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NEUROTROPHIN RECEPTOR STUDIES WITH FUNCTIONAL NERVE GROWTH FACTOR MIMETICS

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Abstract

Polypeptide hormone Nerve Growth Factor (NGF) plays a crucial role in the development and maintenance of certain neuronal populations, both within the central and peripheral nervous systems. NGF receptors and the hormone itself have been attractive pharmacological targets in the treatment of several neuropathologies including Alzheimer Disease, ischemia, brain trauma, peripheral neuropathies, neurotoxicity associated with chemotherapy of cancer and chronic inflammatory pain. NGF and its receptors are also expressed in several non-neuronal tissues and participate in their regulation. In addition NGF can be a survival or differentiation factor for human malignancies of neuroectodermal origin. Accordingly, NGF mimetics can be developed as anti-cancer drugs and non-invasive diagnostic agents.

Two structurally distinct transmembrane proteins TrkA and $p75^{NTR}$ have been characterized as NGF receptors. Each co-receptor is capable of ligand binding and signal transduction, however binding characteristics, activation and signaling of each co-receptor depend on the expression and occupancy state of the other.

Functional interactions between NGF receptors TrkA and $p75^{NTR}$ have been studied using activating anti-receptor monoclonal antibodies (mAbs) as specific ligands that, unlike NGF, can discriminate between the co-receptors. It has been shown that unbound $p75^{NTR}$s could attenuate TrkA activation and trophic signaling, while $p75^{NTR}$ expression and binding with an activating ligand was necessary to achieve optimal trophic signals similar to that induced by NGF. Both negative and positive TrkA regulation occurred within the same cellular context. It has been also shown that the trophic signals of a polypeptide growth factor could be fully mimicked by a combination of two mAbs against its co-receptors.

Different artificial ligands of TrkA have been tested in functional assays alone or in a paradigm of synergy with a selective ligand of $p75^{NTR}$. The studied TrkA ligands included Fab fragments of an anti-TrkA monoclonal antibody, small cyclic peptides derived from an NGF β-turn region, and small molecule compounds selected from a focused β-turn peptidomimetic library based on the pharmacophores of the anti-TrkA

mAb and NGF peptide analogs. In contrast to NGF, all these three classes of NGF mimetics are monomeric, monovalent and can bind only a single defined epitope of the receptor. We have found that, despite of their fundamental differences from the natural ligand, these mimetic could induce agonistic responses in neuronal cell lines and cultured primary neurons. These finding have considerable implications for the rational design and development of agonistic ligands of cell surface receptors with a single transmembrane region, particularly small molecule therapeutics with neurotrophic activity.

Résumé

L'hormone polypeptidique NGF (Nerve Growth Factor) joue un role crucial dans le développement et le maintient de certaines populations neuronales, aussi bien dans le système nerveux central que dans le système nerveux périphérique. Les récepteurs de NGF aussi bien que l'hormone sont des cibles de choix pour le traitement de plusieurs neuropathologies comme l'Alzheimer, ischémies, traumatismes cérébraux, neuropathies périphériques, neurotoxicité associée à la chimiothérapie, et douleurs inflammatoires chroniques. NGF et ses récepteurs sont aussi exprimés dans plusieurs tissus d'origine non-neuronale et participent à leur régulation. De plus, NGF peut être un facteur de survie ou différentiation pour des tumeurs malignes originant du neuroectoderme. En conséquence, des mimétiques de NGF peuvent être développés comme traitements anti-cancer ou agents diagnostic non-invasifs.

Deux protéines transmembranaires structuralement distinctes, TrkA et p75NTR, ont été caractérisées comme étant des récepteurs pour NGF. Chaque co-récepteur peut lier l'hormone et initier une réponse cellulaire. Cependant, les propriétés de ligation, d'activation et de signalisation de chaque co-récepteur dépendent de l'expression et de l'état d'engagement de l'autre.

Les interactions fonctionnelles entre les récepteurs TrkA et p75NTR ont été étudiées en utilisant des anticorps monoclonaux (mAbs) anti-récepteurs capables d'activer ces récepteurs et, contrairement à NGF, pouvant discriminer entre les deux co-récepteurs. Il a été démontré que p75NTR non-lié à NGF peut atténué l'activation et la signalisation cytotrophique de TrkA, alors que l'expression de p75NTR et son engagement avec un ligand activant sont nécessaires pour l'obtention d'une réponse trophique optimale similaire à celle induite par NGF. Cette régulation positive et négative de TrkA a été observée dans le même modèle cellulaire. Il a aussi été prouvé que les sigaux trophiques d'une hormone polypeptidique peuvent être entièrement reproduits par la combinaison de deux mAbs liant ses co-récepteurs.

Plusieurs ligands artificiels de TrkA ont été testés seuls ou en synergie avec un ligand sélectif envers p75NTR dans des essais fonctionnels. Les ligands de TrkA étudiés

inclus les fragments Fab du mAb anti-TrkA, de courts peptides cycliques dérivé d'une boucle β de NGF, et de petites molécules sélectionnées à partir d'une collection de peptidomimétiques basés sur les pharmacophores du mAb anti-TrkA et d'analogues peptidiques de NGF. Contrairement à NGF, ces trois classes de mimétiques de NGF sont des monomères monovalents et ne peuvent s'attacher qu'à un seul épitope défini du récepteur. Nous avons trouvé que, malgré les différences fondamentales avec le ligand naturel, ces mimétiques peuvent induire une réponse agonistique dans des lignées cellulaires neuronales ainsi que dans des cultures primaires de neurones. Ces résultats ont des implications considérables pour le développement et le design d'agonistes de récepteurs cellulaires à région transmembranaire unique, particulièrement en tant que petites molécules thérapeutiques ayant une activité neurotrophique.

Contributions of Authors

This thesis is written in a manuscript-based format. The contributions of each of the authors of the manuscripts are described below.

Chapter I. Maliartchouk S. and Saragovi H.U. 1997. Optimal nerve growth factor trophic signals mediated by synergy of TrkA and $p75^{NTR}$ receptor-specific ligands. J. Neurosci. 17:6031-7

The candidate designed and performed most of the experiments. The initial outline of the study objectives, development of the experimental approach, data analysis, preparation and submission of the manuscript have been done together with Dr. H. Uri Saragovi.

Chapter II. Maliartchouk S., Debeir T., Beglova N., Cuello A.C., Gehring K., and Saragovi H.U. 2000. Genuine monovalent ligands of TrkA nerve growth factor receptors reveal a novel pharmacological mechanism of action. J. Biol. Chem. 275:9946-56.

The candidate designed and performed most of the experiments and participated in the initial outline of the study objectives and data analysis. Dr. Thomas Debeir and Dr. A. Claudio Cuello are responsible for the testing of NGF mimetics on embryonic DRG cultures. Natalia Beglova and Dr. Kalle Gehring are responsible for the physical and chemical characterization of small molecule NGF mimetics. Dr. H. Uri Saragovi carried out initial design, supervision and coordination of the study. All the authors participated in the preparation and submission of the manuscript.

Chapter III. Maliartchouk S., Feng Y., Ivanisevic L. Debeir T., Cuello A.C., Burgess K., and Saragovi H.U. 2000. A designed peptidomimetic agonistic ligand of TrkA nerve growth factor receptors. Mol. Pharmacology, 57:385–91

Drs. Yangbo Feng and Kevin Burgess synthesized focused β-turn peptidomimetic libraries including D3 and D3-biotin compounds. The candidate designed and performed most of the experiments including initial screening of the compound libraries, binding studies and compound testing on neuronal cell lines. Dr. Thomas Debeir and Dr. A. Claudio Cuello are responsible for the testing of compounds on primary neuronal cultures. Ljubica Ivanisevic is responsible for cross-linking experiments and the detection of putative TrkA-TrkA homodimers. Dr. H. Uri Saragovi carried out initial design, supervision and coordination of the study and helped with all the aspects of binding experiments and bioactivity assays. All the authors participated in the preparation and submission of the manuscript.

Contribution to original knowledge

1. It has been shown that the trophic signals of a polypeptide hormone Nerve Growth Factor (NGF) could be fully mimicked by an appropriate combination of monoclonal antibody (mAb)-based ligands of NGF receptors TrkA and $p75^{NTR}$.
2. It has been shown that when NGF receptor TrkA is activated by mAb ligands independently from expression and activation of $p75^{NTR}$, there is a negative regulation of TrkA activation and trophic signaling by unliganded $p75^{NTR}$s.
3. It has been shown that when NGF receptor TrkA is activated by mAb ligands independently from expression and activation of $p75^{NTR}$, there is a positive regulation of TrkA activation and trophic signaling by $p75^{NTR}$s bound with an activating mAb ligand.
4. It has been shown that both positive and negative regulation of NGF receptors TrkA by co-receptors $p75^{NTR}$ can occur within the same cellular context.
5. It has been shown that monomeric and monovalent mAb-based TrkA ligands (Fabs) can be partial agonists.
6. It has been shown that two classes of monomeric and monovalent small molecule TrkA ligands, cyclic peptides and non-peptidic NGF mimetics, can induce agonistic responses in neuronal cell lines and primary neuron cultures.

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I would like to emphasize the role of the colleagues and co-authors, Yangbo Feng, Kevin Burgess, Thomas Debeir, A. Claudio Cuello, Natalia Beglova, Kalle Gehring and Ljubica Ivanisevich, whose contributions made successful the multi-disciplinary studies presented in this thesis.

I am grateful to Lynne LeSauteur who pioneered in the development of peptide and antibody Nerve Growth Factor receptor ligands and helped me a lot at the beginning of my studies. I would like to thank other people I worked with in the laboratory, N. Lavine, M. Gagnon, N. Rebai, S. C. Das, Y. Mowal, L. Ivanisevich, for their help and support. In particular, I would like to thank Natalie Lavine for her expertise and Martin Gagnon for his help with the preparation of this manuscript.

This thesis is dedicated to my wife Irina whose love and continuous support has been a driving force in my studies.

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1. Nerve Growth Factor receptors as pharmacological targets

Receptors and Drugs

Drug is broadly defined as any chemical agent that affects processes of living organism (Goodman and Gilman, 1996). Therapeutics are drugs useful in prevention, diagnostic and treatment of human disease. Drugs assert their therapeutic, adverse and other effects by the means of direct interactions with their receptors. A receptor for a drug can be any functional component of the organism. While for some drugs the receptors have not yet been clearly defined, the concept of drug-receptor interactions provide the basis for rational drug design and their rational clinical use.

The physiological definition of receptor is not as broad as pharmacological, and it includes only the receptors for endogenous regulatory ligands like hormones and neurotransmitters. These receptors make the primary pharmacological targets because they play a crucial role in a flow of information in a multicellular organism. The function of physiological receptors consists of binding the appropriate ligand and, in response, propagating its regulatory signal in the target cell. Thus, these receptors coordinate extracellular signals with cellular metabolism and provide the physical and chemical basis for the integration of the function of specialized tissues in a multicellular organism.

Physiological receptors have been classified into structural and functional families. On a mechanistic level receptors act as biochemical signal amplifiers. Many receptors are localized on a cell surface and possess extracellular, intracellular and membrane spanning portions. Their ligands interact with extracellular parts of the receptor and their binding leads to the changes that are transmitted to the intracellular domains and trigger signal transduction cascades in the cell. Some hormones can penetrate the cell membrane and interact with their respective intracellular receptors. The amplification abilities of signal transducing receptors are so effective that in most cases

ligand binding and activation of only a few hundreds cell surface receptors is required for full biological responses.

The biological effects of physiological receptor ligands are considered agonistic. Accordingly, the receptor-binding drugs are classified into agonistic and antagonistic. Agonistic receptor binding induces biological responses similar to that induced by physiological ligands. Antagonists block the binding of agonistic ligands to the receptor and, therefore, abrogate their biological effects. The mechanisms of antagonistic receptor binding can be competitive or non-competitive, reversible or irreversible.

Binding of a pure antagonist does not induce receptor activation. There are, however, many instances when receptor ligands behave as partial agonists. They induce maximal pharmacological responses that are lower than responses to a full agonist. At the same time partial agonists may antagonize a natural ligand when they are applied together. Often the biological activity of a receptor ligand depends on the level of receptor expression, cellular contest and other factors. This may create uncertainty in a ligand designation as a full or partial agonist or antagonist (reviewed by Hoyer and Boddeke, 1993).

A separate group of receptor ligands were termed inverse agonists (reviewed by Milligan *et al.*, 1995). The biological effects of inverse agonists are similar to that of an antagonist, but their mechanism of action is different. While a pure antagonist simply blocks the binding of a natural agonistic ligand, receptor binding with an inverse agonist leads to changes in the receptor that prevent receptor activation. The most prominent effect of inverse agonists is a reduction of signals induced by unbound receptors. The background receptor activity in the absence of a ligand is usually associated with high levels of receptor expression. Thus, the effects of inverse agonists may strongly depend on the level of receptor expression.

Neurotrophins

This thesis presents studies of the Nerve Growth Factor (NGF) receptor-ligand system. NGF is the first characterized member of the family of polypeptide hormones

termed Neurotrophins. Other mammalian neurotrophins are Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5). Neurotrophins play a crucial role in the development and maintenance of neuronal cells that form central and peripheral nervous systems (CNS and PNS).

During the development of the vertebrate nervous system most neuronal populations undergo a process of selective apoptotic cell death. The surviving cells form complex neuronal networks that appropriately innervate target tissues. The central principle of the Neurotrophic Factor Hypothesis is that target areas regulate their innervation by producing limiting amounts of neurotrophic factors capable of promoting neuronal survival and maintenance (reviewed by Levi-Montalcini, 1987). Series of classical experiments have shown that neuronal survival and growth was regulated by their targets (Levi-Montalcini and Hamburger, 1951, 1953; reviewed by Oppenheim, 1991) and led to the discovery and purification of NGF (Cohen *et al.*, 1954). Much later other members of the Neurotrophin family were discovered and characterized (reviewed by Barbacid, 1994). They are all closely related to NGF in their structure and can support differentiation and survival of certain neuronal sub-populations.

Because of their important physiological functions neurotrophins and neurotrophin receptors have been attractive pharmacological targets in the treatment of several neuropathologies (reviewed by Eide *et al.*, 1993; Saragovi and Gehring, 2000). Neurotrophins and agonistic neurotrophic agents are considered for the treatment of such CNS conditions as Alzheimer Disease (AD), Parkinson's Disease, ischemia and brain trauma. In AD the rationale for neurotrophin therapy arises from the fact that the affected cholinergic neurons express NGF receptors and depend on NGF for their survival and for maintenance of the cholinergic phenotype (reviewed by Olson, 1993). Patients with advanced AD exhibit reduced numbers of NGF receptors on their basal forebrain cholinergic neurons and deficient NGF transport to these cells (Mufson *et al.*, 1997; Narisawa-Saito *et al.*, 1996). Parkinson's disease is characterized by the selective loss of the nigro-striatal dopaminergic neurons that express neurotrophin receptors and can be rescued by BDNF and, possibly, NT-3 (reviewed by Lindsay *et al.*, 1993). Neurotrophic factors may be useful in preventing neurotoxicity associated with chemotherapy of cancer

(Alberts and Noel; 1995). Agents with neurotrophin-like activity are highly promising as potential therapeutics for peripheral (primarily diabetic) neuropathies (reviewed by Apfel, 1999). Neurotrophin antagonists may be useful in the treatment of chronic inflammatory pain (reviewed by Mendell *et al.*, 1999).

Neurotrophins and their receptors expressed in several non-neuronal cells and play important roles in their regulation. These tissues include B and T lymphocytes, insulin-producing pancreatic cells and mast cells (reviewed by Saragovi and Gehring, 2000). Accordingly, both neurotrophin agonists and antagonists can be used in pathologies associated with these tissues. Several neuroectoderm-derived human neoplasias are neurotrophin-dependent or responsive to neurotrophins, some of them synthesize and utilize NGF as an autocrine factor. Thus, neurotrophins and their receptors make a class of molecular targets for the development of anti-cancer drugs (reviewed by Ruggeri *et al.*, 1999; Weeraratna *et al.*, 2000) and non-invasive diagnostic agents (LeSauteur *et al.*, 1996a).

2. NGF Receptors

Binding Sites

NGF receptors were initially characterized on the basis of their affinity. NGF-binding neurons and cell lines of neuronal origin display at list two classes of binding sites with different affinities and association-dissociation kinetics. (Sutter *et al.*, 1979; Landreth and Shooter 1980; Schechter and Bothwell 1981). Two transmembrane cell surface proteins were identified as NGF receptors: p75 Neurotrophin Receptor ($p75^{NTR}$) (Johnson *et al.*, 1986; Chao *et al.*, 1986; Radeke *et al.*, 1987) and Tropomyosin Receptor Kinase (later termed TrkA) proto-oncogene product (Martin-Zanca *et al.*, 1989; Kaplan *et al.*, 1991). When first characterized $p75^{NTR}$ was heterologously expressed in fibroblasts only low affinity ($K_d \sim 10^{-9}$ M) binding sites formed (Chao *et al.*, 1986; Radeke *et al.*, 1987). Other neurotrophins were also shown to bind $p75^{NTR}$ with similar equilibrium dissociation constants (Rodriguez-Tebar *et al.*, 1990; Ernfors*et al.*, 1990; Hallböök *et al.*, 1991;

Rodriguez-Tebar *et al.*, 1992). However, the kinetic characteristics of $p75^{NTR}$ binding were found different for each neurotrophin with BDNF exhibiting the slowest and NGF the fastest association and dissociation rates.

Cells expressing TrkA alone can display a small number of high affinity NGF binding sites (Klein *et al.*, 1991; Jing *et al.*, 1992). These high affinity sites, however, are responsible for the very small fraction of the total binding. Majority binding sites formed by TrkA receptors display low to intermediate binding affinity (K_d 10^{-9} to 10^{-11} M) with very slow rate of association (Kaplan *et al.*, 1991a; Klein *et al.*, 1991; Meakin *et al.*, 1992; Jing *et al.*, 1992; Mahadeo *et al.* 1994, reviewed by Chao, 1994).

Co-expression of TrkA and $p75^{NTR}$ leads to formation of a limited number of high affinity binding sites with $K_d \sim 10^{-12}$ M and a faster association rate (Hempstead *et al.* 1991 Mahadeo *et al.* 1994). High affinity binding is sensitive to the presence of intact functional $p75^{NTR}$ since expression of $p75^{NTR}$ deletion mutants eliminated high affinity sites (Hempstead *et al.*, 1990; Battleman *et al.*, 1993). Formation of high affinity sites also seems to depend on the proper ratio between numbers of TrkA and $p75^{NTR}$ on the cell surface. NGF responsive neurons and neuron derived cell lines (for example PC12 rat pheocromocytoma cells, Greene and Tischler, 1976) have limited levels of TrkA expression and significantly higher levels of $p75^{NTR}$ expression (reviewed by Chao and Hempsted, 1995). These cells display 10-30% of all NGF binding sites as high affinity receptors. In contrast, co-expression of a low number of $p75^{NTR}$ (~ 10^4 receptors per cell) in fibroblasts expressing high number of TrkA (~10^5 receptors per cell) did not result in a formation of a significant number of the high affinity binding sites (Jing *et al.*, 1992). There was also a report that $p75^{NTR}$ alone could form high affinity binding sites for NT-3 on chick sympathetic neurons in the presence of NGF (Dechant *et al.*, 1997). This finding seems to be limited to particular cell type and/or experimental conditions since there were no other reports of high affinity neurotrophin binding to the cells expressing $p75^{NTR}$ only.

Two other members of Trk receptor family termed TrkB and TrkC were discovered and characterized (reviewed by Barbacid, 1994; Bothwell, 1995). Unlike $p75^{NTR}$, Trks bind neurotrophins selectively. TrkA preferentially binds NGF and, to the lesser degree,

depending on cellular context, NT-3. TrkB binds BDNF and NT-4/5, while NT-3 is the preferred ligand of TrkC. Like TrkA, TrkB and TrkC bind their respective neurotrophin ligands with intermediate affinity and $p75^{NTR}$ expression leads to the formation of high affinity binding sites. There are known hormones with neurotrophic activities that structurally do not belong to the Neurotrophin family of proteins. Among them are Basic Fibroblast Growth Factor (bFGF), Glial-Derived Neurotrophic Factor (GDNF) and Ciliary Neurotrophic Factor (CNTF).

TrkA and $p75^{NTR}$

The question of the roles of $p75^{NTR}$ and Trks in transmitting neurotrophic signals has been debated ever since these receptors were identified. Trks belong to the superfamily of receptor tyrosine kinases and their structure is similar to many other growth factor receptors such as the Epidermal Growth Factor (EGF) and Platelet-Derived Growth Factor (PDGF) receptors (reviewed by Ullrich and Schlessinger, 1990). Receptors of this type can undergo autophosphorylation upon ligand binding, phosphorylate second messenger proteins and induce multiple signal transduction pathways leading to trophic and mitogenic responses. $P75^{NTR}$, on the other hand, has no enzymatic activity and is structurally related to the Tumor Necrosis Factor (TNF) receptor family proteins. These receptors are better known for their pro-apoptotic rather than trophic effects.

It was demonstrated that TrkA could mediate most of the cellular responses to NGF. TrkA expression was sufficient for mitogenic responses to NGF and NT-3 in fibroblasts (Cordon-Cardo *et al.*, 1991) and for meiotic maturation of Xenopus oocytes (Nebrada *et al.*, 1991). NGF mutant that binds TrkA but not $p75^{NTR}$ can induce both survival and differentiation of PC12 cells (Ibáñez *et al.*, 1992). The chimeric receptor which contain the extracellular domain of TNF receptor and the transmembrane and cytoplasmic domains of TrkA, induced TNF-dependent neuronal differentiation and survival in PC12 cells (Rovelli *et al.*, 1993). Interestingly, similarly designed chimeras of $p75^{NTR}$ and TNF receptor, two closely related proteins, were found nonfunctional. The studies of phenotypes of knockout mice lacking expression of functional NGF receptors and/or

functional NGF provided additional evidence suggesting that TrkA was a primary mediator of NGF biological effects (reviewed by Klein 1994; Snider 1994; Patel *et al.*, 2000).

These and other findings led to the notion that, while Trks are functional signal transducing neurotrophin receptors, $p75^{NTR}$ is a common accessory co-receptor of Trks. The biological role of $p75^{NTR}$ is complex and many findings in this area remain controversial. The most studied functions of $p75^{NTR}$s are their abilities to participate in the formation of high affinity binding sites for neurotrophins and regulate activation and signal transduction of Trk tyrosine kinases (further discussed in the part 5). However, the mechanisms of these putative $p75^{NTR}$/Trk functional and, possibly, physical interactions remain largely unknown.

Considerable evidence has been accumulated that, apart from its cooperative actions with Trks, $p75^{NTR}$ may induce Trk-independent functional responses and that in some cases these responses may account for apparent regulation of Trk signals by $p75^{NTR}$. As a member of TNF receptor family, $p75^{NTR}$ has been extensively studied for its putative pro-apoptotic effects. The findings in this direction support two seemingly opposite hypotheses. First is that $p75^{NTR}$ exerts a constitutive pro-apoptotic effect and neurotrophin binding to the receptor may, at least in some cases, reverse this effect. The other hypothesis is that neurotrophin-mediated $p75^{NTR}$ activation leads to pro-apoptotic signals. The results of experiments with $p75^{NTR}$ over-expression in TrkA-negative neuronal cell lines (Rabizadeh *et al.*, 1993) and $p75^{NTR}$ down-regulation with anti-sense oligonucleotides in cultured sensory neurons (Barrett and Bartlett, 1994) supported the first theory. The level of $p75^{NTR}$ expression in PC12 cells correlated with the rate and extent of cell death caused by NGF withdrawal (Barrett and Georgiou, 1996). On the other hand, a number of studies have reported ligand-induced pro-apoptotic signals of $p75^{NTR}$ (reviewed by Casaccia-Bonnefil *et al.*, 1999).

Nevertheless, examples abound when $p75^{NTR}$ expression and neurotrophin binding did not induce adverse biological effects or pro-apoptotic signaling cascades (Ladiwala *et al.*, 1998; Bhakar *et al.*, 1999; Barker, 1998; Kume *et al.*, 2000; also this Thesis,

chapter I). The studies of $p75^{NTR}$ deficient mice revealed evidence of both decreased innervation and impaired neuronal apoptosis (further discussed in the part 5).

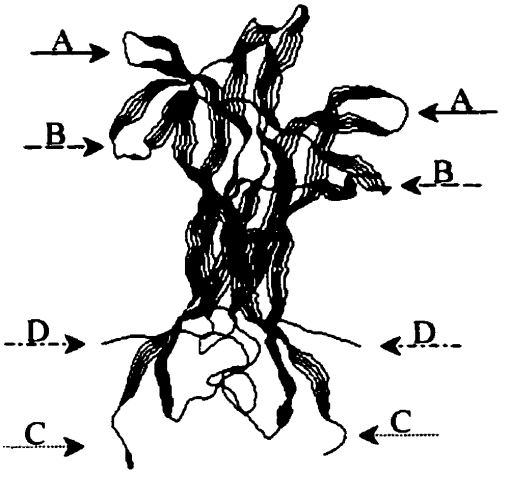
3. NGF mimetics and antagonists

NGF structure

Neurotrophins have a high degree of structural similarity both in their primary structure (55-60%; Hallbook *et al.*, 1991) and in their tertiary structures (Radziejewski *et al.*, 1992). Studies comparing circular dichroism and infrared spectra of NGF, BDNF and NT-3 suggest that these NTs share an overall structural similarity (Narhi *et al.*, 1993; Radziejewski and Robinson, 1993). Furthermore, heterodimers between the neurotrophins have also been reported *in vitro* (Arakawa *et al.*, 1994; Jungbluth *et al.*, 1994). The resolved crystal structures of neurotrophin family members (McDonald *et al.* 1991; Holland *et al.*, 1994; Fandl *et al.*, 1994; Robinson *et al.*, 1995; Robinson *et al.*, 1996; Butte *et al.*, 1998) and of an NT-3/BDNF heterodimer (Robinson *et al.*, 1995) confirmed the close similarity in neurotrophin structures.

The structure of mouse NGF was resolved at 2.3Å using x-ray crystallography (McDonald *et al.* 1991; Holland *et al.*, 1994). The NGF molecule was shown to be a tightly associated non-covalent dimer formed by two identical 118 amino acid polypeptides (Figure 1). NGF protomers are stabilized by three conserved disulfide bonds, which form a core similar to Transforming Growth Factor-β-2 (TGF-β-2) and Platelet-Derived Growth Factor-β (McDonald and Hendrickson, 1993). Each protomer features seven β-strands forming three anti-parallel pairs. The amino acids within these β-strands are important for maintaining the structural motif, and are highly conserved amongst all neurotrophins. The β-strands are linked by four exposed regions, three β-turns (termed A'-A", A"-B, and C-D) and three reverse-turns (termed B-C).

Figure 1. Structure of Nerve Growth Factor



Backbone structure of mouse NGF was rendered based on X-ray crystallography molecular coordinate data (McDonald *et al.* 1991; Holland *et al.*, 1994) using Rasmol 2.6 software. NGF crystallized as a symmetrical non-covalent dimer formed by two identical 118 amino acid protomers.

Important regions are shown with arrows:

A → β-turn C-D corresponding to amino acids 91-99, used in the design of TrkA-targeting peptides (LeSauteur *et al.*, 1995, this thesis Chapter II)

B → β-turn A'-A" corresponding to amino acids 29-35, , used in the design of $p75^{NTR}$-targeting peptides (LeSauteur *et al.*, 1995; Longo *et al.*, 1997)

C → Carboxy termini

D → Amino termini

The β-turns and the reverse-turns in general are widespread features in biologically active polypeptides. Unlike the α-helix and the β-sheet, the backbone conformation of the β-turns is highly variable. Accordingly, the β-turns are commonly thought to act as molecular recognition sites for biological interactions (Amzel *et al.*, 1974; Kabat *et al.*, 1976; Chothia and Lesk. 1987).

Structural features of the β-turns of NGF as well as the amino- and carboxy-termini make them the most attractive candidates for determining receptor binding and specificity toward different neurotrophin receptors. These regions are flexible and solvent accessible and can adapt variations in amino acid sequence without distorting the overall structure of the protein. The comparison of the sequences of β-turns makes them the most variable regions of neurotrophins. Mutagenesis studies have also implicated the β-turns of NGF in binding to NGF receptors (reviewed by Ilag *et al.*, 1994; Ibáñez, 1995). The β-turns A"-B and C-D from NGF are sufficient to impose activity when substituted into the NT-3 molecule (Ibáñez *et al.*, 1991; Kullander and Ebendal, 1994; Kullander *et al.*, 1997). Replacement of the analogous β-turns from BDNF in the corresponding regions of NGF led to a loss of NGF biological effects (Ibáñez *et al.*, 1991). In addition, the carboxy and amino termini of NGF also seems to be necessary for TrkA binding, while they do not define overall structure and stability of the protein (Burton *et al.*, 1992; Kahle *et al.*, 1992; Shih *et al.*, 1994; Drinkwater *et al.*, 1993; Woo *et al.*, 1995).

Development of NGF mimetics and antagonists

Efforts to pharmacologically target NGF and NGF receptors have been made in several directions. First, intact neurotrophins have been considered as potential therapeutic agents. However, therapeutic usage of polypeptides is generally limited by a number of inherent drawbacks such as poor delivery, fast clearance due to proteolysis, potential antigenicity and other adverse side effects (reviewed by Saragovi *et al.*, 1992; Saragovi and Gehring, 2000). Difficulties specific for NGF and other neurotrophins include poor crossing of the blood brain barrier (Poduslo and Curran, 1996), potential adverse effects mediated by p75 neurotrophin receptor (Carter and Lewin, 1997), acute

excitation of sensory neurons and development of hyperalgesia. Accordingly, limited clinical trials of NGF and BDNF have been generally unsuccessful despite the high therapeutic potential of neurotrophin ligands (Olson *et al.*, 1992; Seiger *et al.*, 1993; Petty *et al.*, 1994; Verral 1994; Freeman 1999; reviewed by Olson 1993; Saragovi and Burgess 1999). Recently attempts have been made to chemically modify NGF with polyamines in order to advance its pharmacokinetics (Poduslo *et al.*, 1998). The other means thought to improve neurotrophin delivery include encapsulation, engrafting of neuronal stem cells, and gene therapy (reviewed by Saragovi and Gehring, 2000).

Second, small molecule NGF mimetics have been rationally designed based on the NGF structure, particularly on the structure of the NGF β-turns and reverse-turns. Linear peptides derived from NGF showed limited antagonistic activity in biological assays against low suboptimal concentrations of NGF and did not affect the binding of NGF to PC-12 cells (Romani *et al.*, 1987a,b; Longo *et al.*, 1990; Rashid *et al.*, 1995; Estenne-Bouhtou *et al.*, 1996). It is likely that the linear peptides were lacking the constrain imposed by the protein backbone and did not adapt the native turn-structure from which they were derived.

The approach of designing of small cyclic conformationally restricted peptidomimetics that closely mimic the three-dimensional structure of the turn regions (reviewed by Saragovi *et al.*, 1992) has proved to be more successful. Several small cyclic analogs of NGF turn regions were synthesized and tested in binding and functional assays (LeSauteur *et al.*, 1995). Two of the analogs derived from β-turn C-D (corresponding to amino acids 91-99 of mouse NGF) were selected for their ability to inhibit NGF-mediated neurite outgrowth in PC12 cell line. The effect was specific for NGF, since the peptides did not affect similar responses mediated by β-Fibroblast Growth Factor, and linear or randomized cyclic peptides had no activity. The active peptidomimetics termed C(92-96) and C(92-97) partially inhibited radiolabeled NGF binding to the cells expressing TrkA only or TrkA and $p75^{NTR}$. These compounds were further shown to effectively target TrkA-expressing tumors in an *in vivo* model (LeSauteur *et al.*, 1996a). $P75^{NTR}$ -targeting cyclic peptide mimetics of NGF have also been synthesized and tested (LeSauteur *et al.*, 1995; Van Der Zee *et al.*, 1996; Longo *et*

al., 1997). Their design was based on β-turn A'-A" corresponding to amino acids 30-35 of mouse NGF.

Third, natural and synthetic non-peptide compounds were developed as inhibitors of Trk receptors tyrosine kinase activity. Staurosporine-like compounds K252a and K252b (Kase *et al.*, 1987; reviewed by Lazarovici et. al., 1996) were originally sought as protein kinase C inhibitors. It was found that these compounds could inhibit Trk receptors enzymatic activity at high nanomolar concentrations with some degree of specificity. It was also discovered that they could enhance neurotrophin-mediated signals at low nanomolar concentrations by unclear mechanisms. These compounds have been widely used as reagents in cell biology and regarded as potential therapeutics for both their neurotrophic and anti-neurotrophin activities. However, low degree of specificity and a narrow range of therapeutic concentrations prevented their development as drugs. Efforts are now made to generate chemically modified or synthetic compounds with similar structures that would have higher specificity and, therefore, lower adverse side effects (Camoratto *et al.* 1997; Kaneko *et al.*, 1997; Miknyoczki *et al.* 1999; Pollack *et al.*, 1999).

Another approach to target NGF receptors involves generation of biologically active anti-receptor antibodies (Linthicum *et al* 1988). Antibodies bind a receptor antigen with high affinity. Most of the binding occurs via immunoglobulin domains termed Complementarity Determining Regions (CDRs). CDRs are highly variable, often have β-turn structures and can potentially mimic a binding site of a natural ligand. Most antibodies are bivalent (immunoglobulin M antibodies are poly-valent) and their antigen-binding Fab arms are connected via a flexible hinge region (Kabat, 1976). Therefore, they can mimic a natural ligand in its ability to cause receptor dimerization or higher level aggregation.

A series of monoclonal antibodies against TrkA have been reported (Eager, 1991), however none of them possessed biological activity. This illustrates the fact that oligomerization of tyrosine kinase receptors due to antibody-induced cross-linking is not sufficient for receptor activation (Jiang and Hunter 1999), otherwise all anti-tyrosine

kinase receptor antibodies would be agonistic. A polyclonal antibody against rat TrkA blocked NGF binding to the receptor but exhibited agonistic activity in biological assays at high (micromolar) concentrations (Clary *et al.*, 1994). Ligand-induced receptor cross-linking seemed to play a crucial role in TrkA activation since monovalent Fab fragments of this antibody demonstrated pure antagonistic properties.

Polyclonal anti-receptor antibodies are composed of many different immunoglobulin clones and some clones are likely to closely mimic a natural ligand in its receptor binding epitopes. Therefore, polyclonal antibodies developed against a whole extracellular domain of a receptor (or against receptor-expressing cells) are expected to be partial agonists with low potency. Monoclonal antibodies, on the contrary, bind a defined epitope of a protein antigen. Accordingly, agonistic monoclonal antibodies (mAbs) are very rare, but they are expected to be high affinity potent receptor ligands. Only a few agonistic anti-receptor mAbs have been reported (Trauth *et al.*, 1989; Stancovski *et al.*, 1991; reviewed by Taub and Greene 1992). A mAb against human TrkA has been developed and characterized as a receptor agonist (LeSauteur and al., 1996b). The antibody termed 5C3 was shown to induce tyrosine phosphorylation of TrkA and phoshatidylinositol-3 kinase (PI-3 kinase), receptor internalization and increased cellular transformation of TrkA-expressing cultured fibroblasts. It also protected TrkA-expressing cells from apoptotic death in a growth factor deprivation model. MAb 5C3 binds human TrkA with high (nanomolar) affinity. Interestingly, it only partially (~60%) blocks NGF binding to the receptor and, conversely, mAb 5C3 binding can only be partially blocked by NGF.

Considerable effort has been made in order to identify potential neurotrophin receptor and neurotrophin ligands by high-throughput screening of chemical libraries. Non-peptide agents that inhibit NGF - $p75^{NTR}$ and NGF – TrkA interactions have been reported (Spiegel *et al.*, 1995; Owolabi *et al.* 1999; reviewed by Saragovi and Gehring, 2000). These compounds, however, are more likely to bind to NGF itself and cause adverse pleiotropic effects. Another approach to develop therapeutics with neurotrophic activity is to target neurotrophin synthesis (reviewed by Saragovi and Gehring, 2000).

Vitamin D analogs, purine-like compounds and estrogens may play a role in regulating neurotrophin production in CNS and periphery.

4. TrkA

TrkA structure

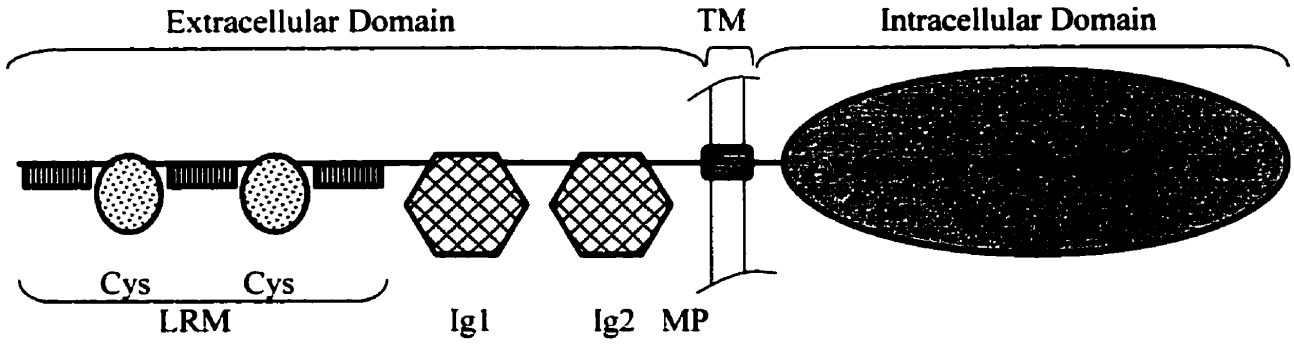
TrkA structure is typical for a growth factor receptor. TrkA is composed of an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic part with protein tyrosine kinase domain (Figure 2, reviewed by Barbacid *et al.*, 1994).

Extracellular domain (ECD) is responsible for ligand binding. It has several glycosylation sites and, when expressed in mammalian cells, bears a carbohydrate component of ~40 kDa. Enzymatic deglycosylation of soluble ECD TrkA leads to destabilization and aggregation of the protein (Woo *et al.*, 1998). Unglycosylated full length TrkA is constitutively active. However, it is not trafficked to the cell membrane and, despite being enzymatically active, cannot induce downstream signaling pathways that are localized to the membrane (Watson *et al.*, 1999).

ECD TrkA structure was divided into subdomains on a basis of their similarity in amino acid sequence to other well characterized proteins (Figure 2). It consists of N-terminal leucine-rich motif (LRM) separated into 3 subdomains by two cysteine-rich clusters, and two immunoglobulin(Ig)-like domains adjacent to the cell membrane. Both LRMs and Ig-like domains are known as common sites of protein-protein interactions. Mutagenesis studies mapped NGF binding to the two different epitopes of ECD TrkA. While the authors of some studies concluded that a single 24-amino acid LRM mediated NGF binding (Windisch *et al.*, 1995; MacDonald and Meakin, 1996), the others suggested that the membrane-proximal Ig-like domain 2 (Ig2) was a docking site of Trks for their respective neurotrophin ligands (Urfer *et al.*, 1995; Perez *et al.*, 1995 Urfer *et al.*, 1998). Taken together results of these and other studies suggest that there may be

multiple docking sites on Trk receptors, with Ig2 domains and membrane-adjacent linker regions playing a crucial role in specificity of the receptors for their respective neurotrophin ligands (reviewed by Friedman and Greene, 1999; O'Connell *et al.*, 2000; Arevalo *et al.*, 2000).

Figure 2. Domain structure of TrkA



Legend:

TM – transmembrane domain; LRM – leucine-rich motif; Cys – cysteine-rich clusters; Ig1, Ig2 – immunoglobulin-like domains 1 and 2; MP – membrane proximal region; Y490-785 – tyrosine residues of intracellular domain important in regulation of tyrosine kinase activity and specificity for its substrates.

The crystal structures of the Ig2 domains of TrkA, TrkB and TrkC and of the NGF- Ig2 TrkA complex have been recently resolved (Ultsch *et al.*, 1999; Wiesmannet *et al.*, 1999). Ig2 domains of all three receptors crystallized as dimers with their amino-terminal strands replacing each other. The authors come to the conclusion that the strand swapping was a refolding artifact. Thus, the issue of the Ig2 domain potential for homodimerization remains open. Two epitopes on NGF were in contact with co-crystallized Ig2 domain of TrkA. First involved L1 loop (β-turn formed by amino acid residues 30-35) and residues from the central β-sheet. The second epitope was formed by amino terminal residues of NGF that were found disordered in the crystal structure of unbound NGF (McDonald *et al.* 1991; Holland *et al.*, 1994). These data contradict the results of the studies showing that the L4 loop of NGF (β-turn formed by amino acid

residues 91-99) plays an important role in NGF binding to TrkA and defining the ligand specificity (Ibáñez *et al.*, 1991; Kullander and Ebendal, 1994; LeSauteur *et al.*, 1995). This controversy may indicate that the L4 loop interacts with a different epitope on TrkA, namely with residues on a membrane-proximal linker region that were not present in the crystals and where L4 loop projects. These results also demonstrate the limitations of the approach of studying a single receptor domain when multiple epitopes may be involved in ligand binding.

Lipophilic transmembrane domain stabilizes the receptor on cell surface and plays an important role in ligand activation of the receptor. It was shown that while substitution of TrkA ECD with the ECD of Tumor Necrosis Factor (TNF) receptor 2 renders a fully functional chimeric receptor, an additional substitution of transmembrane domain leads to the loss of receptor function (Rovelli *et al.*, 1993).

