
9	5C3 50 nM	35	49	39	21

Cells were untreated (lane 1) or treated with the indicated concentrations of NGF (lanes 2–5) or mAb 5C3 (lanes 6–9), for 15 min at 37°C. Ligand concentrations were selected on the basis of survival assays (e.g., Table 3). Equal amounts of protein from whole cell lysates were resolved by SDS-PAGE and analyzed by Western blotting with antiphosphotyrosine (anti-PY) or with α -PY490 blot (DF-49 sera) recognizing specifically the Shc binding site of TrkA. Band intensities were analyzed by densitometry and standardized using the relative optical density of 1 nM NGF treatment as 100%. Data from a representative Western blot are shown.

2*A*, *thin dashed arrow*). With the exception of the ~40 kDa phosphoprotein, mAb MC192 alone did not cause significant and reproducible increases in PY of other proteins within the 15 min treatment (Fig. 2*A*, lane 4). More importantly, mAb MC192 did not affect TrkA PY in a significant and reproducible manner (Fig. 2*A,B*, lane 4; see statistical analysis in C).

Densitometry of the TrkA band of five anti-PY blots as in Figure 2*A* revealed a significant increase in total PY induced by a combination of mAbs 5C3 and MC192 (91% of that induced by optimal NGF) (Fig. 2*C*). The total PY increase induced by treatment with mAb 5C3 alone (56% of that induced by optimal NGF) is significantly higher than untreated control ($p = 0.029$), and it is also significantly different from total PY increases induced by mAb combinations ($p = 0.022$).

Densitometry of the TrkA band of five α -PY490 blots as in Figure 2*B* (DF-49 antibody) revealed an increase after treatment with mAb 5C3 (24% of that induced by optimal NGF), which was significant compared with untreated controls ($p = 0.016$) (Fig. 2*C*). Treatment with mAbs 5C3 + MC192 also increased PY490 (66% of that induced by optimal NGF). The PY490 increases seen after treatment with mAb 5C3 or mAbs 5C3 + MC192 are significantly different from each other ($p = 0.008$). Treatment with mAb MC192 alone did not cause a significant increase in TrkA PY490.

DISCUSSION

Binding of TrkA [with various concentrations of NGF (in PC12 and 4-3.6 cells) or with anti-human TrkA mAb 5C3 (in 4-3.6 cells)] leads to significant trophic signals, as assessed by cell protection in SFM, by increased receptor PY, and by reduced apoptosis and DNA degradation. The signals leading to cell survival in SFM are mediated by a K252a inhibitable tyrosine kinase activity, likely TrkA.

Concomitant binding of TrkA (with the ligands above) and of p75 (with mAb MC192) increase trophic signals synergistically, to levels equivalent to optimal NGF concentrations. When mAbs 5C3 and MC192 are combined, there is a small but significant higher 4-3.6 cell survival over optimal NGF. This is likely attributable to the mAbs being more stable in culture at 37°C than NGF and perhaps to receptor/ligand recycling. The possibility of a small amount of cell division is unlikely, because BrdU incorpo-

Figure 2. Optimal TrkA tyrosine phosphorylation by concomitant binding of p75 and TrkA. 4-3.6 cells were untreated (lane 1) or treated with 1 nM NGF (lane 2), 5 nM mAb 5C3 alone (lane 3), 0.5 nM mAb MC192 alone (lane 4), or a combination of both mAbs (lane 5) for 15 min at 37°C. Ligand concentrations were selected from survival assays (e.g., Table 3) and pilot experiments (e.g., Table 4). Equal amounts of protein from whole cell lysates were resolved by SDS-PAGE and analyzed by Western blotting. *A*, Anti-phosphotyrosine (anti-PY) blot. Short thick arrow indicates p140 TrkA. Notable changes in tyrosine phosphorylation of other cellular proteins can be seen induced by NGF, mAb 5C3, or 5C3 + MC192 (thick dashed arrow), by NGF or mAb 5C3 only (short thin arrow), or by all treatments (thin dashed arrow). *B*, α -PY490 blot (DF-49 sera) recognizing specifically the Shc binding site of TrkA. *C*, Densitometric scanning quantification of band intensities relative to NGF treatment (average $\pm$ SE; $n = 5$). * indicates significant difference from untreated samples (paired Student's *t* tests; $n = 5$; $p < 0.03$).

ration in response to mAb 5C3 or NGF in SFM is undetectable (data not shown).

