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Two New Roles for TrkA:

Endosomal Signaling and Assembly of Coated Pit Proteins

by

Eric C. Beattie

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

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Dedication

This thesis is dedicated to my parents, Nancy and George Beattie, and to my wife Mary. Their love, support, and encouragement throughout these past few years has been unfailing, and for this I will always be grateful.

The faculty, students, and staff of the Department of Physiology and the Program of Biomedical Sciences have all become invaluable resources to me during my stay here at UCSF. With the many gifted and giving people at this campus, I was always able to receive help with a research, administrative, or scholastic problem.

To my orals and thesis committee chairman, Nigel Bunnett, and to the orals and thesis committee members (Frances Brodsky, Regis Kelly, David Bredt, Susan Fisher, Mary Dallman, David Morgan, and William Mobley) I extend a sincere thank you. I will always be grateful not only for their time and guidance but also for treating me as one of their peers and for the collaborations that took this thesis project where it could otherwise not have gone. I especially wish to thank the members of the Bunnett and Brodsky labs for giving generously their time and reagents, and for their helpful discussions.

The members of Mobley lab have been of great assistance in most of this work and I thank in particular my fellow graduate students Chuck Howe, Casey Crawford, and Manish Butte for all their help and for our great lunchtime science sessions. Chuck Howe also contributed experimental work that appears in this thesis, and for his assistance in the clathrin redistribution project I am grateful.

I would especially like to thank my thesis advisor Bill Mobley for providing an excellent research environment, much needed guidance, and focus in my pursuit of the most difficult goal I have ever reached for: that of training myself to think differently.

Preface

Acknowledgements

Chapter 2 of this thesis has been previously published in the Journal of Neuroscience (vol. 16, pp. 7950-64, December 1996, copyright 1996), and represents a collaborative work. As second author of this paper, E. Beattie has made a major contribution to the experiments, to preparing, and writing the paper and performed all the confocal microscopy and radioiodinated NGF cross-linking work described herein. I would like to call attention to the invaluable contributions made by my co-authors, Drs. Mark Grimes, Jie Zhou, Eric Yuen, Deb Hall, Kimberly Top, Jenny LaVail, Nigel Bunnett, William Mobley and Ms. Janice Valletta.

Chapter 3 is a summary of the contributions our lab has made to the TrkA signaling endosome theory, with the exception of the work described in Chapter 4, which was published the following year. This article was previously published in the Cold Spring Harbor Symposia on Quantitative Biology, vol. 61, pp. 389-406, in December of 1996, copyright 1996. As a first author of this paper along with Jie Zhou, E. Beattie has made a major contribution to the experimentation, preparation, and writing of this paper and has performed all confocal microscopy and radioiodinated NGF crosslinking work described herein. As authors, Drs. Mark Grimes, Nigel Bunnett, William Mobley and Chuck Howe made vital contributions to the completion of this work.

Chapter 4 has previously been published in The Proceedings of the National Academy of Sciences, vol. 94, pp. 9909-14, 1997, copyright 1997, National Academy of

Sciences, USA, and represents a collaboration among the authors. As the second author of this paper, E. Beattie has made a major contribution to the writing and completion of this work and has performed all of the radioiodinated NGF cross-linking studies described herein. Drs. Mark Grimes and William Mobley are first and last authors, respectively, on this study.

Written permission from the original publishers to include Chapters 2, 3, and 4 in this thesis follows directly.

The work in Chapter 5 is at this writing, unpublished. All figures represent work done entirely by E. Beattie except for the experiment described in figure 18, which represents a collaboration with Chuck Howe. I would like to gratefully acknowledge his help and the general help of the members of the Mobley lab whose assistance was vital to the completion of this study. We thank Robert Kupta, Lou Reichardt, and David Kaplan for PC12 nnR5 cell lines used in this chapter's work. D283 cells were stabley transfected with TrkA and kindly provided by Janice Valletta. I would like to thank Nigel Bunnett, Frances Brodsky, Mark von Zastrow, Rob Malenka, Roger Nicoll, and the members of their laboratories for their generosity with their reagents, facilities, time and council. Reagents used in this chapter from the Brodsky lab include X22, TD.1;anti-clathrin antibodies, and AP.6, AC1M11; anti-AP2 antibodies.