Intracellular part of TrkA includes a protein tyrosine kinase (PTK) domain structurally similar to kinase domains of other growth factor receptors (Ullrich and Schlessinger. 1990). Its function, in turn, is regulated by phosphorylation of certain tyrosine residues. Phosphorylation of residuesY670, Y675 and Y674 in human TrkA is critical for receptor tyrosine kinase activity (Segal *et al.*, 1996; Cunningham *et al.*, 1997). Phosphorylation of other tyrosine residues often serves as a regulatory signal for binding and activation of specific messenger proteins. Juxtamembrane domains of Trk receptors contain a conserved KFG sequence that is necessary for activation-independent binding of a putative messenger protein SNT (Rabin *et al.*, 1993; Peng *et al.*, 1995). As other protein kinases, TrkA contains an ATP binding site considered as a convenient target epitope for the design of specific PTK inhibitors.

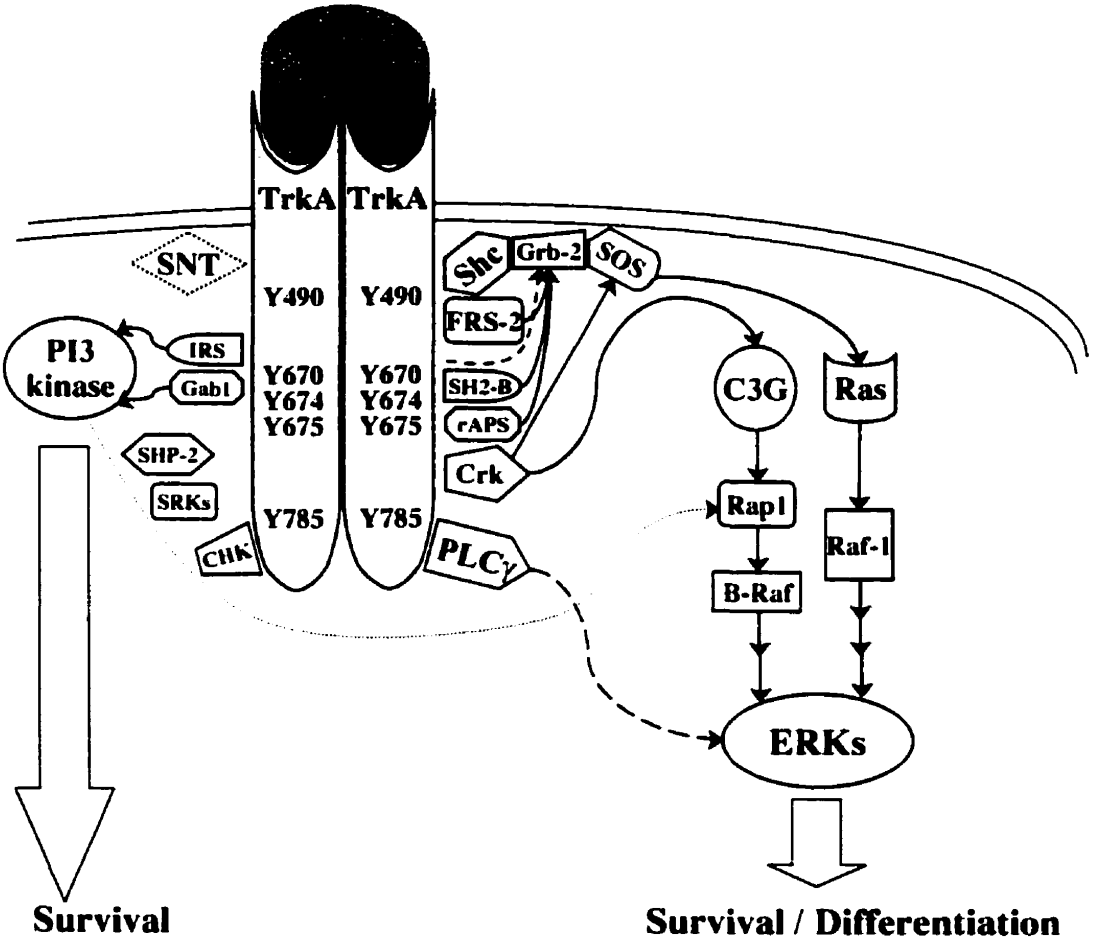
Early events in TrkA activation and signal transduction

It is commonly accepted that the mechanisms of TrkA activation fall into the general conventional scheme established for receptor tyrosine kinases. The first step is a ligand-induced receptor dimerization or oligomerization, universal for cell surface receptors with a single transmembrane region (reviewed by Heldin, 1995). Resulting juxtaposition of the cytoplasmic tyrosine kinase domains in a proper relative orientation leads to trans-phosphorylation of tyrosines in the activation loop (Cunningham *et al.*, 1997) and complete kinase activation. Kinase-active cytoplasmic domains are capable of phosphorylation of specific tyrosine residues outside of the activation loop responsible for the binding and activation of secondary signaling proteins (Segal *et al.*, 1996; reviewed by Friedman and Green, 1999).

Tyrosine residues 670, 674 and 675 in human TrkA have been demonstrated as activation loop tyrosine in mutagenesis studies (Cunningham *et al.*, 1997). Further structure-function studies and molecular modeling based on the resolved crystal structures of the active and inactive forms of the insulin receptor kinase domain lead the authors to the following model (Cunningham and Green, 1998). In an inactive state activation loop blocks substrate access to the kinase catalytic core. Upon activation phosphotyrosines in the activation loop form specific charge pairs with nearby basic residues. The charge pairs stabilize a functionally active conformation in which kinase catalytic center is open to a substrate.

Several secondary signal transduction molecules rapidly become tyrosine phosphorylated upon TrkA activation. Among them phospholipase C-gamma (PLCγ) (Ohmichi *et al.*, 1991; Vetter *et al.*, 1991), Shc (Obermeier *et al.*, 1993; Stephens *et al.*, 1994) and FRS-2 (Kouhara *et al.*, 1997; Meakin *et al.*, 1999) have been shown to associate directly with the activated TrkA. Recently two novel Trk substrates rAPS and SH2-B, have been identified in developing sympathetic neurons (Qian *et al.*, 1998) and a neuroblastoma cell line (Eggert *et al.*, 2000). They are closely related to other Src homolog 2 (SH2) domain-containing signaling molecules.

Figure 3. Early events in TrkA signal transduction



The phosphorylated tyrosine Y490 of human TrkA has been identified as an out of the activation loop residue responsible for the binding and activation of Shc (Obermeier *et al.*, 1993; Obermeier *et al.*, 1994; Segal *et al.*, 1996). Interaction of phosphorylated Shc with Grb2-SOS complexes leads to activation of a small G-protein Ras and Ras-mediated signaling cascades (Basu *et al.*, 1994) involving Raf-1, MEK, MAP kinase and ERKs. Unlike many other similar adapter proteins that interact with receptor tyrosine kinases by the way of their Src homology 2 (SH2) domains, Shc docks to TrkA via its phosphotyrosine-binding (PTB) domain (Dikic *et al.*, 1995). Various isoforms of Shc and Shc analogs that become phosphorylated in response to NGF have been found in neurons and neuronal cell lines. Among them several isoforms of ShcA and one isoform of ShcC in primary dorsal root ganglia (DRG) cultures and a different isoform of ShcA in PC-12 cells (Ganju *et al.*, 1998). N-Shc and Sck were identified in human and rat central nervous systems (Nakamura *et al*, 1998). The apparent diversity in Shc isoform profile may play a role in regulation of neurotrophin-mediated signaling in different cell types and subtypes. However it remains to be demonstrated that activation of different Shc analogs can lead to different functional responses.

FRS-2 is a second signaling adapter protein which activation requires the phosphorylation of tyrosine Y490 of TrkA, that competes with Shc for TrkA binding (Meakin *et al.*, 1999) and which activation by TrkA leads to the recruitment of Grb2 followed by initiation of Ras-mediated signaling pathways. Initially identified as a long sought docking protein that links Fibroblast Growth Factor (FGF) receptor activation to the Ras activation, FRS-2 was also shown to mediate Trk receptor signals (Kouhara *et al.*, 1997; Meakin *et al.*, 1999; Easton *et al.*, 1999; Ong *et al.*, 2000). Like Shc, FRS-2 binds TrkA via its PTB domain. In contrast to the demonstrated mechanisms of FRS-2 interaction with TrkA, its binding to FGF receptor is independent of the receptor activation and occurs via a specific juxtamembrane motif (Xu *et al.*, 1998).

It appears that there is a considerable redundancy in signaling pathways connecting TrkA activation by NGF to the recruitment of Grb2/SOS complexes and activation of Ras-mediated signaling (Figure 2). Besides Shc and FRS-2, the newly identified Trk substrates rAPS and SH2-B can also bind Grb2 (Qian *et al.*, 1998). In

addition to that it was recently shown that Grb2 can interact directly with the activated rat TrkA independently of binding and activation of Shc, FRS-2, PLCgamma-1, rAPS or SH2B (MacDonald *et al.*, 2000). Interestingly, it appears that activation loop tyrosines Y683 and Y684 of mouse TrkA (analogous to human TrkA Y674 and Y675) along with a carboxy-terminal tyrosine Y794 (analogous to human TrkA Y785) participate in substrate binding and not only in substrate activation in this case.

Crk is another adapter protein that interacts with Trk complexes upon NGF activation and becomes tyrosine phosphorylated (Torres and Bogenmann 1996; Ribon and Saltiel 1996). Activated Crk can engage SOS and another guanine nucleotide-releasing protein C3G (Matsuda *et al.*, 1994). While SOS is linked to the Ras pathway, C3G can initiate a parallel cascade that involves Rap1 and B-Raf. (York *et al.*, 1998) and converges with Ras pathway at the level of ERKs activation.

Tyrosine Y785 in the carboxy-terminal part of human TrkA is a component of a consensus site for the PLCγ SH2 domain binding (Mohammadi *et al.*,1991; Larose *et al.*, 1993). It is required for PLCγ interaction with NGF-activated TrkA and subsequent PLCγ tyrosine phosphorylation (Obermeier *et al.*, 1993; Loeb *et al.*, 1994). Activated PLCγ releases lipid second messengers DAG and IP3 that results in a signaling cascade leading to phosphorylation and activation of MAP kinase. MAP kinase, in turn, is one of the intermediates in a pathway initiated with the activation of Ras.

The same tyrosine Y785 epitope of TrkA is responsible for binding and activation of a recently identified regulatory messenger Csk homologous kinase (CHK) (Grgurevich *et al.*, 1997; Yamashita *et al.*, 1999). The Csk tyrosine protein kinases phosphorylate the carboxy-terminal tyrosine of Src-related kinases in vitro and repress their activity. Their functions in mammalian cells and particularly in the nervous system are not well studied yet. It was, however, shown that in PC-12 cells CHK is involved in neurite outgrowth in response to NGF. It was also found that, similarly to PLCγ, CHK docks to TrkA via its SH2 domain and CHK over-expression leads to enhanced activation of the MAP kinase pathway upon NGF stimulation.

Another major signal transduction cascade initiated upon NGF binding to TrkA involves the activation of phosphoinositol 3 (PI3) kinase. The TrkA epitope containing tyrosine Y751 within the kinase domain forms a consensus sequence for PI3 kinase binding. However, experimental evidence suggests that Y751 is not directly involved in PI3 kinase activation but rather plays a role in stabilizing the activated conformation of the receptor (Friedman and Green, 1999). Thus, it appears that PI3 kinase activation occurs indirectly via some secondary messenger(s). The possible candidates for this role are insulin receptor substrates (IRS) and IRS-like Grb-associated binder-1 (Gab1) proteins. It was found that Gab1 in NGF-stimulated PC12 cells (Holgado-Madruga *et al.*, 1997), and IRS-1 and IRS-2 in BDNF-activated cultured cortical neurons (Yamada *et al.*, 1997) underwent tyrosine phosphorylation leading to their association with PI3 kinase and induction of PI3 kinase signaling cascades.

Among the other proteins that undergo rapid phosphorylation upon Trk activation are the protein tyrosine phosphatase SHP-2 (Okada *et al.*, 1996; Goldsmith and Koizumi, 1997; Yamada *et al.*, 1999) and src family members (Sato *et al.*, 1998; Marchetti *et al.*, 1998; Iwasaki *et al.*, 1998). The mechanisms of their activation, like for IRS and Gab1, are unclear. All this proteins can utilize their SH2-SH3 domains to associate directly with Trks or with known primary interactors FRS-2 and Shcs or with each other or with yet unidentified adapter proteins.

Receptor internalization and intracellular transport plays an important role in neurotrophin signaling. Internalization serves as a negative feedback mechanism in the regulation of receptor number on the cell surface. In the case of neurotrophins the hormone is often secreted by a target tissue in the periphery and the signals have to be transported along the axon to the cell body. This retrograde action is carried out by the means of receptor internalization and transportation of receptor-ligand complexes (Bhattacharyya *et al.*, 1997; Grimes *et al.*, 1997; reviewed by Mufson *et al.*, 1999). NGF internalization and transport requires TrkA activation and signal transduction since it can be blocked by the inhibitors of Trk protein tyrosine kinase or PI3 kinase (Reynolds *et al.*, 1998; Reynolds *et al.*, 1999). In addition it was shown that in compartmental cultures

distally applied NGF could trigger rapid TrkA phosphorylation in the cell body before the transport of NGF could occur (Senger and Campenot, 1997).

Taken together these observations indicate that two distinctive features characterize TrkA activation and signal transduction. The first is a multiplicity of primary signaling events that can be initiated upon TrkA activation. As a result, even very early events in TrkA signaling can be regulated at numerous points that include several primary messenger and adapter proteins, potential negative regulators like Shp-2 and CHK and poorly studied mediators of TrkA-NGF internalization and trafficking.

The second feature is that these multiple signaling cascades seem to converge into a few downstream pathways leading to activation of ERKs and PI3 kinase. The later pathway plays a major role in promoting cell survival (Yao and Cooper, 1995; Philpott *et al.*, 1997; Crowder and Freeman, 1998), while the first can mediate both survival and differentiation signals. It has been recently shown that PI3 kinase pathway may also lead to activation of ERKs via a small G protein Rap1 (York *et al.*, 2000) This leaves open an important question of how seemingly opposite trophic/mitogenic and differentiation effects of neurotrophins are so precisely regulated. One apparent answer is that there may be yet unknown or not well studied additional signaling mechanisms. For example, Suc-Associated Neurotrophic Factor-Induced Tyrosine Phosphorylated Target (SNT) has been long considered as a part of Trk signaling machinery (Rabin *et al.*, 1993; Peng *et al.*, 1995). Then, recently cloned FRS-2 adapter protein has been characterized as being equivalent to SNT (Kouhara *et al.*, 1997). However, certain FRS-2 attributes, like intracellular distribution and activation-independent association with Trks, do not match known properties of SNT. This leaves viable the hypothesis that SNT is a separate from FRS-2 messenger specific for differentiation-type neurotrophin signals. The other widely considered notion is that not only ERK activation on itself regulates cell growth and differentiation, but also the kinetics (sustained versus transient) and the level of activation can play decisive roles in cellular responses to NGF. In this case the multiple regulation elements on the early level of TrkA signaling upstream of the points of convergence can provide the necessary means for the fine tuning of cell function.

5. $P75^{NTR}$ structure, signal transduction and functions.

$P75^{NTR}$ neurotrophin receptor belongs to the Tumor Necrosis Factor (TNF) receptor family. It shares common structural features with type I and type II TNF receptors ($p55^{TNFR}$ and $p75^{TNFR}$), CD-40, FAS, OX40, CD30, CD27, DR3, DR4, DR5 and 4-1 BB (reviewed by Naismith and Sprang, 1998). It is a glycoprotein with a single transmembrane domain. Extracellular part contains four cysteine-rich domains (CRDs) all of which are necessary for neurotrophin binding (Yan and Chao, 1991; Baldwin *et al.*, 1992; Baldwin and Shooter; 1994). No direct structural studies of $p75^{NTR}$ ECD have been done and the current models are based on resolved crystal structure of $p55^{TNFR}$ (TNF Receptor 1) ECD (D'Arcy *et al.*, 1993; Banner *et al.*, 1993; Naismith *et al.*, 1996a,b) and limited mutagenesis studies (Baldwin and Shooter; 1994; Baldwin and Shooter; 1995).

Extracellular domain and neurotrophin binding

The results of mapping studies suggest that the $p75^{NTR}$ binding epitopes of neurotrophins consist of a number of juxtaposed positively charged amino acid residues. These residues are located in the two adjacent turn regions L1 (residues 30-35 in human NGF) and L4 (residues 92-98 in human NGF) (Drinkwater *et al.*, 1991; Ibáñez *et al.*, 1992; Ibáñez, 1994; Rydén *et al.*, 1995; Rydén and Ibáñez, 1996). The particular residues crucial for p75 binding vary among neurotrophins. Loop L1 residues Arg31 and His33 or Arg34 and Arg36 mediate binding of NT-3 and NT-4/5 respectively. Loop L4 residues Lys95, Lys96 and Arg97 are critical for BDNF binding, while residues Lys32, Lys34 and Lys95 in both L1 and L4 loops are thought to participate in NGF binding. In addition in the case of NGF loop L3 residues Asp72, Lys74 and His75 may form a second binding epitope (Rydén and Ibáñez, 1997) and residues Trp21, Asp30, Ile31 Glu35, Lys88, Arg100 and Arg103 were found to play a role in $p75^{NTR}$ binding. The variability in $p75^{NTR}$ docking sites among neurotrophins correlates with significantly different kinetics and biological effects of $p75^{NTR}$ binding by different ligands.

Crystallographic studies of $p55^{TNFR}$ ECD and molecular modeling suggest that cysteine-rich domains of TNF family receptors fold independently from each other and form two modular units (Naismith and Sprang, 1998). Two molecular modeling efforts have been reported for neurotrophin binding to $p75^{NTR}$. The first study (Chapman and Kuntz. 1995) suggested that only CRDs III and IV of $p75^{NTR}$ participated in NGF binding. This conclusion is not consistent with the results of a mutagenesis study showing a crucial role of CRD II (Baldwin and Shooter; 1995). The second study resulted in a model suggesting that $p75^{NTR}$ can interact with different binding epitopes of neurotrophins via the same set of negatively charged residues on CRDs I and II. The later model conforms with epitope mapping studies and to the fact that all neurotrophins bind $p75^{NTR}$ with similar affinities. However, it does not offer an explanation for variations in biological effects of $p75^{NTR}$ binding by different ligands. Further crystallographic studies are likely necessary in order to provide more information on the structure of $p75^{NTR}$ ECD and its binding of neurotrophin ligands.

Intracellular domain and signal transduction

The intracellular domain (ICD) of $p75^{NTR}$ can be divided into two subdomains on the basis of their sequence similarity. The membrane-proximal portion has little homology to the other members of TNF receptor family but highly conserved between species (Barrett. 2000). The carboxy-terminal part is similar to the so-called death domains of some TNF receptor family proteins ($p55^{TNFR}$, FAS, DRs) and intracellular pro-apoptotic proteins such as FADD, TRADD and RIP (Chapman, 1995). The solution structure of $p75^{NTR}$ ICD was resolved by nuclear magnetic resonance (NMR) (Liepinsh *et al.*, 1997). The carboxy-terminal death domain folds into two perpendicular sets of three helices packed into a globular structure that is generally similar but subtly different from the resolved structure of Fas death domain (Huang *et al.*, 1996). It was also found that unlike the death domains of known pro-apoptotic proteins, $p75^{NTR}$ ICD does not self-associate *in vitro*. Even though $p75^{NTR}$ has been implicated in induction of apoptosis, it is not clear whether its death domain participates in pro-apoptotic signaling. Homology to

the death domain has been found in diverse proteins with no demonstrated role in regulation of cell death (Feinstein *et al.*, 1995).

The precise mechanisms of $p75^{NTR}$ signal transduction and participation in neurotrophin signaling are still subjects of intense research. Nevertheless, considerable knowledge has been accumulated on signaling pathways induced upon $p75^{NTR}$ expression and binding.

Generation of a lipid second messenger ceramide, a product of sphingomyelin hydrolysis, in response to $p75^{NTR}$ binding with NGF has been reported (Dobrowsky *et al.*, 1994). Interestingly, while all neurotrophins could induce sphingomyelin hydrolysis in the cells that expressed $p75^{NTR}$ but not Trks, NT-3 but not NGF treatment led to elevation in ceramide level in PC-12 cells (Dobrowsky *et al.*, 1995). In cultured neo-natal rat oligodendrocytes only NGF but not BDNF or NT-3 treatment lead to ceramide release (Casaccia-Bonnefil *et al.*, 1996). The functional role(s) for ceramide in neurotrophin signaling is unclear. Elevated ceramide levels have been detected in response to activation of pro-apoptotic $p55^{TNFR}$ (Mathias and Kolesnick, 1993) and Fas (Tepper *et al.*, 1995). Thus, ceramide has been thought as an apoptosis-mediating messenger. However, well characterized pro-apoptotic pathways induced by $p55^{TNFR}$ and Fas do not require or involve ceramide (reviewed by Baker and Reddy, 1998). It is possible that ceramide pathway can modulate main apoptotic signaling cascades or be induced collaterally. In the case of neurotrophin signaling the role of ceramide seems to be complex and dependent on cellular context. Increase in ceramide level coincided with cell death for NGF-treated cultured oligodendrocytes (Casaccia-Bonnefil *et al.*, 1996). In PC-12 cells short-term treatment with the cell-permeable ceramide analog C2-ceramide led to a negative regulation as determined by inhibition of TrkA signaling (MacPhee and Barker, 1997). However long-term exposure to ceramide analogues or ceramide resulted in increased survival of cultured sensory neurons (Ping and Barrett, 1998) and PC-12 cells (Barrett 2000), and enhanced TrkA signaling (MacPhee and Barker, 1999). NGF treatment of neuronal cultures from rat cerebral cortex that express $p75^{NTR}$ and TrkB but not TrkA led to production of ceramides and protected neurons from delayed cytotoxicity induced by brief exposure to glutamate (Kume *et al.*, 2000). Interestingly, ceramide but not ceramide

analogues caused cell death in the presence of an inhibitor of downstream ceramide conversion to sphingosine (Ping and Barrett, 1998). Recent studies have shown that initiation of sphingolipid signaling pathways is often localized to caveolae, small invaginations in cell membrane enriched in cholesterol and cholesterol binding protein caveolin, or so called caveolae-like domains (CLDs) (reviewed by Dobrowsky, 2000). Caveolae and CLDs are thought to be clustering sites of cell surface receptors and membrane-anchored signal transduction molecules, including both $p75^{NTR}$ and TrkA (Huang *et al.*, 1999; Bilderback *et al.*, 1999). Accordingly, $p75^{NTR}$-regulated ceramide release may transduce - $p75^{NTR}$ signals leading to the regulation of TrkA function.

Activation of c-Jun N-terminal kinase (JNK, alternatively called stress-activated terminal kinase – SAPK) has been identified as a part of signal transduction of several cytokine receptors including TNF family receptors (Schulze-Osthoff *et al.*, 1998). JNK is also activated in response to different stress stimuli including heat and cold shock, osmotic shock, ultraviolet irradiation, treatment with toxins and deprivation of growth factors (reviewed by Ip and Davis, 1998). JNK signaling is associated with induction of apoptosis and JNK knockout mice exhibited reduced developmental apoptosis (Kuan *et al.*, 1999), impaired T-cell activation and apoptosis (Dong *et al.*, 1998; Sabapathy *et al.*, 1999) and reduced kainic acid-induced hippocampal neuron apoptosis (Yang *et al.*, 1997). However, since so many stress stimuli lead to JNK activation it is expected that the effects of JNK activation are tightly regulated, context-specific and not always pro-apoptotic (reviewed by Leppa and Bohmann, 1999).

JNK activation and induction of apoptosis in response to NGF treatment has been reported for neo-natal rat oligodendrocytes that express $p75^{NTR}$ but not TrkA (Casaccia-Bonnefil *et al.*, 1996; Yoon *et al.*, 1998). Authors of the later study found that a downstream alkaloid inhibitor of JNK blocked NGF-induced apoptosis. However, in a different study no JNK activation or cell death has been detected for NGF-treated adult human oligodendrocytes (Ladiwala *et al.*, 1998). It is also not clear whether $p75^{NTR}$-mediated activation of JNK can occur in the cells expressing both TrkA and p75. JNK activation upon NGF withdrawal has been observed in cultured sensory neurons (Virdee *et al.*, 1997) and NGF-dependent PC12 cells (Xia *et al.*, 1995). These observations

suggest that in certain cellular context unliganded $p75^{NTR}$ can activate JNK pathway, or that JNK activation upon NGF withdrawal may be induced via mechanisms independent of $p75^{NTR}$. BDNF but not NGF induced c-jun phosphorylation in cultured sympathetic neurons that express $p75^{NTR}$ and TrkA but not TrkB (Bamji *et al.*, 1998). Transfection and expression of TrkA in rat oligodendrocytes resulted in the absence of JNK activation in response to NGF and reversed NGF effects from pro-apoptotic to trophic (Yoon *et al.*, 1998). These observations led the authors to the notion that independent competitive signaling of TrkA and p75NTR determines activation of downstream transduction pathways leading to survival or apoptotic death.

Nuclear Factor-kappaB (NF-κB) is a DNA-binding protein that plays an important role in signal transduction of TNF family receptors. Receptor-induced changes in NF-κB phosphorylation status lead to its translocation from cytoplasm to the nucleus, binding to specific regulatory DNA sequences and regulation of gene expression. NF-κB is activated rapidly after TNF binding to $p55^{TNFR}$ (reviewed by Schutze *et al.*, 1995; Magnusson and Vaux, 1999). NF-κB activation is considered as a trophic signal in TNF signaling, presumably by activating a number of anti-apoptotic genes (reviewed by Van Antwerp *et al.*, 1998). Nevertheless, some studies suggest that the effects of NF-κB activation are context-specific and may be pro-apoptotic in certain circumstances (reviewed by Karin, 1998; Ward *et al.*, 1999; Borset *et al.*, 1999; Kuhnel *et al.*, 2000; Kaltschmidt *et al.*, 2000).

$P75^{NTR}$, like other members of the TNF receptor family, can mediate NF-κB activation. Nuclear translocation and DNA binding of NF-κB in response to NGF treatment have been reported for the cultured Schwann cells and cell lines that express $p75^{NTR}$ but not TrkA (Carter *et al.*, 1996; Gentry *et al.*, 2000). In contrast, in a different study NGF treatment did not activate NF-κB in several cell lines that did not express TrkA and transiently or stably expressed $p75^{NTR}$ (Bhakar *et al.*, 1999). NGF-dependent induction of NF-κB was detected only after cells were subjected to severe stress, while NGF consistently enhanced TNF-dependent NF-κB activation under normal growth conditions. These results led the authors to the conclusion that the reports of direct NF-

κB activation through $p75^{NTR}$ either reflected its modulatory activity on cytokine receptor signaling or were the results of experimental artifacts when cells were subjected to severe stress prior to neurotrophin treatment. Nevertheless, $p75^{NTR}$-mediated NF-κB activation or enhancement of NF-κB activation exerted anti-apoptotic effects. In the case of cultured neurons and neuron-like cell lines that express both TrkA and p75, NF-κB activation was observed only after prolonged (24 hours) treatment (Wood, 1995) but inhibition of NF-κB function led to pro-apoptotic effects (Taglialatela *et al.*, 1997; Maggirwar *et al.*, 1998; Hamanoue *et al.*, 1999). There were, however, reports when $p75^{NTR}$-mediated NF-κB activation associated with apoptosis (Casaccia-Bonnefil *et al.*, 1996; Kuner and Hertel; 1998; Yoon *et al.*, 1998) implying that the anti- or pro-apoptotic effects of NF-κB may be regulated by activated TrkA.

While the signaling complexes of $p55^{TNFR}$ and Fas with primary messenger proteins have been described in considerable detail, until recently very little was known about early events in $p75^{NTR}$ activation and signal transduction. TNF-induced signaling complexes included TNF receptor-associated death domain protein (TRADD), Fas-associated death domain protein (FADD), protein kinase RIP and TNF receptor-associated factor 2 (TRAF2) (reviewed by Baker and Reddy, 1998). FADD and TRADD contain Death Domains capable of homodimerization or heterodimerization with other Death Domain proteins. Recruitment of FADD leads to activation of Pro-caspase-8 and initiation of caspase-mediated pro-apoptotic pathways. No $p75^{NTR}$ signaling complexes with FADD, TRADD or Pro-caspase-8 has been reported.

It was recently shown that human $p75^{NTR}$ could be immunoprecipitated first with rat TRAF6 (Khursigara *et al.*, 1999) and, then, with 5 other TRAF family proteins (Ye *et al.*, 1999). TRAF2 associated with the carboxy-terminal of $p75^{NTR}$ containing death domain. Conversely, TRAF4 and TRAF6 interacted with the juxtamembrane region of the receptor. TRAF2, TRAF4 and TRAF6 were shown to influence cell survival and NF-κB activation associated with $p75^{NTR}$ expression and external dimerization, while no biological effects of other TRAF protein interaction with $p75^{NTR}$ were reported. TRAF6 and TRAF2 enhanced and TRAF4 inhibited limited NF-κB activation induced by $p75^{NTR}$

expression. Accordingly, TRAF6 protected the cells from apoptosis caused by over-expression of unliganded $p75^{NTR}$ and TRAF4 effects were pro-apoptotic However TRAF2 co-expression with $p75^{NTR}$ led to enhanced cell death in the absence of a receptor ligand. It appears that the interaction of TRAFs with $p75^{NTR}$ can be differentially regulated by receptor dimerization. TRAF6 and TRAF4 association with $p75^{NTR}$ required receptor binding with NGF (Khursigara *et al.*, 1999) or artificial ligands capable of dimerizing a tag fused with $p75^{NTR}$ ICD (Ye *et al.*, 1999). TRAF2 preferentially associated with unliganded $p75^{NTR}$. While these studies show that interactions between $p75^{NTR}$ and TRAFs are possible, it is not known whether the observed $p75^{NTR}$/TRAF signaling complexes are physiologically relevant since both $p75^{NTR}$ and TRAFs were transiently over-expressed to high levels.

A novel protein termed the Neurotrophin Receptor Interacting Factor (NRIF) has been isolated and characterized on the basis of its ability to interact with $p75^{NTR}$ ICD (Casademunt *et al.*, 1999). In yeast two hybrid system NRIF interacted with both amino-terminal juxtamembrane portion of $p75^{NTR}$ ICD and carboxy-terminal portion containing death domain. NRIF appears to be involved in pro-apoptotic signaling. Its over-expression in cultured cells led to cell death and the disruption of *nrif* gene resulted in reduced apoptosis in the mouse embryonic neural retina. NRIF sequence includes five zinc finger motifs and a potential nuclear localization signal. NRIF was localized primarily in the nuclei when it was over-expressed in 293 cells. Co-expression of $p75^{NTR}$ in the absence of the receptor ligand led to partial NRIF translocation to the cytoplasm and cell membrane and to reduction in cell death caused by NRIF expression.

A second novel protein containing six zinc finger motifs was also identified on the basis of its ability to interact with $p75^{NTR}$ ICD in yeast two hybrid system (Chittka and Chao, 1999). The protein, termed SC-1, associated with the juxtamembrane domain of the receptor but, unlike NRIF, did not interact with the death domain. In contrast to NRIF, SC-1 localized in the cytoplasm when $p75^{NTR}$ was not expressed or expressed but unbound. $P75^{NTR}$ binding with NGF but not BDNF or NT-3 resulted in predominantly nuclear localization of SC-1. Interestingly, serum starvation also led to nuclear translocation of SC-1, indicating that there may be $p75^{NTR}$-independent regulation of SC-1. TrkA

expression reversed SC-1 distribution to mostly cytoplasmic in both cases. Nuclear localization of SC-1 appeared to associate with growth arrest. Therefore, like in the case of NRIF, it served as a negative regulatory factor.

Two more putative $p75^{NTR}$-interacting proteins have been recently identified in the yeast two hybrid system. Neurotrophin receptor-interacting MAGE homolog (NRAGE) has been shown to associate with $p75^{NTR}$ and translocate to the plasma membrane upon $p75^{NTR}$ binding with NGF (Salehi *et al.*, 2000). Authors suggest that NRAGE can block direct interaction of $p75^{NTR}$ with TrkA and mediate NGF-dependent cell death. $P75^{NTR}$-associated cell death executor (NADE) specifically interacted with the death domain of $p75^{NTR}$ and its co-expression with the receptor led to apoptotic cell death induced by NGF but not BDNF, NT-3 or NT-4/5 (Mukai *et al.*, 2000).

$P75^{NTR}$ and Trks

Apart from putative Trk-independent signaling, arguably the most prominent effects of $p75^{NTR}$ expression remain (i) taking part in the formation of high affinity binding sites for the neurotrophins, (ii) cooperation with Trk receptors in neurotrophin signaling and (iii) regulation of the specificity of Trks for their respective neurotrophin ligands. Examples abound when $p75^{NTR}$ binding enhances biological effects of neurotrophins. When co-expressed with Trks in cultured fibroblasts, $p75^{NTR}$ potentiated functional responses to the corresponding neurotrophins (Hantzopoulos *et al.*, 1994). Interestingly, the truncated form of $p75^{NTR}$ with only a part of a juxtamembrane domain of the ICD remaining was more effective in the potentiation of neurotrophin effects. Blocking the NGF binding to $p75^{NTR}$ with high doses of anti-receptor antibody or BDNF reduced NGF binding affinity and functional responses in PC12 cells (Barker and Shooter, 1994). Co-expression of $p75^{NTR}$ enhanced the NGF-induced tyrosine autophosphorylation of TrkA and neuronal differentiation in a human neuronal progenitor cell line (Verdi, *et al.*, 1994). $P75^{NTR}$ binding with NGF enhanced the TrkA-mediated protein kinase activity in PC12 cells (Canossa *et al.*, 1996). A peptide mimic of the part of the $p75^{NTR}$ death domain enhanced NGF-dependent TrkA phosphorylation and

differentiation in cultured human neuroblastoma cells but had no effect on NGF-mediated cell survival (Wang *et al.*, 1998). Antibodies that block binding of NGF to the $p75^{NTR}$ reduced the NGF survival response in cultured mouse trigeminal neurons and prevented NGF-induced NF-κB activation (Hamanoue *et al.*, 1999).

There are examples when $p75^{NTR}$ ability to potentiate trophic effects of NGF depends on cell type and the developmental state of the cells. While $p75^{NTR}$ antisense oligonucleotides had no effect on NGF-mediated survival of cultured sensory neurons taken from newborn or P2 mice, they blocked the ability of NGF to rescue E15 neurons (Barrett and Bartlett, 1994). The NGF mutant that binds TrkA but not $p75^{NTR}$ was less effective than wild-type NGF in promoting the survival of cultured embryonic sensory neurons and postnatal sympathetic neurons (Horton *et al.*, 1997). Interestingly, the NGF mutant was equally effective as wild-type NGF in promoting the survival of embryonic sympathetic neurons where the level of $p75^{NTR}$ expression was significantly lower. In another study, where authors utilized the same type of $p75^{NTR}$-non-binding NGF mutant, they came to the conclusion that $p75^{NTR}$ enhanced cell responsiveness to NGF, particularly when it was present at limiting concentrations and/or during development in neurons undergoing a down-regulation of NGF receptors (Ryden *et al.*, 1997b).

Mice carrying a mutation of the $p75^{NTR}$ gene that prevents NGF binding have been generated (Lee *et al.*, 1992). These mice had markedly decreased sensory innervation that correlated with loss of heat sensitivity and associated with deficiency in skin maintenance. Crossing a transgenic mice encoding human $p75^{NTR}$ into the mutant animals reversed the observed adverse effects of $p75^{NTR}$ mutation. Cultured sensory and sympathetic neurons from $p75^{NTR}$ deficient mice exhibited reduced sensitivity to NGF but, interestingly, not to other neurotrophins (Davies *et al.*, 1993; Lee *et al.*, 1992). $P75^{NTR}$ deficient mice also displayed significant reduction of basal forebrain volume and had a significant loss in number of forebrain neurons (Peterson *et al.*, 1999). At the same time, as it has been mentioned above, a number of studies indicate that neuronal apoptosis may be impaired in $p75^{NTR}$ deficient mice (Bamji *et al.*, 1999; Frade and Barde, 1999; Ferri and Bisby, 1999; Hannila and Kawaja, 1999).

There is considerable evidence that in addition to regulating neurotrophin binding to Trks and Trk signaling $p75^{NTR}$ may enhance the specificity of the Trks to their preferred neurotrophin ligands. Fibroblasts transfected with *trks* responded better to non-preferred neurotrophins compared to corresponding PC12 transfectants that constitutively express $p75^{NTR}$ (Ip *et al.*, 1993). Co-transfection of *p75*^{NTR} into non-neural A293 cells transfected with *trkB* resulted in higher specificity for BDNF comparing to NT-3 and NT-4/5 (Bibel *et al.*, 1999). Overexpression of a dominant negative $p75^{NTR}$ mutant in PC12 cells or its block with antibodies led to increased responsiveness to NT-3, even though PC12 cells express TrkA but not TrkC (Benedetti *et al.*, 1993; Clary and Reichardt, 1994).

The questions of the mechanisms of $p75^{NTR}$ interaction and mutual regulation with Trks are intriguing. In the case of NGF one traditional explanation is that $p75^{NTR}$ acts as a recruitment factor. In this model $p75^{NTR}$s that expressed in relatively high numbers bind NGF with fast on- and off-rates and present it to TrkA in a way that regulates both NGF binding to TrkA and TrkA activation. The other group of models regards possible functional interactions between TrkA and $p75^{NTR}$ co-receptors. These models, in turn, can be divided into two general groups. One supports the notion that various biological responses to TrkA and $p75^{NTR}$ activation can be explained by independent and often competitive signaling of the receptors (reviewed by Majdan and Miller, 1999). The other concentrates on possible regulation of the co-receptor activation and early events in signal transduction.

6. Rationale and objectives of the research.

Nerve Growth Factor (NGF) receptor is an important pharmacological target in the treatment of several diseases. The functional NGF receptor is a complex system characterized by a number of distinctive features. First, it consists of two co-receptors TrkA and $p75^{NTR}$ each capable of ligand binding and signal transduction. Second, binding characteristics and activation of each co-receptor depend on the expression and occupancy state of the other. The design and development of therapeutic NGF mimetics requires detailed understanding of the structure and function of its receptors. On the other hand, artificial ligands of NGF receptors are the necessary tools for the investigation of their structure and function.

The studies presented in this Thesis pursued two major objectives. The first research objective was to investigate the molecular events required for NGF receptor activation. The second objective was to apply the acquired knowledge to the design of artificial NGF receptor ligands with a therapeutic potential.

There is a strong rationale for combining these objectives within the same research project. The functional interactions between NGF co-receptors have been studied with the artificial ligands selective to TrkA and $p75^{NTR}$ (Chapter I). The results allowed us to further modify NGF receptor ligands and use them to study certain structural requirements for agonistic NGF receptor binding (Chapter II). That, in turn, led to the development and characterization of small molecule agonistic ligands of TrkA with a novel pharmacological mechanism of action (Chapters II and III).

Overview of Chapter I

The study presented in Chapter I focused mainly on putative functional interactions between TrkA and $p75^{NTR}$ NGF receptors. The experimental approach was based on achieving separate activation of TrkA and $p75^{NTR}$ with receptor-specific ligands. This goal was accomplished by the use of agonistic anti-receptor monoclonal antibodies. These antibody-based ligands bound the receptors with high affinity independently of each other and, unlike neurotrophin-based ligands, irrespectively of co-receptor expression. Using this approach we asked the following major questions:

- Is there a functional interaction between TrkA and $p75^{NTR}$ when the co-receptors activated independently?
- Is there a negative or positive modulation of TrkA by unbound $p75^{NTR}$?
- Is there a positive or negative modulation of TrkA by bound $p75^{NTR}$?
- Does $p75^{NTR}$ modulation of TrkA signals take place on the level of TrkA activation or it is limited to downstream transduction pathways?

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

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ABSTRACT

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

INTRODUCTION

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 ~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 ~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 signals, 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 have 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 (LeSauteur 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.

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 FACScan 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) (LeSauteur 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 (LeSauteur et al., 1996). Further characterization of mAb 5C3 is published in LeSauteur 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, Mississagua, 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: $[(OD_{test}-OD_{untreated})/(OD_{\ 1\ nM\ NGF}-OD_{untreated})] \times 100$. 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 (LeSauteur 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.

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.

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

	TREATMENT ADDED TO 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 2 μg/ml	2 ± 1.2	0.7 ± 0.8	1.2 ± 2.3
11	MC192 0.2 μg/ml	-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.

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 (5pM) + 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.

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 2 μg/ml	2 ± 1.5	1 ± 4.1
5	MC192 0.2 μg/ml	6 ± 2.7 [a]	6 ± 3.5 [a]
6	MC192 0.02 μg/ml	1 ± 2.0	1 ± 4.2
7	5 pM NGF + MC192 2 μg/ml	49 ± 3.3	85 ± 6.5
8	5 pM NGF + MC192 0.2 μg/ml	86 ± 7.4	108 ± 5.6
9	5 pM NGF + MC192 0.02 μg/ml	26.2 ± 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 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 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).

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	MC192 1 μg/ml	16 ± 5.7 [a]	1 ± 1.2
7	MC192 0.1 μg/ml	8 ± 4.0	0 ± 1.8
8	5C3 1 μg/ml	42 ± 3.0	79 ± 5.2
9	5C3 0.1 μg/ml	20 ± 5.5	64 ± 5.3
10	5C3 1 μg/ml + MC192 1 μg/ml	78 ± 2.7	73 ± 3.8 [c]
11	5C3 1 μg/ml + MC192 0.1 μg/ml	118 ± 3.1 [b]	59 ± 4.9 [c]
12	5C3 0.1 μg/ml + MC192 1 μg/ml	65 ± 6.8	not tested
13	5C3 0.1 μg/ml + MC192 0.1 μg/ml	96 ± 2.3	not tested

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.

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).

Table 4. K252a inibits 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.