Synergy of mAb MC192 and NGF in protection from apoptosis can be explained partially by increased binding of NGF to p75 receptors (Chandler et al., 1984); however, several arguments suggest that affinity considerations are not the sole mechanism by which p75 ligands modulate TrkA function. First, although NGF increases its affinity for p75 approximately threefold in the presence of MC192, the functional enhancement is $\sim$ 200-fold (survival with 5 pM NGF + MC192 is nearly equivalent to 1 nM NGF). Second, enhancement of p75 affinity by mAb MC192 ought to sequester NGF from TrkA (Barker and Shooter, 1994), and therefore a reduction in TrkA-mediated survival should occur rather than the observed increase. Third, and most important, mAb MC192 enhances the biological and biochemical function of TrkA stimulated with mAb 5C3. Synergy between these mAb ligands was not caused by a change in affinity or binding properties of the mAbs, because each mAb binds its receptor regardless of, and is unaffected by, the other (see Materials and Methods).

Functional synergy between p75 ligands and TrkA ligands (in cells expressing both receptors), together with decreased TrkA-mediated signals in TrkA⁺ p75⁺ cells compared with TrkA⁺ p75⁻ cells, suggests functional interactions. Two nonexclusive mechanisms may account for the p75 effect. (1) Bound p75 positively enhances TrkA signals directly or indirectly, and (2) unbound p75 negatively modulates TrkA-mediated trophic signals directly or indirectly. Our data provide stronger support for the latter mechanism, based on the following three arguments.

First, decreased trophic signals in response to TrkA binding by mAb 5C3 were detected in 4-3.6 cells (TrkA⁺ p75⁺) when compared with C10 cells (TrkA⁺ p75⁻). Comparable data were published using fibroblasts transfected with trkA cDNA (LeSauter et al., 1996).

Second, synergistic effects occur between TrkA ligands and mAb MC192 only when the concentration of MC192 is optimized to achieve bivalent binding of all or most receptors. At low

concentrations (subsaturating), mAb MC192 does not synergize with TrkA ligands. At very high mAb MC192 concentrations, poor synergy is observed, likely because of high dose inhibition (the probability of mAb binding in a monovalent fashion). This is consistent with reports that high doses of mAb MC192 (8 μ g/ml; $\sim$ 40-fold higher than our optimal concentrations) can antagonize the effect of NGF on PC12 cells (Barker and Shooter, 1994). The issue of monovalent versus bivalent receptor binding has also been examined (our unpublished observations).

Third, protection from apoptotic death in SFM was very limited or undetectable after binding of p75 alone with NGF (in B104 cells) or with MC192 mAb (in B104, PC12, and 4-3.6 cells) and undetectable after binding with BDNF (in B104 and 4-3.6 cells). The simplest interpretation is that detectable p75 trophic signals in SFM require pre- or coactivation of TrkA. This would be consistent with reports of a protein kinase that associates with p75 receptors only after TrkA activation (Canossa et al., 1996).

The mechanism by which p75 controls TrkA function probably does not involve TrkA-p75 heterodimers, because they are not likely to be induced by binding of the mAb-based ligands; however, the possibility that receptor heterodimers preexist on the cell membrane and are not ligand dependent cannot be ruled out (Wolf et al., 1995; Ross et al., 1996). Furthermore, it is also possible that a positive modulation of bound p75 on TrkA occurs (Verdi et al., 1994; Canossa et al., 1996).

Previously, polyclonal anti-TrkA antiserum was used to achieve $\sim$ 70% of the neuronal survival afforded by optimal NGF (Clary et al., 1994). The neurons expressed TrkA and p75, but potential synergy on p75 binding was not studied. Our results are consistent with and expand on that data.