The Appendix summarizes a collaborative work among the authors that has been submitted for publication. As second author, E. Beattie has made a major contribution to the planning, writing, experimental design, and conceptual design of this work. Andrew Wilde and Frances Brodsky are first and last authors of this paper and as such, provided much of the direction and performed most of the experimentation. As co-authors on this

paper, the contributions of Lawrence Lem and William Mobley towards the completion of this work were vital.

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Abstract **Eric C. Beattie**

Nerve growth factor (NGF) is required for the proper growth and maintenance of specific sets of central and peripheral neurons. This polypeptide growth factor binds two receptors, the receptor tyrosine kinase (RTK) $gp140^{TrkA}$ (TrkA), and the single transmembrane glycoprotein, p75. NGF binding initiates the retrograde transport of a trophic signal from the tips of neuronal processes to the cell's nucleus. The nature of this retrogradely trafficked trophic signal has been unclear.

This thesis presents data that support signaling of TrkA from endosomal structures and suggest that these signaling endosomes are a key component of the retrogradely trafficked trophic signal. Our biochemical analyses of endosomal fractions from NGF-treated pheochromocytoma (PC12) cells provide the following indicators of TrkA signaling activity: 1) TrkA and NGF can be chemically cross-linked; 2) TrkA is tyrosine phosphorylated; and 3) the downstream signaling molecules, PLC-γ and Shc, can be found phosphorylated and associated with TrkA.

Confocal microscopy studies were performed to investigate the initial steps of TrkA's trophic signaling journey into the cell. These studies show increased colocalization of TrkA and the coated pit endocytosis proteins, clathrin and AP2, after NGF application. These data suggest that these proteins contribute to TrkA endocytosis. Further studies demonstrated a NGF-induced redistribution of clathrin from the cytosol to the plasma membrane that was dependent on TrkA kinase activity.

Clathrin and AP2 have been shown to be involved with the endocytosis of other RTKs (e.g.-EGF-R), but the control of this process has been poorly understood. We

hypothesized that TrkA, p75, or both mediate clathrin's redistribution. Our biochemical and confocal studies indicated that: 1) TrkA, and not p75, is responsible for this redistribution; and 2) disruption of signaling by TrkA mutation prevents this action. It was found that NGF treatment caused clathrin heavy chain phosphorylation and the formation of complexes containing TrkA and the following proteins: clathrin, AP2, and the non-receptor kinase $pp60^{src}$. Studies involving EGF-R effects on clathrin redistribution and phosphorylation showed that EGF acted similarly, confirming that at least these two RTK's induce redistribution and phosphorylation of clathrin. These studies also identify $pp60^{src}$ as the kinase responsible for clathrin phosphorylation.

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List of Abbreviations

RTK	Receptor tyrosine kinase.
NGF	Nerve growth factor, a neurotrophin.
BDNF	Brain derived neurotrophic factor, a neurotrophin.
TrkA	$gp140^{TrkA}$, the receptor tyrosine kinase for NGF.
TrkB	$gp140^{TrkB}$, the receptor tyrosine kinase for BDNF.
p75	A transmembrane glycoprotein receptor which binds all neurotrophins.
PC12 cells	Pheochromocytoma cells (rat).
PLC-γ	Phospholipase C-γ, part of TrkA's signal transduction cascade.
Shc	Src homology protein, part of TrkA's signal transduction cascade.
EGF	Epidermal growth factor.
EGF-R	Epidermal growth factor receptor.
$pp60^{src}$	A non-receptor kinase of the SRC family.
CHC	Clathrin heavy chain
AP2	Adaptor protein 2; specific to plasma membrane endocytosis.
α-adaptin	A subunit specific to AP2
CREB	Cyclic adenosine monophosphate response element-binding protein.
LCa, LCb	Two isoforms of clathrin light chain.
DiI	A lipohilic carbocyanine dye used to mark plasma membrane.
EM	Electron microscopy.

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Chapter 1

Introduction

NGF and NGF Receptors

Nerve growth factor (NGF) is a member of the neurotrophin family, which includes brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. These polypeptides bind specifically to members of the Trk receptor tyrosine kinase (RTK) family. NGF binds to $gp140^{TrkA}$ (TrkA), BDNF and NT4/5 bind TrkB, while NT-3 and NT4/5 bind with highest affinity to TrkC with low levels of crossover binding to the other Trks (Yuen et al., 1996). All neurotrophins also bind to the single transmembrane glycoprotein receptor, p75. A number of distinct signaling receptor complexes may exist for each of the neurotrophins, and high and low-affinity binding has been detected. High affinity ($Kd=10^{-11}$) complexes have been best characterized for NGF; they demonstrate very slow ligand dissociation and have been called slowly-dissociating receptors. They are likely to be formed by heterodimeric complexes of Trk and p75 and by Trk homodimers. Monomeric complexes involving just Trk or p75 show low-affinity binding ($Kd=10^{-9}$) (Ross et al., 1996; Wolf et al., 1995; Wolf et al., 1998) (Yuen et al., 1996).