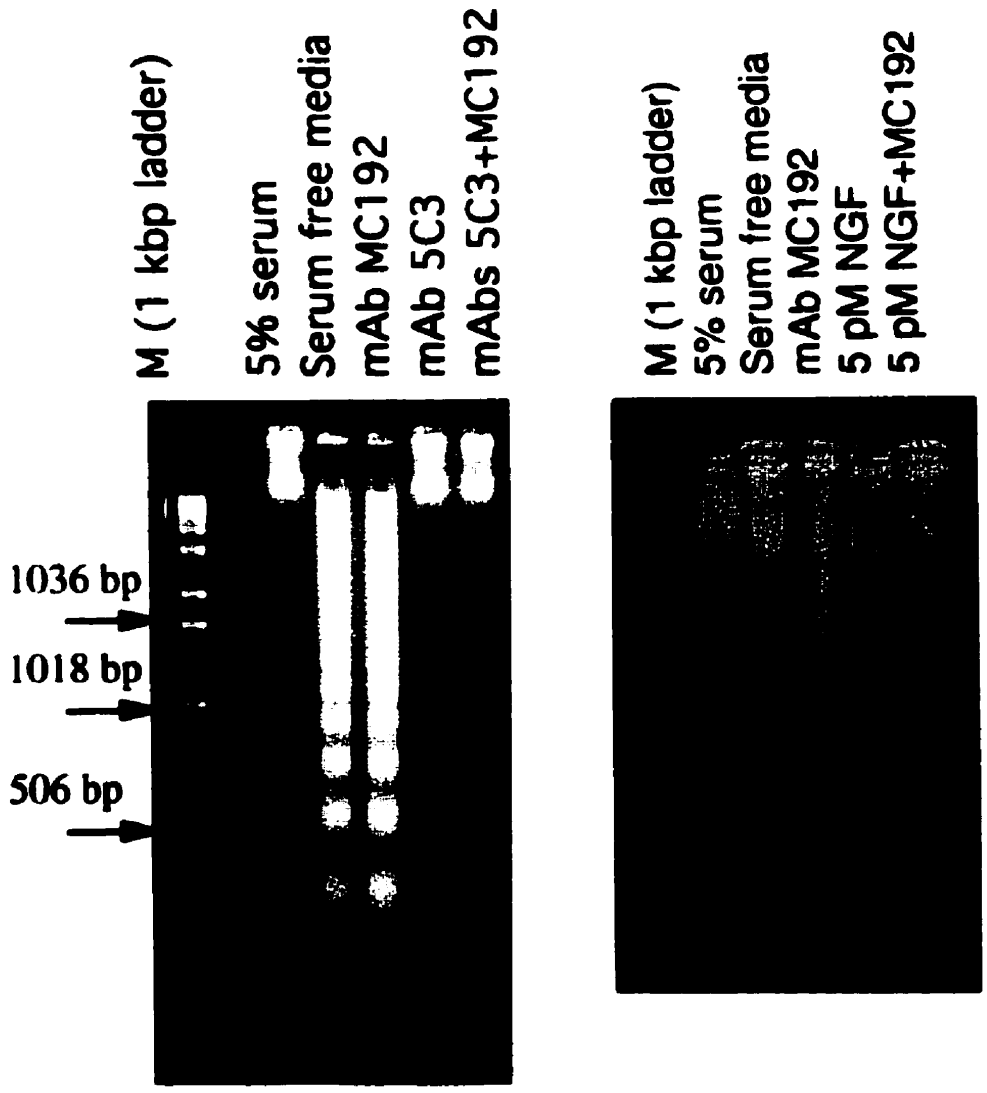

Fig. 1. Changes in apoptotic DNA degradation. Genomic DNA was extracted from (*A*) 4-3.6 or (*B*) PC12 cells cultured as indicated for 48 hr in SFM. Equal amounts from each sample were resolved on a 1.5% agarose gel and visualized with ethidium bromide. Standard molecular markers (*M*) are shown. A typical 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.

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. 1*A*). In contrast, extensive apoptotic DNA degradation is seen when 4-3.6 cells are cultured with SFM or mAb MC192 alone (Fig. 1*A*).

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. 1*B*), as expected, because this concentration of NGF affords suboptimal survival. PC12 cells cultured with SFM or mAb MC192 alone show extensive DNA degradation (Fig. 1*B*).

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 (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.

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

Cells		C10 Cells		4-3.6 Cells	
		PY total	PY 490	PY total	PY 490
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 -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.

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. 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.

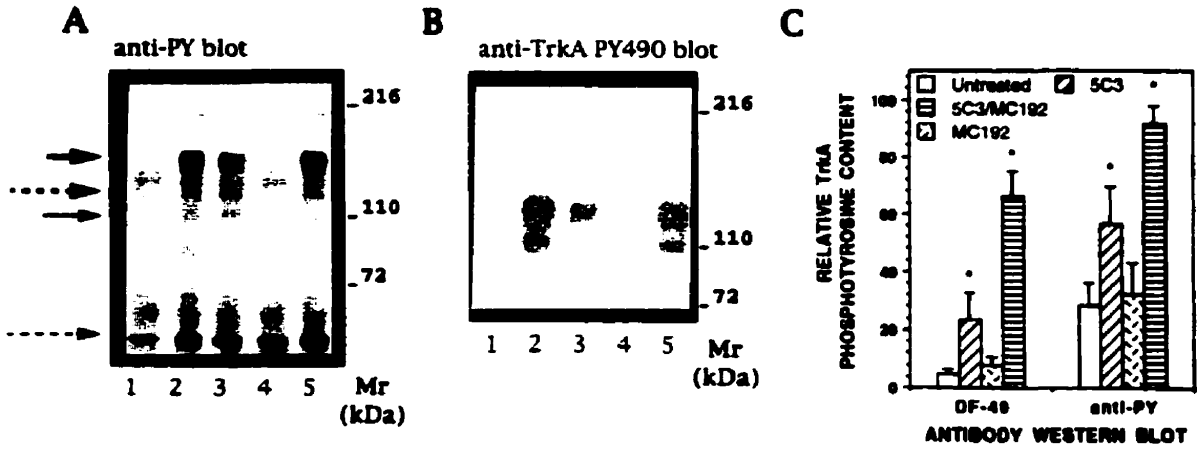

Fig. 2. Optimal TrkA tyrosine phosphorylation by concomitant binding of p75 and TrkA. 4-3.6 cells were untreated (*lane 1*) or treated with 1 nM NGF (*lane 2*), 5 nM mAb 5C3 alone (*lane 3*), 0.5 nM mAb MC192 alone (*lane 4*), or a combination of both mAbs (*lane 5*) for 15 min at 37°C. Ligand concentrations 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*, -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 ± SE; n = 5).

* indicates significant difference from untreated samples (paired Student's t tests; n = 5; $p < 0.03$).

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 incorporation 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 ~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 (LeSauteur 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 μg/ml; ~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 ~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 antimitotic 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- (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 (LeSauteur 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.

FOOTNOTES

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Connection to Chapter II.

In a study presented in Chapter I a complex two receptor system was investigated with a usage of artificial mAb ligands that bind the co-receptors irrespectively of each other expression, binding and activation. Alternative approaches to achieve a selective activation of TrkA and $p75^{NTR}$ involved the use of engineered forms of NGF incapable of binding either co-receptor or selective blocking of TrkA and $p75^{NTR}$ with an antibody (reviewed by Ibáñez, 1994). Receptor-binding and, importantly, receptor-activating properties of NGF mutants can still depend on expression of co-receptors, while blocking antibodies may display partial agonistic or inverse agonistic properties. In contrast, mAb receptor ligands afford truly independent binding and activation of TrkA and $p75^{NTR}$.

The results clearly show the existence of functional interactions between TrkA and $p75^{NTR}$. In a model based on cultured cells, TrkA activation and trophic signals were negatively regulated by $p75^{NTR}$ expression, even though TrkA ligand binding and occupancy state were not affected. At the same time the optimal trophic signals, similar to that induced by NGF, were only achieved when $p75^{NTR}$s were expressed and bound with a $p75^{NTR}$-specific ligand. Taken together these findings point out that both negative regulation of TrkA by unbound $p75^{NTR}$ and positive regulation of TrkA by $p75^{NTR}$ bound with an activating ligand can take place within the same cellular context. $P75^{NTR}$ regulation of TrkA signals occurred, at least in part, directly on the level of TrkA activation.

These findings have important implications for discovery and development of artificial neurotrophic ligands. First, it has been demonstrated that a trophic action of NGF could be mimicked by a combination of TrkA-specific and $p75^{NTR}$-specific ligands. In more general terms, this study has shown that in a two-receptor system an appropriate combination of receptor-selective artificial ligands can produce the same signals as a polypeptide hormone. The same methodology can be adapted to other therapeutically important two- or multi-receptor complexes. The examples of such systems include erb-B receptor complexes involved in the development of several malignances, Interleukin (IL)-

4 / IL-13 and IL-5 receptor complexes thought to be important in the development of asthma.

The second implication is that characterization of NGF mimetics should involve the use of models that include expression and activation of both TrkA and $p75^{NTR}$. It is still unclear what molecular mechanisms are responsible for TrkA - $p75^{NTR}$ interactions and even what constitutes $p75^{NTR}$ activation. However, from a practical point of view, it is apparent that in the design and development of NGF mimetics both co-receptors must be taken into account.

Monoclonal antibodies (mAbs) have been used as agonistic ligands of NGF receptors. Unlike NGF, mAbs dock into a small epitope rather than interact with an extended surface. And, unlike polyclonal antibodies, they are capable of a single defined mode of binding. In addition, mAbs can imitate bivalent neurotrophins in their ability to form dimeric (or higher order) complexes. The success of this methodology made possible to ask important questions pertinent to development of artificial neurotrophic ligands:

- What are the valency requirements for agonistic ligands of NGF receptors?
- Is it possible to imitate NGF binding with a small molecule ligand that, like a mAb, docks into a single small defined epitope?

These issues are remarkably significant in the design and development of therapeutic receptor ligands. Natural hormones are usually oligomeric and/or oligovalent (reviewed by Heldin, 1995). It has been traditionally thought that external cross-linking by an agonistic ligand was necessary for activation of cell surface receptors with a single transmembrane region. However, from a pharmacological point of view good drug candidates are small non-peptidic molecules, easily bioavailable and proteolytically stable. These compounds are likely to be monomeric, monovalent and able to interact only with a single defined epitope of a target receptor.

The two mentioned above questions are the main issues of the studies presented in the Chapter II. Monovalent and bivalent mAb-based ligands were used in order to model monomeric or dimeric or higher order receptor complexes. Small cyclic peptides based on

a structure of an NGF turn region were previously shown to bind TrkA and interfere with NGF binding. In the present study they were characterized as defined monomeric and monovalent TrkA ligands capable of inducing agonistic effects. The main focus was on TrkA ligands since TrkA activation leads to neurotrophic biological effects. And, importantly, potential TrkA-binding NGF mimetics were tested in a paradigm of synergy with an activating $p75^{NTR}$ ligand.

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Genuine Monovalent Ligands of TrkA Nerve Growth Factor Receptors Reveal a Novel Pharmacological Mechanism of Action*

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ABSTRACT

Developing small molecule agonistic ligands for tyrosine kinase receptors has been difficult and it is generally thought that such ligands require bivalency. Moreover, multisubunit receptors are difficult to target because each subunit contributes to ligand affinity, and each subunit may have distinct and sometimes opposing functions. Here, the NGF receptor subunits p75 and the tyrosine kinase TrkA were studied using artificial ligands that bind specifically to their extracellular domain. Bivalent TrkA ligands afford robust signals. However, genuine monomeric and monovalent TrkA ligands afford partial agonism, activate the tyrosine kinase activity, cause receptor internalization, and induce survival and differentiation in cell lines and primary neurons. Monomeric and monovalent TrkA ligands can synergize with ligands that bind the p75 subunit. However the p75 ligands used in this study must be bivalent, monovalent p75 ligands have no effect. These findings will be useful in designing and developing screens of small molecules selective for tyrosine kinase receptors, and indicate that strategies for designing agonists of multisubunit receptors require consideration of the role of each subunit. Lastly, the strategy of using anti-receptor mAbs and small molecule hormone mimics as receptor ligands could be applied to the study of many other heteromeric cell surface receptors.

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Nerve Growth Factor (NGF)[1] is a dimeric hormone composed of two identical protomers. NGF binds to either or both of two receptors termed TrkA and p75. Cells expressing TrkA bind NGF with intermediate affinity (K_d ~10^{-10} M) (1-3), and cells expressing p75 bind NGF with lower affinity (K_d ~10^{-9} M) (4). Co-expression of TrkA and p75 creates high affinity NGF binding sites (K_d ~10^{-12} M) (3), indicative of physical and functional interactions (5-8).

Agonists that activate TrkA afford protection from apoptotic cell death and neuronal differentiation and axonal growth (9). The p75 receptor mediates apoptosis in some neuronal and non-neuronal cells (reviewed in (10,11)), but it is unclear whether p75-mediated death is constitutive, induced by agonistic p75 ligands, or can be antagonized by other ligands. Culture studies where Trk-specific ligands were mixed with p75-specific ligands have shown synergy and reciprocal regulation of function (6-8,12).

TrkA is a tyrosine kinase receptor that transduces NGF signals. The dimeric NGF protein induces TrkA dimerization leading to activation of the kinase (13), as expected by analogy with other receptor tyrosine kinases (14). However, dimeric ligands do not always lead to receptor activation (15-17). Hence, the possibility that monomeric ligands could induce conformational changes leading to receptor dimerization or activation remains an attractive hypothesis (18). Since no biological studies have been done with *defined and genuine* monomeric ligands of TrkA or any other tyrosine kinase receptor, this is one aim of the present study.

Functional synergy between bivalent Trk ligands and bivalent p75 ligands, leading to enhanced Trk activation and cell survival have been reported (6,8). However, no functional studies of synergy have been done with defined and genuine monovalent ligands of p75 and TrkA. This is another aim of the present study.

To answer both aims, we used defined monovalent and monomeric ligands that bind to the extracellular domain of TrkA and p75 receptors. Specifically, we asked: (*i*)

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[1] The abbreviations used are: NGF, nerve growth factor; mAb, monoclonal antibody; DRG, dorsal root ganglia; FPLC, fast protein liquid chromatography; N-Ac, N-acetyl; SFM, serum-free medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

whether monovalent and monomeric ligands of TrkA can act as partial agonists; (*ii*) whether monomeric ligands of TrkA can synergize with ligands of p75; and (*iii*) what the valency requirement is for p75 ligands to synergize with TrkA ligands. Three sets of ligands that bind the extracellular domain of NGF receptors were available. Each ligand was used in its bivalent or monovalent state, alone or in combinations, to probe receptor function in biological and biochemical assays.

Anti-TrkA mAb 5C3 is an agonist of TrkA (19); anti-p75 mAb MC192 can synergize with TrkA ligands (6); and small molecule peptide mimics of NGF. The peptide mimics are small, conformationally constrained analogs of a single β-turn of a single NGF protomer. Peptides termed C(92-96) and C(92-97) bind TrkA *in vitro* and target TrkA-expressing cells *in vivo* (20-22). When TrkA is engaged by these peptide analogs, binding of the natural ligand NGF is antagonized (21), but a possible intrinsic activity of the peptide analogs upon binding TrkA had not been studied.

Biophysical characterization of C(92-96), described herein, defines it as a genuine monomeric and monovalent TrkA ligand. We report that genuine monovalent TrkA ligands are partial agonists and induce TrkA activation and internalization, and cell survival and differentiation. Expectedly, bivalent TrkA ligands afford more robust signals. These data challenge the exclusive notion of ligand bivalency postulated for activation of tyrosine kinase receptors. For p75, only bivalent ligands afford signals that synergize with TrkA-mediated signals. This suggests that TrkA and p75 differ in their requisites for ligand-activation. Lastly, oligomerizing ligands afford the same signals as homodimerizing ligands.

The insight that monovalent small molecule ligands can be partial agonists will be useful for screening and designing pharmacological agents, and the approach described can be adapted to the study of other receptors.

MATERIALS AND METHODS

Cell lines

Rat PC12 cells express low levels of rat TrkA and 40,000-100,000 p75 receptors/cell ($TrkA^{+}$ $p75^{+++}$). B104 rat neuroblastomas express ~50,000 p75 receptors/cell but do not express Trks ($TrkA^{-}$ $p75^{+++}$). The 4-3.6 cells are B104 cells stably transfected with human *trkA* cDNA, and express equal surface levels of p75 and TrkA ($TrkA^{+++}$ $p75^{+++}$) (23). The 6-24 cells are PC12 cells stably transfected with human *trkA* cDNA and overexpressing TrkA ($TrkA^{+++}$ $p75^{+++}$). Cell surface expression of each of NGF receptors was routinely controlled in all cells by quantitative FACScan assays (Becton Dickinson, CA) (data not shown). These cells do not express detectable mRNA for neurotrophins (data not shown, and reference 23), and undergo apoptosis when neurotrophins or serum are withdrawn.

Dissociated neuronal dorsal root ganglia cultures

Fetal rat dorsal root ganglia (DRG) primary cultures were established essentially as described (24) from Sprague Dawley day 17 rat embryos. All ganglia were dissected and dissociated first enzymatically with trypsin and then mechanically. Dissociated cells were cultured (100,000 cells/well) in 96 well plates pre-coated with collagen, and grown for a total of 8 days in Neuro Basal Medium containing N2 supplement (GIBCO, Toronto), antibiotics, and L-glutamine. These DRG cultures are ~85% TrkA-expressing and are heavily dependent on TrkA signals for survival (25,26).

Antibody and fragment preparation

The activity of anti-human TrkA IgG mAb 5C3 and anti-rat p75 IgG mAb MC192 have been described (6,19). MAbs 5C3 and MC192 do not cross-block each other's binding. Purified IgGs were digested with papain (GIBCO, Toronto) to yield monovalent fragments (F_{ab}s). For further purification, first papain was inactivated, second the Fc fragments were removed in protein G-Sepharose columns (HiTrap, Pharmacia), and third the F_{ab}s containing kappa light chains were purified to >98% purity in KappaLock-

Sepharose columns (Upstate Biotechnology, Lake Placid, NY) and by preparative FPLC with sizing columns (Pharmacia). No IgG was detected in F_{ab} preparations. FPLC spectrometry and size exclusion analysis under native conditions did not reveal the presence of aggregates, even at 40 μM F_{ab} concentrations (bioassays use nM concentrations). The conditions used would have detected <0.2% of F_{ab} aggregates. Binding competition assays between 5C3 F_{ab}s and the intact antibody indicated that the affinity of F_{ab}s (K_d 10 nM) is within 5-fold of the intact IgG (K_d 2 nM). The affinity of the MC192 F_{ab}s were not measured directly. However, FACScan assays demonstrated that MC192 F_{ab}s and MC192 IgG (Figure 1A and 1B), and 5C3 F_{ab}s and 5C3 IgG (Figure 1 C and 1D) bound their cellular targets in a specific and saturable fashion indistinguishable from each other (Figure 1).

Cyclic NGF mimics

The NGF mimic C(92-96) is an N-acetylated (N-Ac) cyclic peptide with primary sequence N-Ac-Y<u>CTDEKQC</u>Y. The NGF mimic C(92-97) is N-Ac-Y<u>CTDEKQAC</u>Y. The C(92-96) and C(92-97) peptides are cyclized by intrachain disulfide bonds (indicated by underline) (21). These peptides are structural mimics of the C-D β-turn of NGF (27). *Linear* peptides with the same sequences do not bind TrkA, and were prepared as controls by substituting Cys with Met (primary sequence YMTDEKQMY). The linear peptides do not cyclize and NMR spectroscopy indicated lack of conformation (data not shown). The $C(92\text{-}97)_{dimer}$ is a tethered covalently linked dimer of C(92-97). HPLC and mass spectroscopy analysis confirmed the expected retention time and mass for a dimer.

Peptide sythesis and characterization

N-Ac peptides were synthesized by FMOC chemistry. Purification, quality control and characterization of the peptides were done as described (21), and by nuclear magnetic resonance (NMR) diffusion studies (this report). More convincingly, the full NMR spectra of NAc-C(92-96) were analyzed (27). Assignment of all resonances and distances, and resolution of the structure showed the peptide to be monomeric. Therefore it is extremely unlikely that NAc-C(92-96) is a non-covalent dimer. With respect to a

possible covalent dimer, mass spectroscopy (API III MS System, Sciex, Thornhill, Ontario) by electrospray ionization quadrupole (data points every 0.1 Da) verified the chemical composition and monomeric state of NAc-C(92-96) with 1192.3±0.3 atomic mass units measured, which is the theoretical mass (1192.2) for an oxidized monomer. No trace of a covalent peptide dimer was detected even after prolonged signal averaging, using conditions that would have detected 1% of dimer. Therefore, it is extremely unlikely that NAc-C(92-96) is a covalent dimer

NMR spectroscopy

NMR samples contained 5 mM N-Ac-C(92-96) in distilled water at pH 5.7 containing 10% (v/v) of D_2O for the deuterium lock. When D_2O was used as a solvent, the peptide was twice lyophilized and redissolved in D_2O. Spectra were acquired at 500 MHz proton frequency on a three channel Bruker DRX500 spectrometer equipped with pulsed field gradients. Standard experimental protocols were used for the acquisition of NMR spectra and spectral assignments. Isotropic self-diffusion measurements used NMR pulse field gradients at different peptide concentrations (28.29). Seventeen 1-D assays were done at each concentration with gradient strengths from 0.67 to 63.65 G/cm, gradient duration 3.5 milliseconds, and a diffusion time of 150 milliseconds. Peptide signal decay was measured at nine different frequencies. Data were fit to the equation $I=I^{\circ}\exp(-(\gamma\delta G)^2(\tau-\delta/3)\delta]$, where I is the experimentally measured signal intensity attenuated by diffusion, γ is ^1H gyromagnetic ratio, δ is gradient duration, G is the gradient strength, τ is time between gradient pulses, and δ is diffusion coefficient. Results were averaged.

Ligand concentrations and valency

Antibodies are defined as "artificial receptor ligands" because they are specific, they bind with high affinity, with saturable and reversible kinetics, and they are bioactive. Responses to a full dose range (from pM to high μM) were studied previously for some ligands; and the same dose range for all ligands were studied herein (data not shown). Usually, only optimal concentrations of NGF mimics, mAbs, or F_{abs} that afford trophic signals are shown for clarity. The NGF mimic C(92-96) is monomeric and monovalent. It

is water soluble and does not aggregate even at 18 mM (this manuscript). The $[C(92\text{-}97)]_{dimer}$ is a covalent dimer and bivalent analog of the C(92-97) NGF mimic. Intact IgGs are dimeric and bivalent, and F_{ab}s are monomeric and likely monovalent. Where indicated, F_{ab}s were cross-linked with goat-anti-mouse F_{ab} antibody (α-F_{ab}, Sigma, St. Louis, MO) at a 2:1 ratio of F_{ab}•α-F_{ab}. This cross-linking ratio affords optimal dimerization (one α-F_{ab} can bind two F_{ab}s). Higher cross-linking of F_{ab}s using F_{ab}• α-F_{ab} ratios of 1:1 or 1:5 (each F_{ab} bound by many α-F_{ab}s), leading to ligand oligomerization. achieved results comparable as dimerizing ratios of 2:1 F_{ab}• α-F_{ab} (data not shown).

Protection from apoptotic death

Primary DRG cultures: After a total of 8 days of culture with NGF (Prince Labs, Toronto) or the indicated test or control ligands, cell survival were studied using the 3(4,5-Dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide colorimetric (MTT) assay and by microscopic observation.

Cell lines: 5,000 cells/well in protein-free media (PFHM-II, GIBCO, Toronto) containing 0.2% bovine serum albumin (BSA) (Crystalline fraction V, Sigma, St. Louis, MO) were seeded in 96 well plates (Falcon, Mississauga, Ontario). The cultures were untreated, or treated with the indicated test or control ligands. Cell viability was quantitated using the MTT assay after 56-72 hours of culture, and apoptotic death was confirmed by analysis of DNA fragmentation patterns. Percent protection was standardized from MTT optical density (OD) readings relative to optimal NGF (1 nM) = 100%. The OD of untreated cells were subtracted and were <15% for cell lines and <30% for primary cultures. The higher survival of untreated primary cultures is likely due to endogenous production of limiting amounts of growth factors.

Tyrosine phosphorylation assays

TrkA tyrosine phosphorylation was assayed after a 15 min. treatment of intact cells with the indicated agent(s), and revealed by western blotting of whole cell lysates as described (6). Anti-phosphotyrosine (α-pTyr) mAb 4G10 (UBI, NY), or antiserum α-pTyr490

against the pTyr490 of TrkA (within the Shc docking site) (30) were used as a primary antibody. Bands in X-ray films were quantified by densitometry, and intensities standardized relative to 1 nM NGF. Densitometry of 4-5 independent gels were analyzed statistically by paired Student t-tests with Bonferroni corrections.

TrkA internalization measurements

Live 4-3.6 cells were treated as indicated for 45 min at 4°C in the presence or absence of 0.25% sodium azide. Cells were maintained at 4°C or shifted to 37°C for another 20 min to allow ligand-induced receptor internalization (9). Then, cells were washed and immunostained with mAb 5C3 at 4°C (PBS, 0.5% BSA, 0.1% sodium azide), for analysis of surface TrkA expression by FACScan immunofluorescence as described (9). In each assay, 5.000 cells were acquired, and the mean channel fluorescence (MCF) of bell-shaped histograms were analyzed (LYSIS II, Becton Dickinson, CA). Percent inhibition of mAb 5C3 binding was calculated as a change in MCF with respect to control untreated cells. Rapid loss of surface TrkA is interpreted as receptor internalization, which is delayed or inhibited by low temperatures or sodium azide (9).

Chemical cross-linking

Live 4-3.6 single cell suspensions were bound by the indicated ligand(s) for 45 min at 4°C. Cells were then washed in PBS, cross-linked with 1 mM disuccinimidyl suberate (DSS, Pierce) for 15 minutes at 15°C as described (31). Unreacted DSS was quenched with 5 mM ammonium acetate and whole cells were lysed directly in SDS sample buffer. Equal amounts of protein for each sample were resolved in a 5-10% SDS-PAGE gradient, transferred to nitrocellulose, and western blotted with anti-Trk polyclonal antibody 203 (a gift of Dr. David Kaplan, McGill University) that recognizes the intracellular domain of Trk. This antibody was selected because of high specificity towards Trk in western blots, and because its epitopes are not affected by DSS cross-linking.

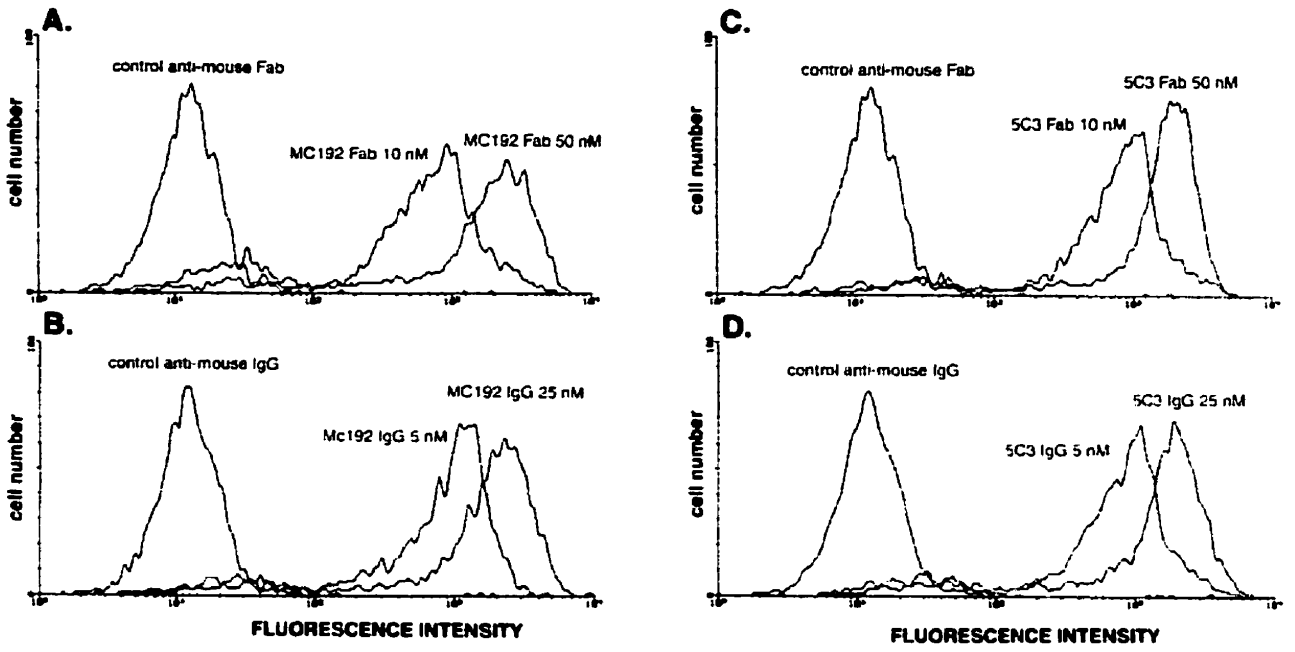


Figure 1. Binding profile of mAb versus F_{ab} fragments

4-3.6 cells expressing equal levels of TrkA and p75 were analyzed by FACScan binding as described in the Methods section. Binding of intact IgG was revealed with fluorescein (FITC)-coupled goat anti-mouse IgG, and binding of F_{ab}s with FITC-coupled goat anti-mouse F_{ab}. Controls excluded the specific primary. Saturating concentrations are achieved at 50 nM F_{ab}s and at 25 nM IgG. These concentrations have equal number of receptor-binding units because IgGs are bivalent. Decreasing the saturating dose 5 fold results in similar immunostaining patterns for IgG and F_{ab}s (compare A versus B, and C versus D), suggesting similar binding properties. Note that FACScan immunostaining conditions (10^5 cells stained/test, binding primary for 30 min at 4°C) are not identical to the conditions used for survival assays hence saturating concentrations for the latter can not be extrapolated.

RESULTS

Cell survival induced by monovalent and bivalent TrkA ligands

Previously, we showed that anti-human TrkA mAb 5C3 significantly protected cells from apoptosis when cultured in serum free media (SFM), but anti-p75 mAb MC192 did not promote cell survival. Combinations of anti-TrkA mAb 5C3 and anti-p75 mAb MC192 synergized to protect cells optimally, to levels comparable to 1 nM NGF; as did combinations of mAb MC192 and 10 pM NGF (6). Therefore, we tested 4-3.6 cells (human $TrkA^{+++}$ rat $p75^{+++}$) (Figure 2A) or PC12 cels (rat $TrkA^{+}$ rat $p75^{+++}$) (Figure 2B) in the same paradigm, but using putative monovalent ligands.

Significant protection was afforded by 5C3 F_{ab}s, in a dose dependent manner. 5C3 F_{ab}s at 1 nM-10 nM afford protection comparable to 10 pM NGF. More robust protection was afforded by 1-10 nM 5C3 F_{ab}•α-F_{ab} complexes. Negative controls α-F_{ab} or mouse IgG did not afford survival. Positive control bivalent mAb 5C3 at the optimal concentration of 0.5 nM protected ~50% of the cells. Interestingly, 10 nM 5C3 F_{ab}•α-F_{ab} complexes afforded significantly higher protection than 0.5 nM 5C3 IgG, possibly because 5C3 F_{ab}•α-F_{ab} complexes are more flexible than IgG, or oligomerize TrkA more efficiently.

Monovalent p75 ligands do not potentiate NGF signals

1 nM NGF protects PC12 cells (expressing rat $TrkA^{+}$ rat $p75^{+++}$) from apoptosis induced by culture in SFM. Low concentrations of NGF (10 pM) as a high affinity ligand afforded ~30% survival. Monovalent MC192 F_{ab}s failed to synergize with 10 pM NGF, and protection was not significantly different than 10 pM NGF alone. Synergy did occur with MC192 F_{ab}•α-F_{ab} complexes + 10 pM NGF. The effect was dependent on the concentration of MC192 F_{ab}•α-F_{ab} complexes (data not shown) and was optimal at 1 nM cross-linked MC192 F_{ab}.

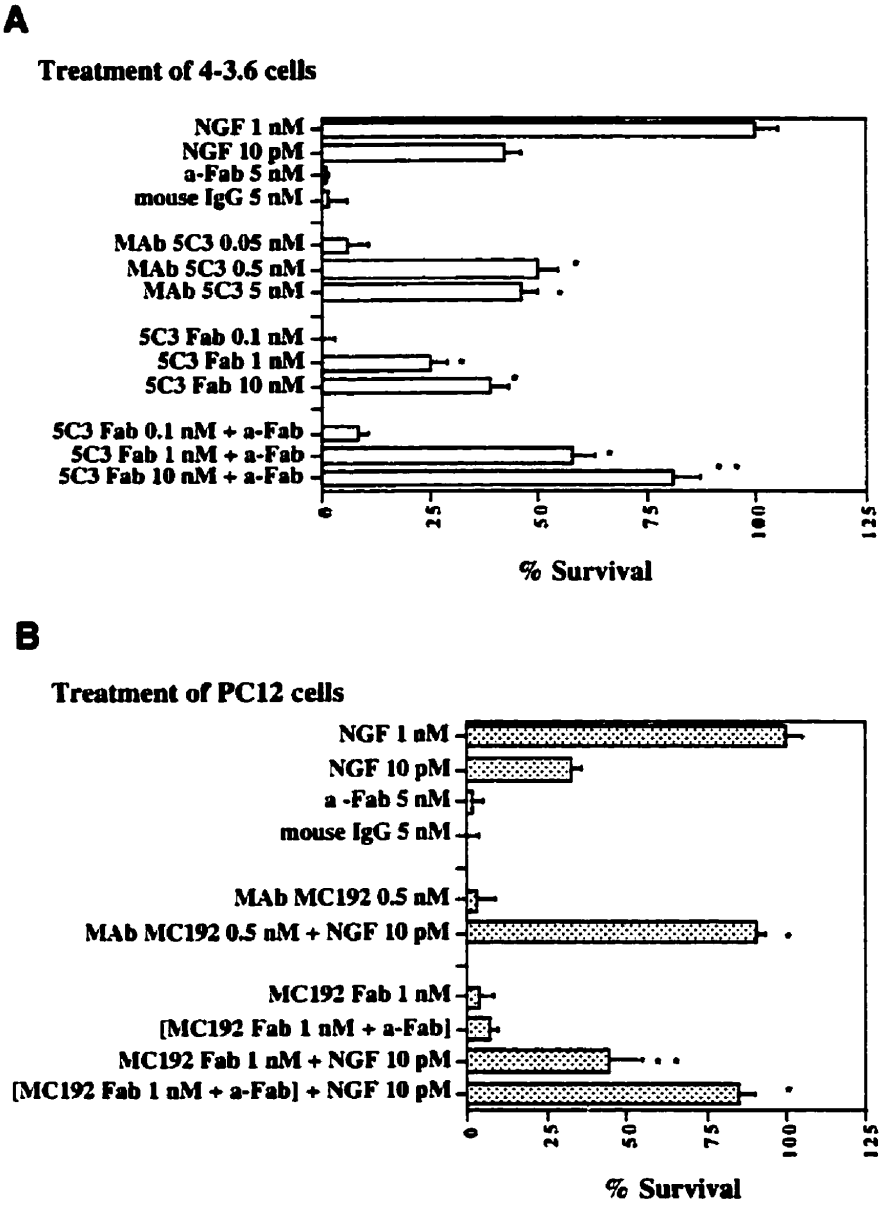


Figure 2. Trophic Protection by Monovalent and Bivalent TrkA or p75 ligands

Cells were cultured in SFM supplemented with the indicated ligands for ~68 hours. Cross-linking of F_{ab}s was achieved with a 2 fold molar excess of α-F_{ab} antibody. Cell survival was measured in MTT assays. Protection from apoptotic death was calculated relative to that of optimal NGF (1 nM, 100% protection). Average ± standard error of mean (sem), n=4. Representative from at least 3 experiments. (A) 4-3.6 cells. * significant protection compared to control mouse IgG. ** significantly higher than 0.5 nM mAb 5C3. (B) PC12 cells. * significantly higher than 10 pM NGF. ** not significantly different from 10 pM NGF. $p < 0.01$.

As positive control, bivalent MC192 synergized with 10 pM NGF increasing protection from ~30% to ~90%. Synergy was dependent on the concentration of mAb, and was optimal at 0.5 nM MC192 (data not shown). Controls α-F_{ab}, mouse IgG, bivalent MC192, monovalent MC192 F_{ab}s, and MC192 F_{ab}•α-F_{ab} complexes alone did not protect PC12 cells substantially from apoptosis. In all permutations of these experiments, apoptotic cell death was confirmed by analysis of DNA fragmentation patterns (data not shown).

Synergy of bivalent and monovalent ligands of NGF receptors

To analyze the valency requirement of each NGF receptor, combinations of bivalent and monovalent antibody-based ligands were tested on 4-3.6 cells for synergy in protection of apoptotic cell death (Figure 3). In these assays it was encouraging to observe that comparable biological responses by different ligands (e.g. 1 nM cross-linked 5C3 F_{ab}s afford the same protection as 0.5 nM 5C3 bivalent IgG) also result in equivalent receptor occupancy (e.g. 1 nM F_{ab}s bind the same number of receptors as 0.5 nM IgG). Positive controls of bivalent 5C3 combined with bivalent MC192 were synergistic and afforded 100% protection. Negative controls α-F_{ab} alone and mouse IgG alone did not afford cell survival in SFM (data not shown).

A combination of monovalent 5C3 F_{ab} with either monovalent MC192 F_{ab} or with bivalent MC192 did not result in synergy; the ~25% protection was not significantly different than seen with monovalent 5C3 F_{ab} alone. However, 5C3 F_{ab}•α-F_{ab} complexes synergized with bivalent MC192. A combination of monovalent MC192 F_{ab} with bivalent 5C3 did not result in synergy; the ~50% protection was not significantly different than that afforded by bivalent 5C3 alone. In contrast, MC192 F_{ab}s•α-F_{ab} complexes synergized with bivalent 5C3 and afforded ~110% protection. One bias in this assay is that α-F_{ab} cross-linking does not occur exclusively at MC192 F_{ab}s but also occurs upon bivalent 5C3, resulting in some multivalent 5C3 oligomers. However, α-F_{ab} cross-linking of bivalent 5C3 IgG does not enhance its activity (data not shown), therefore the biological effect of cross-linking occurs at the MC192 F_{ab}s.

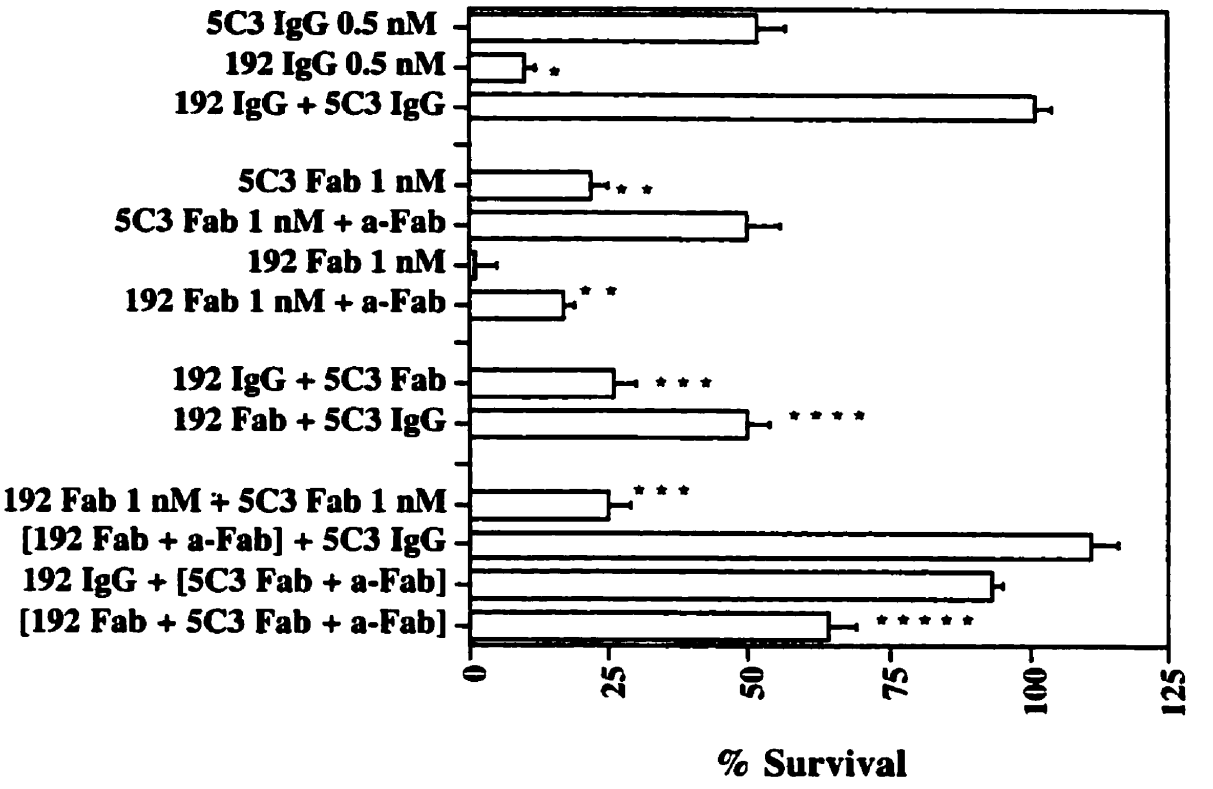


Figure 3. Synergistic trophic protection by p75 and TrkA Ligands

Experiments using 4-3.6 cells were as described in Figure 2. Cross-linking of F_{ab}s was achieved with a 2 fold excess of α-F_{ab} antibody. Only optimal doses for each of the TrkA or p75 ligands (0.5 nM IgG and 1 nM F_{ab}s) are shown. Protection from apoptotic death was calculated relative to that of optimal NGF (1 nM, 100% protection). Average ± sem, n=4. Data representative from at least 3 independent experiments. * not significantly higher than control mouse IgG. ** significantly higher than control mouse IgG. *** not significantly higher than 5C3 F_{ab}s alone. **** not significantly higher than mAb 5C3 alone. ***** significantly lower than all dimeric combinations of MC192 + 5C3. $p < 0.01$.