Although p75 has been reported to signal in the absence of TrkA binding (for review, see Chao, 1994; also see Carter et al., 1996; Cortazzo et al., 1996), those p75-mediated signals do not lead to trophic responses or to increased PY of TrkA as studied herein. Our results contrast with other reports wherein unbound p75 receptors did not modulate TrkA-mediated signals (Verdi et

al., 1994), and p75 binding in the absence of TrkA binding did protect from apoptosis induced by antimetabolic agents (Cortazzo et al., 1996). The different results likely are attributable to the presence of growth factors in these other experiments. Our results also differ to some extent from a report by Rabizadeh et al. (1993) in which p75-mediated TrkA-independent protection from apoptosis was described in NR5D (a line derived from PC12 cells) and CSM14.1 (immortalized neuronal cells), purported to lack TrkA as assessed by Northern blot analysis. These cells, however, may express very low levels of TrkA, which may help to explain the discrepancy.

Analysis of TrkA PY, particularly the Shc docking site PY490, confirmed that higher activity is induced after concomitant binding of TrkA and p75. This likely is attributable to increased kinase kinetics, to lower tyrosine phosphatase activity, or to sustained phosphorylation of PY490 (Segal et al., 1996). Any one of these alternatives supports the hypothesis of a negative modulation of TrkA enzymatic activity by unbound p75.

On the basis of our Western blot experiments, the putative negative modulation by p75 seems to be released within a few minutes. Thus, it is unlikely that this modulation involves NF κ - β (Carter et al., 1996) or JNK (Xia et al., 1995) transcriptional pathways. Perhaps the regulation of TrkA by p75 is more direct and acts via phospholipid hydrolysis (Dobrowsky et al., 1995) or other kinases (Canossa et al., 1996).

Important changes in the PY of cellular proteins other than TrkA are also seen induced by ligands that afford optimal protection from apoptotic death. Some of these proteins are tyrosine-phosphorylated in a ligand-specific manner. The identification of these phosphoproteins may reveal differences or specificities in signal transduction induced by NGF versus antibody-based ligands and will aid in understanding whether the putative negative modulation of TrkA is direct or indirect via adapter or regulatory proteins.

Very few anti-receptor mAbs with agonistic activity exist (Taub and Greene, 1992), and even agonistic polyclonal antisera are rare. Thus, given the dimerizing ability of antibodies, it seems that although receptor dimerization is required (Heldin, 1995), it alone cannot account for agonistic function. Likely, a conformational change(s) in the structure of the receptor must also occur (Posner et al., 1992; Carraway and Cerione, 1993; Cadena et al., 1994; Arakawa et al., 1995). We predict that mAb 5C3 affords TrkA homodimerization as well as a partial receptor conformational change(s) that leads to partial agonistic signals.

Partial conformational changes are expected from the fact that mAb 5C3 likely docks onto a region of TrkA and affects the receptor differently than NGF (Perez et al., 1995; Windisch et al., 1995). This is also supported by published observations that mAb monovalent 5C3 Fabs function as agonists in bioassays using fibroblasts transfected with human TrkA (LeSauter et al., 1996). Furthermore, treatment of C10 cells (TrkA⁺ p75⁻) with mAb 5C3 affords only ~80% of the trophic survival afforded by treatment NGF, suggesting that mAb 5C3 and NGF are not identical TrkA ligands.

Structural analysis of mAb 5C3-TrkA and NGF-TrkA complexes may reveal the nature of the differences and perhaps putative receptor conformational changes that occur on ligand binding. Furthermore, medulloblastomas engineered to express TrkA undergo apoptotic death after NGF treatment (Muragaki et al., 1997), and it would be of interest to test whether mAb 5C3 affects these cells in the same manner.

An important and novel concept is the demonstration that

functional agonism in a multireceptor system could be optimally achieved by a combination of a natural ligand and an anti-receptor antibody or by two antibodies against different constituents of the complex. This information might be useful in the design of artificial receptor agonists and antagonists, particularly for neurotrophin or other multireceptor systems.

Our work will continue using monovalent fragments of the mAbs to assess the role of dimerization. Future work will focus on how different NGF receptor-ligand complexes affect early events of neurotrophin signaling, internalization, and activation of second messengers.