Significantly, signaling arises from the Trks in Trk homodimers. Signaling from TrkA is initiated when a dimer of NGF brings together two TrkA receptors on the neuron's cell surface. This allows the kinase domains of TrkA to transphosphorylate specific tyrosine residues on the receptor's carboxy-terminal which in turn allows for the binding of cytosolic signaling molecules. Shc (SRC homology protein), and

phospholipase C-gamma (PLC-γ) are two such proteins that function in the initial steps of TrkA's signal cascade. These proteins feed into the Ras/MAP kinase and protein kinase C (PKC) pathways respectively, which ultimately results in the translation of survival and differentiation-promoting proteins (Kaplan, 1997).

Signaling from p75, and the receptor complex responsible, is not well characterized, but various groups have shown NGF-induced stimulation of the sphingomyelin pathway through this NGF receptor (Dobrowsky et al., 1994). Other groups supply evidence for the antagonistic signaling roles of p75 and TrkA with regards to control over apoptosis (Bredesen and Rabizadeh, 1997).

A signaling endosome hypothesis

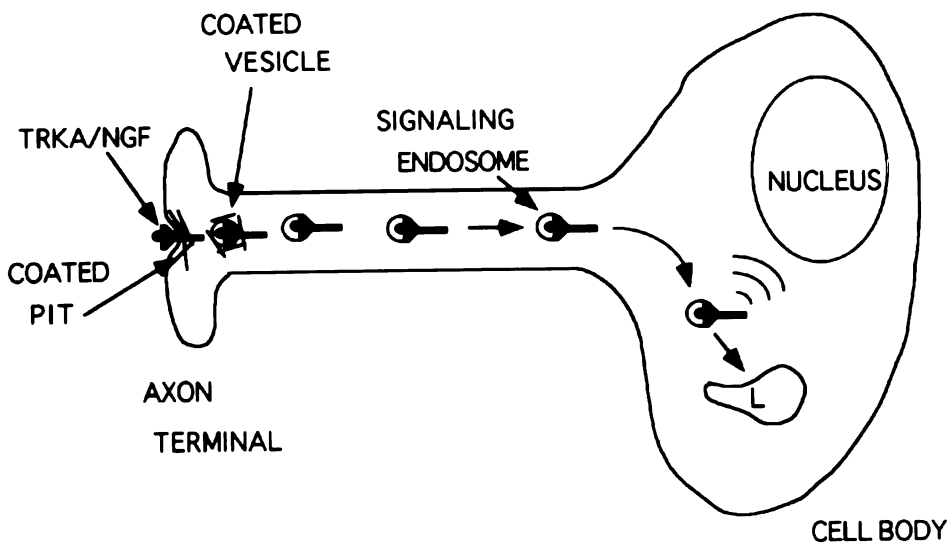
When bound by NGF, TrkA signals to support the survival and differentiation of a subset of neurons in the central and peripheral nervous system (Kaplan and Miller, 1997; Yuen et al., 1996). NGF is a target-derived factor. Thus, signaling must begin at receptors on axon terminals. The mode of delivery of this survival signal along neuronal processes to the cell nucleus has been unclear until now. Axon terminals may be a great distance from the cell's soma and nucleus (Hendry, 1992). Simple diffusion of cytosolic signaling proteins stimulated by activated TrkA is unlikely to account for retrograde trophic signaling.

We hypothesized that TrkA acts as this retrograde signal. Our studies in Chapters 2, 3, and 4 suggest a TrkA signaling endosome as the means for retrogradely transporting the NGF signal. This scenario is modeled in the diagram below that depicts the journey of a TrkA-containing vesicle down the axon of a neuron to the cell body. Our data suggest

that this journey begins with the internalization of NGF-bound TrkA in clathrin coated pits prior to the formation of NGF/TrkA-containing coated vesicles and transport vesicles. As our studies in Chapter 3 suggest, when these vesicles reach the cell body TrkA may be quickly degraded in the lysosome (marked as L in the diagram). However, before downregulation occurs, active TrkA signaling may reach the nucleus to promote neuronal growth and maintenance. Though our analysis of cultured PC12 cells cannot directly prove this model, our data supporting the formation of TrkA signaling-competent vesicles in a neuron-like cell, and data from other labs strongly supports this proposal. For example, studies done by Riccio et al. (1997) (discussed in the introduction of Chapter 2) using compartmentalized cultures of sympathetic neurons showed that the arrival of a NGF/TrkA-containing vesicle to the cell body corresponded to phosphorylation of CREB (cyclic adenosine monophosphate response element-binding protein), a nuclear transcription factor participating in the trophic response.