Lastly, α-F_{ab} cross-linking of 1 nM MC192 F_{ab} + 5C3 F_{ab} afforded ~65% protection. This activity can be ascribed to 4 theoretical ligand mixtures: 5C3 dimers (25%), MC192 dimers (25%), and 5C3/MC192 heterodimers (50%). If the 5C3/MC192 heterodimers are indeed formed, they seem to be inactive because we observed that reducing the concentration of bivalent mAbs 5C3 and MC192 to 0.125 nM (the concentrations in the theoretical mixtures above) results in synergy and ~65% protection (data not shown).

While the data is suggestive that heterodimers are inactive, this is an unclear issue because we have no evidence that the heterodimers indeed form. The putative heterodimeric ligands can not be isolated and analyzed because they dissociate and re-associate during purification, nor can they be stabilized because binding activity is lost upon chemical cross-linking. Additionally, it is noteworthy that more extensive cross-linking with higher ratios of α-F_{ab} did not increase protection although higher oligomerization of ligands is expected (data not shown).

For clarity, only nearly optimal concentrations of ligands are presented; but responses from μM to pM concentrations were studied (see Methods). Thus, optimal protection is afforded by combinations that result in homodimerizing ligands, and no increased protection is seen with oligomerizing ligands. In all permutations of these experiments, apoptotic cell death was confirmed by analysis of DNA fragmentation patterns (data not shown). Moreover, the ligands mediate trophic effects in a TrkA-dependent manner, because no concentration or combination of NGF and antibody could induce significant protection of B104 cells ($TrkA^-$, $p75^{+++}$) (data not shown).

Ligand valency and TrkA tyrosine phosphorylation

TrkA tyrosine phosphorylation (TrkA-pTyr) was studied as a biochemical correlate of cell survival in SFM (Figure 4). Analysis was done by western blotting with mAb 4G10 against phosphotyrosine (total pTyr), or with antibodies against phosphorylated tyrosine 490 of TrkA (pTyr490) which is the Shc binding site of TrkA. A representative western blot of total pTyr is shown in Figure 4A. Statistical analysis of densitometry for several blots of total TrkA-pTyr (Figure 4B upper panel), and for several

blots of TrkA-pTyr490 (Figure 4B lower panel) were used to quantify the TrkA-pTyr data. Western blotting with anti-TrkA antibodies, done in parallel, demonstrated that all lanes contained the same amount of receptor (data not shown).

Monovalent 5C3 F_{ab}s induced small but significant increases in TrkA-pTyr (Figure 4A, lane 2) and TrkA-pTyr490 compared with untreated control (Figure 4A, lane 1) or MC192 F_{ab}s (Figure 4A, lane 4). Much higher signals were induced by 5C3 F_{ab}•α-F_{ab} complexes (Figure 4A, lane 3). Quantification showed that ~80% of total TrkA-pTyr and ~55% of TrkA-pTyr490 were induced compared with optimal NGF-induced signals (Figure 4B). In contrast, no significant TrkA-pTyr or TrkA-pTyr490 were induced by p75 ligands bivalent MC192, MC192 F_{ab}•α-F_{ab} complexes, or monovalent MC192 F_{ab}s (Figure 4A, lanes 10, 5 and 4). For quantitative statistical analysis of these data see Figure 4B. All of these findings are consistent with the survival data.

There were no significant differences between treatments with 5C3 F_{ab}s + MC192 F_{ab}s (Figure 4A, lane 6) versus 5C3 F_{ab}s alone (Figure 4A, lane 2), indicating lack of synergy. Cross-linking of 5C3 F_{ab}s + MC192 F_{ab}s with α-F_{ab} afforded an increase in total pTyr (Figure 4A, lane 7).

Approximately 85% of total pTyr and ~65% of pTyr490 of TrkA were induced compared with optimal NGF-induced levels (Figure 4B). However, the increases in TrkA pTyr and pTyr490 induced by [5C3 F_{ab}•MC192 F_{ab}•α-F_{ab}] complexes were not statistically different from increases induced by 5C3 F_{ab}•α-F_{ab} complexes (Figure 4B). All of these findings are consistent with the survival data.

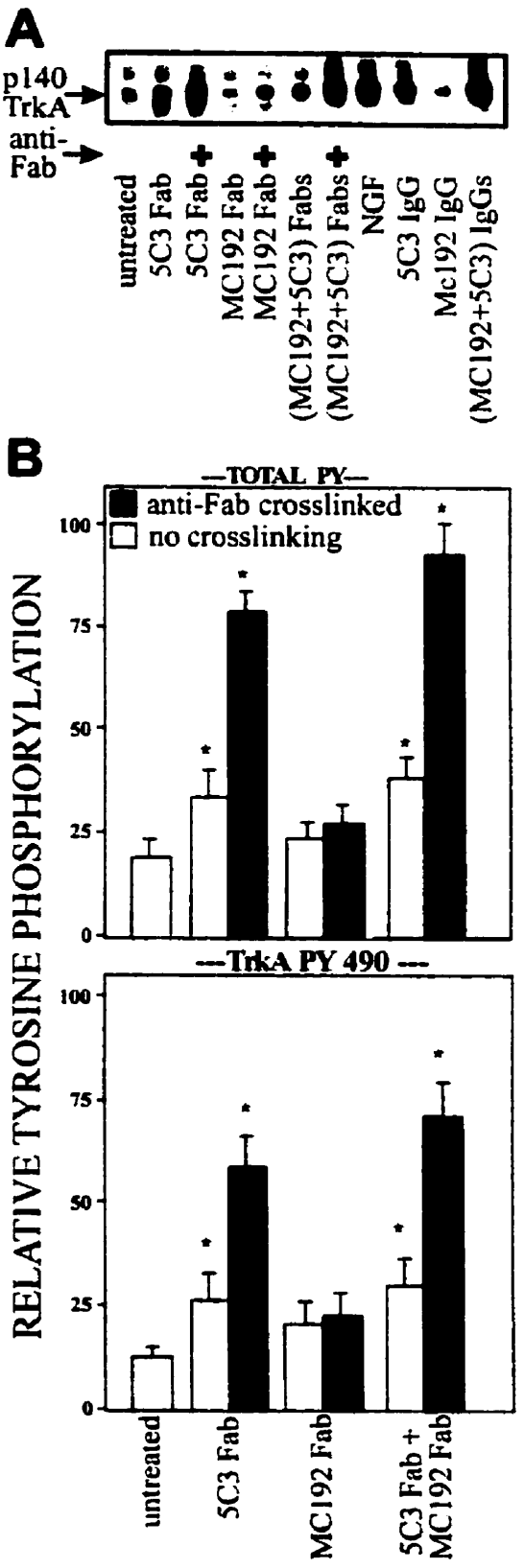
Thus, 5C3 F_{ab}s are partial agonists monovalent ligands of TrkA, that induce receptor activation and lead to trophic cell survival. Although the evidence that mAb 5C3 F_{ab}s are indeed monomeric is compelling (see Methods) it is possible that these large F_{ab} molecules of ~50 kDa could aggregate. Hence, other ligands were tested.

Figure 4. Bivalent TrkA ligands induce optimal TrkA tyrosine phosphorylation

Equal amounts of protein from whole cell lysates were resolved by SDS-PAGE and analyzed by western blotting with anti-pTyr mAb 4G10 (total pTyr) or with an antibody recognizing pTyr490 within the Shc binding site of TrkA (TrkA pTyr490) (not shown). Blot shown is representative of at least three independent total pTyr experiments.

A. 4-3.6 cells were untreated (lane 1) or treated with indicated ligands for 15 min at 37°C. In lanes 3, 5 and 7 monovalent F_{ab}s were cross-linked with ♿-F_{ab} antibodies as indicated (+). Homodimeric binding of p75 and TrkA (lane 11) with intact IgGs enhances TrkA pTyr over each IgG alone (lanes 9 and 10). Ligand concentrations are as in Table 1B. Asterisks (*) indicate significant difference from untreated samples (paired t-tests, n=6, $p<0.05$).

B. Densitometric scanning quantification of TrkA total pTyr (upper panel) and pTyr490 (lower panel) intensities relative to optimal NGF treatment (average ± sem, n=6). Filled bars indicate anti-F_{ab} cross-linking. Asterisks (*) indicate significant difference from untreated samples (paired t-tests, n=6, $p<0.05$).



Characterization of small molecule monomeric TrkA ligands

C(92-96) is a small molecule (~1 kDa), cyclic and conformationally constrained peptide analog of the C-D β-turn region of a single NGF protomer. Therefore, the C(92-96) mimic of NGF was studied as a candidate genuine monovalent and monomeric TrkA ligand.

To address the valency of C(92-96) we determined the solution structure of the pharmacophore to better than 0.5 Å root mean square deviation (RMSD). NOESY and TOCSY spectra were consistent with a monomeric, non-aggregated state and a 5 residue pharmacophore within a β-turn (27). Five chemical moieties are too few to bind two receptors simultaneously as a bivalent agent, hence this ligand is monovalent.

The following criteria indicate that C(92-96) is monomeric. *First*, mass spectroscopy of C(92-96) demonstrated that there were no covalent dimers or oligomers (see Materials and Methods). *Second*, the aggregation state of the peptide at *millimolar* concentrations *in solution* was resolved by high resolution proton NMR spectroscopy (Figure 5A). *Third*, natural ^{13}C abundance NMR relaxation parameters were measured for the α carbon atom, heteronuclear NOEs, and the molecular correlation time of C(92-96) was assessed. The overall correlation time detected of 1.76 nanoseconds at 5°C is expected for a monomer. *Fourth*, the translational self-diffusion constant in solution unequivocally identified C(92-96) as monomeric.

Pulse field gradient NMR measurements of the self-diffusion coefficient (δ) were determined at various peptide concentrations of 2, 6, or 18 mM; T=278 K. Values of $\delta = 1.01 \pm 0.07$ (10^{-6} cm^2/s); $\delta = 1.00 \pm 0.06$ (10^{-6} cm^2/s); and $\delta = 1.04 \pm 0.05$ (10^{-6} cm^2/s) were measured for 18 mM, 6 mM and 2 mM samples respectively (Figure 5B). These δ values are essentially the same, indicating an identical state for the peptide. Thus, the samples remain monomeric, and peptide aggregates are undetectable in solution even at concentrations as high as 18 mM. We estimate that the self-association constant for any putative aggregate cannot be larger than 10 M^{-1}. Thus, a 10 μM solution of C(92-96) (used hereafter) could not contain >1 nM self-aggregated dimers, if any aggregate at all.

Figure 5. NMR spectroscopy of NAc(92-96) peptide mimetic.

Small molecule monomeric and monovalent agonists of TrkA

Four questions were addressed. *First*, the genuine monovalent and monomeric TrkA ligand C(92-96) was tested for trophic support of cells in SFM. *Second*, a covalent dimeric analog of C(92-96) termed $C(92\text{-}97)_{dimer}$ was also evaluated to directly compare the potency and efficacy of monomeric *versus* dimeric small molecule TrkA ligands. *Third*, to study whether surface density of TrkA receptors influences trophic signals, these agents were assayed in parallel on cell lines that differ only in TrkA density (PC12 *versus* 6-24, and B104 *versus* 4-3.6 cells). *Fourth*, to study whether the ligands activate receptors in normal neurons, primary cultures of dissociated dorsal root ganglia from day 17 rat embryos were tested. These cells express TrkA and p75 receptors and their survival and differentiation are dependent on TrkA activation. Growth and survival were studied first in MTT assays (Table 1). Differentiation was studied morphometrically (Figure 6).

The trophic response was dependent on NGF dose and was optimal to 1 nM NGF in all cell types (Table 1, row 1). Better survival was seen at 10 pM NGF for 6-24 and 4-3.6 cells (Table 1, row 3) suggesting that high TrkA expression affords better efficacy when ligand concentrations are limiting. B104 cells did not respond to any dose of NGF (data not shown). Negligible survival to 10 pM NGF in DRG cultures is due to the fact that DRG cultures are heterogeneous and secrete growth factors, masking the effect of low concentrations of exogenous NGF.

The C(92-96) NGF mimic did not afford significant survival of PC12, 6-24, or 4-3.6 cells; compared to control linear peptides; but did afford significant survival of DRG cultures (Table 1, rows 4 *versus* 6). This effects was dose dependent, and C(92-96) at 1 μM afforded ~10% growth of DRG (data not shown). In contrast, the $C(92\text{-}97)_{dimer}$ peptide afforded good trophic support for 6-24 and 4-3.6 cells, and very low but statistically significant support for PC12 cells (Table 1, row 5). The 6-2.4 and 4-3.6 cells express comparable numbers of TrkA receptors, suggesting a TrkA density-dependent response.

MAb MC192 alone afforded very low or insignificant trophic support of cell lines (Table 1, row 7); but as a bivalent p75 ligand it synergizes with TrkA ligands (e.g. see Figure 2). High DRG survival in response to mAb MC192 alone (Table 1, rows 7 and 10)

is explained by the mAb potentiating endogenously produced growth factors. Furthermore, bivalent MC192 potentiated the activity of C(92-96) (Table 1, row 8). In cell lines the combination is synergistic, while in DRG cultures the combination is additive due to high protection afforded by each ligand alone.

Table 1. NGF peptide mimics evoke trophic responses alone, and in synergy with p75 dimerizing ligands.

Experiments were performed as described in Figure 2. Cell lines PC12 ($p75^{+++}$ $TrkA^{+}$), 6-24 (PC12 cells overexpressing TrkA, $p75^{+++}$ $TrkA^{+++}$), and 4-3.6 ($p75^{+++}$ $TrkA^{+++}$), or dissociated primary neuronal cultures from embryonic day 17 rat DRGs cells were used. Cell lines were treated for 3 days, and DRGs were treated for 8 days with the indicated ligands. Cell growth/survival was studied by the MTT method. Growth was calculated relative to that of optimal NGF (1 nM, 100% protection) subtracting the O.D. of untreated cells. % growth: mean ± sem, averaged from at least 3 independent experiments, each experiment n=4.

	TREATMENT	% protection in serum-free media			
		PC12	6-24	4-3.6	DRG
1	1 nM NGF	100 ± 1.8	100 ± 3.7	100 ± 4.2	100 ± 6
2	500 pM NGF	NT	NT	NT	68 ± 4
3	10 pM NGF	30 ± 3.6	60 ± 3.1	47 ± 2.5	7 ± 2
4	C(92-96) 10 μM	0 ± 2.7	0.6 ± 3.1	0.7 ± 0.9	38 ± 3
5	$C(92\text{-}97)_{dimer}$ 10 μM	5 ± 1.6	23 ± 2.4	19 ± 3.1	NT
6	linear peptide 10 μM	1 ± 1.3	8 ± 1.2	0.3 ± 2.6	0 ± 4
7	MC192 IgG 1 nM	4 ± 2.5	7 ± 1.8	5 ± 0.6	25 ± 6
8	C(92-96) + MC192	28 ± 2.8	52 ± 5.2	40 ± 3.0	55 ± 5
9	$C(92\text{-}97)_{dimer}$ + MC192	39 ± 6.3	72 ± 3.1	59 ± 3.2	NT
10	linear peptide + MC192	5 ± 2.0	7 ± 3.8	2 ± 1.0	26 ± 2

[a]NT: not tested.

As a control, bivalent MC192 did not synergize with linear peptides (Table 1, row 10). Further controls using B104 cells ($TrkA^-$, $p75^{+++}$ parental to 4-3.6) demonstrated no protection by the peptide NGF mimics, alone or in combination with mAb MC192 (data not shown), suggesting that the activity requires TrkA expression.

Monovalent TrkA ligands induce the differentiation of embryonic DRG cultures

The differentiation of dissociated primary DRG cultures was studied (Figure 6). Untreated DRG cultures had sparse, bipolar, and poorly differentiated neurons (Figure 6, panel A). At 20 pM NGF the increase in the number and the length of neurites and branches was very low (Figure 6, panel B); at 1 nM NGF the increase was optimal (Figure 6, panel C). Treatment with control linear peptide did not induce differentiation (not shown). Treatment with 0.5 nM MC192 alone (Figure 6, panel D); or with 10 μM C(92-96) alone (Figure 6, panel E) induced substantial differentiation. However, treatment with a combination of 10 μM C(92-96) + 0.5 nM MC192 (Figure 6, panel F) induced higher differentiation, comparable to that induced by 1 nM NGF. These differentiation data is consistent with synergy in survival seen for cell lines and primary cultures (see Table 1).

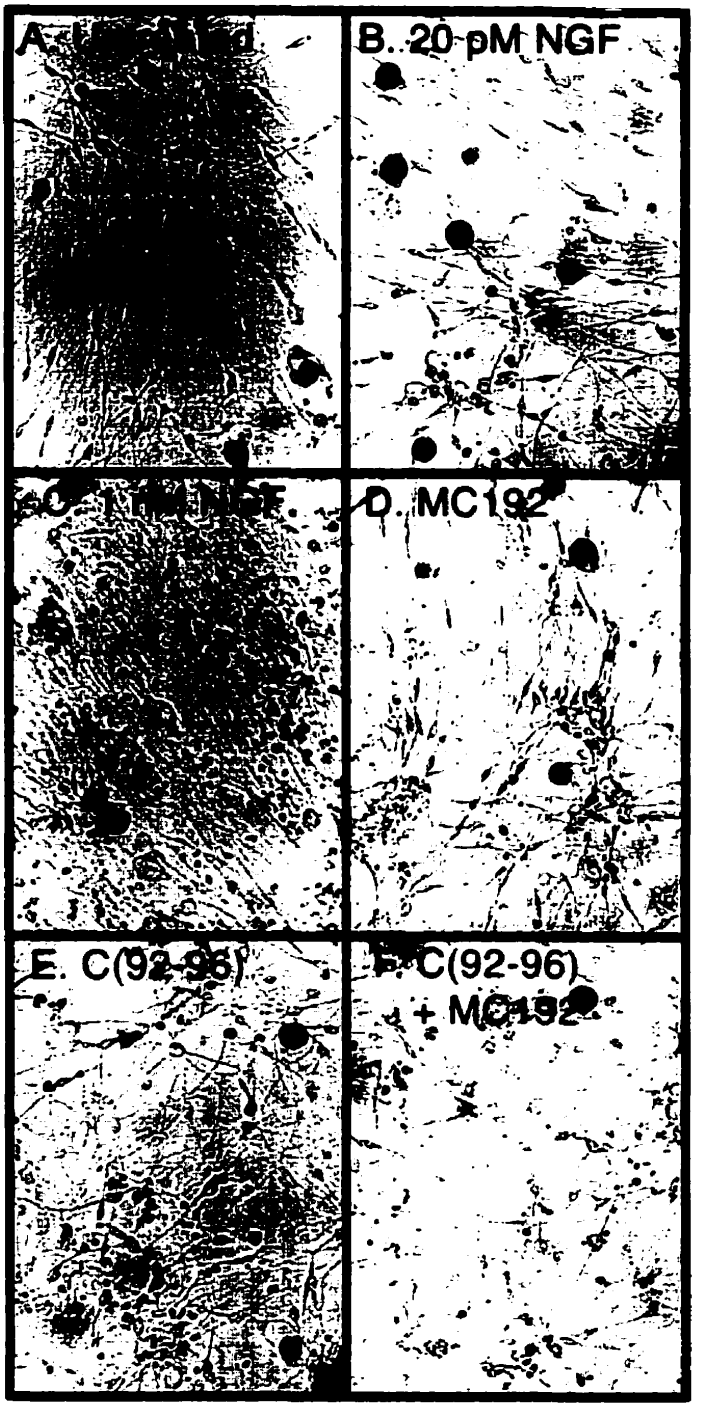
Monovalent TrkA ligands induce TrkA tyrosine phosphorylation in synergy with bivalent p75 ligands

To further assess whether the signals induced by small cyclic peptides are mediated by TrkA, tyrosine phosphorylation of the receptor was studied in 4-3.6 cells (Figure 7). Representative anti-pTyr western blots are shown in Figure 7A. A summary of densitometric analysis from several blots is given in Figure 7B.

Figure 6. Monovalent TrkA ligands induce the differentiation of embryonic DRG cultures

Primary neuronal cultures from embryonic day 17 rat DRGs were treated with the indicated ligands for 8 days, and cell differentiation was studied morphometrically. Magnification 60x.

Data representative of 3 independent experiments.



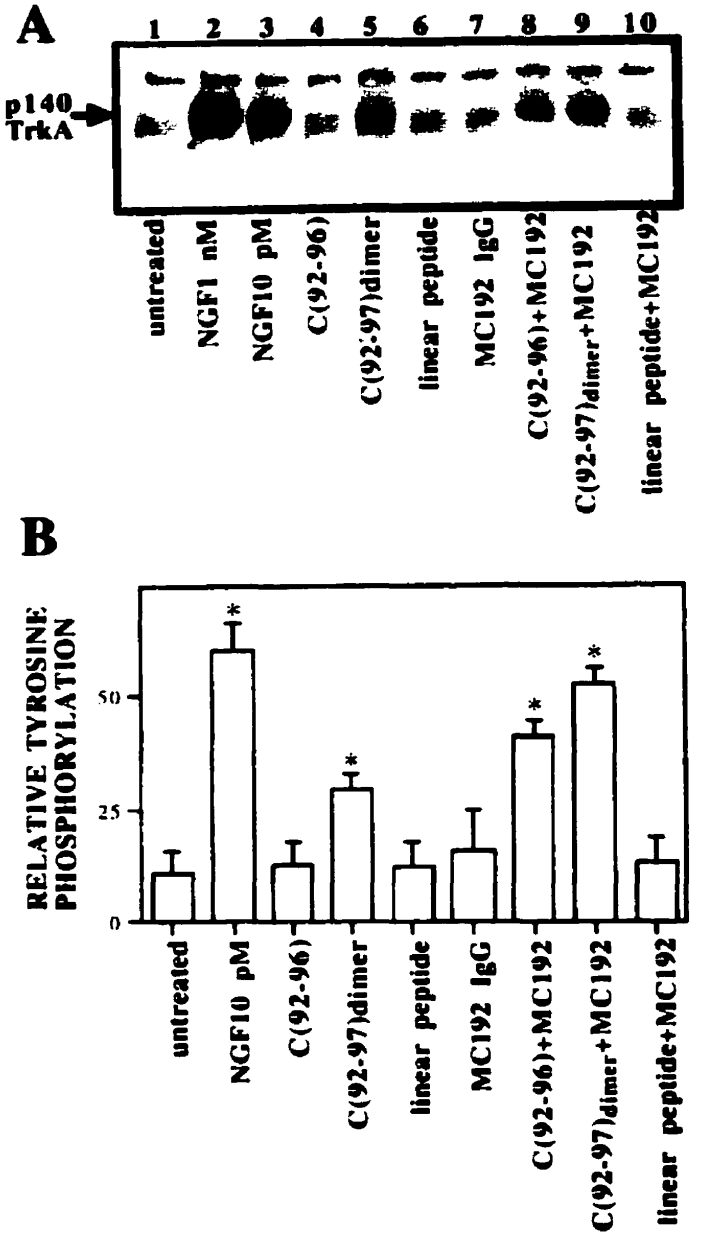


Figure 7. Monovalent ligands induce TrkA tyrosine phosphorylation

Treatment and analysis of 4-3.6 cells was as in Fig. 4; ligand concentrations are as per Table 1.

A. Cells were untreated (lane 1) or treated with 1 nM NGF (lane 2), 10 pM NGF (lane 3), 10 μM C(92-96) (lane 4), 10 μM $C(92\text{-}97)_{dimer}$ (lane 5), 10 μM control linear peptide (lane 6), 1 nM MC192 mAb (lane 7), 10 μM C(92-96) + 1 nM MC192 (lane 8), 10 μM $C(92\text{-}97)_{dimer}$ + 1 nM MC192 (lane 9), or 10 μM control linear peptide + 1 nM MC192 (lane 10).

B. Densitometric scanning quantification of band intensities relative to optimal NGF treatment (average ± sem, n=6). Asterisks (*) indicate significant difference from untreated samples (paired t-tests, n=6, $p<0.05$).

C(92-96) alone did not induce an increase in TrkA-pTyr compared with untreated cells, cells treated with control linear peptide, or bivalent MC192 (Figure 7A, lane 4 versus 1, 6 and 7). Significant TrkA-pTyr was induced by treatment with $C(92\text{-}97)_{dimer}$ (Figure 7A, lane 5), representing ~30% of that induced by 1 nM NGF (Figure 7A, lane 2). Combinations of mAb MC192 and C(92-96) peptide (Figure 7A, lane 8), or mAb MC192 and $C(92\text{-}97)_{dimer}$ peptide (Figure 7A, lane 9) afforded notable increases in TrkA-pTyr, comparable to those induced by 10 pM NGF (Figure 7A, lane 3). These results are consistent with the survival data. In contrast, treatment with a combination of bivalent MC192 and linear peptide controls (Figure 7A, lane 10) did not result in significant increases in TrkA-pTyr. For statistics of of densitometric analysis see Figure 7B.

Small molecule monovalent ligands induce TrkA receptor homodimerization

TrkA tyrosine phosphorylation leading to trophic and differentiative signals require TrkA homodimerization. To study whether monovalent C(92-96) peptide induces TrkA homodimerization, chemical cross-linking studies of the receptor were done in 4-3.6 cells (Figure 8). Cells were treated as indicated followed by chemical cross-linking, and then were detergent solubilized and analyzed by western blotting with anti-Trk polyclonal antibody 203.

A doublet consistent with previously reported TrkA monomers of p110 and p140 were seen in all samples (Figure 8, arrows). Samples from NGF-treated cells and in C(92-96) + MC192-treated cells had a band of ~280 kDa, consistent with the molecular weight of TrkA-TrkA homodimers (Figure 8, lanes 2 and 5). This band was also detected, albeit weakly, in samples from cells treated with C(92-96) alone (Figure 8, lane 4). A second band of ~220 kDa (that may be consistent with cross-linked p140-p75 heterodimers or p110 homodimers) was seen in samples from NGF-treated cells, and more weakly in C(92-96) + MC192-treated cells (Figure 8, lanes 2 and 5). The 280 kDa and 220 kDa bands were not seen in untreated cross-linked cells (Figure 8, lane 1), or in cells treated with MC192 alone (Figure 8, lane 3), or in linear peptide control with or without MC192 treatment (data not shown). Similar data were obtained whether whole cell lysates were

analyzed, or cell lysates were immunoprecipitated with anti-TrkA antibodies prior to western blotting (data not shown).

Given that the efficiency of chemical cross-linking is <5% of the TrkA expressed on the cell surface, we have not studied the individual components of the 280 kDa and 220 kDa bands, other than the fact that they contain TrkA. However, it is unlikely that these bands comprise NGF because they are detected in the C(92-96)+MC192-treated cells.

Small molecule monovalent ligands induce TrkA receptor internalization

Next, we assessed whether the monomeric C(92-96) peptide binds to a receptor domain that overlaps with mAb 5C3. This study was done by attempting to block mAb 5C3 binding with C(92-96). Moreover, since agonistic ligands are expected to cause receptor internalization, it was of interest to study whether monomeric ligands such as C(92-96) can induce TrkA internalization.

We studied ligand-dependent receptor internalization as a decrease of surface receptor density, which can be inhibited by low temperature or by poisons such as sodium azide. 4-3.6 cells were treated with NGF, C(92-96) peptide, or control peptides; in the presence or absence of sodium azide; at different temperatures. Surface TrkA receptors were quantitated by FACScan analysis with mAb 5C3 before and after 20 min of internalization (Table 2). This time was selected because the $t_{1/2}$ for ^{125}I[NGF] internalization is ~10 min (9).

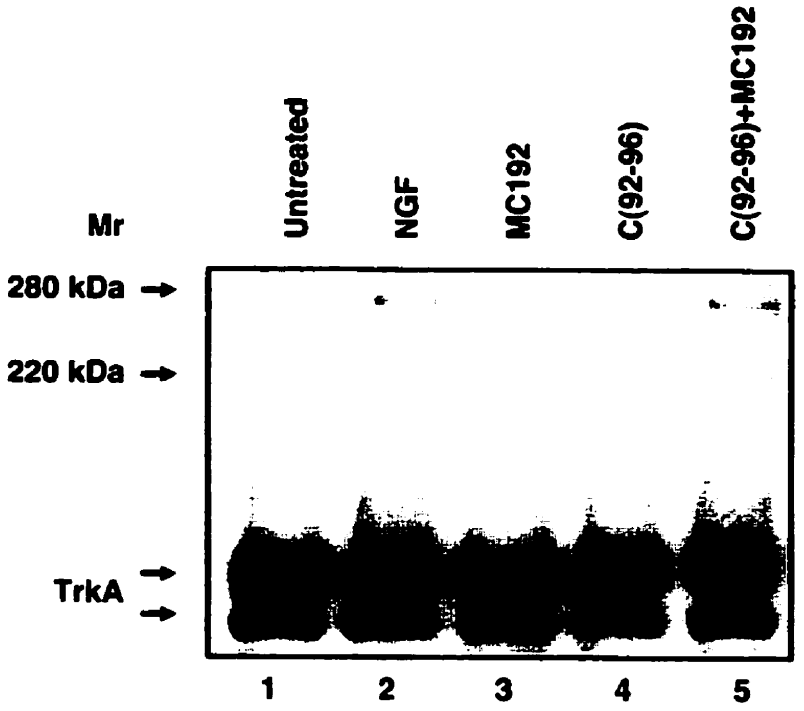


Figure 8. Monovalent ligands induce or stabilize putative TrkA homodimers

4-3.6 cells were untreated (lane 1) or treated with 2 nM NGF (lane 2), 1 nM MC192 (lane 3), 10 μM C(92-96) (lane 4), or 10 μM C(92-96) + 1 nM MC192 (lane 5). Cells were then chemically cross-linked with DSS, lysed, resolved by SDS-PAGE and analyzed by western blotting with anti-TrkA polyclonal antisera.

Table 2. NGF peptide mimics induce TrkA internalization.

4-3.6 cells were treated with the indicated ligands or controls at 4°C in the presence (+) or absence (-) of sodium azide. Cells were then maintained at 4°C or shifted to 37°C to allow internalization. Analysis was done by FACScan, immunostaining surface TrkA with mAb 5C3. Percentage loss of binding was calculated as a change in mean channel fluorescence (MCF) with respect to untreated cells. Average ± sem, n=3, 5000 cells acquired each assay.

	TREATMENT	Na azide	% loss of mAb 5C3 binding sites	
			at 4°C	at 37°C
1	2 nM NGF	-	21 ± 6	46 ± 9
2	10 μM C(92-96)	-	1 ± 7	23 ± 8
3	10 μM C(92-96)	+	4 ± 7	-4 ± 6
4	10 μM linear peptide	-	4 ± 4	3 ± 2
5	10 μM linear peptide	+	5 ± 2	8 ± 3

C(92-96) did not block surface mAb 5C3 binding sites at 4°C (Table 2, row 2). Treatment with C(92-96) at 37°C caused a ~23% loss of surface mAb 5C3 binding sites (Table 2, row 2). This effect was sensitive to sodium azide (Table 2, row 3). A 23% loss of surface TrkA represents ~11,000 receptors that presumably internalized, out of ~50,000 expressed at the surface. Similar results were obtained with C(92-97) (data not shown). Negative control linear peptides did not affect the number of mAb 5C3 binding sites (Table 2, rows 4 and 5).

In positive control studies, treatment with NGF at 4°C blocked ~21% of the surface mAb 5C3 binding sites (Table 2, row 1; also published in (19)), suggesting that NGF partially blocks mAb 5C3. Treatment with NGF at 37°C increased loss of surface mAb 5C3 binding sites from ~21% to ~46% (Table 2, row 1), likely because of TrkA internalization.

DISCUSSION

We demonstrate that artificial ligands selective for subunits of receptor complexes can be used to study receptor structure-activity relationships in systems where each subunit has distinct or unclear functions. For NGF receptors, the main novel findings of this study are: (*i*) genuine monomeric and monovalent ligands of TrkA can be partial agonists, suggesting that bivalent ligands are not the sole mechanism for dimerizing and/or activating tyrosine kinase receptors; (*ii*) the monovalent p75 ligands used in this study do not enhance TrkA-mediated signals, suggesting that p75 ligands may require bivalency. Lastly, bivalent ligands that induce TrkA-TrkA and p75-p75 homodimers afford optimal signals. Putative ligand-induced TrkA-p75 heterodimers do not seem to afford signals, and receptor oligomerization does not result in enhanced signals.

While it is well-established that physiological ligands activate signal transduction by inducing receptor dimerization, allosteric models of receptor activation have also been proposed (15,32-36). There are 2 major obstacles to study allosteric models experimentally. First, paucity of monovalent ligands that activate receptor tyrosine kinases (18). Second, the role of each subunit must be considered in the analysis of heteromeric receptors, and agents that bind to and affect the activity of each subunit must be available. We postulate that the strategy of using growth factor-derived and antibody-based artificial ligands can be easily adapted to study other multisubunit receptors, or for receptors where a subunit has unclear function or no defined ligand. Also, strategies that develop monovalent small molecule agonists that bind to the extracellular domain of receptors will be useful for the discovery of pharmacological agents.

Valency, avidity, and aggregation state of agonistic ligands

Monovalent ligands 5C3 F_{ab}s and C(92-96) are partial agonists. For T cell receptor complexes and G-coupled receptors it has been shown that sometimes ligands with high affinity can overcome low avidity and lead to activation or to conformational changes (37-39). This does not seem to be the case for the ligands 5C3 F_{ab}s or C(92-96) because

their affinity ranges from nM to μM. Hence NAc-C(92-96) is a ligand of relative low affinity and avidity that can induce or stabilize putative TrkA homodimers. Since receptor dimerization alone does not necessarily cause activation (15,17) the simplest interpretation is that the TrkA ligands induce allosteric or conformational changes, as shown for other receptors (16,18,35). However this report would be a case where monomeric and monovalent ligands induce allosteric or conformational changes

Arguably 5C3 F_{ab}s could aggregate in solution as do other large peptides (40), but this is unlikely to occur at *nanomolar* F_{ab} concentrations in 0.2% BSA and did not occur at *micromolar* F_{ab} concentrations in related studies (41). Hence we conclude that F_{ab}s are monomeric. It is also unlikely that 5C3 F_{ab}s are bivalent, and sequence analysis of the variable complementarity determining regions of mAb 5C3 excluded this possibility (our unpubished data).

More conclusively, the *genuine* monomeric small peptide C(92-96) affords trophic signals, although the dimeric $C(92\text{-}97)_{dimer}$ is more efficient. Monomeric C(92-96) is monovalent because it has a 5-residue pharmacophore (27), and could not interact with two receptors simultaneously. The intriguing possibility that C(92-96) could dimerize *after* docking is unlikely (see below), but it remains unexplored and would require structural analysis of receptor-ligand complexes.

Ligand density at the cell surface

The optimal functional concentration of the peptide NAc-C(92-96) and of mAb 5C3 and 5C3 F_{ab}s approximates their K_d (respectively 10 μM, 2 nM and 5 nM). In contrast, the optimal functional concentration of 1 nM NGF is 2-3 orders of magnitude above its K_d ($\sim 10^{-11}$ - 10^{-12} M). It is unlikely that these differences reflect requirements for receptor occupancy. It is more likely that NGF, the mAbs, or the peptides have different half lives in solution at 37°C; or that they are bound by matrix proteoglycans or carrier proteins.

In some cases the local concentration of a ligand at the cell surface can be higher than in solution, making the ligands more propense to dimerize or to aggregate. While the

mobility of a ligand is reduced to two dimensions when bound to a receptor, ligand mobility within the plane of the membrane is still exclusively dependent on the receptor. Therefore, monovalent ligands could only dimerize subsequent to inducing their receptors to dimerize. In addition, we estimate that a cell expressing 50,000 receptors out of which 5% are bound would achieve a local ligand concentration of ~10 mM. This concentration was tested by NMR for monovalent C(92-96) with no evidence of aggregation. Lastly, we demonstrated that the monovalent ligands induce rapid receptor internalization, which will effectively reduce possible high local concentrations of ligand at the cell surface. It is noteworthy that self aggregation of C(92-96) beyond the detection of NMR is unlikely to account for the effects because $C(92\text{-}97)_{dimer}$ at high nM concentrations did not afford signals.

Mechanism of action of TrkA and p75 ligands

How can conformational changes induced by monovalent ligands lead to receptor dimerization? While the function of the monovalent ligands is defined by comparison with the natural ligand NGF, their mechanism of action may differ. We hypothesize three possible mechanisms: (i) conformational changes that favor direct dimerization; (ii) a reduction of the rate at which preformed receptor dimers disengage; (iii) increased receptor mobility with a consequent increase in spontaneously dimerized receptors.

The most attractive explanation is that the monovalent ligands could be inverse antagonists (32,33). Inverse antagonists can stabilize receptor conformation(s) which are favorable to subsequent TrkA-TrkA interactions. Indeed, one criterion for defining inverse antagonists is that their potency increases with increased receptor density on the cell surface; and this was observed for the activity of C(92-96) and $C(92\text{-}97)_{dimer}$ in PC12 cells that have low TrkA numbers *versus* 6-24 and 4-3.6 cells that have high TrkA numbers. Furthermore, an inverse antagonist would antagonize the natural agonist NGF, and NGF blocking properties have been shown previously for C(92-96) (20,21). Lastly, as expected, the $C(92\text{-}97)_{dimer}$ affords higher signals which would be predicted for a bivalent ligand that induced receptor dimerization directly.

With respect to p75 receptors, bivalent ligands potentiate the effects of all *bivalent* TrkA ligands (NGF, mAb 5C3, 5C3 F_{ab}•α-F_{ab} complexes, and $C(92\text{-}97)_{dimer}$). However, bivalent p75 ligands did not potentiate all *monovalent* TrkA ligands. MAb MC192 potentiated the activity of C(92-96) but it did not potentiate the activity of 5C3 F_{ab}s. These data suggest that these monovalent TrkA ligands likely have different mechanisms of activation, and this possibility is supported by the fact that C(92-96) and 5C3 F_{ab}s bind to non-overlapping sites.

We speculate that a small molecule like C(92-96) docks onto a small pocket of TrkA and may therefore be sensitive to a documented p75-mediated regulation (6,7) or internalization (9,42,43) of TrkA. Consequently, C(92-96) may be sensitive to ligands binding p75 concomitantly; whereas larger molecules like 5C3 F_{ab}s possess more extended TrkA binding surfaces do not exhibit this sensitivity. Hence, two classes of partial agonism (or inverse antagonism) by monomeric TrkA ligands may have been uncovered in this study.

Agonistic ligand binding sites

MAb 5C3 and the NGF analog C(92-96) do not block each other's binding, hence they bind to non-overlapping sites of TrkA. Ligands docking onto restricted receptor pockets or "hot spots" are presumed to be more efficient at mediating (ant)agonistic function (34,44). Reportedly, there are at least two activating TrkA "hot spots" (45-48) encompassing the IgC-2 like domain and the Leucine Rich Motif (LRM).

MAb 5C3 binds an epitope within the IgC-2 like domain of TrkA, and the epitope is stabilized by disulfide-bonds (19). TrkA and other tyrosine kinase receptors have a dimer interface stabilized by disulfide bonds (49,50). The agonistic "hot spot" of mAb 5C3 and 5C3 derivatives may be at the dimer interface of this receptor. However, 5C3 and C(92-96) do not block each other, hence the data suggest that C(92-96) binds elsewhere. NGF may utilize both sites to fully activate the receptor.

Conclusions

The screening of functional small molecule ligands that bind multisubunit receptors may require testing ligand combinations that target all subunits. It would be of interest to test other NGF receptor ligands or activators in this paradigm of synergy, including peptidic small molecule p75 ligands (21) or alike peptides reproduced by others (51,52); organic p75 ligands; gangliosides; and alkaloid derivatives that activate TrkA (53-55).

With respect to NGF receptors, our results support the hypothesis that functional receptors consist of TrkA homodimers and p75 homodimers. Our results also demonstrate that genuine monovalent and monomeric ligands of TrkA tyrosine kinase receptors can be functionally agonistic. Recently, two small molecule ligands of other receptors were shown to be agonistic. In one, a mimic of granulocyte-colony-stimulating factor (GCSF) activated the GCSF receptor (56) but no studies of the aggregation state of the ligand were performed. In another, a small molecule activated the insulin receptor tyrosine kinase (57). However, this insulinomimetic ligand is a symmetrical lipophylic agent, in principle capable of dimerizing the receptor as shown for similar ring structures (16). Hence, our study is the first formal proof, to our knowledge, of genuine monovalent ligands of the extracellular domain of a tyrosine kinase acting as partial agonists by inducing or stabilizing receptor homodimers.

Neurotrophins and their receptors play a role in neurodegenerative diseases, pain and neoplasias (44). Internalizing TrkA ligands could be exploited to deliver radioligands, toxins, oligonucleotides or membrane impermeable molecules selectively to receptor-expressing cells. This study has implications for the design and screening of small molecules with pharmacological, diagnostic, or targeting activity for neurotrophin receptors.

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Connection to Chapter III.

It has been shown in a study presented in Chapter II that defined monovalent ligands of TrkA NGF receptor could act as partial agonists alone or in synergy with selective ligands of $p75^{NTR}$. They induced TrkA activation, TrkA internalization and trophic cellular responses. These findings are of significant importance for the development of agonistic ligands of TrkA, other Trk family neurotrophin receptors and cell surface receptors with a single transmembrane region in general.

However, the nature of the developed and characterized artificial ligands (antibody fragments and small cyclic peptides) does not make them good drug candidates. Proteins and peptides have inherent drawbacks that hinder their wide use as therapeutic agents. They have insufficient bioavailability and must be delivered directly into the bloodstream. Rapid proteolytic degradation reduces their half-lives to minutes and seconds. They do not cross readily blood-brain barrier that is especially important for neurotrophic agents. They also can be highly antigenic and are relatively expensive to produce.