REFERENCES

- Arakawa T, Holst P, Narhi LO, Philo JS, Wen J, Prestrelski SJ, Zhu Rees DC, Fox GM (1995) The importance of Arg40 and 45 in the mitogenic activity and structural stability of basic fibroblast growth factor: effects of acidic amino acid substitutions. *J Protein Chem* 14:263–74.
- Barker PA, Shooter EM (1994) Disruption of NGF binding to the low affinity neurotrophin receptor p75^{NTR} reduces NGF binding to TrkA on PC12 cells. *Neuron* 13:203–215.
- Barrett GL, Georgiou A (1996) The low affinity Nerve Growth Factor Receptor p75^{NGFR} mediates death of PC12 cells after Nerve Growth Factor withdrawal. *J Neurosci Res* 45:117–128.
- Benedetti M, Levi A, Chao MV (1993) Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. *Proc Natl Acad Sci USA* 90:7859–7863.
- Bogenmann E, Torres M, Matsushima H (1995) Constitutive N-myc gene expression inhibits TrkA mediated neuronal differentiation. *Oncogene* 10:1915–1925.
- Buck H, Winter J (1996) K252a modulates the expression of nerve growth factor-dependent capsaicin sensitivity and substance P levels in cultured adult rat dorsal root ganglion neurones. *J Neurochem* 67:345–351.
- Cadena DL, Chan CL, Gill GN (1994) The intracellular tyrosine kinase domain of the epidermal growth factor receptor undergoes a conformational change upon autophosphorylation. *J Biol Chem* 269:260–265.
- Canossa M, Twiss JL, Verity AN, Shooter EM (1996) p75^{NGFR} and TrkA receptors collaborate to rapidly activate a p75^{NGFR}-associated protein kinase. *EMBO J* 15:3369–3376.
- Carraway III KL, Cerione RA (1993) Fluorescent-labeled growth factor molecules serve as probes for receptor binding and endocytosis. *Biochemistry* 32:12039–12045.
- Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhauser N, Bohm-mathaei-R, Baeuerle PA, Barde YA (1996) Selective activation of NF- κ B by nerve growth factor through the neurotrophin receptor p75. *Science* 272:542–545.
- Chandler CE, Parsons LM, Hosang M, Shooter EM (1984) A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. *J Biol Chem* 259:6882–6889.
- Chao MV (1994) The p75 neurotrophin receptor. *J Neurobiol* 25:1373–1385.
- Chastagner P, Moreau JL, Jacques Y, Tanaka T, Miyasaka M, Kondo M, Sugamura K, Theze J (1996) Lack of intermediate-affinity interleukin-2 receptor in mice leads to dependence on interleukin-2 receptor alpha, beta and gamma chain expression for T cell growth. *Eur J Immunol* 26:201–206.
- Clary DO, Weskamp G, Austin LR, Reichardt LF (1994) TrkA cross-linking mimics neuronal responses to nerve growth factor. *Mol Biol Cell* 5:549–563.
- Cortazzo MH, Kassis ES, Sproul KA, Schor NF (1996) Nerve Growth Factor (NGF)-mediated protection of neural crest cells from antimetabolic agent-induced apoptosis: the role of the low-affinity NGF receptor. *J Neurosci* 16:3895–3899.
- Dobrowsky RT, Jenkins GM, Hannun YA (1995) Neurotrophins induce sphingomyelin hydrolysis: modulation by co-expression of p75^{NTR} with Trk receptors. *J Biol Chem* 270:22135–22142.
- Heldin C-H (1995) Dimerisation of cell surface receptors in signal transduction. *Cell* 80:213–223.
- Hempstead BL, Schleifer LS, Chao MV (1989) Expression of functional nerve growth factor receptors after gene transfer. *Science* 243:373–375.
- Hempstead BL, Martin-Zanca D, Kaplan DR, Parada LF, Chao MV (1991) High affinity NGF binding requires co-expression of the trk proto-oncogene and the low affinity NGF receptor. *Nature* 350:678–683.