Herein we give evidence that these TrkA signaling vesicles carry the NGF trophic signal. Chapters 2, 3 and 4 outline biochemical and confocal microscopic evaluation of PC12 cells and derivative endosomal fractions that suggest that TrkA signals from endosomal structures in which NGF remains bound to these receptors. These signaling endosomes likely make up the retrogradely trafficked signal that supports NGF-induced neuronal health and survival (Beattie et al., 1996; Grimes et al., 1997; Grimes et al., 1996a; Yuen et al., 1996).

A Model For The Signaling Endosome Hypothesis



Coated Pit Endocytosis

The nature of the process of signaling endosome production was suggested by our observations in confocal studies that TrkA and clathrin were colocalized following NGF treatment. The model we tested was that TrkA and its bound ligand, NGF, are internalized from the plasma membrane in clathrin coated pits (see diagram above). Confocal and biochemical analysis of various cell lines described in Chapters 2-4 point to

a role for clathrin (a well characterized protein involved in coated pit endocytosis; see Introduction, Chapter 5), as a contributor to the process of TrkA internalization. These analyses also point to TrkA signaling as being responsible for rapid NGF-induced redistribution, phosphorylation, and receptor association of clathrin. They raise the possibility that TrkA signaling induces a general increase in clathrin coated pit traffic, which may regulate endocytosis not just of TrkA, but other receptors as well

The non-receptor kinase, $pp60^{src}$ has been shown to be activated by NGF treatment (Hilborn et al., 1998) (Kremer et al., 1991). We asked if $pp60^{src}$ could play a role in NGF-induced clathrin redistribution and phosphorylation. In collaboration with our lab, Brodsky and Wilde have shown that $pp60^{src}$ overexpression in A431 increases phosphorylation of the clathrin heavy chain (CHC) (Appendix). Chapter 5 describes studies in which NGF treatment was shown to result in the formation of complexes containing TrkA together with AP2, clathrin, and $pp60^{src}$. The studies in Chapter 5 and the Appendix represent the first to define such a complex and ligand-induced tyrosine phosphorylation of CHC.

These findings support a model in which TrkA activation recruits not only signaling molecules, but endocytosis-promoting proteins as well. In this model, TrkA is able to serve as a signaling scaffold for these proteins, thus enhancing endocytosis of itself and possibly other receptors. Our collaborative studies with Brodsky and Wilde suggest that this endocytosis model may share many similar features with the EGF receptor system. These studies describe the ability of TrkA and EGF-R to recruit and phosphorylate coated pit proteins (Chapter 5 and the Appendix), and makes the re-

examination of many other receptors systems utilizing clathrin-mediated internalization an attractive pursuit.

Chapter Two

Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes

Introduction

We hypothesized that TrkA continues to signal subsequent to the binding by NGF at the plasma membrane and internalization by clathrin coated pits. This chapter describes the initial work done to support the theory that TrkA signals from endosomes and proposes TrkA to be the best candidate for the retrogradely trafficked neurotrophic signal. Elucidation of the true nature of the retrogradely trafficked NGF-induced survival signal has been a goal of neuroscientists ever since it was determined that basal forebrain cholinergics required unperturbed connections to their NGF source in the cortex (Yuen et al., 1996) (Koliatsos et al., 1991). Earlier studies demonstrated that the NGF receptors TrkA and p75 have been candidates for carrying the signal, but no direct support was provided until relatively recently (Ehlers et al., 1995). This chapter contains data that establish TrkA in internalized vesicles as signaling competent.

First, TrkA and clathrin were shown with confocal microscopy to become colocalized at the cell surface of PC12 cells minutes after NGF application. Clathrin staining was also seen to increase at the plasma membrane after NGF addition. These observations gave clues to the nature of TrkA endocytosis, the first step in the formation of NGF-induced signaling endosomes.