The technology of constructing of cyclic non-peptide mimetics of protein turn regions have been developed and applied to different targets (Saragovi *et al.*, 1991; Chen *et al.*, 1992; Andrade-Gordon *et al.*, 1999; reviewed by Saragovi *et al.*, 1992). These mimetics are conformationally restricted and can incorporate amino acid residues and amino acid-like moieties. In a study presented in Chapter III similar technology was used to create a focused library of macrocyclic ring-based compounds designed to mimic a β-turn C-D of NGF. These compounds were screened in a paradigm of synergy with a $p75^{NTR}$-activating ligand. This led to selection and characterization of a small proteolytically stable compound that bound human TrkA and exhibited agonistic properties in cellular assays.

Chapter III. **Maliartchouk S., Feng Y., Ivanisevic L., Debeir T., Cuello A.C., Burgess K., and Saragovi H.U. 2000. A designed peptidomimetic agonistic ligand of TrkA nerve growth factor receptors. Mol. Pharmacology, 57:385–91**

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A Designed Peptidomimetic Agonistic Ligand of TrkA Nerve Growth Factor Receptors

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Abstract

A proteolytically stable small molecule β-turn peptidomimetic, termed D3, was identified as an agonist of the TrkA neurotrophin receptor. D3 binds the immunoglobulin-like C2 region of the extracellular domain of TrkA, competes the binding of another TrkA agonist, affords selective trophic protection to TrkA-expressing cell lines and neuronal primary cultures, and induces the differentiation of primary neuronal cultures. These results indicate that a small β-turn peptidomimetic can activate a tyrosine kinase neurotrophin receptor that normally binds a relatively large protein ligand. Agents such as D3 that bind the extracellular domain of Trk receptors will be useful pharmacological agents to address disorders where Trk receptors play a role, by targeting populations selectively.

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TrkA is a transmembrane tyrosine kinase receptor with high selectivity for the neurotrophin nerve growth factor (NGF*). Related neurotrophins include Brain Derived Neurotrophic Factor (BDNF) which binds TrkB receptors, and Neurotrophin-3 (NT-3) which prefers binding to TrkC receptors (Barbacid, 1994).

Docking of TrkA with NGF initiates receptor dimerization, catalytic phosphorylation of cytoplasmic tyrosine residues on the receptor, and a cascade of cell signaling events (Kaplan and Stephens, 1994). These signals lead to prevention of apoptotic cell death (Maliartchouk and Saragovi, 1997), to promotion of cellular differentiation and axon elongation, and upregulation of choline acetyl transferase (ChAT) (Hefti et al., 1985).

Several neuronal cell types that are implicated in important disease states express TrkA and therefore respond to NGF, including sensory, sympathetic and cholinergic neurons. It has been suggested that NGF therapy may delay the onset of Alzheimer's disease (Barinaga, 1994; Lindsay, 1996), and ameliorate peripheral diabetic neuropathies (Ebadi et al., 1997). Other applications proposed for NGF include treatment of neuronal damage (Hughes et al., 1997), and targeting of neuroectoderm-derived tumors (Cortazzo et al., 1996; LeSauteur et al., 1996). For a review of disease targets see (Saragovi and Burgess, 1999).

Despite the therapeutic potential of NGF clinical trials featuring this protein have been disappointing (Saragovi and Burgess, 1999; Verrall, 1994). There are several reasons for this: inherent drawbacks associated with the use of polypeptides applied as drugs (Saragovi et al., 1992), *in vivo* instability (Barinaga, 1994), and pleiotrophic effects due to activation of signals that were not intentionally targeted (e.g those mediated via the

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*ABBREVIATIONS: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; BOC, tert-butoxycarbonyl; ChAT, choline acetyl trans-ferase; DMF, dimethylformamide; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbent assay; FACScan, fluorescent activated cell scanner; FITC, fluorescein isothiocyanate; FMOC, fluorenyloxycarbonyl; MCF, mean channel fluorescence; MTT, 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide; NT-3, neurotrophin-3; RIA, radioimmunoassay; TFA, trifluoroacetic acid; Trt, trityl; ECD, extracellular domain; mAb, monoclonal antibody; HEK, human embryonic kidney; BB, binding buffer; OD, optical density.

low affinity NGF receptor p75 (Carter and Lewin, 1997)). Moreover, the NGF protein is relatively expensive to produce for medicinal applications.

For these reasons, we aimed to design small, proteolytically stable molecules with neurotrophic activity, selective for cells expressing TrkA. However, strategies that result in agonists of tyrosine kinase receptors have not been well established. Previously, we used ligand mimicry and antibody mimicry strategies (Saragovi et al., 1991; Saragovi et al., 1992) to generate peptide analogs of two agonists directed to the extracellular domain of TrkA: the natural ligand NGF (Debeir et al., 1999; LeSauteur et al., 1996; LeSauteur et al., 1995), and monoclonal antibody (mAb) 5C3 (LeSauteur et al., 1996). TrkA binding is mediated by discrete β-turn regions of these ligands. Only certain cyclic β-turn analogs were active (Beglova et al., 1998), and other conformers or linear peptides were inactive.

Based on the pharmacophores of the mAb 5C3 and NGF peptide analogs described above, we synthesized a focussed β-turn peptidomimetic library of ~60 members. We report the identification of compound D3, a small, selective, and proteolytically stable agonist of the TrkA receptor. Furthermore, the docking site of D3 onto TrkA was studied. Our findings support the notion that a small peptidomimetic ligand can agonize a tyrosine kinase neurotrophin receptor that normally binds a relatively large protein ligand. These agents may offer an alternative therapeutic strategy with pharmacological agents that selectively target neuronal populations expressing specific receptors on the cell surface.

Materials and Methods

Preparation of D3 and D3-biotin. Compound D3 was prepared according to methods previously outlined for related compounds (Feng et al., 1998). FMOC-Gly, FMOC-Hse(Trt), FMOC-Lys(BOC), FMOC-Glu(OtBu), then 2-fluoro-5-nitrobenzoyl chloride were coupled (di-iso-propylcarbodiimide activation, 20 % piperidine in DMF to remove FMOC groups) to TentaGel S PHB resin at 0.18 mmol/g loading. The supported peptide was treated six times with 1 % TFA/4 5 $HSiiPr_3$ in CH_2Cl_2 for 5 min to remove only the Trt-protection. Cyclization was effected by treatment with 5.0 equivalents of K_2CO_3 in DMF for 40 h. After 90% TFA / 5% H_2O / 5% $HSiiPr_3$ cleavage, the final product was

purified by reverse phase HPLC. D3 and its derivatives were soluble in water to 5 mg/ml (the highest concentration tested).

D3-biotin was prepared in the same way as D3, except that after the cyclization the nitro group was reduced by treatment with 10 equivalents of $SnCl_2.2H_2O$ in DMF for 20 h. After reduction, FMOC-Gly, then biotin-N-hydroxysuccinimide was coupled to the newly formed arylamine. The product was then cleaved from the resin. The final product was purified by reverse phase HPLC.

Cell lines: B104 rat neuroblastomas express p75 receptors but do not express any of the Trks (TrkA- p75+). The 4-3.6 cells are B104 cells stably transfected with human trkA cDNA, and express equal levels of p75 and TrkA (TrkA+ p75+) (kindly provided by Dr. E. Bogenmann) (Maliartchouk and Saragovi, 1997). Surface expression of each of NGF receptor was routinely controlled in all cells by quantitative FACScan assays (Becton Dickinson, CA) (data not shown) using anti-TrkA mAb 5C3 and anti-p75 mAb MC192.

Generation of human TrkA-rat TrkB chimeras in HEK293 cells The IgG-C2 domain of human TrkA was generated by PCR as described (Perez et al., 1995) using unique restriction sites in the primers to allow exchange with the corresponding rat TrkB domain. The chimeric receptors were constructed by subcloning the human TrkA IgG-C2 domain into the corresponding restriction sites of the rat *trkB* cDNA reported in a previous work (Perez et al., 1995). Chimeric constructs (kindly provided by Dr. P. Perez) were confirmed by sequencing, and were cloned into the pCDNA3 expression vector that contains a selection gene providing resistance to neomycin (G418, GIBCO). HEK293 cells were transfected using the lipofectamine plus method (GIBCO), selected with neomycin (0.5 mg/ml) and at least 3 independent subclones were obtained by limiting dilution techniques (293-TrkB/A-IgC2 chimera). Western blot analysis with polyclonal antibody 203 directed to the Trk intracellular domain (a gift of Dr. D. Kaplan), and cell surface FACScan analysis with polyclonal antibody 1001 directed to the TrkA extracellular domain (our unpublished data) indicated that all stable subclones express comparable levels of chimeric receptors (data not shown).

Dissociated neuronal dorsal root ganglia cultures: Fetal rat DRG primary cultures were established essentially as described (Kimpinski et al., 1997) from Sprague Dawley day 17

rat embryos. All ganglia were dissected and dissociated first enzymatically with trypsin and then mechanically. Dissociated cells were cultured (10^5 cells/well) in 96 well plates pre-coated with collagen, and grown for a total of 8 days in Neuro Basal Medium containing N2 supplement (GIBCO, Toronto), antibiotics, and L-glutamine. These DRG cultures are ~85% TrkA-expressing and are heavily dependent on TrkA signals for survival (Vogelbaum et al., 1998).

Septal Neuronal Cultures: Cell cultures were established from the septal area of 17-day-old rat embryos as described (Hefti et al., 1985). In brief, tissue was incubated in PBS containing trypsin and DNase. Tissue pieces then were mechanically dissociated. After centrifugation, the pellet was suspended in Leibovitz's L-15 medium. Cells were plated onto 96-multiwell NUNC dishes (10^5 cells/well) coated with poly-D-lysine (5 μg/ml). Mixed cultures of septal neurons were treated 1 day after plating. Drugs, prepared in medium, were added directly to the cells without changing the initial medium. The incubation continued for 8 days, at which time ChAT activity was evaluated.

D3•TrkA binding assays.

Direct binding studies: were done as described (Saragovi et al., 1998) using 6 ng/well of recombinant baculovirus TrkA-extracellular domain protein (TrkA-ECD) or control bovine serum albumin (BSA, Fraction V, Boehringer Mannheim) immobilized onto 96-well microtest plates. Wells were blocked with binding buffer (BB: PBS with 1% BSA) for 1 hour. Then, 50 ng/well of biotinylated D3 were added as primary reagent in BB for 40 min in the absence or presence of excess non-biotinylated D3 as competitor. Wells were washed 5 times with BB, and horseradish peroxidase (HRP)-coupled avidin (Sigma) was added as secondary reagent for 30 min. Plates were washed in BB, and peroxidase activity was determined colorimetrically using 2,2-azinobis (3-ethylbenzthiazoline sulfonic acid) (ABTS, Sigma). The optical density (OD) was measured at 414 nm in a Microplate reader (Bio-Rad). Assays were repeated at least three times, n=4.

FACScan binding assays: 4-3.6 cells ($2x10^5$) in FACScan binding buffer (PBS, 0.5% BSA, and 0.1% NaN3) were immunostained as described (LeSauteur et al., 1996; Saragovi et al., 1998). Saturating anti-TrkA mAb 5C3, or anti-p75 mAb MC192, or

control non-binding IgGs were added to cells for 1 hour at 4°C, in the presence or absence of D3 as competitor. Excess primary antibody was washed off, and cells were immunostained with fluorescinated goat-anti-mouse IgG secondary antibody. Cells were acquired on a FACScan and mean channel fluorescence of bell-shaped histograms were analyzed using the LYSIS II program.

Binding Competition: studies were as described for direct binding assays to TrkA-ECD, except that as primary reagent 50 ng anti-TrkA mAb 5C3/well were added in BB, in the presence or absence of D3 or controls as competitors as described (Saragovi et al., 1998). Wells were washed 5 times with BB, and HRP-coupled goat anti-mouse was added as secondary reagent for 30 min. Plates were washed in BB, and peroxidase activity were determined. Assays were repeated at least three times, n=4.

Cell survival assays.

Primary DRG cultures: After a total of 8 days of culture with the indicated test or control ligands, cell survival were studied using the 3(4,5-Dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide colorimetric (MTT) assay, and by microscopic observation as described (Debeir et al., 1999).

Cell lines: 5,000 cells/well in protein-free media (PFHM-II, GIBCO, Toronto) containing 0.2% bovine serum albumin (BSA) (Crystalline fraction V, Sigma, St. Louis, MO) were seeded in 96 well plates (Falcon, Mississauga, Ontario). The cultures were untreated, or treated with the indicated test or control ligands. Cell viability was quantitated using the MTT assay after 56-72 hours of culture, as described (Maliartchouk and Saragovi, 1997). Percent protection was standardized from optical density (OD) readings relative to optimal NGF (1 nM) = 100%. The OD of untreated cells were subtracted. The higher optical density of untreated primary cultures is likely due to cellular heterogeneity and to endogenous production of limiting amounts of growth factors.

Measurement of ChAT Activity. At day 8 of culture, the medium was aspirated, and ice-cold lysis buffer (10 mM sodium phosphate, pH 7.4/0.1% TritonX-100) was added. ChAT activity assays were performed directly in the wells using Fonnum's method (Fonnum, 1975).

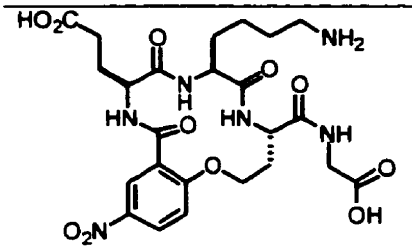
Detection of putative TrkA•TrkA homodimers. Live 4-3.6 cells suspended in PBS were treated with the indicated ligand(s) for 40 min at 4°C to allow binding. Cells were then washed in PBS, cross-linked with the membrane impermeable cross-linker disuccinimidyl suberate (DSS, Pierce; 1 mM DSS, 15 minutes at 15°C). Unreacted DSS was quenched with 5 mM ammonium acetate. Then cells were either lysed directly in SDS sample buffer (whole cell lysate), or lysed in non-ionic detergent NP-40 and immunoprecipitated with anti-Trk or anti-p75 antibodies as described (LeSauteur et al., 1996). Similar results were obtained with either method. For western blot analysis, equal amounts of protein or cell equivalents for each sample were resolved in a 5-10% SDS-PAGE gradient, transferred to nitrocellulose membranes (Xymotech Biosystems, Montréal, Qc), and blotted with anti-Trk polyclonal antibody 203 that recognizes the intracellular domain of Trk. Blots were visualized using the enhanced chemiluminescence (ECL) system (New England Nuclear, Boston, MA).

Results

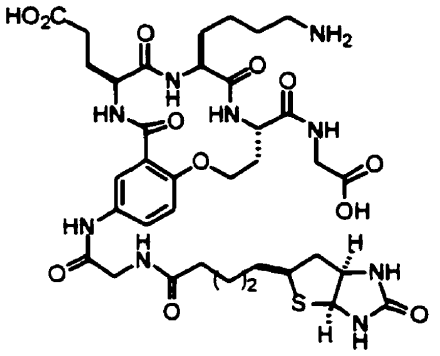
Synthesis of focussed β-turn peptidomimetic libraries

A solid phase synthesis was developed to yield a macrocyclic ring with the i + 1 and i + 2 residues of a β-turn in the appropriate conformation. Approximately 60 compounds of this type were prepared (Feng et al., 1998), with amino acid side chains incorporated to correspond to β-turns of NGF and mAb 5C3 implicated in docking to TrkA (LeSauteur et al., 1996; LeSauteur et al., 1996; LeSauteur et al., 1995; Debeir et al., 1999). TrkA binding is mediated by discrete β-turn regions of these ligands. Cyclic peptide β-turn analogs of NGF and of mAb 5C3 were active only in the appropriate conformation (Beglova et al., 1998).

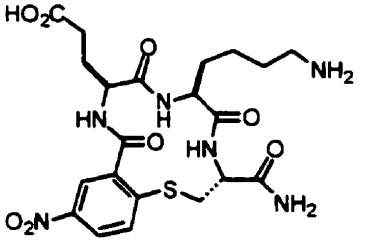
Figure 1 shows the molecular structure of D3 and that of a similar, but inactive, molecule called C59. C59 was used as a negative control. A biotinylated form of D3, termed D3-biotin, was synthesized to carry out direct binding studies to TrkA. All ligands were highly soluble in physiological buffers and did not require organic solvents.



D3



D3-Biotin



C59

Figure 1. Structures of D3, D3-biotin, and C59.

D3 is a selective ligand of TrkA

FACScan analysis featuring the secondary fluorescent agent avidin-FITC was used to detect binding of D3-biotin to the cell surface (Table 1). The 4-3.6 cells (p75+TrkA+) had fluorescence approximately 4 times greater for D3-biotin than for a background control peptide-biotin. Moreover, a 10-fold molar excess of D3 abolished binding of D3-biotin. In contrast, no specific binding was measured for B104 cells (p75+TrkA-). Since 4-3.6 cells are B104 cells stably transfected with *TrkA* cDNA and

these cell lines are otherwise identical, the data indicate that D3-biotin and D3 bind cell surface TrkA.

Similar binding data for D3-biotin was obtained by ELISA using pure soluble TrkA extracellular domain (TrkA-ECD) produced in baculovirus (data not shown, also see Table 3). These data further indicate that D3 binds to the extracellular domain of TrkA, and that membrane lipids are not required.

Table 1. D3 and D3-biotin bind TrkA.

Binding of biotin-D3 to B104 cells (p75+ TrkA-) or 4-3.6 cells (p75+ TrkA+) was quantitated by FACScan analysis. Ligands are control-biotin (an inactive biotinylated peptide) (row 2), D3-biotin (row 3), or D3-biotin with a 10-fold molar excess of D3 (row 4). All ligands were followed with avidin-FITC as a fluorescent label. Data shown are mean channel fluorescence (MCF) of bell-shaped histograms, 5,000 events acquired. MCF data ± sem are averaged from 3 independent experiments.

Ligand	MCF	
	B104	4-3.6
untreated	10 ± 3	13 ± 2
control-biotin 20 μM	11 ± 1	10 ± 3
D3-biotin 20 μM	10 ± 4	53 ± 4
D3-bio 20 μM + D3 200 μM	11 ± 2	17 ± 7

D3 binds within an agonistic site of TrkA

Previously, mAb 5C3 was shown to act as a full TrkA agonist. MAb 5C3 binds with K_d 2 nM (LeSauteur et al., 1996) at an epitope within the IgC2 domain of TrkA near the NGF binding site. This site is postulated to define a receptor "hot spot" (Wells, 1996). We tested whether D3 and mAb 5C3 bind to overlapping receptor sites.

Table 2. D3 specifically blocks mAb 5C3 binding to cell surface TrkA.

4-3.6 cells were analyzed by FACScan for binding of anti-TrkA mAb 5C3 or anti-p75 mAb MC192. Cells exposed to control primary mouse IgG with or without 40 μM D3 afford identical background staining (data not shown). For each condition 5,000 cells were acquired. Percentage maximal bindings were calculated from the MCF of bell-shaped histograms, using the formula:

$(TEST_{MCF}–background_{MCF})*100 / (MAXIMAL_{MCF}–background_{MCF})$.

MCF ± sem are averaged from 3 independent experiments.

	MAb (1 nM)	Competitor	Dose (μM)	% Maximal binding
1	5C3	none	0	100 ± 0
2	5C3	D3	0.20	95 ± 4
3	5C3	D3	1	80± 3
4	5C3	D3	5	53 ± 5
5	5C3	D3	40	33 ± 4
6	5C3	C59 control	40	97 ± 6
7	MC192	none	0	100 ± 0
8	MC192	D3	40	101 ± 2

Two related assays tested the ability of D3 to compete for the binding of the full TrkA agonist mAb 5C3. In the first test, a FACScan-based assay using intact cells, D3-induced a dose-dependent competitive decrease of mAb 5C3•TrkA interactions (Table 2, rows 2-5). On average, D3 exhibited an IC_{50} of 4 μM. From experimental conditions we estimate a K_d ~2 μM for D3•TrkA interactions. Blocking of 5C3•TrkA interactions by D3 is selective because the binding of mAb MC192 directed to the p75 NGF receptor subunit was not blocked (Table 2, rows 7 vs 8). Furthermore, inactive control C59 peptidomimetic did not inhibit the binding of either mAb 5C3 (Table 2, row 6) or mAb MC192 (data not shown).

The second test used purified recombinant TrkA extracellular domain (TrkA-ECD) immobilized onto ELISA plates to assay competitive blocking of 5C3•TrkA-ECD by D3.

Table 3. D3 inhibits 5C3•TrkA interactions *in vitro.*

The binding of mAb 5C3 (at constant 2 nM) to purified TrkA-ECD immobilized onto ELISA plates was measured in the absence or presence of competitors. Background (<2%) was the optical density of wells with all reactants except immobilized TrkA-ECD. Data are averaged from 3 experiments, each experiment n=4.

	Competitor added	**Concentration (μM)**	**% Binding ± sem**
1	-	-	100 ± 3
2	D3	0.05	100 ± 14
3	D3	0.2	89 ± 8
4	D3	1	64 ± 10
5	D3	5	43 ± 12
6	D3	20	38 ± 7
7	D3	40	31 ± 4
8	C59	40	96 ± 9

D3 exhibited a dose-dependent inhibition of 5C3•TrkA-ECD interactions, but control inactive C59 peptidomimetic had no effect (Table 3). Since a K_d ~2 nM was measured for 5C3•TrkA interactions , from the experimental IC_{50} a K_d ~2 μM was calculated for D3•TrkA-ECD interactions. This calculation is consistent with the data shown in Table II. Interestingly, similar ELISA and RIA binding assays revealed that D3 did not substantially block NGF•TrkA-ECD interactions (data not shown).

D3 affords trophic activity selectively via TrkA, and is proteolytically stable

Since D3 binds at or near an agonistic site of TrkA, trophic effects were probed in cell survival assays using the quantitative MTT method (Maliartchouk and Saragovi, 1997). Several doses of D3 were tested. However, for clarity only near optimal concentrations are shown, which approximate the estimated K_d.

Table 4. D3 protects TrkA-expressing primary neurons from apoptosis, and potentiates NGF.

NGF-dependent primary neuronal cultures from embryonic rat DRGs were treated with the indicated ligands for a total of 8 days. Cell survival was measured by MTT assays. Protection was calculated relative to optimal NGF (1 nM, 100% protection) with subtraction of the O.D. of untreated cells. Shown is the O.D. from one experiment, mean ± sem. n=4. % protection was averaged from 3 experiments.

	Treatment	Optical Density	% Protection
1	untreated	256 ± 15	0 ± 2
2	NGF 1 nM	823 ± 28	100 ± 4
3	NGF 20 pM	316 ± 11	9 ± 1
4	NGF 500 pM	535 ± 19	68 ± 3
5	D3 10 μM	405 ± 22	38 ± 2
6	Control C59 10 μM	271 ± 8	0 ± 1
7	D3 10 μM + NGF 20 pM	471 ± 28	48 ± 3
8	D3 10 μM + NGF 500 pM	603 ± 26	84 ± 3
9	D3 10 μM + NGF 1 nM	977 ± 38	120 ± 7

Dissociated primary neuronal cultures from fetal dorsal root ganglia (DRG) are dependent on TrkA agonists for survival (Vogelbaum et al., 1998). Exogenous NGF

showed a dose-dependent trophic effect (Table 4, rows 2-4). D3 alone had a significant protective effect on DRG cultures (Table 4, row 5) but control C59 did not (Table 4, row 6). Primary cultures are heterogeneous and low levels of neurotrophins are made endogenously (Kimpinski et al., 1997), which explains a relatively high optical density for untreated cultures (Table 4, row 1).

Since D3 does not block NGF binding, potential synergy between NGF and D3 was assessed. D3 combined with different concentrations of exogenous NGF demonstrated an additive or potentiating effect on DRG survival (Table 4, rows 7-9).

Similar results were obtained with other neuronal cell lines, wherein D3 potentiated the effect of low NGF concentrations (Table 5). Optimal protection of 4-3.6 cells (p75+TrkA+) and HEK293-TrkB/A-IgC2 chimeras corresponded to treatment with 1 nM NGF (Table 5, row 2) whereas 10 pM NGF gave significantly less protection (Table 5, row 3). D3 alone afforded low but significant protection (Table 5, row 4), and protection was enhanced with a combination of 10 pM NGF + 10 μM D3 (Table 5, row 6). The negative control C59 compound had no effect alone or in enhancing 10 pM NGF (Table 5, rows 5 and 7).

In other controls (data not shown), neither D3 nor NGF protected B104 cells, wild type HEK293 cells, or TrkB-expressing HEK293 cells from apoptosis. Hence the trophic activity of NGF and D3 require TrkA expression, or at least the IgG-C2 domain of TrkA. Additionally, D3 did not enhance the trophic effect of EGF suggesting that it may be NGF selective. Lastly, D3 enhanced NGF protection of NIH3T3 cells stably transfected with *TrkA* cDNA (data not shown) but did not enhance NT-3 protection of NIH3T3 cells stably transfected with *trkC* cDNA. These data indicate that D3 selectively accentuates the trophic effect of NGF, and that p75 expression is not required.

The proteolytic stability of D3 versus trypsin and papain was assessed. D3 was first exposed to enzymatic treatment as described previously (Saragovi et al., 1991; Saragovi et al., 1992), followed by gauging its biological activity on 4-3.6 cells. Compound D3 remained fully active in trophic assays even after 1 hour of exposure to trypsin or pepsin, whereas NGF lost all activity within minutes under the same conditions (data not shown).

Table 5. D3 potentiates NGF in protecting TrkA-expressing cell lines from apoptosis by binding to the IgC2 domain of the receptor.

4-3.6 cells or HEK 293 cells expressing TrkB/TrkA IgG-C2 chimeric receptor were treated with the indicated ligands for a total of 72 hours. Survival was measured by MTT assays. % Protection was calculated as in Table IV. Shown is the O.D. from one experiment, mean ± sem, n=4. Percent protection was averaged from 6 (4-3.6 cells) or 3 (293-IgG-C2 chimera) independent experiments.

	Treatment	4-3.6 cells		HEK 293-TrkB/TrkA chimera	
		Optical Density	% Protection	Optical Density	% Protection
1	untreated	64 ± 7	0 ± 2	32 ± 5	0 ± 4
2	1 nM NGF	412 ± 24	100 ± 6	350 ± 12	100 ± 4
3	10 pM NGF	205 ± 19	40 ± 5	88 ± 8	18 ± 5
4	10 μM D3	95 ± 9	8 ± 2	69 ± 7	9 ± 3
5	10 μM C59	76 ± 4	2 ± 1	30 ± 7	-1 ± 2
6	10 μM D3 + 10 pM NGF	255 ± 14	55 ± 3	165 ± 11	42 ± 5
7	10 μM C59 + 10 pM NGF	209 ± 17	41 ± 4	90 ± 9	21 ± 6

D3 induces differentiation of primary cultures of fetal DRG and fetal septal neurons

The effect of D3 on TrkA-mediated cellular differentiation was assessed using two independent assays: morphometric analysis of DRG dissociated neurons and induction of ChAT activity in septal neuronal cultures. In the first of these assays, data indicate that DRG neuronal cultures undergo neurite outgrowth in response to D3, and that D3 potentiates the effect of NGF (Figure 2). In the second assay, ChAT activity was found to increase in response to NGF (Table 6, rows 1 and 2) and to D3 alone (Table 6, rows 3-5),

whereas C59 control had no effect (Table 6, row 6). Increases in ChAT activity in response to 2 μM D3 alone were comparable to 10 pM exogenous NGF. Moreover, combinations of 2 μM D3 + 10 pM NGF markedly increased ChAT activity, and were more effective than 400 pM NGF (Table 6, rows 8-10).

Table 6. D3 induces ChAT synthesis.

Septal neuronal cultures were treated as indicated for a total of 8 days. ChAT activity (pmol Ach / min / well ± sem) was measured at day 8. Average ± sem. Data averaged from 3 independent experiments, each experiment n-=4.

	Treatment	ChAT Activity	Fold Increase
1	10 pM NGF	0.42 ± 0.07	1.4
2	400 pM NGF	0.72 ± 0.10	2.41
3	0.2 μM D3	0.37 ± 0.05	1.23
4	2 μM D3	0.44 ± 0.02	1.47
5	20 μM D3	0.48 ± 0.06	1.56
6	20 μM C59 control	0.30 ± 0.05	1
7	untreated	0.31 ± 0.07	1
8	0.2 μM D3 + 10 pM NGF	0.60 ± 0.04	2.00
9	2 μM D3 + 10 pM NGF	0.76 ± 0.03	2.53
10	20 μM D3 + 10 pM NGF	0.79 ± 0.04	2.63

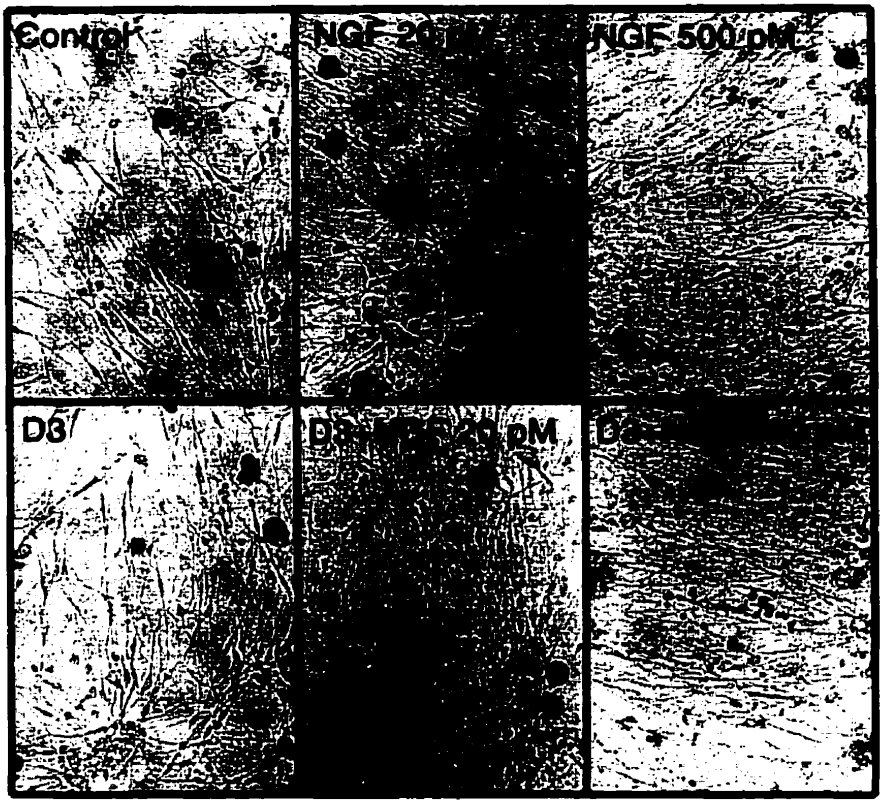


Figure 2. D3 induces the differentiation of embryonic DRG cultures.

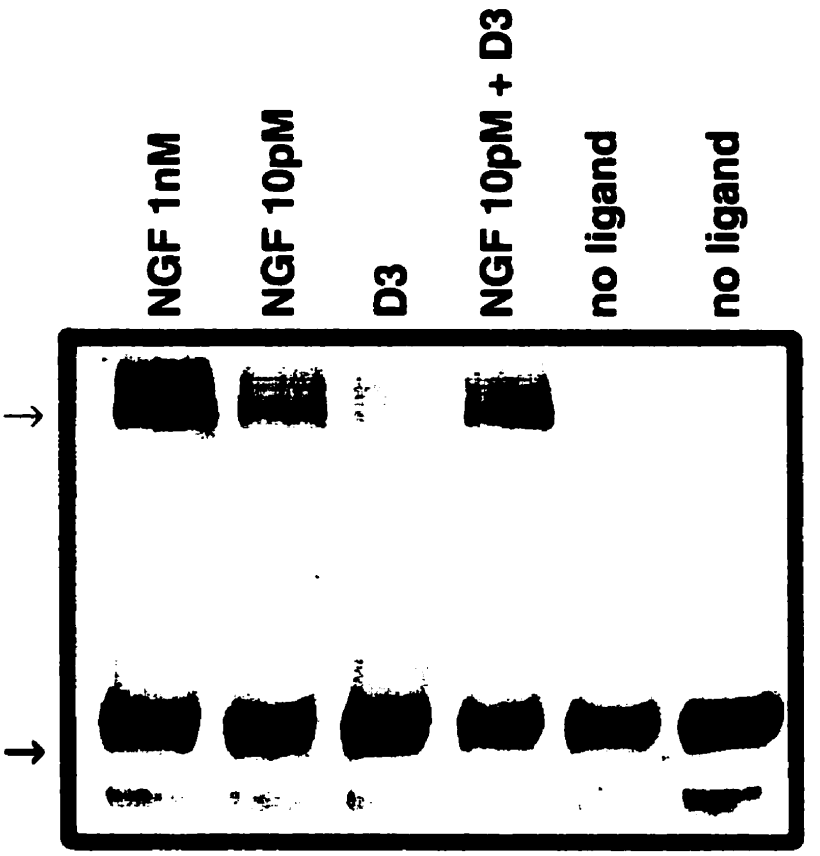
Primary neuronal DRG cultures were treated as indicated for 8 days, and cell differentiation was studied morphometrically. Magnification 60x. Pictures representative of 3 independent experiments.

D3 enhances or stabilizes putative TrkA•TrkA homodimers.

Based on the data above, it was expected that D3 would induce or stabilize TrkA•TrkA interactions. This hypothesis was studied biochemically in 4-3.6 cells exposed to ligands, followed by cell surface chemical cross-linking (Figure 3).

The expected doublet consistent with previously reported TrkA monomers of p110 and p140 were seen in all samples (Figure 3, thick arrow). Bands of ~300 kDa, consistent with the molecular weight of TrkA•TrkA homodimers (Figure 3, thin arrow), were seen in samples from cells treated with TrkA ligands 1 nM NGF, 10 pM NGF, or 10 pM NGF + 10 μM D3, and was also detected (albeit very weakly) in cells treated with 10 μM D3 alone. The intensity of the band M_r 300 kDa, presumed to be TrkA dimers, was analyzed densitometrically from 4 independent experiments standardized to 1 nM NGF (100%). There was a consistent increase in dimers after treatment with D3 alone (21 ± 4%) or 10 pM NGF alone (52 ± 6%), which was higher after treatment with 10 pM NGF + 10 μM D3 (77 ± 7%). Control cells cross-linked in the absence of ligand or cells exposed to ligand but not-cross-linked (data not shown) did not have putative dimers.

TrkA homodimers are stable to SDS denaturation because of covalent cross-linking. Given that the efficiency of chemical cross-linking is ~1-4% of the total TrkA pool, we were precluded from further biochemical characterization of the complexes, other than the fact that they contain TrkA. The complexes may contain cross-linked NGF. However, it is unlikely that the bands comprise p75 because immunoprecipitations with anti-p75 antibodies did not reveal any material in the M_r of TrkA homodimers (data not shown). Furthermore, material of M_r 215 kDa that would comprise p75-TrkA heterodimers was not seen consistently.



cross-linking	+	+	+	+	+	-
% TrkA dimers	100	52±6	21±4	77±7	0	0

Figure 3. D3 enhances cell surface TrkA•TrkA homodimers.

4-3.6 cells were exposed to TrkA ligands as per Table V (lanes 1-4) or no ligand (lanes 5 and 6), and chemically cross-linked (lanes 1-5) or not cross-linked (lane 6). Cell lysates were western blotted with anti-TrkA 203 antisera. The intensity of the M_r 300 kDa band was analyzed densitometrically from 4 experiments standardized to 1 nM NGF.

Discussion

We report on a proteolytically stable β-turn peptidomimetic small molecule agonist of the TrkA neurotrophin receptor. We showed that D3 binds TrkA, competes the binding of the TrkA agonist mAb 5C3, selectively potentiates trophic protection of TrkA-expressing cell lines and neuronal primary cultures, and induces the differentiation of primary neuronal cultures. These results indicate that a small β-turn peptidomimetic can activate a tyrosine kinase neurotrophin receptor that normally binds a relatively large protein ligand.

Recent advances in ligand mimicry have resulted from screening large phage or peptide libraries (Reddy et al., 1996; Wrighton et al., 1996), natural products (Zhang et al., 1999), or chemical libraries (Owolabi et al., 1999). However, most of the ligands described are antagonists, or otherwise require the dimerization of relatively large peptides, have a 2-fold axis of symmetry that resemble a dimer, or are poorly soluble in physiological buffers. In contrast, D3 is a small, non-symmetrical, proteolytically stable, highly water soluble peptidomimetic that binds the extracellular domain of TrkA.

Recently a symmetrical alkaloid-like molecule screened from fungi was found to potentiate, at μM concentrations, the action of insulin presumably by binding near the catalytic domain of insulin receptors (Zhang et al., 1999). Hence, with regards to agonistic activity and optimal concentration our compound D3 is analogous to the insulinomimetic ligand. In addition, binding and ligand competition studies demonstrate selective interaction of D3 with the extracellular domain of TrkA rather than the catalytic domain. Hence, the water solubility and extracellular targeting of D3 mean that toxic organic solvents are not required to permeate the cell membrane.

Mechanism of action of D3

It is surprising that D3 is an agonist because the natural ligand NGF is a symmetrical dimer known to activate TrkA via homodimerization (Kaplan and Stephens, 1994). D3 is not a dimer and, from NMR studies, it has no detectable propensity to dimerize even at high mM concentrations in solution (data not shown). Then, why does D3 behave as an agonist of TrkA? One hypothesis is that D3 stabilizes the signaling

conformation of preformed TrkA homodimers without *per se* inducing efficient receptor dimerization. This hypothesis is supported by the data because, as would be predicted, exposure to low levels of NGF enhanced D3 activity in bioassays and in receptor cross-linking assays.

What is the role of pM concentrations of NGF? Given the low concentrations used in synergy with D3, it is unlikely that the effect of NGF was mediated by docking with the low affinity receptor p75. We speculate that NGF acts by increasing TrkA•TrkA interactions whereas D3 stabilizes the homodimers or reduces the rate of separation of receptor homodimers by inducing conformational changes. Indeed, there are precedents for ligands of serpentine receptors acting in this manner (Milligan et al., 1995), and recent models of single transmembrane receptor dimerization and activation are compatible with this view (Livnah et al., 1999; Remy et al., 1999; Tian et al., 1998).

In the present study, the biological data shown are with low μM concentrations of D3, which are optimal. As expected from the affinity estimated for TrkA•D3 interactions, lower D3 concentrations afford lower efficacy. It is noteworthy that while NGF•TrkA affinity is $\sim 10^{-11}$ M, optimal activity requires 2 nM NGF concentrations. Hence, D3 is optimal at concentrations that approximate its K_d while NGF is optimal at concentrations ~100 fold over its K_d. We interpret this difference to mean that D3 is more stable in solution, and this notion is supported by D3 resistance to proteolysis.

Ligand binding sites

D3 competitively blocks the binding of mAb 5C3 but it does not block NGF. Moreover, the optimal agonistic activity of mAb 5C3 (Maliartchouk and Saragovi, 1997) was inhibited by D3 in a dose-dependent manner (data not shown), while the agonistic effect of NGF was enhanced. These results are intriguing since previously we reported that mAb 5C3 can block ~50% of the NGF binding sites on a cell expressing TrkA, while NGF can block ~25% of the mAb 5C3 binding sites (LeSauteur et al., 1996). It is unlikely that D3 does not block NGF because of affinity differences, because NGF•TrkA-ECD and 5C3•TrkA-ECD interactions are both in the nM range.

Two factors could account for this result. First, both mAb 5C3 and D3 dock onto a single and continuous epitope within the IgG-C2 domain of TrkA, whereas NGF binds a discontinuous epitope within the IgG-C1 and IgG-C2 domains of TrkA (Perez et al., 1995), and at least one other domain (Windisch et al., 1995). This would facilitate mAb 5C3 blocking by D3 whereas NGF could bind via its second docking site. Second, mAb 5C3 and NGF bind TrkA at sites partially overlapping but not identical (LeSauteur et al., 1996). Hence the data suggest that D3 binds TrkA at an epitope overlapping the agonistic mAb 5C3 "hot spot" of the IgG-C2 domain of TrkA, near the NGF docking site. These observations may account for D3 synergizing with NGF and blocking mAb 5C3.

The fact that D3 is bioactive and was selected from a relatively small pool of β-turn based compounds has broad implications for many research initiatives involving protein-protein interactions. Other small molecules with neurotrophic activity have been reported (Maroney et al., 1998; Steiner et al., 1997). However, the molecular targets of these ligands are ubiquitous intracellular proteins, and the mechanisms of action are often unclear. Thus, these other molecules are not Trk ligands, and are not defined as peptidomimetics of known ligands.

In contrast, we report a small molecule peptidomimetic that binds and activates TrkA. In the present report of D3, we show that a hybrid of a peptide and a small organic molecule designed to hold key amino acid residues in a turn conformation within a small framework offers a means to transform a peptide lead into an active organic small molecule. Hence, D3 represents the validation of the peptidomimetic concept for the Trk family of tyrosine kinase receptors. This small molecule peptidomimetic ligand of TrkA that has neurotrophic activity may be useful to address neurodegenerative disorders, pain, neoplasias, and other pathologies (reviewed in Saragovi and Burgess, 1999) where TrkA receptors play a role.

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Discussion

Agonistic properties of monovalent TrkA ligands.

The data presented in this Thesis demonstrated that three different monovalent artificial TrkA ligands could act as partial agonists. Namely, these are (i) monomeric Fab fragments of an anti-TrkA monoclonal antibody (Chapters I and II), (ii) small cyclic peptides derived from an NGF β-turn (Chapter II) and (iii) a small mimetic composed of a macrocyclic ring with incorporated side chains designed to mimic an NGF β-turn (Chapter III). The exact mechanisms underlying their agonistic activity yet remain to be investigated. However, recent developments in receptor biology and receptor pharmacology can provide some insights into this phenomenon.