- Ibáñez CF, Ebendal T, Barbany G, Murray-Rust J, Blundell TL, Persson H (1992) Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the *trk* gene product. *Cell* 69:329–341.
- Kaplan DR, Hempstead BL, Martin-Zanca B, Chao MV, Parada LF (1991) The *trkA* proto-oncogene product: a signal transducing receptor for Nerve Growth Factor. *Science* 252:554–558.
- Klein R, Jing S, Nanduri V, O'Rourke E, Barbacid M (1991) The *trkA* proto-oncogene encodes a receptor for Nerve Growth Factor. *Cell* 65:189–197.
- LeSauter L, Maliartchouk S, LeJeune H, Quirion R, Saragovi HU (1996) Potent human p140-TrkA agonists derived from an anti-receptor antibody. *J Neurosci* 16:1308–1316.
- Mahadeo D, Kaplan DR, Chao MV, Hempstead BL (1994) High affinity nerve growth factor binding displays a faster rate of association than p140 TrkA binding. *J Biol Chem* 269:6884–6991.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63.
- Muragaki Y, Chou TT, Kaplan DR, Trojanowski JQ, Lee VM-Y (1997) Nerve growth factor induces apoptosis in human medulloblastoma cell lines that express TrkA receptors. *J Neurosci* 17:530–542.
- Nebreda AR, Martin-Zanca D, Kaplan DR, Parada LF, Santos E (1991) Induction by NGF of mitotic maturation of *Xenopus* oocytes expressing the *trk* proto-oncogene product. *Science* 252:558–563.
- Perez P, Coll PM, Hempstead BL, Martin-Zanca D, Chao MV (1995) NGF binding to the *trk* tyrosine kinase receptor requires the extracellular immunoglobulin-like domains. *Mol Cell Neurosci* 6:97–105.
- Pinkas-Kramarski R, Soussan L, Waterman H, Levkowitz G, Alroy I, Klapper L, Lavi S, Seger R, Ratzkin BJ, Sela M, Yarden Y (1996) Diversification of neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J* 15:2452–2467.
- Posner I, Engel M, Levitzki A (1992) Kinetic model of the epidermal growth factor (EGF) receptor tyrosine kinase and a possible mechanism of its activation by EGF. *J Biol Chem* 267:20638–20647.
- Rabizadeh S, Oh J, Zhong L-T, Young J, Bitler CM, Butcher LL, Bredesen DE (1993) Induction of apoptosis by the low affinity NGF receptor. *Science* 261:345–348.
- Ross AH, Daou MC, McKinnon CA, Condon PJ, Lachyankar MB, Stephens RM, Kaplan DR, Wolf DE (1996) The neurotrophin receptor, gp75, forms a complex with the receptor tyrosine kinase TrkA. *J Cell Biol* 132:945–953.
- Rovelli G, Heller RA, Canossa M, Shooter EM (1993) Chimeric tumor necrosis factor-TrkA receptors reveal that ligand-dependent activation of the TrkA tyrosine kinase is sufficient for differentiation and survival of PC12 cells. *Proc Natl Acad Sci USA* 90:8717–8721.
- Ryden M, Murray-Rust J, Glass D, Ilag LL, Trupp M, Yancopoulos GD, McDonald NQ, Ibanez CF (1995) Functional analysis of mutant neurotrophins deficient in low-affinity binding reveals a role for p75^{LNGFR} in NT-4 signalling. *EMBO J* 14:1979–1990.
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd Ed, pp 9.16–9.19. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Segal RA, Bhattacharyya A, Rua RA, Alberta JA, Stephens RM, Kaplan DR, Stiles CD (1996) Differential utilization of Trk autophosphorylation sites. *J Biol Chem* 271:20175–20181.
- Taub R, Greene MI (1992) Functional validation of ligand mimicry by anti-receptor antibodies: structural and therapeutic implications. *Biochemistry* 31:7431–7435.
- Verdi JM, Birren SJ, Ibáñez CF, Persson H, Kaplan DR, Benedetti M, Chao MV, Andersson DJ (1994) p75^{LNGFR} regulates signal transduction and NGF induced neuronal differentiation in MAH cells. *Neuron* 12 733–745.
- Windisch JM, Marksteiner R, Schneider R (1995) Nerve growth factor binding site on TrkA mapped to a single 24-amino acid leucine-rich motif. *J Biol Chem* 270:28133–28138.
- Wolf DE, McKinnon CA, Daou MC, Stephens RM, Kaplan DR, Ross AH (1995) Interaction with TrkA immobilizes gp75 in the high affinity nerve growth factor receptor complex. *J Biol Chem* 270:2133–2138.
- Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270:1326–1330.