Second, NGF and TrkA could be chemically cross-linked in isolated endosomal fractions from PC12 cells, showing that NGF remained bound to TrkA and suggesting the

possibility that TrkA remained active in these internalized membranes. This possibility was confirmed by experiments showing TrkA in these endosomal fractions was tyrosine phosphorylated and bound to tyrosine phosphorylated PLC-γ.

Studies from separate laboratories were published during the progress of our work that confirmed a role for TrkA-containing endosomes in conveying the retrograde signal (Riccio et al., 1997a). In these studies, the authors used compartmentalized cultures of sympathetic neurons to show that the arrival of a NGF/TrkA-containing vesicle to the cell body corresponded to activation of a key target of the NGF-stimulated signaling pathway, CREB. Microspheres bound covalently to NGF were used to show that this source of NGF was capable of initiating TrkA phosphorylation when brought in contact with neurons. However, microsphere-bound NGF applied to axon terminals was incapable of inducing CREB phosphorylation in the nucleus. Citing our earlier studies detailed in Chapter 2, and noting that NGF itself has been ruled out as the retrograde signal (Heumann et al., 1984), the authors concluded that retrogradely trafficked vesicles containing active TrkA elicit the retrograde trophic signal.

Endocytosis of Activated TrkA: Evidence that Nerve Growth Factor Induces Formation of Signaling Endosomes

Mark L. Grimes,[1] Jie Zhou,[2a] Eric C. Beattie,[6a] Eric C. Yuen,[2] Deborah E. Hall,[2] Janice S. Valletta,[2] Kimberly S. Topp,[3,5] Jennifer H. LaVail,[3,4] Nigel W. Bunnett,[6] and William C. Mobley[2,3]

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The survival, differentiation, and maintenance of responsive neurons are regulated by nerve growth factor (NGF), which is secreted by the target and interacts with receptors on the axon tip. It is uncertain how the NGF signal is communicated retrogradely from distal axons to neuron cell bodies. Retrograde transport of activated receptors in endocytic vesicles could convey the signal. However, little is known about endocytosis of NGF receptors, and there is no evidence that NGF receptors continue to signal after endocytosis. We have examined early events in the membrane traffic of NGF and its receptor, $gp140^{TrkA}$ (TrkA), in PC12 cells. NGF induced rapid and extensive endocytosis of TrkA in these cells, and the receptor subsequently moved into small organelles located near the plasma membrane. Some of these organelles contained clathrin and α-adaptin, which implies that TrkA is internalized by clathrin-mediated endocytosis. Using mechanical permeabilization and fractionation, intracellular organelles derived from endocytosis were separated from the plasma membrane. After NGF treatment, NGF was bound to TrkA in endocytic organelles, and TrkA was tyrosine-phosphorylated and bound to PLC-γ1, suggesting that these receptors were competent to initiate signal transduction. These studies raise the possibility that NGF induces formation of signaling endosomes containing activated TrkA. They are an important first step in elucidating the molecular mechanism of NGF retrograde signaling.

Key words: NGF; TrkA; signaling; endosome; clathrin; PLC-γ1

Nerve growth factor (NGF), a polypeptide neurotrophic factor of the neurotrophin gene family, acts physiologically to enhance the survival and differentiation of specific populations of neurons in the central (CNS) and peripheral nervous systems (PNS) (Levi-Montalcini, 1987; Yuen and Mobley, 1995). NGF actions are mediated by its receptors, $p75^{NTR}$ and $gp140^{TrkA}$ (TrkA). $p75^{NTR}$, a single-transmembrane glycoprotein, is a receptor for all of the neurotrophins (Bothwell, 1995). The role that $p75^{NTR}$ plays in NGF signaling is not well defined (Bothwell, 1996; Carter et al., 1996); however, $p75^{NTR}$ modulates NGF binding and activation of TrkA (Meakin and Shooter, 1992; Davies et al., 1993; Barker and Shooter, 1994; Hantzopoulos et al., 1994; Lee et al., 1994; Mahadeo et al., 1994; Verdi et al., 1994). TrkA is a receptor tyrosine kinase whose activation has been shown *in vitro* to induce many of the typical neuronal responses to NGF (Loeb and Greene, 1993). NGF binding to TrkA causes dimerization of the receptor with resulting activation of its kinase domain (Kaplan et al., 1991; Klein et al., 1991; Meakin and Shooter, 1991; Jing et al., 1992). Autophosphorylation of certain tyrosine residues in the intracellular domain of the receptor creates sites for binding and activation of signaling intermediates that continue the signal transduction cascade (Stephens et al., 1994). Known intermediaries are PLC-γ1, SHC, and PI-3 kinase (Kaplan and Stephens, 1994; Stephens et al., 1994). Significantly, TrkA plays an important role in NGF signaling *in vivo*. Animals in which the gene for TrkA was disrupted showed marked abnormalities in the development of NGF-responsive neurons in the CNS and PNS (Smeyne et al., 1994).