It is widely accepted that the activation of cell surface receptors with a single transmembrane region is achieved due to external cross-linking by a ligand. This notion has been confirmed in the results presented in this Thesis, where the maximal TrkA activation was induced by either bivalent NGF or monovalent anti-TrkA Fabs externally cross-linked by an anti-Fab antibody. There is, nevertheless, evidence for a different model that implies the existence of pre-formed unliganded inactive receptor dimers that can be activated and/or stabilized by an agonistic ligand. The direct evidence in support of this hypothesis was found in the case of erythropoietin (Epo) receptor (EpoR). EpoR belongs to a family of cytokine receptors that possess no catalytic activity but associate with JAK tyrosine kinases via their intracellular domains. The crystal structure of the extracellular domain of EpoR in its unliganded form has revealed a dimeric structure. Remarkably, the membrane-proximal domains of receptors were oriented away from each other at an angle of 135°, so the transmembrane and intracellular domains would be positioned too far apart to permit cross-phosphorylation by JAK2 tyrosine kinase (Livnah *et al.*, 1996; Livnah *et al.*, 1999). The relative orientation of the receptors changed to an angled at 45° in a co-crystals with a partially activating peptide ligand and to the parallel in a complex with EPO. Importantly, these findings were confirmed in a separate study

where authors employed *in vivo* protein fragment complementation assays (Remy *et al.*, 1999). Crystallographic studies of the tumor necrosis factor receptor $p55^{TNFR}$ ECDs also indicate that these receptors, when unliganded, may form inactive dimers (Naismith *et al.*, 1995; Naismith *et al.*, 1996). The existence of pre-formed oligomeric complexes of TNF family receptors $p55^{TNFR}$, $p75^{TNFR}$, Fas, TRAIL receptor 1 and CD-40 on the cell surface was recently confirmed in studies where authors utilized chemical cross-linking and fluorescence resonance energy transfer (FRET) techniques (Chan *et al.*, 2000; Siegel *et al.*, 2000). In the case of TNFRs the inter-receptor interactions were attributed to the so-called pre-ligand-binding assembly domain (PLAD) of the ECD that is distinct from the ligand-binding domain, but is necessary and sufficient for the assembly of unliganded TNFR complexes.

The indirect evidence in support of the existence of inactive receptor dimers on the cell surface comes from several observations showing that receptor dimerization is often not enough for its activation. One of the artificial peptide ligands of EpoR can induce receptor dimerization but exhibits pure antagonistic properties (Livnah *et al.*, 1998). Neu/ErbB-2/Her-2 is a tyrosine kinase growth factor receptor similar to TrkA. It was found that a chimeric form of Neu with altered transmembrane domain formed ligand-independent dimers but did not induce biological responses (Burke *et al.*, 1997), while different mutations in the transmembrane domain produce constitutively active receptors (Weiner *et al.*, 1989; Burke and Stern, 1998). Insulin and insulin-like growth factor-1 (IGF-1) receptors are found as two ($\alpha\beta$) subunits constitutively cross-linked by disulfide bonds (reviewed by Lee and Pilch, 1994). Thus, these receptors could be considered as preformed covalent inactive dimers that are activated due to a conformational change upon ligand binding.

Certain observations indicate that Trk receptors may also form ligand-independent dimers. They, as many other tyrosine kinase receptors, possess a putative conserved sequence motif in the transmembrane region that can promote dimerization (Sternberg and Gullick, 1990). Overexpression of TrkA can lead to detectable receptor autophosphorylation in the absence of activating ligands (Hempstead *et al.*, 1992; also this Thesis, chapter I, figure 2C). Glycolipids such as ganglioside GM1 (Mutoh *et al.*,

1995; reviewed by Ferrari and Greene) and ceramide (MacPhee and Barker, 1999) can cause autophosphorylation of unliganded TrkA and TrkA-mediated biological responses, arguably by interacting with lypophilic transmembrane domains and stabilizing pre-formed receptor dimers. Recent studies have shown that TrkA tended to localize in caveolae or caveolae-like domains (CLDs) at the plasma membrane (Huang *et al.*, 1999; Bilderback *et al.*, 1999). Receptor aggregation in caveolae and CLDs together with glycolipids may create more favorable conditions for TrkA-TrkA interactions, even though it may facilitate TrkA interaction with $p75^{NTR}$, which also is found in caveolae and CLDs.

Taken together these observations support an idea that agonistic monovalent TrkA ligands, while unable to directly cross-link the receptors, can induce conformational changes that lead to receptor cross-phosphorylation and initiation of signaling cascades. On a mechanistic level these conformational changes can cause three types of alterations in receptor behavior: (i) enhanced inter-receptor interactions, i.e. increased probability of a homodimer formation upon random encounter of two receptors; (ii) increased receptor mobility, i.e. increased probability of random receptor encounter; and (iii) enhanced stability of pre-formed receptor dimers due to reduced rate of dissociation.

Mechanisms of TrkA modulation by $p75^{NTR}$

Experimental data suggests that the regulation of TrkA-mediated signals by $p75^{NTR}$-selective ligands occurs, at least in part, on the level of TrkA activation (Chapter I, figure 2 and Chapter II, figure 4). There may be a number of possible points of regulation. First, as it discussed above, the formation of unliganded inactive TrkA dimers and conformational changes may play an essential role in TrkA activation. Interference with TrkA conformation and TrkA-TrkA interactions may account for the negative TrkA regulation by unbound $p75^{NTR}$. A closely related issue is that $p75^{NTR}$ may reduce TrkA mobility on the cell membrane (Wolf *et al.*, 1998), thus reducing the probability of random inter-receptor interactions and dimer formation. The $p75^{NTR}$-mediated release of

ceramide (MacPhee and Barker, 1999) can serve as a mechanism for positive TrkA regulation by ligand-activated $p75^{NTR}$.

$P75^{NTR}$ may also regulate TrkA signals by interfering with kinetics of TrkA activation and with internalization of receptor-ligand complexes. For example, we used an anti-NGF mAb that bound NGF with 1:1 stoichiometry and abolished NGF binding to NGF but not to TrkA (Saragovi *et al.*, 1998). Addition of this antibody changed the time course of NGF-induced TrkA activation from sustained to transient and enhanced NGF-induced TrkA internalization. The kinetic analysis of TrkA activation by combinations of TrkA-specific and $p75^{NTR}$-specific ligands (same mAb ligands as described in Chapter I) revealed that $p75^{NTR}$ binding changed not only the extent but also the kinetics of TrkA autophosphorylation (Saragovi *et al.*, unpublished observations).

The molecular mechanisms underlying $p75^{NTR}$ – Trk interactions remain unknown. The most plausible explanation of the requirement of both $p75^{NTR}$ and Trks for high affinity neurotrophin binding and of mutual regulation of the receptor function would be the formation of molecular complexes involving both $p75^{NTR}$ and Trks. Indirect evidence for physical association between $p75^{NTR}$ and TrkA comes from co-patching studies of fluorescently labeled receptors that show decrease in a receptor mobility due to expression of a co-receptor (Wolf *et al.*, 1995; Wolf *et al.*, 1998). However, most of the attempts to characterize such complexes in the cells expressing $p75^{NTR}$ and Trks at physiologically relevant levels were unsuccessful (reviewed by Barrett, 2000). $P75^{NTR}$ – Trk complexes were only detected when both co-receptors had been over-expressed at very high levels in insect cells and chemically cross-linked (Gargano *et al.*, 1997) or over-expressed in a mammalian cell line as fusion proteins with hemagglutinin tag (Bibel *et al.*, 1999). Intracellular and extracellular domains of both co-receptors were found to contribute to $p75^{NTR}$ – Trk interactions. In both studies complexes were detected in the absence of receptor ligands. Interestingly, in a later study $p75^{NTR}$ – Trk complexes could only be immunoprecipitated from the lysates of the cells co-expressing both $p75^{NTR}$ and Trks, but not from mixtures of cell lysates containing individual co-receptors. In another study complexes containing $p75^{NTR}$ and TrkA were detected following NGF binding and chemical cross-linking but not in unliganded state (Huber and Chao, 1995). Taken

together, these observations suggest that direct stable associations of Trks and $p75^{NTR}$ in physiologically relevant situations are unlikely. Nevertheless, it is possible that complex indirect and/or transient interactions between Trks and $p75^{NTR}$ take place.

Development of NGF mimetics.

Three different types of artificial ligands of NGF receptor TrkA have been characterized as agonistic NGF mimetics in the studies presented in Chapters II and III of this Thesis. First, monovalent Fab fragments of an anti-receptor mAb 5C3 have been shown to activate agonistic trophic signals in TrkA-expressing cultured cells. External cross-linking of Fabs led to further enhancement of trophic effects, however, even without cross-linking 5C3 Fabs could induce cellular responses similar to that of caused by intact bivalent mAb 5C3. 5C3 Fabs exhibited agonistic properties alone or in synergy with activating bivalent ligands of $p75^{NTR}$.

Second, small cyclic peptides mimicking β-turn C-D of NGF were characterized as partial TrkA agonists. These peptides have been previously shown to bind TrkA and partially antagonize NGF at optimal concentrations (LeSauteur *et al.*, 1995). However, when tested in a paradigm of synergy with an activating of $p75^{NTR}$ ligands, these peptides exhibited agonistic properties. They induced trophic responses in neuronal cell lines and enhanced survival and differentiation of primary cultured neurons.

The third newly developed TrkA agonist is a small non-peptide compound termed D3 selected from a library based on the pharmacophores of the mAb 5C3 and NGF peptide analogs. D3 has been shown to bind membrane-proximal Ig-like domain 2 of TrkA that is thought to be a docking site for NGF. D3 can block TrkA interaction with mAb 5C3 but not with NGF, while mAb 5C3 can partially block NGF binding to TrkA (LeSauteur *et al.*, 1996b). Agonistic properties of D3 include its ability to induce trophic responses in neuronal cell lines and enhance survival and differentiation of primary cultured neurons.

All three artificial neurotrophic ligands possess common features that distinguish them from natural polypeptide hormones. First, they are monovalent. D3 and cyclic peptide NGF mimetics were characterized as genuine monomeric and monovalent compounds. Anti-receptor Fabs can theoretically be bivalent and/or aggregate, however it is extremely unlikely, especially within the used experimental conditions. Second, all three NGF mimetics can dock onto small defined epitopes of TrkA, in contrast to natural ligands that interact with extended surfaces of the receptor. This notion is confirmed by the fact that these ligands can only partially cross-block each other and NGF in TrkA binding assays.

These finding have important implications for the design of neurotrophic therapeutics and artificial receptor ligands in general. It has been traditionally thought that small molecule compounds can only be developed as antagonistic ligands of cell surface receptors with a single transmembrane domain because their docking into a small defined epitope could only disrupt receptor binding and activation with a natural ligand. The finding presented in this Thesis indicate that the interaction of small molecules with "hot spots" of the receptor may in some cases lead to receptor dimerization and activation, possibly by inducing conformational changes that enhance inter-receptor interactions or activate pre-formed inactive receptor dimers.

Summary

Functional interactions between NGF receptors TrkA and $p75^{NTR}$ have been studied using artificial receptor-specific mAb-based ligands. It has been shown that while unliganded $p75^{NTR}$ negatively regulated TrkA activation and trophic signaling, $p75^{NTR}$ expression and binding with an activating ligand led to increased TrkA activation and trophic responses. It has been also shown that the trophic signals of NGF could be fully mimicked by a combination of TrkA-specific and $p75^{NTR}$-specific ligands.

The study of valency requirements for NGF receptor ligands revealed that an activating anti-TrkA mAb remained partially agonistic in its monovalent (Fab) form. Further studies led to characterization of a small NGF-based cyclic peptide as a partial TrkA agonist and to the development of a small molecule proteolytically stable NGF mimetic with agonistic properties.

These three agonistic artificial TrkA ligands display a novel pharmacological mechanism of action. First, unlike natural polypeptide hormones and bivalent anti-receptor antibodies, these ligands are monomeric and monovalent. And second, these ligands interact with a small defined epitope of the receptor.

The characterized agonistic NGF mimetics do not yet meet requirements for good drug candidates because of their peptide nature or low potency. Nevertheless, they can be used for further optimization and molecular modeling on the basis of their pharmacophores. More importantly, the revealed novel pharmacological mechanism of action opens new possibilities in the rational design of small molecule agonistic mimetics of polypeptide hormones, particularly small molecule therapeutics with neurotrophic activity.

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Appendices: reprints of Chapters I, II and III

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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 signals, 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 (LeSauteur 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 FACScan 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) (LeSauteur 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 (LeSauteur et al., 1996). Further characterization of mAb 5C3 is published in LeSauteur 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, Mississagua, 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: $[(OD_{test}\text{-}OD_{untreated})/(OD_{1\ nM\ NGF}\text{-}OD_{untreated})] \times 100$. 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 (LeSauteur 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. 1*A*). In contrast, extensive apoptotic DNA degradation is seen when 4-3.6 cells are cultured with SFM or mAb MC192 alone (Fig. 1*A*).

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. 1*B*), as expected, because this concentration of NGF affords suboptimal survival. PC12 cells cultured with SFM or mAb MC192 alone show extensive DNA degradation (Fig. 1*B*).

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 inibits 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.

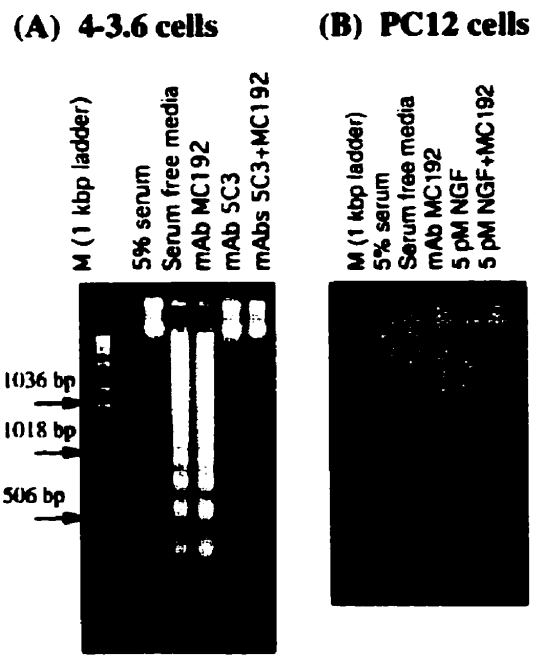

Figure 1. Changes in apoptotic DNA degradation. Genomic DNA was extracted from (*A*) 4-3.6 or (*B*) PC12 cells cultured as indicated for 48 hr in SFM. Equal amounts from each sample were resolved on a 1.5% agarose gel and visualized with ethidium bromide. Standard molecular markers (*M*) are shown. A 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 She 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-

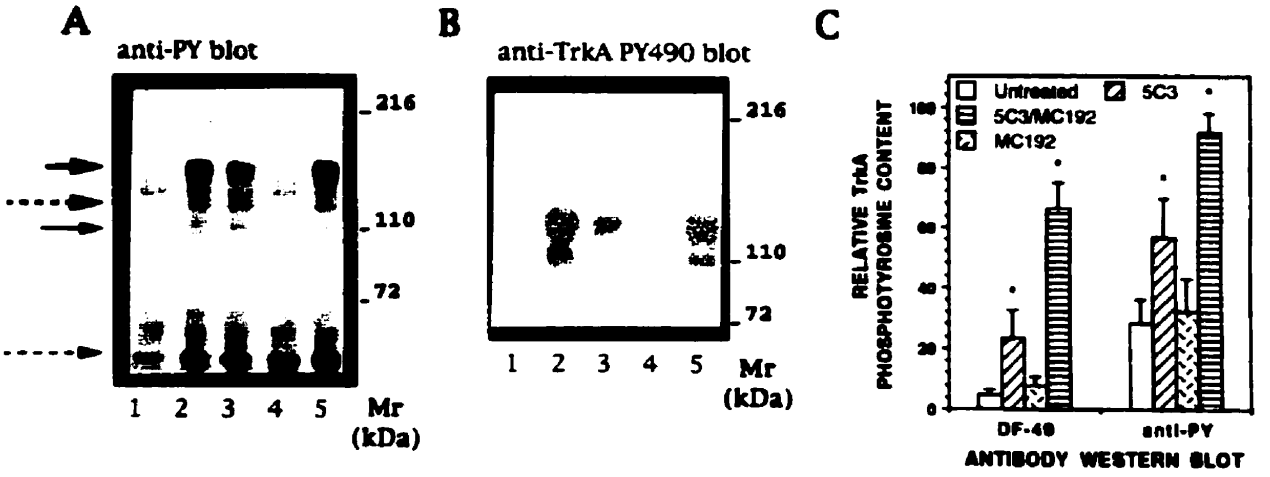

Figure 2. Optimal TrkA tyrosine phosphorylation by concomitant binding of p75 and TrkA. 4-3.6 cells were untreated (*lane 1*) or treated with 1 nM NGF (*lane 2*), 5 nM mAb 5C3 alone (*lane 3*), 0.5 nM mAb MC192 alone (*lane 4*), or a combination of both mAbs (*lane 5*) for 15 min at 37°C. Ligand concentrations 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 ± 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 ~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 (LeSauteur 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 μg/ml; ~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 ~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 antimitotic 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 NFκ-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 (LeSauteur 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.

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Genuine Monovalent Ligands of TrkA Nerve Growth Factor Receptors Reveal a Novel Pharmacological Mechanism of Action*

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Developing small molecule agonistic ligands for tyrosine kinase receptors has been difficult, and it is generally thought that such ligands require bivalency. Moreover, multisubunit receptors are difficult to target, because each subunit contributes to ligand affinity, and each subunit may have distinct and sometimes opposing functions. Here, the nerve growth factor receptor subunits p75 and the tyrosine kinase TrkA were studied using artificial ligands that bind specifically to their extracellular domain. Bivalent TrkA ligands afford robust signals. However, genuine monomeric and monovalent TrkA ligands afford partial agonism, activate the tyrosine kinase activity, cause receptor internalization, and induce survival and differentiation in cell lines and primary neurons. Monomeric and monovalent TrkA ligands can synergize with ligands that bind the p75 subunit. However, the p75 ligands used in this study must be bivalent, and monovalent p75 ligands have no effect. These findings will be useful in designing and developing screens of small molecules selective for tyrosine kinase receptors and indicate that strategies for designing agonists of multisubunit receptors require consideration of the role of each subunit. Last, the strategy of using anti-receptor mAbs and small molecule hormone mimics as receptor ligands could be applied to the study of many other heteromeric cell surface receptors.

Nerve growth factor (NGF)[1] is a dimeric hormone composed of two identical protomers. NGF binds to either or both of two receptors termed TrkA and p75. Cells expressing TrkA bind NGF with intermediate affinity ($K_d \sim 10^{-10}$ M) (1–3), and cells expressing p75 bind NGF with lower affinity ($K_d \sim 10^{-9}$ M) (4). Co-expression of TrkA and p75 creates high affinity NGF binding sites ($K_d \sim 10^{-12}$ M) (3), indicative of physical and functional interactions (5–8).

Agonists that activate TrkA afford protection from apoptotic cell death and neuronal differentiation and axonal growth (9). The p75 receptor mediates apoptosis in some neuronal and nonneuronal cells (reviewed in Refs. 10 and 11)), but it is unclear whether p75-mediated death is constitutive, induced by agonistic p75 ligands, or can be antagonized by other ligands. Culture studies where Trk-specific ligands were mixed with p75-specific ligands have shown synergy and reciprocal regulation of function (6–8, 12).

TrkA is a tyrosine kinase receptor that transduces NGF signals. The dimeric NGF protein induces TrkA dimerization leading to activation of the kinase (13), as expected by analogy with other receptor tyrosine kinases (14). However, dimeric ligands do not always lead to receptor activation (15–17). Hence, the possibility that monomeric ligands could induce conformational changes leading to receptor dimerization or activation remains an attractive hypothesis (18). Since no biological studies have been done with *defined and genuine* monomeric ligands of TrkA or any other tyrosine kinase receptor, this is one aim of the present study.

Functional synergy between bivalent Trk ligands and bivalent p75 ligands, leading to enhanced Trk activation and cell survival, have been reported (6, 8). However, no functional studies of synergy have been done with defined and genuine monovalent ligands of p75 and TrkA. This is another aim of the present study.

To answer both aims, we used defined monovalent and monomeric ligands that bind to the extracellular domain of TrkA and p75 receptors. Specifically, we asked (i) whether monovalent and monomeric ligands of TrkA can act as partial agonists, (ii) whether monomeric ligands of TrkA can synergize with ligands of p75, and (iii) what the valency requirement is for p75 ligands to synergize with TrkA ligands. Three sets of ligands that bind the extracellular domain of NGF receptors were available. Each ligand was used in its bivalent or monovalent state, alone or in combinations, to probe receptor function in biological and biochemical assays.

Anti-TrkA mAb 5C3 is an agonist ligand of TrkA (19). Anti-p75 mAb MC192 is a p75 ligand that can synergize with TrkA ligands (6). Peptides termed C(92–96) and C(92–97) bind TrkA *in vitro* and target TrkA-expressing cells *in vivo* (20–22). When TrkA is engaged by these peptide analogs, binding of the natural ligand NGF is antagonized (21), but a possible intrinsic activity of the peptide analogs upon binding TrkA had not been studied.

Biophysical characterization of C(92–96), described herein, defines it as a genuine monomeric and monovalent TrkA ligand. We report that genuine monovalent TrkA ligands are partial agonists and induce TrkA activation and internalization and cell survival and differentiation. Expectedly, bivalent TrkA ligands afford more robust signals. These data challenge

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[1] The abbreviations used are: NGF, nerve growth factor; mAb, monoclonal antibody; DRG, dorsal root ganglia; FPLC, fast protein liquid chromatography; *N*-Ac, *N*-acetyl; SFM, serum-free medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

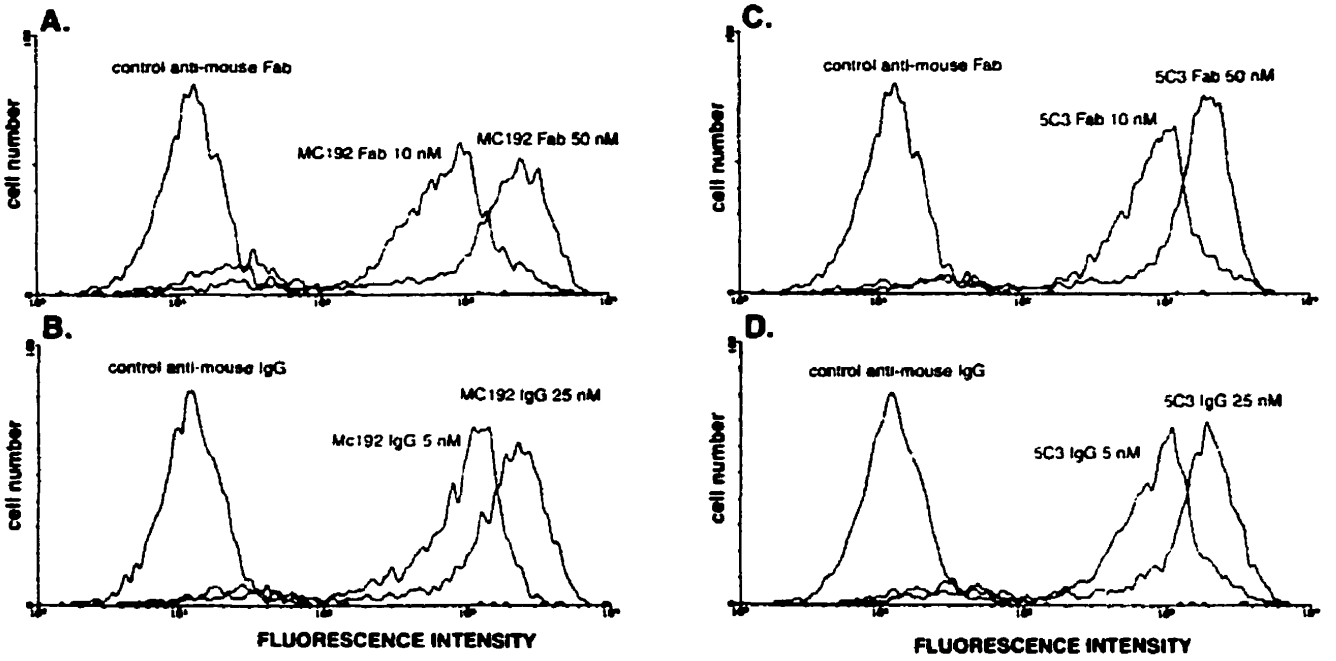

FIG. 1. **Binding profile of mAb *versus* Fab fragments.** 4-3.6 cells expressing equal levels of TrkA and p75 were analyzed by FACScan binding as described under "Materials and Methods." Binding of intact IgG was revealed with fluorescein isothiocyanate-coupled goat anti-mouse IgG and binding of Fabs with fluorescein isothiocyanate-coupled goat anti-mouse Fab. Controls excluded the specific primary. Saturating concentrations are achieved at 50 nM Fabs and at 25 nM IgG. These concentrations have equal number of receptor-binding units because IgGs are bivalent. Decreasing the saturating dose 5-fold results in similar immunostaining patterns for IgG and Fabs (compare A *versus* B and C *versus* D), suggesting similar binding properties. Note that FACScan immunostaining conditions (10^5 cells stained/test, binding primary for 30 min at 4 °C) are not identical to the conditions used for survival assays; hence, saturating concentrations for the latter cannot be extrapolated.

the exclusive notion of ligand bivalency postulated for activation of tyrosine kinase receptors. For p75, only bivalent ligands afford signals that synergize with TrkA-mediated signals. This suggests that TrkA and p75 differ in their requisites for ligand activation. Last, oligomerizing ligands afford the same signals as homodimerizing ligands.

The insight that monovalent small molecule ligands can be partial agonists will be useful for screening and designing pharmacological agents, and the approach described can be adapted to the study of other receptors.

MATERIALS AND METHODS

Cell Lines

Rat PC12 cells express low levels of rat TrkA and 40,000–100,000 p75 receptors/cell (TrkA$^+$ p75^{+++}). B104 rat neuroblastomas express ~50,000 p75 receptors/cell but do not express Trks (TrkA$^-$ p75^{+++}). The 4-3.6 cells are B104 cells stably transfected with human *trkA* cDNA and express equal surface levels of p75 and TrkA (TrkA^{+++} p75^{+++}) (23). The 6–24 cells are PC12 cells stably transfected with human *trkA* cDNA and overexpressing TrkA (TrkA^{+++} p75^{+++}). Cell surface expression of each of the NGF receptors was routinely controlled in all cells by quantitative FACScan assays (Becton Dickinson) (data not shown). These cells do not express detectable mRNA for neurotrophins (data not shown; Ref. 23) and undergo apoptosis when neurotrophins or serum are withdrawn.

Dissociated Neuronal Dorsal Root Ganglia Cultures

Fetal rat dorsal root ganglia (DRG) primary cultures were established essentially as described (24) from Harlan Sprague-Dawley day 17 rat embryos. All ganglia were dissected and dissociated first enzymatically with trypsin and then mechanically. Dissociated cells were cultured (100,000 cells/well) in 96-well plates precoated with collagen and grown for a total of 8 days in Neuro Basal Medium containing N2 supplement (Life Technologies, Inc.), antibiotics, and L-glutamine. These DRG cultures are ~85% TrkA-expressing and are heavily dependent on TrkA signals for survival (25, 26).

Antibody and Fragment Preparation

The activities of anti-human TrkA IgG mAb 5C3 and anti-rat p75 IgG mAb MC192 have been described (6, 19). mAbs 5C3 and MC192 do not cross-block each other's binding. Purified IgGs were digested with papain (Life Technologies, Inc.) to yield monovalent fragments (Fabs). For further purification, first papain was inactivated; second, the Fc fragments were removed in protein G-Sepharose columns (HiTrap; Amersham Pharmacia Biotech); and third, the Fabs containing κ light chains were purified to >98% purity in KappaLock-Sepharose columns (Upstate Biotechnology, Lake Placid, NY) and by preparative FPLC with sizing columns (Amersham Pharmacia Biotech). No IgG was detected in Fab preparations. FPLC spectrometry and size exclusion analysis under native conditions did not reveal the presence of aggregates, even at 40 μM Fab concentrations (bioassays use nanomolar concentrations). The conditions used would have detected <0.2% of Fab aggregates. Binding competition assays between 5C3 Fabs and the intact antibody indicated that the affinity of Fabs (K_d 10 nM) is within 5-fold of the intact IgG (K_d 2 nM). The affinities of the MC192 Fabs were not measured directly. However, FACScan assays demonstrated that MC192 Fabs and MC192 IgG (Fig. 1, *A* and *B*) and 5C3 Fabs and 5C3 IgG (Fig. 1, *C* and *D*) bound their cellular targets in a specific and saturable fashion indistinguishable from each other (Fig. 1).

Cyclic NGF Mimics

The NGF mimic C(92–96) is an *N*-acetylated (*N*-Ac) cyclic peptide with primary sequence *N*-Ac-YCTDEKQCY. The NGF mimic C(92–97) is *N*-Ac-YCTDEKQACY. The C(92–96) and C(92–97) peptides are cyclized by intrachain disulfide bonds (indicated by the underlines) (21). These peptides are structural mimics of the C-D β-turn of NGF (27). *Linear* peptides with the same sequences do not bind TrkA and were prepared as controls by substituting Cys with Met (primary sequence YMTDEKQMY). The linear peptides do not cyclize, and NMR spectroscopy indicated a lack of conformation (data not shown). The $C(92–97)_{dimer}$ is a tethered covalently linked dimer of C(92–97). High pressure liquid chromatography and mass spectroscopy analysis confirmed the expected retention time and mass for a dimer.

Peptide Synthesis and Characterization

N-Ac peptides were synthesized by Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. Purification, quality control, and characterization of the peptides were done as described (21) and by NMR diffusion studies (this report). More convincingly, the full NMR spectra of *N*-Ac-C(92–96) were analyzed (27). Assignment of all resonances and distances and resolution of the structure showed the peptide to be monomeric. Therefore, it is extremely unlikely that *N*-Ac-C(92–96) is a noncovalent dimer. With respect to a possible covalent dimer, mass spectroscopy (API III MS System, Sciex, Thornhill, Ontario) by electrospray ionization quadrupole (data points every 0.1 Da) verified the chemical composition and monomeric state of *N*-Ac-C(92–96) with 1192.3 ± 0.3 atomic mass units measured, which is the theoretical mass (1192.2) for an oxidized monomer. No trace of a covalent peptide dimer was detected even after prolonged signal averaging, using conditions that would have detected 1% of dimer. Therefore, it is extremely unlikely that *N*-Ac-C(92–96) is a covalent dimer.

NMR Spectroscopy

NMR samples contained 5 mM *N*-Ac-C(92–96) in distilled water at pH 5.7 containing 10% (v/v) of D_2O for the deuterium lock. When D_2O was used as a solvent, the peptide was twice lyophilized and redissolved in D_2O. Spectra were acquired at 500-MHz proton frequency on a three-channel Bruker DRX500 spectrometer equipped with pulsed field gradients. Standard experimental protocols were used for the acquisition of NMR spectra and spectral assignments. Isotropic self-diffusion measurements used NMR pulse field gradients at different peptide concentrations (28, 29). Seventeen one-dimensional assays were done at each concentration with gradient strengths from 0.67 to 63.65 G/cm, gradient duration of 3.5 ms, and a diffusion time of 150 ms. Peptide signal decay was measured at nine different frequencies. Data were fit to the equation $I = I^0\exp(-(\gamma\delta G)^2(\tau - \delta/3)\delta)$, where I is the experimentally measured signal intensity attenuated by diffusion, γ is the ^1H gyromagnetic ratio, δ is gradient duration, G is the gradient strength, τ is time between gradient pulses, and δ is diffusion coefficient. Results were averaged.

Ligand Concentrations and Valency

Antibodies are defined as "artificial receptor ligands" because they are specific, they bind with high affinity, with saturable and reversible kinetics, and they are bioactive. Responses to a full dose range (from picomolar to high micromolar) were studied previously for some ligands, and responses for the same dose range for all ligands were studied herein (data not shown). Usually, only optimal concentrations of NGF mimics, mAbs, or Fabs that afford trophic signals are shown for clarity. The NGF mimic C(92–96) is monomeric and monovalent. It is water-soluble and does not aggregate even at 18 mM (this paper). The $C(92–97)_{dimer}$ is a covalent dimer and bivalent analog of the C(92–97) NGF mimic. Intact IgGs are dimeric and bivalent, and Fabs are monomeric and probably monovalent. Where indicated, Fabs were cross-linked with goat anti-mouse Fab antibody (α-Fab; Sigma) at a 2:1 ratio of Fab to α-Fab. This cross-linking ratio affords optimal dimerization (one α-Fab can bind two Fabs). Higher cross-linking of Fabs using Fab/α-Fab ratios of 1:1 or 1:5 (each Fab bound by many α-Fabs), leading to ligand oligomerization, achieved results comparable with dimerizing ratios of 2:1 Fab/α-Fab (data not shown).

Protection from Apoptotic Death

Primary DRG Cultures—After a total of 8 days of culture with NGF (Prince Labs, Toronto, Canada) or the indicated test or control ligands, cell survival were studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and by microscopic observation.

Cell Lines—5,000 cells/well in protein-free medium (PFHM-II; Life Technologies, Inc.) containing 0.2% bovine serum albumin (crystalline fraction V; Sigma) were seeded in 96-well plates (Falcon, Mississauga, Canada). The cultures were untreated or were treated with the indicated test or control ligands. Cell viability was quantitated using the MTT assay after 56–72 h of culture, and apoptotic death was confirmed by analysis of DNA fragmentation patterns. Percentage of protection was standardized from MTT OD readings relative to optimal NGF (1 nM) = 100%. The OD values of untreated cells were subtracted and were <15% for cell lines and <30% for primary cultures. The higher survival of untreated primary cultures is probably due to endogenous production of limiting amounts of growth factors.

Tyrosine Phosphorylation Assays

TrkA tyrosine phosphorylation was assayed after a 15-min treatment of intact cells with the indicated agent(s) and revealed by Western blotting of whole cell lysates as described (6). Anti-phosphotyrosine (α-Tyr(P)) mAb 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) or antiserum α-Tyr(P)490 against the $Tyr(P)^{490}$ of TrkA (within the Shc docking site) (30) was used as a primary antibody. Bands in x-ray films were quantified by densitometry, and intensities were standardized relative to 1 nM NGF. Densitometry of four to five independent gels was analyzed statistically by paired Student's *t* tests with Bonferroni corrections.

TrkA Internalization Measurements

Live 4-3.6 cells were treated as indicated for 45 min at 4 °C in the presence or absence of 0.25% sodium azide. Cells were maintained at 4 °C or shifted to 37 °C for another 20 min to allow ligand-induced receptor internalization (9). Then, cells were washed and immunostained with mAb 5C3 at 4 °C (phosphate-buffered saline, 0.5% bovine serum albumin, 0.1% sodium azide), for analysis of surface TrkA expression by FACScan immunofluorescence as described (9). In each assay, 5,000 cells were acquired, and the mean channel fluorescence of bell-shaped histograms was analyzed (LYSIS II, Becton Dickinson, CA). Percentage inhibition of mAb 5C3 binding was calculated as a change in mean channel fluorescence with respect to control untreated cells. Rapid loss of surface TrkA is interpreted as receptor internalization, which is delayed or inhibited by low temperatures or sodium azide (9).

Chemical Cross-linking

Live 4-3.6 single cell suspensions were bound by the indicated ligand(s) for 45 min at 4 °C. Cells were then washed in phosphate-buffered saline, cross-linked with 1 mM disuccinimidyl suberate (Pierce) for 15 min at 15 °C as described (31). Unreacted disuccinimidyl suberate was quenched with 5 mM ammonium acetate, and whole cells were lysed directly in SDS sample buffer. Equal amounts of protein for each sample were resolved in a 5–10% SDS-polyacrylamide gel electrophoresis gradient, transferred to nitrocellulose, and Western blotted with anti-Trk polyclonal antibody 203 (a gift of Dr. David Kaplan, McGill University) that recognizes the intracellular domain of Trk. This antibody was selected because of high specificity toward Trk in Western blots and because its epitopes are not affected by disuccinimidyl suberate cross-linking.

RESULTS

Cell Survival Induced by Monovalent and Bivalent TrkA Ligands—Previously, we showed that anti-human TrkA mAb 5C3 significantly protected cells from apoptosis when cultured in serum-free medium (SFM), but anti-p75 mAb MC192 did not promote cell survival. Combinations of anti-TrkA mAb 5C3 and anti-p75 mAb MC192 synergized to protect cells optimally to levels comparable with 1 nM NGF, as did combinations of mAb MC192 and 10 pM NGF (6). Therefore, we tested 4-3.6 cells (human $TrkA^{+++}$ rat $p75^{+++}$) (Fig. 2*A*) or PC12 cels (rat $TrkA^{+}$ rat $p75^{+++}$) (Fig. 2*B*) in the same paradigm but using putative monovalent ligands.

Significant protection was afforded by 5C3 Fabs, in a dose-dependent manner. 5C3 Fabs at 1–10 nM afford protection comparable with 10 pM NGF. More robust protection was afforded by 1–10 nM 5C3 Fab-α-Fab complexes. Negative control α-Fab or mouse IgG did not afford survival. Positive control bivalent mAb 5C3 at the optimal concentration of 0.5 nM protected ~50% of the cells. Interestingly, 10 nM 5C3 Fab-α-Fab complexes afforded significantly higher protection than 0.5 nM 5C3 IgG, possibly because 5C3 Fab-α-Fab complexes are more flexible than IgG or oligomerize TrkA more efficiently.

Monovalent p75 Ligands Do Not Potentiate NGF Signals—1 nM NGF protects PC12 cells (expressing rat $TrkA^{+}$ rat $p75^{+++}$) from apoptosis induced by culture in SFM. Low concentrations of NGF (10 pM) as a high affinity ligand afforded ~30% survival. Monovalent MC192 Fabs failed to synergize with 10 pM NGF, and protection was not significantly different than 10 pM NGF alone. Synergy did occur with MC192 Fab-α-Fab complexes plus 10 pM NGF. The effect was dependent on the con-

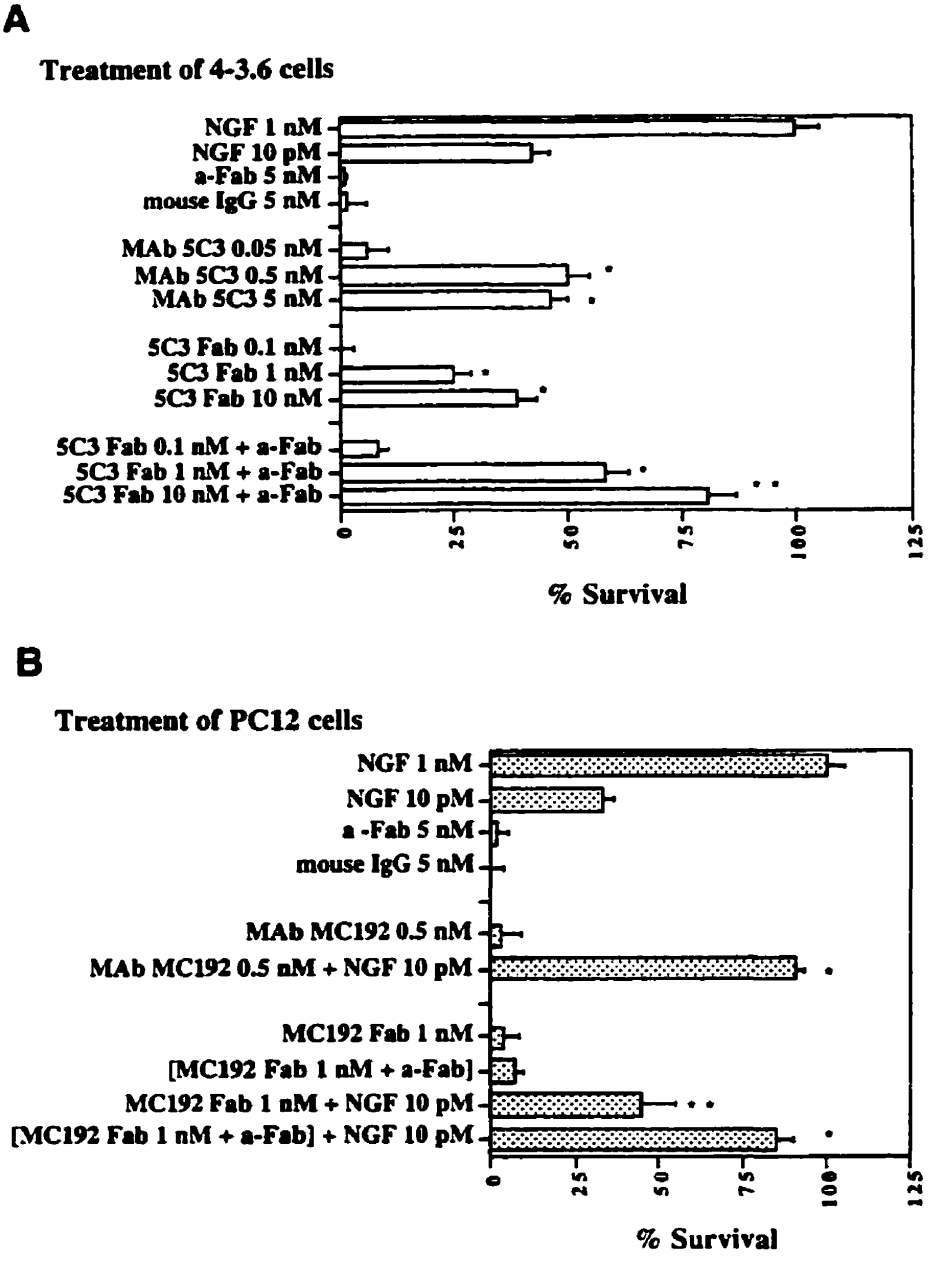

FIG. 2. **Trophic protection by monovalent and bivalent TrkA or p75 ligands.** Cells were cultured in SFM supplemented with the indicated ligands for ~68 h. Cross-linking of Fabs was achieved with a 2-fold molar excess of α-Fab antibody. Cell survival was measured in MTT assays. Protection from apoptotic death was calculated relative to that of optimal NGF (1 nM, 100% protection). Results shown are the average ± S.E., $n = 4$, and representative from at least three experiments. *A*, 4-3.6 cells. *, significant protection compared with control mouse IgG. **, significantly higher than 0.5 nM mAb 5C3. *B*, PC12 cells. *, significantly higher than 10 pM NGF. **, not significantly different from 10 pM NGF. $p < 0.01$.

centration of MC192 Fab·α-Fab complexes (data not shown) and was optimal at 1 nM cross-linked MC192 Fab.

As positive control, bivalent MC192 synergized with 10 pM NGF increasing protection from ~30 to ~90%. Synergy was dependent on the concentration of mAb and was optimal at 0.5 nM MC192 (data not shown). Controls α-Fab, mouse IgG, bivalent MC192, monovalent MC192 Fabs, and MC192 Fab·α-Fab complexes alone did not protect PC12 cells substantially from apoptosis. In all permutations of these experiments, apoptotic cell death was confirmed by analysis of DNA fragmentation patterns (data not shown).

Synergy of Bivalent and Monovalent Ligands of NGF Receptors—To analyze the valency requirement of each NGF receptor, combinations of bivalent and monovalent antibody-based ligands were tested on 4-3.6 cells for synergy in protection of apoptotic cell death (Fig. 3). In these assays, it was encouraging to observe that comparable biological responses by different ligands (*e.g.* 1 nM cross-linked 5C3 Fabs afford the same protection as 0.5 nM 5C3 bivalent IgG) also result in equivalent receptor occupancy (*e.g.* 1 nM Fabs bind the same number of receptors as 0.5 nM IgG). Positive controls of bivalent 5C3 combined with bivalent MC192 were synergistic and afforded 100% protection. Negative controls α-Fab alone and mouse IgG alone did not afford cell survival in SFM (data not shown).

A combination of monovalent 5C3 Fab with either monovalent MC192 Fab or with bivalent MC192 did not result in synergy; the −25% protection was not significantly different from that seen with monovalent 5C3 Fab alone. However, 5C3 Fab·α-Fab complexes synergized with bivalent MC192. A combination of monovalent MC192 Fab with bivalent 5C3 did not result in synergy; the −50% protection was not significantly different than that afforded by bivalent 5C3 alone. In contrast, MC192 Fabs·α-Fab complexes synergized with bivalent 5C3 and afforded −110% protection. One bias in this assay is that α-Fab cross-linking does not occur exclusively at MC192 Fabs but also occurs upon bivalent 5C3, resulting in some multiva-

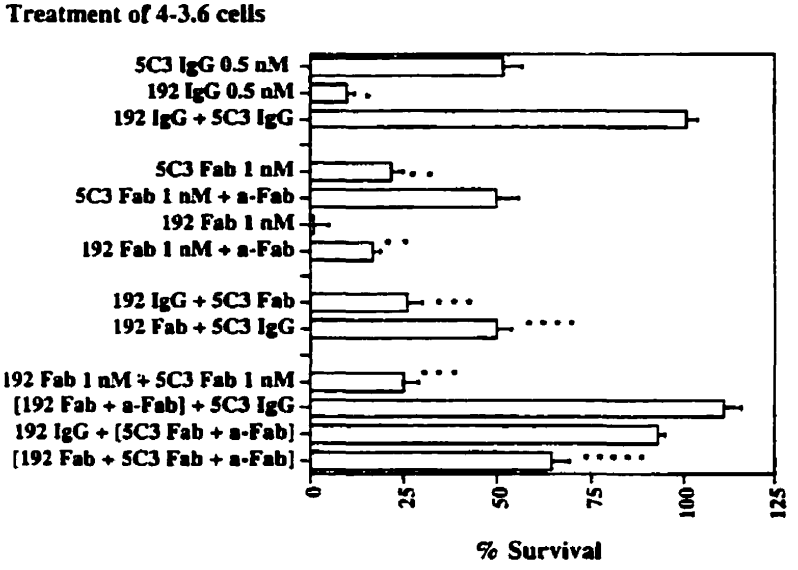

FIG. 3. **Synergistic trophic protection by p75 and TrkA Ligands.** Experiments using 4-3.6 cells were as described in Fig. 2. Cross-linking of Fabs was achieved with a 2-fold excess of α-Fab antibody. Only optimal doses for each of the TrkA or p75 ligands (0.5 nM IgG and 1 nM Fabs) are shown. Protection from apoptotic death was calculated relative to that of optimal NGF (1 nM, 100% protection). Results shown are average ± S.E., $n = 4$, and are representative from at least three independent experiments. *, not significantly higher than control mouse IgG. **, significantly higher than control mouse IgG; ***, not significantly higher than 5C3 Fabs alone. ****, not significantly higher than mAb 5C3 alone. *****, significantly lower than all dimeric combinations of MC192 and 5C3. $p < 0.01$.

lent 5C3 oligomers. However, α-Fab cross-linking of bivalent 5C3 IgG does not enhance its activity (data not shown), therefore the biological effect of cross-linking occurs at the MC192 Fabs.

Last, α-Fab cross-linking of 1 nM MC192 Fab plus 5C3 Fab afforded ~65% protection. This activity can be ascribed to four theoretical ligand mixtures: 5C3 dimers (25%), MC192 dimers (25%), and 5C3/MC192 heterodimers (50%). If the 5C3/MC192 heterodimers are indeed formed, they seem to be inactive, because we observed that reducing the concentration of bivalent mAbs 5C3 and MC192 to 0.125 nM (the concentrations in the theoretical mixtures above) results in synergy and ~65% protection (data not shown).

While the date is suggestive that heterodimers are inactive, this is an unclear issue, because we have no evidence that the heterodimers indeed form. The putative heterodimeric ligands cannot be isolated and analyzed because they dissociate and reassociate during purification; nor can they be stabilized, because binding activity is lost upon chemical cross-linking. Additionally, it is noteworthy that more extensive cross-linking with higher ratios of α-Fab did not increase protection, although higher oligomerization of ligands is expected (data not shown).

For clarity, only nearly optimal concentrations of ligands are presented, but responses from micromolar to picomolar concentrations were studied (see "Materials and Methods"). Thus, optimal protection is afforded by combinations that result in homodimerizing ligands, and no increased protection is seen with oligomerizing ligands. In all permutations of these experiments, apoptotic cell death was confirmed by analysis of DNA fragmentation patterns (data not shown). Moreover, the ligands mediate trophic effects in a TrkA-dependent manner, because no concentration or combination of NGF and antibody could induce significant protection of B104 cells ($TrkA^-$, $p75^{+++}$) (data not shown).

Ligand Valency and TrkA Tyrosine Phosphorylation—TrkA tyrosine phosphorylation (TrkA-Tyr(P)) was studied as a biochemical correlate of cell survival in SFM (Fig. 4). Analysis was done by Western blotting with mAb 4G10 against phosphotyrosine (total Tyr(P)), or with antibodies against phosphorylated tyrosine 490 of TrkA ($Tyr(P)^{490}$), which is the Shc binding site of TrkA. A representative Western blot of total Tyr(P) is shown in Fig. 4*A*. Statistical analysis of densitometry for several blots of total TrkA-Tyr(P) (Fig. 4*B*, *upper panel*), and for several blots of TrkA-$Tyr(P)^{490}$ (Fig. 4*B*, *lower panel*) were used to quantify the TrkA-Tyr(P) data. Western blotting with anti-TrkA antibodies, done in parallel, demonstrated that all lanes contained the same amount of receptor (data not shown).

Monovalent 5C3 Fabs induced small but significant increases in TrkA-Tyr(P) (Fig. 4*A*, *lane 2*) and TrkA-$Tyr(P)^{490}$ compared with untreated control (Fig. 4*A*, *lane 1*) or MC192 Fabs (Fig. 4*A*, *lane 4*). Much higher signals were induced by 5C3 Fab-α-Fab complexes (Fig. 4*A*, *lane 3*). Quantification showed that ~80% of total TrkA-Tyr(P) and ~55% of TrkA-$Tyr(P)^{490}$ were induced compared with optimal NGF-induced signals (Fig. 4*B*). In contrast, no significant TrkA-Tyr(P) or TrkA-$Tyr(P)^{490}$ was induced by p75 ligands bivalent MC192, MC192 Fab-α-Fab complexes, or monovalent MC192 Fabs (Fig. 4*A*, *lanes 10*, *5*, and *4*). For quantitative statistical analysis of these data, see Fig. 4*B*. All of these findings are consistent with the survival data.

There were no significant differences between treatments with 5C3 Fabs plus MC192 Fabs (Fig. 4*A*, *lane 6*) *versus* 5C3 Fabs alone (Fig. 4*A*, *lane 2*), indicating a lack of synergy. Cross-linking of 5C3 Fabs plus MC192 Fabs with α-Fab afforded an increase in total Tyr(P) (Fig. 4*A*, *lane 7*).

Approximately 85% of total Tyr(P) and ~65% of $Tyr(P)^{490}$ of TrkA were induced compared with optimal NGF-induced levels (Fig. 4*B*). However, the increases in TrkA Tyr(P) and $Tyr(P)^{490}$ induced by 5C3 Fab-MC192 Fab-α-Fab complexes were not statistically different from increases induced by 5C3 Fab-α-Fab complexes (Fig. 4*B*). All of these findings are consistent with the survival data.

Thus, 5C3 Fabs are partial agonist monovalent ligands of TrkA that induce receptor activation and lead to trophic cell survival. Although the evidence that mAb 5C3 Fabs are indeed monomeric is compelling (see "Materials and Methods"), it is possible that these large Fab molecules of ~50 kDa could aggregate. Hence, other ligands were tested.

Characterization of Small Molecule Monomeric TrkA Ligands—C(92–96) is a small molecule (~1 kDa), cyclic and conformationally constrained peptide analog of the C-D β-turn region of a single NGF protomer. Therefore, the C(92–96) mimic of NGF was studied as a candidate genuine monovalent and monomeric TrkA ligand.

To address the valency of C(92–96), we determined the solution structure of the pharmacophore to better than 0.5 Å root mean square deviation. Nuclear Overhauser effect and total

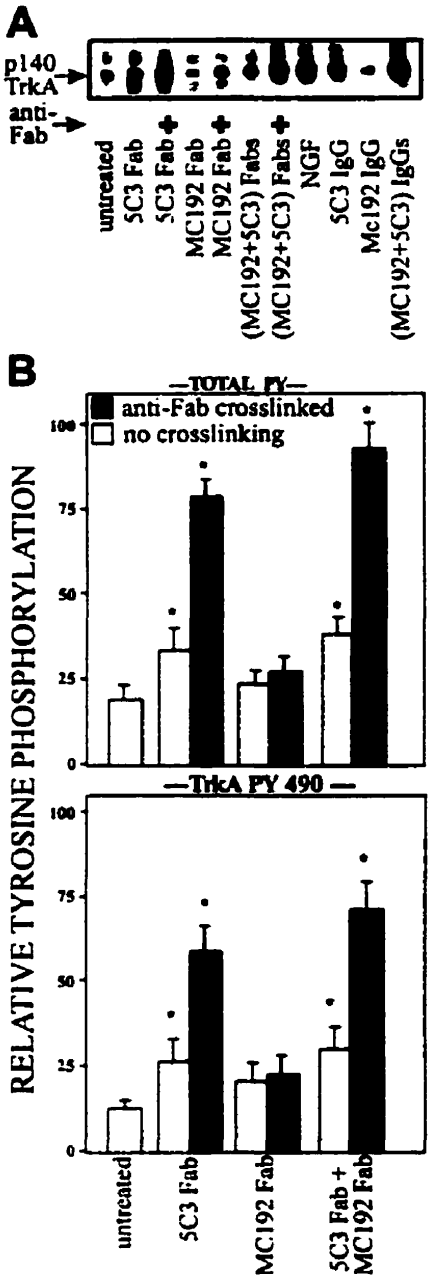

FIG. 4. **Bivalent TrkA ligands induce optimal TrkA tyrosine phosphorylation.** Equal amounts of protein from whole cell lysates were resolved by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-Tyr(P) mAb 4G10 (total Tyr(P)) or with an antibody recognizing $Tyr(P)^{490}$ within the Shc binding site of TrkA ($TrkA\ Tyr(P)^{490}$) (not shown). Blot shown is representative of at least three independent total Tyr(P) experiments. *A*, 4-3.6 cells were untreated (*lane 1*) or treated with indicated ligands for 15 min at 37 °C. In *lanes 3*, *5*, and *7*, monovalent Fabs were cross-linked with α-Fab antibodies as indicated (+). Homodimeric binding of p75 and TrkA (*lane 11*) with intact IgGs enhances TrkA Tyr(P) over each IgG alone (*lanes 9* and *10*). Ligand concentrations are as in Table I. *, significant difference from untreated samples (paired *t* tests, $n = 6$, $p < 0.05$). *B*, densitometric scanning quantification of TrkA total Tyr(P) (*upper panel*) and $Tyr(P)^{490}$ (*lower panel*) intensities relative to optimal NGF treatment (average ± S.E., $n = 6$). *Filled bars* indicate anti-Fab cross-linking. *, significant difference from untreated samples (paired *t* tests, $n = 6$, $p < 0.05$).

correlation spectroscopy spectra were consistent with a monomeric, nonaggregated state and a five-residue pharmacophore within a β-turn (27). Five chemical moieties are too few to bind two receptors simultaneously as a bivalent agent, hence this ligand is monovalent.

The following criteria indicate that C(92–96) is monomeric. First, mass spectroscopy of C(92–96) demonstrated that there were no covalent dimers or oligomers (see "Materials and Methods"). Second, the aggregation state of the peptide at millimolar concentrations in solution was resolved by high resolution proton NMR spectroscopy (Fig. 5*A*). Third, natural ^{13}C abundance NMR relaxation parameters were measured for the α-carbon atom, heteronuclear NOEs, and the molecular correlation time of C(92–96) was assessed. The overall correlation time detected of 1.76 ns at 5 °C is expected for a monomer. Fourth, the translational self-diffusion constant in solution unequivocally identified C(92–96) as monomeric.

Pulse field gradient NMR measurements of the self-diffusion coefficient (δ) were determined at various peptide concentrations of 2, 6, or 18 mM; $T = 278$ K. Values of $\delta = 1.01 \pm 0.07$ (10^{-6} cm^2/s); $\delta = 1.00 \pm 0.06$ (10^{-6} cm^2/s); and $\delta = 1.04 \pm 0.05$ (10^{-6} cm^2/s) were measured for 18, 6, and 2 mM samples, respectively (Fig. 5*B*). These δ values are essentially the same, indicating an identical state for the peptide. Thus, the samples remain monomeric, and peptide aggregates are undetectable in solution even at concentrations as high as 18 mM. We estimate that the self-association constant for any putative aggregate cannot be larger than 10 M^{-1}. Thus, a 10 μM solution of C(92–96) (used hereafter) could not contain >1 nM self-aggregated dimers, if any aggregate at all.

Small Molecule Monomeric and Monovalent Agonists of TrkA—Four questions were addressed. First, the genuine monovalent and monomeric TrkA ligand C(92–96) was tested for trophic support of cells in SFM. Second, a covalent dimeric analog of C(92–96) termed $C(92–97)_{dimer}$ was also evaluated to directly compare the potency and efficacy of monomeric *versus* dimeric small molecule TrkA ligands. Third, to study whether surface density of TrkA receptors influences trophic signals, these agents were assayed in parallel on cell lines that differ only in TrkA density (PC12 *versus* 6-24, and B104 *versus* 4-3.6 cells). Fourth, to study whether the ligands activate receptors in normal neurons, primary cultures of dissociated dorsal root ganglia from day 17 rat embryos were tested. These cells express TrkA and p75 receptors, and their survival and differentiation are dependent on TrkA activation. Growth and survival were studied first in MTT assays (Table I). Differentiation was studied morphometrically (Fig. 6).

The trophic response was dependent on NGF dose and was optimal to 1 nM NGF in all cell types (Table I, row 1). Better survival was seen at 10 pM NGF for 6-24 and 4-3.6 cells (Table I, row 3), suggesting that high TrkA expression affords better efficacy when ligand concentrations are limiting. B104 cells did not respond to any dose of NGF (data not shown). Negligible survival to 10 pM NGF in DRG cultures is due to the fact that DRG cultures are heterogeneous and secrete growth factors, masking the effect of low concentrations of exogenous NGF.

The C(92–96) NGF mimic did not afford significant survival of PC12, 6-24, or 4-3.6 cells compared with control linear peptides, but it did afford significant survival of DRG cultures (Table I, row 4 *versus* row 6). This effect was dose-dependent, and C(92–96) at 1 μM afforded ~10% growth of DRG (data not shown). In contrast, the $C(92–97)_{dimer}$ peptide afforded good trophic support for 6-24 and 4-3.6 cells and very low but statistically significant support for PC12 cells (Table I, row 5). The 6-2.4 and 4-3.6 cells express comparable numbers of TrkA receptors, suggesting a TrkA density-dependent response.

MAb MC192 alone afforded very low or insignificant trophic support of cell lines (Table I, row 7); but as a bivalent p75 ligand, it synergizes with TrkA ligands (*e.g.* see Fig. 2). High

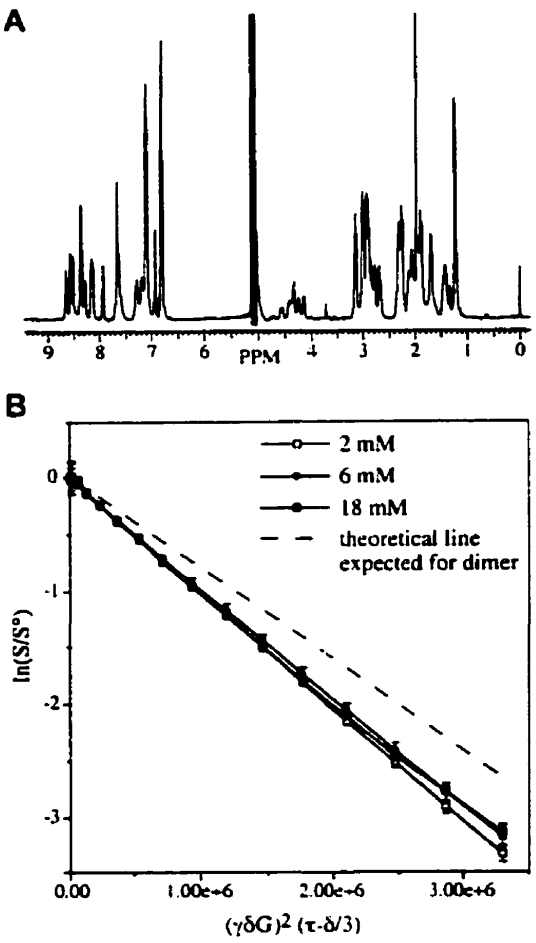

FIG. 5. **NMR spectroscopy of *N*-Ac(92–96) peptide mimetic.** *A*, one-dimensional spectrum of the monomeric Nac-C(92–96) peptide in H_2O solution, pH 5.6, 278 K. *B*, the absence of concentration-dependent aggregation as measured by the translational diffusion rate. Measurements were made by the pulsed field gradient technique of Stejskal and Tanners (reviewed in Ref. 29) at three different peptide concentrations. The *dashed line* indicates the expected diffusion rate for a dimeric peptide of the same shape as *N*-Ac-C(92–96). The plot shows the attenuation of the signal intensity (*vertical axis*) as a function of molecular size and shape (monomer *versus* dimer) and as a function of gradient duration (δ), strength (G), diffusion delay (τ), and gyromagnetic ratio (γ). The diffusion constant for the monomer is $1.02 \pm 0.06 \times 10^{-6}$ (cm^2 s^{-1}), and for the dimer it is predicted to be 0.81×10^{-6} (cm^2 s^{-1}).

DRG survival in response to mAb MC192 alone (Table I, rows 7 and 10) is explained by the mAb potentiating endogenously produced growth factors. Furthermore, bivalent MC192 potentiated the activity of C(92–96) (Table I, row 8). In cell lines the combination is synergistic, while in DRG cultures the combination is additive due to high protection afforded by each ligand alone.

As a control, bivalent MC192 did not synergize with linear peptides (Table I, row 10). Further controls using B104 cells ($TrkA^-$, $p75^{+++}$ parental to 4-3.6) demonstrated no protection by the peptide NGF mimics, alone or in combination with mAb MC192 (data not shown), suggesting that the activity requires TrkA expression.

Monovalent TrkA Ligands Induce the Differentiation of Embryonic DRG Cultures—The differentiation of dissociated primary DRG cultures was studied (Fig. 6). Untreated DRG cultures had sparse, bipolar, and poorly differentiated neurons (Fig. 6*A*). At 20 pM NGF the increase in the number and the length of neurites and branches was very low (Fig. 6*B*); at 1 nM NGF the increase was optimal (Fig. 6*C*). Treatment with control linear peptide did not induce differentiation (not shown). Treatment with 0.5 nM MC192 alone (Fig. 6*D*) or with 10 μM C(92–96) alone (Fig. 6*E*) induced substantial differentiation. However, treatment with a combination of 10 μM C(92–96) plus 0.5 nM MC192 (Fig. 6*F*) induced higher differentiation, comparable with that induced by 1 nM NGF. These differentiation data are consistent with synergy in survival seen for cell lines and primary cultures (see Table I).

Monovalent TrkA Ligands Induce TrkA Tyrosine Phosphorylation in Synergy with Bivalent p75 Ligands—To further assess whether the signals induced by small cyclic peptides are mediated by TrkA, tyrosine phosphorylation of the receptor was studied in 4-3.6 cells (Fig. 7). Representative anti-Tyr(P) Western blots are shown in Fig. 7*A*. A summary of densitometric analysis from several blots is given in Fig. 7*B*.

C(92–96) alone did not induce an increase in TrkA-Tyr(P) compared with untreated cells or cells treated with control linear peptide or bivalent MC192 (Fig. 7*A*, *lane 4 versus lanes 1*, *6*, and *7*). Significant TrkA-Tyr(P) was induced by treatment with $C(92–97)_{dimer}$ (Fig. 7*A*, *lane 5*), representing ~30% of that induced by 1 nM NGF (Fig. 7*A*, *lane 2*). Combinations of mAb MC192 and C(92–96) peptide (Fig. 7*A*, *lane 8*) or mAb MC192 and $C(92–97)_{dimer}$ peptide (Fig. 7*A*, *lane 9*) afforded notable increases in TrkA-Tyr(P), comparable with those induced by 10 pM NGF (Fig. 7*A*, *lane 3*). These results are consistent with the survival data. In contrast, treatment with a combination of bivalent MC192 and linear peptide controls (Fig. 7*A*, *lane 10*) did not result in significant increases in TrkA-Tyr(P). For statistics of densitometric analysis, see Fig. 7*B*.

Small Molecule Monovalent Ligands Induce TrkA Receptor Homodimerization—TrkA tyrosine phosphorylation leading to trophic and differentiative signals require TrkA homodimerization. To study whether monovalent C(92–96) peptide induces TrkA homodimerization, chemical cross-linking studies of the receptor were done in 4-3.6 cells (Fig. 8). Cells were treated as indicated, followed by chemical cross-linking, and then were detergent-solubilized and analyzed by Western blotting with anti-Trk polyclonal antibody 203.

A doublet consistent with previously reported TrkA monomers of p110 and p140 was seen in all samples (Fig. 8, *arrows*). Samples from NGF-treated cells and in C(92–96) plus MC192-treated cells had a band of ~280 kDa, consistent with the molecular mass of TrkA-TrkA homodimers (Fig. 8, *lanes 2* and *5*). This band was also detected, albeit weakly, in samples from cells treated with C(92–96) alone (Fig. 8, *lane 4*). A second band of ~220 kDa (that may be consistent with cross-linked p140-p75 heterodimers or p110 homodimers) was seen in samples from NGF-treated cells and more weakly in C(92–96) plus MC192-treated cells (Fig. 8, *lanes 2* and *5*). The 280-kDa and 220-kDa bands were not seen in untreated cross-linked cells (Fig. 8, *lane 1*), in cells treated with MC192 alone (Fig. 8, *lane 3*), or in linear peptide control with or without MC192 treatment (data not shown). Similar data were obtained whether whole cell lysates were analyzed or cell lysates were immunoprecipitated with anti-TrkA antibodies prior to Western blotting (data not shown).

Given that the efficiency of chemical cross-linking is <5% of the TrkA expressed on the cell surface, we have not studied the individual components of the 280- and 220-kDa bands other than the fact that they contain TrkA. However, it is unlikely that these bands comprise NGF, because they are detected in the C(92–96) plus MC192-treated cells.

Small Molecule Monovalent Ligands Induce TrkA Receptor Internalization—Next, we assessed whether the monomeric C(92–96) peptide binds to a receptor domain that overlaps with

TABLE I

NGF peptide mimics evoke trophic responses alone and in synergy with p75-dimerizing ligands

Experiments were as described in the legend to Fig. 2. Cell lines PC12 ($p75^{+++}$ $TrkA^{+}$), 6–24 (PC12 cells overexpressing TrkA, $p75^{+++}$ $TrkA^{+++}$), and 4–3.6 ($p75^{+++}$ $TrkA^{+++}$) or dissociated primary neuronal cultures from embryonic day 17 rat DRGs cells were used. Cell lines were treated for 3 days, and DRGs were treated for 8 days with the indicated ligands. Cell growth/survival was studied by the MTT method. Growth was calculated relative to that of optimal NGF (1 nM, 100% protection) subtracting the OD of untreated cells. Results are mean ± S.E. averaged from at least three independent experiments; for each experiment $n = 4$.

Treatment	Protection in serum-free media			
	PC12	6–24	4–3.6	DRG
	%	%	%	%
1. 1 nM NGF	100 ± 1.8	100 ± 3.7	100 ± 4.2	100 ± 6
2. 500 pM NGF	NT[a]	NT	NT	68 ± 4
3. 10 pM NGF	30 ± 3.6	60 ± 3.1	47 ± 2.5	7 ± 2
4. C(92–96), 10 μM	0 ± 2.7	0.6 ± 3.1	0.7 ± 0.9	38 ± 3
5. $C(92–97)_{dimer}$, 10 μM	5 ± 1.6	23 ± 2.4	19 ± 3.1	NT
6. Linear peptide, 10 μM	1 ± 1.3	8 ± 1.2	0.3 ± 2.6	0 ± 4
7. MC192 IgG, 1 nM	4 ± 2.5	7 ± 1.8	5 ± 0.6	25 ± 6
8. C(92–96) + MC192	28 ± 2.8	52 ± 5.2	40 ± 3.0	55 ± 5
9. $C(92–97)_{dimer}$ + MC192	39 ± 6.3	72 ± 3.1	59 ± 3.2	NT
10. Linear peptide + MC192	5 ± 2.0	7 ± 3.8	2 ± 1.0	26 ± 2

[a] NT, not tested.

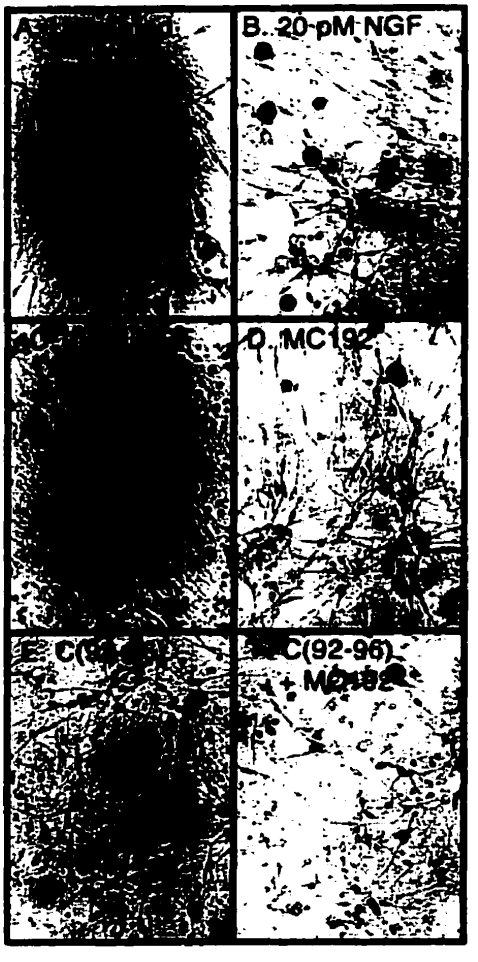

FIG. 6. **Monovalent TrkA ligands induce the differentiation of embryonic DRG cultures.** Primary neuronal cultures from embryonic day 17 rat DRGs were treated with the indicated ligands for 8 days, and cell differentiation was studied morphometrically. Magnification was × 60. Data are representative of three independent experiments.

mAb 5C3. This study was done by attempting to block mAb 5C3 binding with C(92–96). Moreover, since agonistic ligands are expected to cause receptor internalization, it was of interest to study whether monomeric ligands such as C(92–96) can induce TrkA internalization.

We studied ligand-dependent receptor internalization as a decrease of surface receptor density, which can be inhibited by low temperature or by poisons such as sodium azide. 4-3.6 cells were treated with NGF, C(92–96) peptide, or control peptides in the presence or absence of sodium azide at different temperatures. Surface TrkA receptors were quantitated by FACScan analysis with mAb 5C3 before and after 20 min of internalization (Table II). This time was selected because the t for ^{125}I[NGF] internalization is ~10 min (9).

C(92–96) did not block surface mAb 5C3 binding sites at 4 °C (Table II, row 2). Treatment with C(92–96) at 37 °C caused a ~23% loss of surface mAb 5C3 binding sites (Table II, row 2). This effect was sensitive to sodium azide (Table II, row 3). A 23% loss of surface TrkA represents ~11,000 receptors that presumably internalized out of ~50,000 expressed at the surface. Similar results were obtained with C(92–97) (data not shown). Negative control linear peptides did not affect the number of mAb 5C3 binding sites (Table II, rows 4 and 5).

In positive control studies, treatment with NGF at 4 °C blocked ~21% of the surface mAb 5C3 binding sites (Table II, row 1; also published in Ref. 19), suggesting that NGF partially blocks mAb 5C3. Treatment with NGF at 37 °C increased loss of surface mAb 5C3 binding sites from ~21 to ~46% (Table II, row 1), probably because of TrkA internalization.

DISCUSSION

We demonstrate that artificial ligands selective for subunits of receptor complexes can be used to study receptor structure-activity relationships in systems where each subunit has distinct or unclear functions. For NGF receptors, the main novel findings of this study are as follows: (i) genuine monomeric and monovalent ligands of TrkA can be partial agonists, suggesting that bivalent ligands are not the sole mechanism for dimerizing and/or activating tyrosine kinase receptors; (ii) the monovalent p75 ligands used in this study do not enhance TrkA-mediated signals, suggesting that p75 ligands may require bivalency; and (iii) bivalent ligands that induce TrkA-TrkA and p75-p75 homodimers afford optimal signals. Putative ligand-induced TrkA-p75 heterodimers do not seem to afford signals, and receptor oligomerization does not result in enhanced signals.

While it is well established that physiological ligands activate signal transduction by inducing receptor dimerization, allosteric models of receptor activation have also been proposed (15, 32–36). There are two major obstacles to studying allosteric models experimentally. First, there is a paucity of monovalent ligands that activate receptor tyrosine kinases (18). Second, the role of each subunit must be considered in the

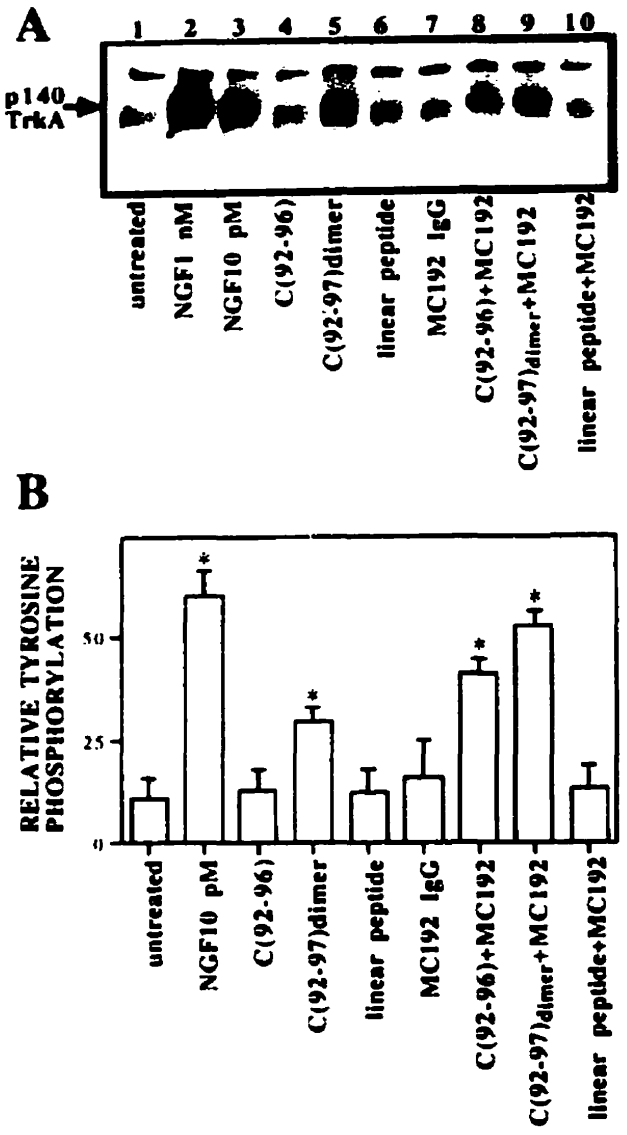

FIG. 7. **Monovalent ligands induce TrkA tyrosine phosphorylation.** Treatment and analysis of 4-3.6 cells was as in Fig. 4; ligand concentrations are as per Table I. *A*, cells were untreated (*lane 1*) or treated with 1 nM NGF (*lane 2*), 10 pM NGF (*lane 3*), 10 μM C(92–96) (*lane 4*), 10 μM $C(92–97)_{dimer}$ (*lane 5*), 10 μM control linear peptide (*lane 6*), 1 nM MC192 mAb (*lane 7*), 10 μM C(92–96) plus 1 nM MC192 (*lane 8*), 10 μM $C(92–97)_{dimer}$ plus 1 nM MC192 (*lane 9*), or 10 μM control linear peptide plus 1 nM MC192 (*lane 10*). *B*, densitometric scanning quantification of band intensities relative to optimal NGF treatment (average ± S.E., $n = 6$). *, significant difference from untreated samples (paired *t* tests, $n = 6$, $p < 0.05$).

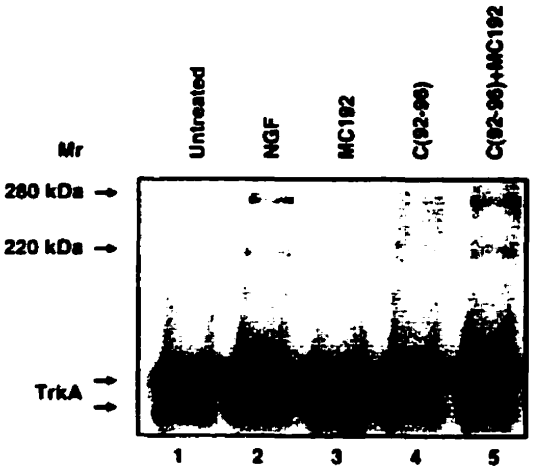

FIG. 8. **Monovalent ligands induce or stabilize putative TrkA homodimers.** 4-3.6 cells were untreated (*lane 1*) or treated with 2 nM NGF (*lane 2*), 1 nM MC192 (*lane 3*), 10 μM C(92–96) (*lane 4*), or 10 μM C(92–96) plus 1 nM MC192 (*lane 5*). Cells were then chemically crosslinked with disuccinimidyl suberate, lysed, resolved by SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting with anti-TrkA polyclonal antiserum.

TABLE II

NGF peptide mimics induce TrkA internalization

4–3.6 cells were treated with the indicated ligands or controls at 4 °C in the presence (+) or absence (−) of sodium azide. Cells were then maintained at 4 °C or shifted to 37 °C to allow internalization. Analysis was done by FACScan, immunostaining surface TrkA with mAb 5C3. Percentage loss of binding was calculated as a change in mean channel fluorescence with respect to untreated cells. Results are average ± S.E., $n = 3$, 5000 cells were acquired for each assay.

	Treatment	Sodium azide	Loss of mAb 5C3 binding sites	
			At 4 °C	At 37 °C
			%	%
1.	2 nM NGF	−	21 ± 6	46 ± 9
2.	10 μM C(92–96)	−	1 ± 7	23 ± 8
3.	10 μM C(92–96)	+	4 ± 7	−4 ± 6
4.	10 μM linear peptide	−	4 ± 4	3 ± 2
5.	10 μM linear peptide	+	5 ± 2	8 ± 3

analysis of heteromeric receptors, and agents that bind to and affect the activity of each subunit must be available. We postulate that the strategy of using growth factor-derived and antibody-based artificial ligands can be easily adapted to study other multisubunit receptors, or for receptors where a subunit has unclear function or no defined ligand. Also, strategies that develop monovalent small molecule agonists that bind to the extracellular domain of receptors will be useful for the discovery of pharmacological agents.

Valency, Avidity, and Aggregation State of Agonistic Ligands—Monovalent ligands 5C3 Fabs and C(92–96) are partial agonists. For T cell receptor complexes and G-coupled receptors, it has been shown that sometimes ligands with high affinity can overcome low avidity and lead to activation or to conformational changes (37–39). This does not seem to be the case for the ligands 5C3 Fabs or C(92–96) because their affinity ranges from nanomolar to micromolar. Hence, *N*-Ac-C(92–96) is a ligand of relative low affinity and avidity that can induce or stabilize putative TrkA homodimers. Since receptor dimerization alone does not necessarily cause activation (15, 17), the simplest interpretation is that the TrkA ligands induce allosteric or conformational changes, as shown for other receptors (16, 18, 35). However, this report would be a case where monomeric and monovalent ligands induce allosteric or conformational changes.

Arguably, 5C3 Fabs could aggregate in solution as do other large peptides (40), but this is unlikely to occur at nanomolar Fab concentrations in 0.2% bovine serum albumin and did not occur at micromolar Fab concentrations in related studies (41). Hence, we conclude that Fabs are monomeric. It is also unlikely that 5C3 Fabs are bivalent, and sequence analysis of the variable complementarity-determining regions of mAb 5C3 excluded this possibility.[2]

More conclusively, the *genuine* monomeric small peptide C(92–96) affords trophic signals, although the dimeric C(92–

[2] H. U. Saragovi, unpublished data.

$97)_{dimer}$ is more efficient. Monomeric C(92–96) is monovalent, because it has a 5-residue pharmacophore (27) and could not interact with two receptors simultaneously. The intriguing possibility that C(92–96) could dimerize *after* docking is unlikely (see below), but it remains unexplored and would require structural analysis of receptor-ligand complexes.

Ligand Density at the Cell Surface—The optimal functional concentration of the peptide *N*-Ac-C(92–96) and of mAb 5C3 and 5C3 Fabs approximates their K_d (10 μM, 2 nM, and 5 nM, respectively). In contrast, the optimal functional concentration of 1 nM NGF is 2–3 orders of magnitude above its K_d ($\sim 10^{-11}$ to 10^{-12} M). It is unlikely that these differences reflect requirements for receptor occupancy. It is more likely that NGF, the mAbs, or the peptides have different half-lives in solution at 37 °C or that they are bound by matrix proteoglycans or carrier proteins.

In some cases, the local concentration of a ligand at the cell surface can be higher than in solution, making the ligands more likely to dimerize or to aggregate. While the mobility of a ligand is reduced to two dimensions when bound to a receptor, ligand mobility within the plane of the membrane is still exclusively dependent on the receptor. Therefore, monovalent ligands could only dimerize subsequent to inducing their receptors to dimerize. In addition, we estimate that a cell expressing 50,000 receptors out of which 5% are bound would achieve a local ligand concentration of ~10 mM. This concentration was tested by NMR for monovalent C(92–96) with no evidence of aggregation. Last, we demonstrated that the monovalent ligands induce rapid receptor internalization, which will effectively reduce possible high local concentrations of ligand at the cell surface. It is noteworthy that self-aggregation of C(92–96) beyond the detection of NMR is unlikely to account for the effects, because $C(92–97)_{dimer}$ at high nM concentrations did not afford signals.

Mechanism of Action of TrkA and p75 Ligands—How can conformational changes induced by monovalent ligands lead to receptor dimerization? While the function of the monovalent ligands is defined by comparison with the natural ligand NGF, their mechanism of action may differ. We hypothesize three possible mechanisms: (i) conformational changes that favor direct dimerization; (ii) a reduction of the rate at which preformed receptor dimers disengage; and (iii) increased receptor mobility with a consequent increase in spontaneously dimerized receptors.

The most attractive explanation is that the monovalent ligands could be inverse antagonists (32, 33). Inverse antagonists can stabilize receptor conformation(s) that are favorable to subsequent TrkA-TrkA interactions. Indeed, one criterion for defining inverse antagonists is that their potency increases with increased receptor density on the cell surface, and this was observed for the activity of C(92–96) and $C(92–97)_{dimer}$ in PC12 cells that have low TrkA numbers *versus* 6-24 and 4-3.6 cells that have high TrkA numbers. Furthermore, an inverse antagonist would antagonize the natural agonist NGF, and NGF blocking properties have been shown previously for C(92–96) (20, 21). Last, as expected, the $C(92–97)_{dimer}$ affords higher signals, which would be predicted for a bivalent ligand that induced receptor dimerization directly.

With respect to p75 receptors, bivalent ligands potentiate the effects of all *bivalent* TrkA ligands (NGF, mAb 5C3, 5C3 Fab-α-Fab complexes, and $C(92–97)_{dimer}$). However, bivalent p75 ligands did not potentiate all *monovalent* TrkA ligands. mAb MC192 potentiated the activity of C(92–96), but it did not potentiate the activity of 5C3 Fabs. These data suggest that these monovalent TrkA ligands probably have different mechanisms of activation, and this possibility is supported by the fact that C(92–96) and 5C3 Fabs bind to nonoverlapping sites.