An important question is how NGF signaling in axons is communicated to neuronal cell bodies. For many NGF-responsive populations, the principal source of NGF is the target field of innervation (Longo et al., 1993). Thus, NGF is available to bind and activate its receptors only on distal axons. The importance of signaling through these receptors was demonstrated by Campenot (1977), who showed that NGF present only at the tips of neurites was sufficient to maintain the viability of cell bodies. This indicates that a signal(s) created by receptor activation on distal axons must be communicated to the cell body. For neurons with long axons, the distance through which the retrograde signal must be moved may be >1000 times the width of the cell body. In earlier studies to define the NGF retrograde signal, the following were discovered. (1) NGF itself was taken up in the target in a dose-

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Abbreviations: APP, amyloid precursor protein; BS^3, bis(sulfosuccinimidyl)suberate; DSS, disuccinimidyl suberate; HRP, horseradish peroxidase; PNS, peripheral nervous system; $p75^{NTR}$, low-affinity neurotrophin receptor; TrkA, receptor tyrosine kinase activated by NGF; PLC-γ1, phospholipase C-γ1; PI-3 kinase, phosphatidylinositol 3′-kinase.

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dependent, saturable, and stereospecific manner (Hendry et al., 1974a). (2) NGF was retrogradely transported to the cell body at ~2500 μm/hr (Hendry et al., 1974b; Claude et al., 1982). (3) A response to NGF was registered in only the neurons in which it was transported (Hendry, 1977). (4) The response to NGF coincided temporally with the arrival of NGF at the cell body (Hendry and Bonyhady, 1980). (5) Retrograde movement of NGF was abolished by colchicine (Hendry et al., 1974b; Claude et al., 1982). (6) Colchicine also blunted the retrograde signal caused by NGF (Paravicini et al., 1975). Importantly, although transport of NGF marked retrograde signaling, NGF itself was not the signal. NGF injection into the cell body did not create responses, and NGF antibodies failed to suppress them (Heumann et al., 1984). We suggest that the retrograde signaling entity must be (1) present in distal axons, (2) activated by NGF, (3) retrogradely transported to the cell body, and (4) that its activity must be sufficient to initiate the NGF response in the cell body.

Our hypothesis is that activated TrkA in endocytic vesicles is a retrograde signal. In support, the following are known. (1) TrkA is present in axons in NGF-responsive neurons (Holtzman et al., 1995). (2) TrkA in axons can be activated by NGF treatment (Knüsel et al., 1994; Li et al., 1995). (3) *In vitro*, surface TrkA is rapidly downregulated in response to NGF binding (Hosang and Shooter, 1987; Zhou et al., 1995). (4) TrkA, including the tyrosine-phosphorylated active form of the receptor, is retrogradely transported in axons (Ehlers et al., 1995). Significantly, the amount of TrkA that accumulated distal to a sciatic nerve ligature, and the extent of its tyrosine phosphorylation, was increased by applying NGF to the foot pad, the target of sciatic nerve sensory neurons (Ehlers et al., 1995). These experiments showed that target-derived NGF regulates retrograde transport of TrkA. However, they did not examine the mechanism by which NGF accomplishes this effect or its significance for signaling. Important, as yet untested, predictions of the hypothesis are that NGF induces endocytosis of TrkA, that internalized receptors continue to be activated, and that they are moved retrogradely in axons and are capable of signaling.

We have examined TrkA receptors internalized in response to NGF binding. PC12 cells have been widely used to study NGF responses (Tischler and Greene, 1975; Stephens et al., 1994; Zhou et al., 1995) and provide a model for neuronal signal transduction and membrane traffic. In this paper, we show that NGF rapidly induced internalization of TrkA receptors, that these receptors were localized in endocytic vesicles, and that intracellular receptors were activated as judged by the presence of phosphotyrosine and association with PLC-γ1. These data suggest that by inducing activation and endocytosis of TrkA, NGF creates signaling endosomes that could be used to convey the retrograde signal.

MATERIALS AND METHODS

Materials. Bis(sulfosuccinimidyl)suberate (BS^3), disuccinimidyl suberate (DSS), and NHS-SS-biotin were obtained from Pierce (Rockford, IL). Potassium hydroxide (99%) was from Aldrich (Milwaukee, WI). Type IV collagen was from Collaborative Biomedical Products (Bedford, MA). Normal goat serum and Vectastain mounting medium were from Vector Laboratories (Burlingame, CA). Except as noted, Sigma (St. Louis, MO) was the source of all other reagents and chemicals.

NGF was prepared as described previously (Mobley et al., 1986). NGF was labeled with ^{125}I (Amersham, Arlington Heights, IL) using lactoperoxidase, as modified from Vale and Shooter (1985). Iodinated protein was separated from free ^{125}I on a PD-10 column (Pharmacia, Uppsala, Sweden) precquilibrated with binding buffer [PBS, pH 7.4, containing 1 mg/ml glucose and 1 mg/ml bovine serum albumin (BSA)]. Final specific activity was 25–100 cpm/pg. Radioactivity was quantified using a Beckman 2000 gamma counter.

1088 is a rabbit antibody against the C terminus of human TrkA. It was purified using protein A–Sepharose (Pierce) and has been characterized previously (Zhou et al., 1995). Sc11 is another rabbit antibody to the C terminus of human TrkA (Santa Cruz Biotechnology, Santa Cruz, CA). Both antibodies recognize full-length receptors whose kinase domains can be activated. RTA is a rabbit antibody against the extracellular domain of rat TrkA (Clary et al., 1994; Lucidi-Phillipi et al., 1996). X22 is a mouse monoclonal antibody to the clathrin heavy chain (Brodsky, 1985). AP.6 is a mouse monoclonal antibody to the α-adaptin 100 kDa subunits (Chin et al., 1989). GM10 is a mouse monoclonal antibody that stains lysosomes (Grimaldi et al., 1987; Grady et al., 1995). The antibody to PLC-γ1 was a mixed monoclonal antibody; 4G10 is a mouse monoclonal antibody to phosphorylated tyrosine (both from UBI, Lake Placid, NY). ^{125}I-labeled goat anti-mouse was prepared using Na-^{125}I (Amersham), iodobeads (Pierce), and goat anti-mouse (Pierce) according to the manufacturer's instructions, and desalted on a PD-10 column (Pharmacia Biotech, Piscataway, NJ).

NGF binding and cross-linking to surface receptors. PC12 cells (a gift of Lloyd A. Greene, Columbia University) were grown on collagen-coated plates in RPMI 1640 with 10% horse serum and 5% fetal calf serum (both from HyClone Laboratories, Logan, UT). Cells were washed and harvested in warm PBS (without Ca^{2+} and Mg^{2+}) and resuspended in cold (4°C) binding buffer. In all experiments, equal amounts of cells were aliquoted for each condition. Cells were incubated with [^{125}I]NGF (1 nM = 26.5 ng/ml) at 4°C for 1 or 2 hr, warmed to 37°C in the presence of [^{125}I]NGF for 5, 10, or 30 min, and then rapidly chilled (4°C). For cross-linking [^{125}I]NGF to surface receptors, the membrane-impermeant cross-linking reagent BS^3 was added at a final concentration of 0.8 mM, while rotating at 4°C for 30 min. To correct for nonspecific binding and cross-linking, unlabeled NGF (1 μM = 26.5 μg/ml) was included during binding and cross-linking. The reactions were quenched with 1 mM lysine for 10 min. Cells were pelleted and then lysed for 20 min on ice in 1 ml of lysis buffer #1 (20 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 10% glycerol, and proteinase inhibitors: 1 mM PMSF, 10 μg/ml benzamidine, 1 μg/ml *O*-phenantholine, and 0.1 μg/ml each pepstatin, chymostatin, leupeptin, and aprotinin). After centrifuging at 16,000 × *g* for 30 min, the supernatant was removed and assayed for protein (BCA Assay, Pierce). Samples were normalized for protein and immunoprecipitated with 1088 (12 μg/ml), rotating overnight at 4°C. Protein A–Sepharose beads (Pierce), 120 μl of a 20% solution per ml of lysate, were added and incubated at 4°C for 2 hr. After washing two times in lysis buffer #1 and once in H_2O, 50 μl of 7 M urea SDS-PAGE sample buffer (125 mM Tris, pH 6.95, 7 M urea, 2% SDS, 1 mM EDTA, 0.1% Bromphenol Blue) with 100 mM DTT was added, and the sample was heated to 65°C for 15 min. Samples were loaded onto 8–12% SDS-PAGE. Fixed gels (10% acetic acid, 10% isopropanol, 20% methanol) were dried and exposed to the PhosphorImager and then to XAR-5 film (Eastman Kodak, Rochester, NY).