We speculate that a small molecule like C(92–96) docks onto a small pocket of TrkA and may therefore be sensitive to a documented p75-mediated regulation (6, 7) or internalization (9, 42, 43) of TrkA. Consequently, C(92–96) may be sensitive to ligands binding p75 concomitantly, whereas larger molecules like 5C3 Fabs possess more extended TrkA binding surfaces and do not exhibit this sensitivity. Hence, two classes of partial agonism (or inverse antagonism) by monomeric TrkA ligands may have been uncovered in this study.

Agonistic Ligand Binding Sites—MAb 5C3 and the NGF analog C(92–96) do not block each other's binding; hence, they bind to nonoverlapping sites of TrkA. Ligands docking onto restricted receptor pockets or "hot spots" are presumed to be more efficient at mediating (ant)agonistic function (34, 44). Reportedly, there are at least two activating TrkA "hot spots" (45–48) encompassing the IgC-2-like domain and the leucine-rich motif.

mAb 5C3 binds an epitope within the IgC-2-like domain of TrkA, and the epitope is stabilized by disulfide bonds (19). TrkA and other tyrosine kinase receptors have a dimer interface stabilized by disulfide bonds (49, 50). The agonistic "hot spot" of mAb 5C3 and 5C3 derivatives may be at the dimer interface of this receptor. However, 5C3 and C(92–96) do not block each other; hence, the data suggest that C(92–96) binds elsewhere. NGF may utilize both sites to fully activate the receptor.

Conclusions—The screening of functional small molecule ligands that bind multisubunit receptors may require testing ligand combinations that target all subunits. It would be of interest to test other NGF receptor ligands or activators in this paradigm of synergy, including peptidic small molecule p75 ligands (21) or similar peptides reproduced by others (51, 52), organic p75 ligands, gangliosides, and alkaloid derivatives that activate TrkA (53–55).

With respect to NGF receptors, our results support the hypothesis that functional receptors consist of TrkA homodimers and p75 homodimers. Our results also demonstrate that genuine monovalent and monomeric ligands of TrkA tyrosine kinase receptors can be functionally agonistic. Recently, two small molecule ligands of other receptors were shown to be agonistic. In one, a mimic of granulocyte colony-stimulating factor activated the granulocyte colony-stimulating factor receptor (56), but no studies of the aggregation state of the ligand were performed. In another, a small molecule activated the insulin receptor tyrosine kinase (57). However, this insulinomimetic ligand is a symmetrical lipophylic agent, in principle capable of dimerizing the receptor as shown for similar ring structures (16). Hence, our study is the first formal proof, to our knowledge, of genuine monovalent ligands of the extracellular domain of a tyrosine kinase acting as partial agonists by inducing or stabilizing receptor homodimers.

Neurotrophins and their receptors play a role in neurodegenerative diseases, pain, and neoplasias (44). Internalizing TrkA ligands could be exploited to deliver radioligands, toxins, oligonucleotides, or membrane-impermeable molecules selectively to receptor-expressing cells. This study has implications for the design and screening of small molecules with pharmacological, diagnostic, or targeting activity for neurotrophin receptors.

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A Designed Peptidomimetic Agonistic Ligand of TrkA Nerve Growth Factor Receptors

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ABSTRACT

A proteolytically stable small molecule β-turn peptidomimetic, termed D3, was identified as an agonist of the TrkA neurotrophin receptor. D3 binds the Ig-like C2 region of the extracellular domain of TrkA, competes the binding of another TrkA agonist, affords selective trophic protection to TrkA-expressing cell lines and neuronal primary cultures, and induces the differentiation of primary neuronal cultures. These results indicate that a small β-turn peptidomimetic can activate a tyrosine kinase neurotrophin receptor that normally binds a relatively large protein ligand. Agents such as D3 that bind the extracellular domain of Trk receptors will be useful pharmacological agents to address disorders where Trk receptors play a role, by targeting populations selectively.

TrkA is a transmembrane tyrosine kinase receptor with high selectivity for the neurotrophin nerve growth factor (NGF). Related neurotrophins include brain-derived neurotrophic factor (BDNF), which binds TrkB receptors, and neurotrophin-3 (NT-3), which prefers binding to TrkC receptors (Barbacid, 1994).

Docking of TrkA with NGF initiates receptor dimerization, catalytic phosphorylation of cytoplasmic tyrosine residues on the receptor, and a cascade of cell-signaling events (Kaplan and Stephens, 1994). These signals lead to prevention of apoptotic cell death (Maliartchouk and Saragovi, 1997), promotion of cellular differentiation and axon elongation, and up-regulation of choline acetyl transferase (ChAT) (Hefti et al., 1985).

Several neuronal cell types that are implicated in important disease states express TrkA and therefore respond to NGF, including sensory, sympathetic, and cholinergic neurons. It has been suggested that NGF therapy may delay the onset of Alzheimer's disease (Barinaga, 1994; Lindsay, 1996) and ameliorate peripheral diabetic neuropathies (Ebadi et al., 1997). Other applications proposed for NGF include treatment of neuronal damage (Hughes et al., 1997) and targeting of neuroectoderm-derived tumors (Cortazzo et al., 1996; LeSauteur et al., 1996a). For a review of disease targets, see Saragovi and Burgess (1999).

Despite the therapeutic potential of NGF, clinical trials featuring this protein have been disappointing (Verrall, 1994; Saragovi and Burgess, 1999). There are several reasons for this: inherent drawbacks associated with the use of polypeptides applied as drugs (Saragovi et al., 1992), in vivo instability (Barinaga, 1994), and pleiotrophic effects due to activation of signals that were not intentionally targeted (e.g., those mediated via the low-affinity NGF receptor p75 (Carter and Lewin, 1997)). Moreover, the NGF protein is relatively expensive to produce for medicinal applications.

For these reasons, we aimed to design small, proteolytically stable molecules with neurotrophic activity, selective for cells expressing TrkA. However, strategies that result in agonists of tyrosine kinase receptors have not been well established. Previously, we used ligand mimicry and antibody mimicry strategies (Saragovi et al., 1991; Saragovi et al., 1992) to generate peptide analogs of two agonists directed to the extracellular domain (ECD) of TrkA: the natural ligand NGF (LeSauteur et al., 1995; LeSauteur et al., 1996a; Debeir et al., 1999), and monoclonal antibody (mAb) 5C3 (LeSauteur et al., 1996b). TrkA binding is mediated by discrete β-turn regions of these ligands. Only certain cyclic

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ABBREVIATIONS: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; BOC, tert-butoxycarbonyl; ChAT, choline acetyl transferase; DMF, dimethylformamide; DRG, dorsal root ganglia; ELISA, enzyme-linked immunosorbent assay; FACScan, fluorescent activated cell scanner; FITC, fluorescein isothiocyanate; FMOC, fluorenyloxycarbonyl; MCF, mean channel fluorescence; MTT, 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide; NT-3, neurotrophin-3; RIA, radioimmunoassay; TFA, trifluoroacetic acid; Trt, trityl; ECD, extracellular domain; mAb, monoclonal antibody; HEK, human embryonic kidney; BB, binding buffer; OD, optical density.

β-turn analogs were active (Beglova et al., 1998), and other conformers or linear peptides were inactive.

Based on the pharmacophores of the mAb 5C3 and NGF peptide analogs described above, we synthesized a focused β-turn peptidomimetic library of ~60 members. We report the identification of compound D3, a small, selective, and proteolytically stable agonist of the TrkA receptor. Furthermore, the docking site of D3 onto TrkA was studied. Our findings support the notion that a small peptidomimetic ligand can agonize a tyrosine kinase neurotrophin receptor that normally binds a relatively large protein ligand. These agents may offer an alternative therapeutic strategy with pharmacological agents that selectively target neuronal populations expressing specific receptors on the cell surface.

Materials and Methods

Preparation of D3 and D3-Biotin. Compound D3 was prepared according to methods previously outlined for related compounds (Feng et al., 1998). fluorenyloxycarbonyl (FMOC)-Gly, FMOC-Hse-(Trt), FMOC-Lys(*tert*-butoxycarbonyl (BOC)), FMOC-Glu(OtBu), then 2-fluoro-5-nitrobenzoyl chloride were coupled (di-iso-propylcarbodiimide activation, 20% piperidine in dimethylformamide (DMF) to remove FMOC groups) to TentaGel S PHB resin at 0.18 mmol/g loading. The supported peptide was treated six times with 1% trifluoroacetic acid (TFA)/4% $HSiiPr_3$ in CH_2Cl_2 for 5 min to remove only the trityl (Trt) protection. Cyclization was effected by treatment with 5.0 equivalents of K_2CO_3 in DMF for 40 h. After 90% TFA/5% H_2O/5% $HSiiPr_3$ cleavage, the final product was purified by reversed-phase HPLC. D3 and its derivatives were soluble in water to 5 mg/ml (the highest concentration tested).

D3-biotin was prepared in the same way as D3, except that after the cyclization the nitro group was reduced by treatment with 10 equivalents of $SnCl_2 \cdot 2H_2O$ in DMF for 20 h. After reduction, FMOC-Gly, then biotin-*N*-hydroxysuccinimide was coupled to the newly formed arylamine. The product was then cleaved from the resin. The final product was purified by reversed-phase HPLC.

Cell Lines. B104 rat neuroblastomas express p75 receptors but do not express any of the Trks (TrkA$^-$ p75$^+$). The 4-3.6 cells are B104 cells stably transfected with human TrkA cDNA and express equal levels of p75 and TrkA (TrkA$^+$ p75$^+$) (kindly provided by Dr. E. Bogenmann) (Maliartchouk and Saragovi, 1997). Surface expression of each of NGF receptor was routinely controlled in all cells by quantitative fluorescence-activated cell sorter scanner (FACScan) assays (Becton Dickinson, CA) (data not shown) using anti-TrkA mAb 5C3 and anti-p75 mAb MC192.

Generation of Human TrkA-Rat TrkB Chimeras in Human Embryonic Kidney (HEK) 293 Cells. The IgG-C2 domain of human TrkA was generated by PCR as described (Perez et al., 1995) using unique restriction sites in the primers to allow exchange with the corresponding rat TrkB domain. The chimeric receptors were constructed by subcloning the human TrkA IgG-C2 domain into the corresponding restriction sites of the rat *trkB* cDNA reported in a previous work (Perez et al., 1995). Chimeric constructs (kindly provided by Dr. P. Perez) were confirmed by sequencing and were cloned into the pCDNA3 expression vector that contains a selection gene providing resistance to neomycin (G418; Life Technologies, Rockville, MD). HEK293 cells were transfected using the lipofectamine plus method (Life Technologies), selected with neomycin (0.5 mg/ml), and at least three independent subclones were obtained by limiting dilution techniques (293-TrkB/A-IgC2 chimera). Western blot analysis with polyclonal antibody 203 directed to the Trk intracellular domain (a gift of Dr. D. Kaplan) and cell-surface FACScan analysis with polyclonal antibody 1001 directed to the TrkA-ECD (our unpublished data) indicated that all stable subclones express comparable levels of chimeric receptors (data not shown).

Dissociated Neuronal Dorsal Root Ganglia (DRG) Cultures. Fetal rat DRG primary cultures were established essentially as described (Kimpinski et al., 1997) from Sprague-Dawley day 17 rat embryos. All ganglia were dissected and dissociated first enzymatically with trypsin and then mechanically. Dissociated cells were cultured (10^5 cells/well) in 96-well plates precoated with collagen and grown for a total of 8 days in Neuro Basal Medium containing N2 supplement (Life Technologies), antibiotics, and L-glutamine. These DRG cultures are ~85% TrkA-expressing and are heavily dependent on TrkA signals for survival (Vogelbaum et al., 1998).

Septal Neuronal Cultures. Cell cultures were established from the septal area of 17-day-old rat embryos as described (Hefti et al., 1985). In brief, tissue was incubated in PBS containing trypsin and DNase. Tissue pieces then were mechanically dissociated. After centrifugation, the pellet was suspended in Leibovitz's L-15 medium. Cells were plated onto 96-multiwell Nunc dishes (10^5 cells/well) coated with poly-D-lysine (5 μg/ml). Pure cultures of septal neurons were treated 1 day after plating. Drugs, prepared in medium, were added directly to the cells without changing the initial medium. The incubation continued for 8 days, at which time ChAT activity was evaluated.

D3-TrkA Binding Assays

Direct Binding Studies. Direct binding studies were done as described (Saragovi et al., 1998) using 6 ng/well of recombinant baculovirus TrkA-ECD or control BSA (Fraction V; Boehringer Mannheim, Mannheim, Germany) immobilized onto 96-well microtest plates. Wells were blocked with binding buffer (BB: PBS with 1% BSA) for 1 h. Then, 50 ng/well of biotinylated D3 was added as primary reagent in BB for 40 min in the absence or presence of excess nonbiotinylated D3 as competitor. Wells were washed five times with BB, and horseradish peroxidase-coupled avidin (Sigma, St. Louis, MO) was added as secondary reagent for 30 min. Plates were washed in BB, and peroxidase activity was determined colorimetrically using 2,2-azinobis (3-ethylbenzthiazoline sulfonic acid) (Sigma). The optical density (OD) was measured at 414 nm in a Microplate reader (Bio-Rad, Richmond, CA). Assays were repeated at least three times ($n = 4$).

FACScan binding assays. 4-3.6 cells (2×10^5) in FACScan binding buffer (PBS, 0.5% BSA, and 0.1% NaN3) were immunostained as described (LeSauteur et al., 1996; Saragovi et al., 1998). Saturating anti-TrkA mAb 5C3, or anti-p75 mAb MC192, or control nonbinding IgGs were added to cells for 1 h at 4°C, in the presence or absence of D3 as competitor. Excess primary antibody was washed off, and cells were immunostained with fluorescinated goat-anti-mouse IgG secondary antibody. Cells were acquired on a FACScan, and mean channel fluorescence (MCF) of bell-shaped histograms were analyzed using the LYSIS II program.

Binding competition. Binding competition studies were as described for direct binding assays to TrkA-ECD, except that as primary reagent 50 ng anti-TrkA mAb 5C3/well were added in BB, in the presence or absence of D3 or controls as competitors as described (Saragovi et al., 1998). Wells were washed five times with BB, and horseradish peroxidase-coupled goat anti-mouse was added as secondary reagent for 30 min. Plates were washed in BB, and peroxidase activity were determined. Assays were repeated at least three times ($n = 4$).

Cell Survival Assays

Primary DRG Cultures. After a total of 8 days of culture with the indicated test or control ligands, cell survival were studied using the 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide colorimetric (MTT) assay and by microscopic observation as described (Debeir et al., 1999).

Cell lines. A total of 5,000 cells/well in protein-free media (PFHM-II; Life Technologies) containing 0.2% BSA (crystalline fraction V; Sigma) were seeded in 96-well plates (Falcon, Mississauga, Ontario,

Canada). The cultures were untreated or treated with the indicated test or control ligands. Cell viability was quantitated using the MTT assay after 56 to 72 h of culture, as described (Maliartchouk and Saragovi, 1997). Percent protection was standardized from OD readings relative to optimal NGF (1 nM) = 100%. The OD of untreated cells were subtracted. The higher OD of untreated primary cultures is likely due to cellular heterogeneity and to endogenous production of limiting amounts of growth factors.

Measurement of ChAT Activity. At day 8 of culture, the medium was aspirated, and ice-cold lysis buffer (10 mM sodium phosphate, pH 7.4/0.1% Triton X-100) was added. ChAT activity assays were performed directly in the wells using Fonnum's method (Fonnum, 1975).

Detection of Putative TrkA·TrkA Homodimers. Live 4-3.6 cells suspended in PBS were treated with the indicated ligand(s) for 40 min at 4°C to allow binding. Cells were then washed in PBS, cross-linked with the membrane impermeable cross-linker disuccinimidyl suberate (Pierce, Rockford, IL; 1 mM, 15 min at 15°C). Unreacted disuccinimidyl suberate was quenched with 5 mM ammonium acetate. Then cells were either lysed directly in SDS sample buffer (whole-cell lysate) or lysed in nonionic detergent Nonidet P-40 and immunoprecipitated with anti-Trk or anti-p75 antibodies as described (LeSauteur et al., 1996b). Similar results were obtained with either method. For Western blot analysis, equal amounts of protein or cell equivalents for each sample were resolved in a 5 to 10% SDS-polyacrylamide gel electrophoresis gradient, transferred to nitrocellulose membranes (Xymotech Biosystems, Montréal, Quebec, Canada), and blotted with anti-Trk polyclonal antibody 203 that recognizes the intracellular domain of Trk. Blots were visualized using the enhanced chemiluminescence system (New England Nuclear, Boston, MA).

Results

Synthesis of Focused β-Turn Peptidomimetic Libraries. A solid-phase synthesis was developed to yield a macrocyclic ring with the i + 1 and i + 2 residues of a β-turn in the appropriate conformation. Approximately 60 compounds of this type were prepared (Feng et al., 1998), with amino acid side chains incorporated to correspond to β-turns of NGF and mAb 5C3 implicated in docking to TrkA (LeSauteur et al., 1995; LeSauteur et al., 1996a,b; Debeir et al., 1999). TrkA binding is mediated by discrete β-turn regions of these ligands. Cyclic peptide β-turn analogs of NGF and of mAb 5C3 were active only in the appropriate conformation (Beglova et al., 1998).

Figure 1 shows the molecular structure of D3 and that of a similar, but inactive, molecule called C59. C59 was used as a negative control. A biotinylated form of D3, termed D3-biotin, was synthesized to carry out direct binding studies to TrkA. All ligands were highly soluble in physiological buffers and did not require organic solvents.

D3 Is a Selective Ligand of TrkA. FACScan analysis featuring the secondary fluorescent agent avidin-fluorescein isothiocyanate (FITC) was used to detect binding of D3-biotin to the cell surface (Table 1). The 4-3.6 cells ($p75^+$ $TrkA^+$) had fluorescence approximately four times greater for D3-biotin than for a background control peptide-biotin. Moreover, a 10-fold molar excess of D3 abolished binding of D3-biotin. In contrast, no specific binding was measured for B104 cells ($p75^+$ $TrkA^-$). Because 4-3.6 cells are B104 cells stably transfected with *TrkA* cDNA and these cell lines are otherwise identical, the data indicate that D3-biotin and D3 bind cell-surface TrkA.

Similar binding data for D3-biotin was obtained by enzyme-linked immunosorbent assay (ELISA) using pure soluble TrkA-ECD produced in baculovirus (data not shown, also see Table 3). These data further indicate that D3 binds to the ECD of TrkA and that membrane lipids are not required.

D3 Binds Within an Agonistic Site of TrkA. Previously, mAb 5C3 was shown to act as a full TrkA agonist. Monoclonal antibody 5C3 binds with K_d 2 nM (LeSauteur et al., 1996b) at an epitope within the IgC2 domain of TrkA near the NGF binding site. This site is postulated to define a receptor "hot spot" (Wells, 1996). We tested whether D3 and mAb 5C3 bind to overlapping receptor sites.

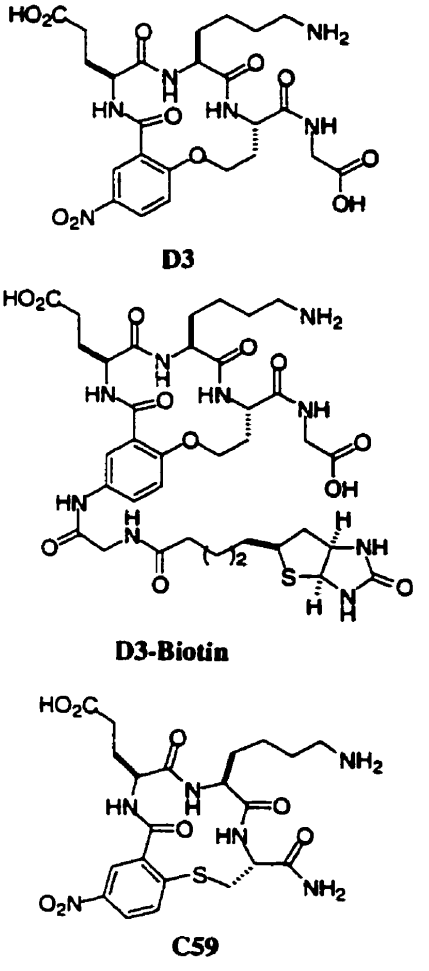

Fig. 1. Structures of D3, D3-biotin, and C59.

TABLE 1

D3 and D3-biotin bind TrkA

Binding of D3-biotin to B104 cells ($p75^+$ $TrkA^-$) or 4-3.6 cells ($p75^+$ $TrkA^+$) was quantitated by FACScan analysis. Ligands are control-biotin (an inactive biotinylated peptide) (row 2), D3-biotin (row 3), or D3-biotin with a 10-fold molar excess of D3 (row 4). All ligands were followed with avidin-FITC as a fluorescent label. Data shown are MCF of bell-shaped histograms, 5000 events acquired. MCF data ± S.E.M. are averaged from three independent experiments.

Ligand	MCF	
	B104	4-3.6
Untreated	10 ± 3	13 ± 2
Control-biotin 20 μM	11 ± 1	10 ± 3
D3-biotin 20 μM	10 ± 4	53 ± 4
D3-bio 20 μM + D3 200 μM	11 ± 2	17 ± 7

Two related assays tested the ability of D3 to compete for the binding of the full TrkA agonist mAb 5C3. In the first test, a FACScan-based assay using intact cells, D3-induced a dose-dependent competitive decrease of mAb 5C3-TrkA interactions (Table 2, rows 2–5). On average, D3 exhibited an IC_{50} of 4 μM. From experimental conditions, we estimate a K_d ~2 μM for D3-TrkA interactions. Blocking of 5C3-TrkA interactions by D3 is selective because the binding of mAb MC192 directed to the p75 NGF receptor subunit was not blocked (Table 2, rows 7 versus 8). Furthermore, inactive control C59 peptidomimetic did not inhibit the binding of either mAb 5C3 (Table 2, row 6) or mAb MC192 (data not shown).

The second test used purified recombinant TrkA-ECD immobilized onto ELISA plates to assay competitive blocking of 5C3-TrkA-ECD by D3. D3 exhibited a dose-dependent inhibition of 5C3-TrkA-ECD interactions, but control inactive C59 peptidomimetic had no effect (Table 3). Because a K_d ~2 nM was measured for 5C3-TrkA interactions, from the experimental IC_{50} a K_d ~2 μM was calculated for D3-TrkA-ECD interactions. This calculation is consistent with the data shown in Table 2. Interestingly, similar ELISA and radioimmunoassay (RIA) binding assays revealed that D3 did not substantially block NGF-TrkA-ECD interactions (data not shown).

D3 Affords Trophic Activity Selectively via TrkA, and Is Proteolytically Stable. Because D3 binds at or near an agonistic site of TrkA, trophic effects were probed in cell survival assays using the quantitative MTT method (Maliartchouk and Saragovi, 1997). Several doses of D3 were tested. However, for clarity only near optimal concentrations are shown, which approximate the estimated K_d.

Dissociated primary neuronal cultures from fetal DRG are dependent on TrkA agonists for survival (Vogelbaum et al., 1998). Exogenous NGF showed a dose-dependent trophic effect (Table 4, rows 2–4). D3 alone had a significant protective effect on DRG cultures (Table 4, row 5), but control C59 did not (Table 4, row 6). Primary cultures are heterogeneous and low levels of neurotrophins are made endogenously (Kimpinski et al., 1997), which explains a relatively high OD for untreated cultures (Table 4, row 1).

Because D3 does not block NGF binding, potential synergy between NGF and D3 was assessed. D3 combined with different concentrations of exogenous NGF demonstrated an additive or potentiating effect on DRG survival (Table 4, rows 7–9).

Similar results were obtained with other neuronal cell lines, wherein D3 potentiated the effect of low NGF concentrations (Table 5). Optimal protection of 4-3.6 cells ($p75^+$ $TrkA^+$) and HEK293-TrkB/A-IgC2 chimeras corresponded to treatment with 1 nM NGF (Table 5, row 2), whereas 10 pM NGF gave significantly less protection (Table 5, row 3). D3 alone afforded low but significant protection (Table 5, row 4), and protection was enhanced with a combination of 10 pM NGF plus 10 μM D3 (Table 5, row 6). The negative control C59 compound had no effect alone or in enhancing 10 pM NGF (Table 5, rows 5 and 7).

In other controls (data not shown), neither D3 nor NGF protected B104 cells, wild-type HEK293 cells, or TrkB-expressing HEK293 cells from apoptosis. Hence the trophic

TABLE 2

D3 specifically blocks mAb 5C3 binding to cell-surface TrkA

4-3.6 cells were analyzed by FACScan for binding of anti-TrkA mAb 5C3 or anti-p75 mAb MC192. Cells exposed to control primary mouse IgG with or without 40 μM D3 afford identical background staining (data not shown). For each condition, 5000 cells were acquired. Percentage maximal bindings were calculated from the MCF of bell-shaped histograms, using the formula ($TEST_{MCF}$ − $background_{MCF}$) × 100/($MAXIMAL_{MCF}$ − $background_{MCF}$). MCF ± S.E.M. are averaged from three independent experiments.

	mAb (1 nM)	Competitor	Dose (μM)	% Maximal Binding
1	5C3	None	0	100 ± 0
2	5C3	D3	0.20	95 ± 4
3	5C3	D3	1	80 ± 3
4	5C3	D3	5	53 ± 5
5	5C3	D3	40	33 ± 4
6	5C3	C59 control	40	97 ± 6
7	MC192	None	0	100 ± 0
8	MC192	D3	40	101 ± 2

TABLE 3

D3 inhibits 5C3 · TrkA interactions in vitro

The binding of mAb 5C3 (at constant 2 nM) to purified TrkA-ECD immobilized onto ELISA plates was measured in the absence or presence of competitors. Background (<2%) was the OD of wells with all reactants except immobilized TrkA-ECD. Data are averaged from three experiments (n = 1).

	Competitor Added	Concentration (μM)	% Binding ± S.E.M.
1	–	–	100 ± 3
2	D3	0.05	100 ± 14
3	D3	0.2	89 ± 8
4	D3	1	64 ± 10
5	D3	5	43 ± 12
6	D3	20	38 ± 7
7	D3	40	31 ± 4
8	C59	40	96 ± 9

TABLE 4

D3 protects TrkA-expressing primary neurons from apoptosis and potentiates NGF

NGF-dependent primary neuronal cultures from embryonic rat DRGs were treated with the indicated ligands for a total of 8 days. Cell survival was measured by MTT assays. Protection was calculated relative to optimal NGF (1 nM, 100% protection) with subtraction of the OD of untreated cells. Shown is the OD from one experiment, mean ± S.E.M. (n = 1). Percentage protection was averaged from three experiments.

Treatment	OD	% Protection
1 Untreated	256 ± 15	0 ± 2
2 NGF 1 nM	823 ± 28	100 ± 4
3 NGF 20 pM	316 ± 11	9 ± 1
4 NGF 500 pM	535 ± 19	68 ± 3
5 D3 10 μM	405 ± 22	38 ± 2
6 Control C59 10 μM	271 ± 8	0 ± 1
7 D3 10 μM + NGF 20 pM	471 ± 28	48 ± 3
8 D3 10 μM + NGF 500 pM	603 ± 26	84 ± 3
9 D3 10 μM + NGF 1 nM	977 ± 38	120 ± 7

TABLE 5

D3 potentiates NGF in protecting TrkA-expressing cell lines from apoptosis by binding to the IgC2 domain of the receptor

4-3.6 cells or HEK293 cells expressing TrkB/TrkA IgG-C2 chimeric receptor were treated with the indicated ligands for a total of 72 h. Survival was measured by MTT assays. Percentage protection was calculated as in Table 4. Shown is the OD from one experiment, mean ± S.E.M. (n = 4). Percent protection was averaged from six (4-3.6 cells) or three (293-IgG-C2 chimera) independent experiments.

Treatment	4-3.6 Cells		HEK293-TrkB/TrkA Chimera	
	OD	% Protection	OD	% Protection
1 Untreated	64 ± 7	0 ± 2	32 ± 5	0 ± 4
2 1 nM NGF	412 ± 24	100 ± 6	350 ± 12	100 ± 4
3 10 pM NGF	205 ± 19	40 ± 5	88 ± 8	18 ± 5
4 10 μM D3	95 ± 9	8 ± 2	69 ± 7	9 ± 3
5 10 μM C59	76 ± 4	2 ± 1	30 ± 7	−1 ± 2
6 10 μM D3 + 10 pM NGF	255 ± 14	55 ± 3	165 ± 11	42 ± 5
7 10 μM C59 + 10 pM NGF	209 ± 17	41 ± 4	90 ± 9	21 ± 6

activity of NGF and D3 require TrkA expression, or at least the IgG-C2 domain of TrkA. Additionally, D3 did not enhance the trophic effect of epidermal growth factor, suggesting that it may be NGF selective. Lastly, D3 enhanced NGF protection of NIH3T3 cells stably transfected with *TrkA* cDNA (data not shown), but did not enhance NT-3 protection of NIH3T3 cells stably transfected with *trkC* cDNA. These data indicate that D3 selectively accentuates the trophic effect of NGF, and that p75 expression is not required.

The proteolytic stability of D3 versus trypsin and papain was assessed. D3 was first exposed to enzymatic treatment as described previously (Saragovi et al., 1991; Saragovi et al., 1992), followed by gauging its biological activity on 4-3.6 cells. Compound D3 remained fully active in trophic assays even after 1 h of exposure to trypsin or pepsin, whereas NGF lost all activity within minutes under the same conditions (data not shown).

D3 Induces Differentiation of Primary Cultures of Fetal DRG and Fetal Septal Neurons. The effect of D3 on TrkA-mediated cellular differentiation was assessed using two independent assays: morphometric analysis of DRG dissociated neurons and induction of ChAT activity in septal neuronal cultures. In the first of these assays, data indicate that DRG neuronal cultures undergo neurite outgrowth in response to D3, and that D3 potentiates the effect of NGF (Fig. 2). In the second assay, ChAT activity was found to increase in response to NGF (Table 6, rows 1 and 2) and to D3 alone (Table 6, rows 3–5), whereas C59 control had no effect (Table 6, row 6). Increases in ChAT activity in response to 2 μM D3 alone were comparable with 10 pM exogenous NGF. Moreover, combinations of 2 μM D3 plus 10 pM NGF markedly increased ChAT activity and were more effective than 400 pM NGF (Table 6, rows 8–10).

D3 Enhances or Stabilizes Putative TrkA-TrkA Homodimers. Based on the data above, it was expected that D3 would induce or stabilize TrkA-TrkA interactions. This hypothesis was studied biochemically in 4-3.6 cells exposed to ligands, followed by cell-surface chemical cross-linking (Fig. 3).

The expected doublet consistent with previously reported TrkA monomers of p110 and p140 were seen in all samples (Fig. 3, thick arrow). Bands of ~300 kDa, consistent with the molecular mass of TrkA-TrkA homodimers (Fig. 3, thin arrow), were seen in samples from cells treated with TrkA ligands 1 nM NGF, 10 pM NGF, or 10 pM NGF plus 10 μM D3 and was also detected (albeit very more weakly) in cells treated with 10 μM D3 alone. The intensity of the 300-kDa band, presumed to be TrkA dimers, was analyzed densitometrically from four independent experiments standardized to 1 nM NGF (100%). There was a consistent increase in dimers after treatment with D3 alone (21 ± 4%) or 10 pM NGF alone (52 ± 6%), which was higher after treatment with 10 pM NGF plus 10 μM D3 (77 ± 7%). Control cells cross-linked in the absence of ligand or cells exposed to ligand but not-cross-linked (data not shown) did not have putative dimers.

TrkA homodimers are stable to SDS denaturation because of covalent cross-linking. Given that the efficiency of chemical cross-linking is ~1 to 4% of the total TrkA pool, we were precluded from further biochemical characterization of the complexes, other than the fact that they contain TrkA. The complexes may contain cross-linked NGF. However, it is unlikely that the bands comprise p75 because immunoprecipitations with anti-p75 antibodies did not reveal any material in the molecular mass of TrkA homodimers (data not shown). Furthermore, material of 215 kDa that would comprise p75-TrkA heterodimers was not seen consistently.

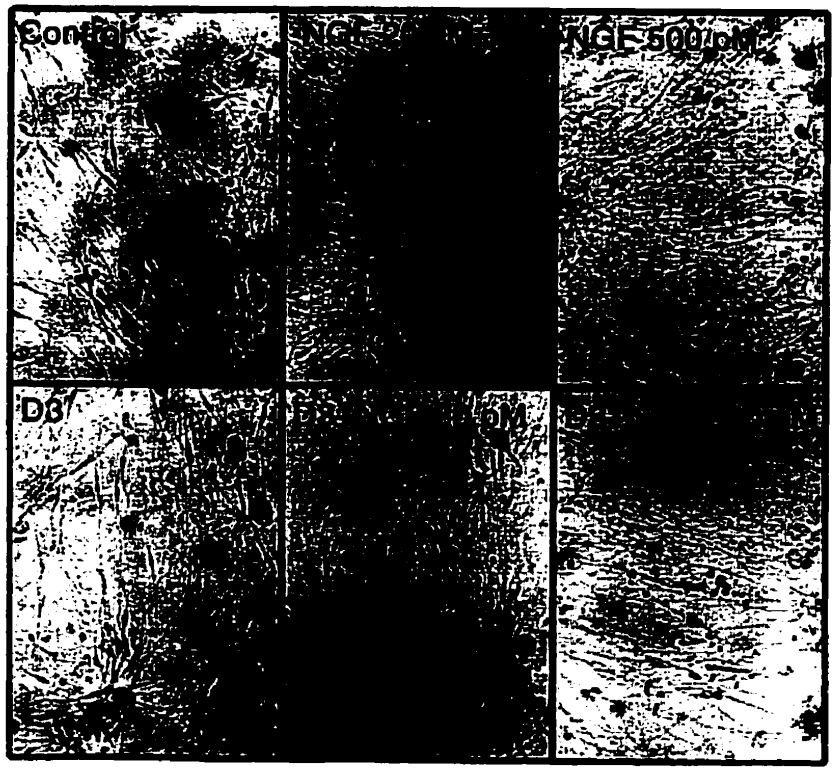

Fig. 2. D3 induces the differentiation of embryonic DRG cultures. Primary neuronal DRG cultures were treated as indicated for 8 days, and cell differentiation was studied morphometrically. Magnification, 60×. Pictures representative of three independent experiments.

Discussion

We report on a proteolytically stable β-turn peptidomimetic small molecule agonist of the TrkA neurotrophin receptor. We showed that D3 binds TrkA, competes the binding of the TrkA agonist mAb 5C3, selectively potentiates trophic protection of TrkA-expressing cell lines and neuronal primary cultures, and induces the differentiation of primary neuronal cultures. These results indicate that a small β-turn peptidomimetic can activate a tyrosine kinase neurotrophin receptor that normally binds a relatively large protein ligand.

Recent advances in ligand mimicry have resulted from screening large phage or peptide libraries (Reddy et al., 1996; Wrighton et al., 1996), natural products (Zhang et al., 1999), or chemical libraries (Owolabi et al., 1999). However, most of the ligands described are antagonists, or otherwise require the dimerization of relatively large peptides, have a 2-fold axis of symmetry that resemble a dimer, or are poorly soluble in physiological buffers. In contrast, D3 is a small, nonsymmetrical, proteolytically stable, highly water soluble peptidomimetic that binds the ECD of TrkA.

Recently, a symmetrical alkaloid-like molecule screened from fungi was found to potentiate, at micromolar concentrations, the action of insulin presumably by binding near the catalytic domain of insulin receptors (Zhang et al., 1999). Hence, with regards to agonistic activity and optimal concentration our compound D3 is analogous to the insulinomimetic ligand. In addition, binding and ligand competition studies demonstrate selective interaction of D3 with the ECD of TrkA rather than the catalytic domain. Hence, the water solubility and extracellular targeting of D3 mean that toxic organic solvents are not required to permeate the cell membrane.

Mechanism of Action of D3. It is surprising that D3 is an agonist because the natural ligand NGF is a symmetrical dimer known to activate TrkA via homodimerization (Kaplan and Stephens, 1994). D3 is not a dimer and, from NMR studies, it has no detectable propensity to dimerize even at high millimolar concentrations in solution (data not shown). Then, why does D3 behave as an agonist of TrkA? One hypothesis is that D3 stabilizes the signaling conformation of preformed TrkA homodimers without per se inducing efficient receptor dimerization. This hypothesis is supported by the data because, as would be predicted, exposure to low levels of NGF enhanced D3 activity in bioassays and in receptor cross-linking assays.

TABLE 6

D3 induces ChAT synthesis

Septal neuronal cultures were treated as indicated for a total of 8 days. ChAT activity (pmol Ach/min/well ± S.E.M.) was measured at day 8. Average ± S.E.M. Data averaged from three independent experiments (n = 4).

Treatment	ChAT Activity	Fold Increase
1 10 pM NGF	0.42 ± 0.07	1.4
2 400 pM NGF	0.72 ± 0.10	2.41
3 0.2 μM D3	0.37 ± 0.05	1.23
4 2 μM D3	0.44 ± 0.02	1.47
5 20 μM D3	0.48 ± 0.06	1.56
6 20 μM C59 control	0.30 ± 0.05	1
7 Untreated	0.31 ± 0.07	1
8 0.2 μM D3 + 10 pM NGF	0.60 ± 0.04	2.00
9 2 μM D3 + 10 pM NGF	0.76 ± 0.03	2.53
10 20 μM D3 + 10 pM NGF	0.79 ± 0.04	2.63

What is the role of picomolar concentrations of NGF? Given the low concentrations used in synergy with D3, it is unlikely that the effect of NGF was mediated by docking with the low-affinity receptor p75. We speculate that NGF acts by increasing TrkA·TrkA interactions whereas D3 stabilizes the homodimers or reduces the rate of separation of receptor homodimers by inducing conformational changes. Indeed, there are precedents for ligands of serpentine receptors acting in this manner (Milligan et al., 1995), and recent models of single transmembrane receptor dimerization and activation are compatible with this view (Tian et al., 1998; Livnah et al., 1999; Remy et al., 1999).

In the present study, the biological data shown are with low micromolar concentrations of D3, which are optimal. As expected from the affinity estimated for TrkA·D3 interactions, lower D3 concentrations afford lower efficacy. It is noteworthy that whereas NGF·TrkA affinity is $\sim 10^{-11}$ M, optimal activity requires 2 nM NGF concentrations. Hence, D3 is optimal at concentrations that approximate its K_d, whereas NGF is optimal at concentrations ~100-fold over its K_d. We interpret this difference to mean that D3 is more stable in solution, and this notion is supported by D3 resistance to proteolysis.

Ligand Binding Sites. D3 competitively blocks the binding of mAb 5C3, but it does not block NGF. Moreover, the optimal agonistic activity of mAb 5C3 (Maliartchouk and Saragovi, 1997) was inhibited by D3 in a dose-dependent manner (data not shown), whereas the agonistic effect of NGF was enhanced. These results are intriguing because previously we reported that mAb 5C3 can block ~50% of the NGF binding sites on a cell expressing TrkA, whereas NGF can block ~25% of the mAb 5C3 binding sites (LeSauteur et al., 1996b). It is unlikely that D3 does not block NGF because of affinity differences, because NGF·TrkA-ECD and 5C3·TrkA-ECD interactions are both in the nanomolar range.

Two factors could account for this result. First, both mAb 5C3 and D3 dock onto a single and continuous epitope within the IgG-C2 domain of TrkA, whereas NGF binds a discontin-

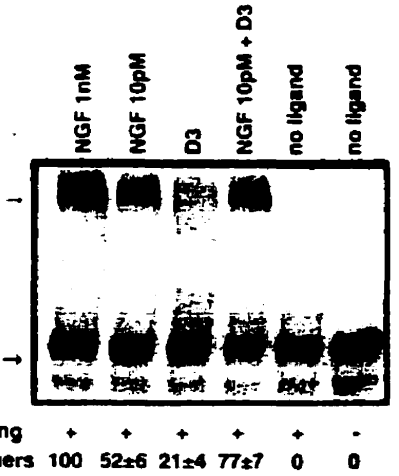

Fig. 3. D3 enhances cell-surface TrkA·TrkA homodimers. 4-3.6 cells were exposed to TrkA ligands as per Table 5 (*lanes 1–4*) or no ligand (*lanes 5 and 6*) and chemically cross-linked (*lanes 1–5*) or not cross-linked (*lane 6*). Cell lysates were Western blotted with anti-TrkA 203 antisera. The intensity of the 300-kDa band was analyzed densitometrically from four experiments standardized to 1 nM NGF.

uous epitope within the IgG-C1 and IgG-C2 domains of TrkA (Perez et al., 1995), and at least one other domain (Windisch et al., 1995). This would facilitate mAb 5C3 blocking by D3, whereas NGF could bind via its second docking site. Second, mAb 5C3 and NGF bind TrkA at sites partially overlapping but not identical (LeSauteur et al., 1996b). Hence, the data suggest that D3 binds TrkA at an epitope overlapping the agonistic mAb 5C3 "hot spot" of the IgG-C2 domain of TrkA, near the NGF docking site. These observations may account for D3 synergizing with NGF and blocking mAb 5C3.

The fact that D3 is bioactive and was selected from a relatively small pool of β-turn-based compounds has broad implications for many research initiatives involving protein-protein interactions. Other small molecules with neurotrophic activity have been reported (Steiner et al., 1997; Maroney et al., 1998). However, the molecular targets of these ligands are ubiquitous intracellular proteins, and the mechanisms of action are often unclear. Thus, these other molecules are not Trk ligands, and are not defined as peptidomimetics of known ligands.

In contrast, we report a small molecule peptidomimetic that binds and activates TrkA. In the present report of D3, we show that a hybrid of a peptide and a small organic molecule designed to hold key amino acid residues in a turn conformation within a small framework offers a means to transform a peptide lead into an active organic small molecule. Hence, D3 represents the validation of the peptidomimetic concept for the Trk family of tyrosine kinase receptors. This small molecule peptidomimetic ligand of TrkA that has neurotrophic activity may be useful to address neurodegenerative disorders, pain, neoplasias, and other pathologies (reviewed by Saragovi and Burgess, 1999) where TrkA receptors play a role.

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