Cell surface biotinylation. Cells were incubated with or without NGF (1 nM) for 30 min at 4°C in PBS with 1 mg/ml glucose, and then NHS-SS-biotin (0.5 mg/ml) was added. The mixture was incubated with gentle rocking for 90 min at 4°C. Cells were pelleted (1000 rpm for 5 min) and then washed three times in cold PBS containing 1 mM lysine. Samples were resuspended in binding buffer, and equal amounts were aliquoted for three different treatments. One sample (designated 100%) was held at 4°C. Another (bkg = background) was treated at 4°C with 50 mM reduced glutathione in 50 mM Tris, pH 8.6, 100 mM NaCl, 1 mg/ml glucose, and 1 mg/ml BSA for 30 min. This treatment was repeated twice. The third sample (int = internalized) was warmed to 37°C for either 10 or 20 min to allow endocytosis, and then treated with glutathione as above. All samples were then incubated 1 hr on ice in 0.2 ml of lysis buffer #2 (20 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing 1% BSA and 1 mg/ml iodoacetamide. The lysates were centrifuged 10 min at 10,000 × *g*. SDS (final concentration 0.5%) was added to the supernatant, and the lysates were boiled 5 min. Lysis buffer #2 (0.8 ml) was then added, and TrkA was immunoprecipitated with 1088 (12 μg). Each lysate was divided into two aliquots. The first was used to detect the amount of biotinylated TrkA. Proteins were separated on nonreducing 7.5% SDS-PAGE, transferred to nitrocellulose (Hoefer Pharmacia Biotech, San Francisco, CA), and blotted with [^{125}I]streptavidin (Amersham). Biotinylated TrkA was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The second aliquot was submitted to 7.5% SDS-PAGE in a reducing environment, transferred to nitrocellulose, and blotted with 1088 (1:500). The signal was developed using [^{125}I]protein A (Amersham) and quantified using the PhosphorImager. The signal for biotinylated TrkA was normalized to the amount of TrkA protein. The TrkA available for internalization was (100% − bkg). TrkA internalized during warming

was not susceptible to reduction with glutathione. The amount of TrkA internalized was (int − bkg). The percent of TrkA internalized was (int − bkg)/(100% − bkg) × 100.

Immunostaining and confocal microscopy. Cells were plated on 8-well chamber slides (Nunc, Naperville, IL) that had been coated with Matrigel (Collaborative Biomedical Products) using a 1:200 dilution in PBS (Ca^{2+}- and Mg^{2+}-free) overnight at 4°C. Wells were washed three times with cold PBS. PC12 cells were plated in DME H-21 medium with 10% horse serum and 5% fetal calf serum 1–2 d before experiments. After aspirating the medium, NGF (2 nM = 53 ng/ml) was added to cells for 30 sec, 2 min, or 60 min in 300 μl of DME H-21 containing 0.5 mg/ml BSA and 10 mM HEPES at 37°C. This medium, without NGF, was added to controls.

Cells were fixed on ice with 1% paraformaldehyde in PBS for 15 min. Cells were permeabilized in PSS (PBS with 10% normal goat serum and saponin at 1 mg/ml) at room temperature for 30 min, changing the solution every 10 min. Primary antibodies were diluted in PSS (sc11 at 1 μg/ml; X22 at 3.1 μg/ml; AP.6 at 8 μg/ml; GM10 at 1:6000) and were incubated with cells overnight at 4°C. After three 10 min washes with PSS, secondary antibodies diluted in PSS (FITC-conjugated goat anti-rabbit IgG at 1:100; Texas Red-conjugated goat anti-mouse immunoglobulins, 1:200; both from Cappel Research Products, Durham, NC) were applied for 45 min at room temperature. After three washes in PBS, coverslips were mounted using Vectashield mounting solution. No staining was evident when primary antibodies were excluded. For Sc11, preliminary incubation (overnight, 4°C) with the peptide immunogen (10 μg/ml) eliminated staining.

Cells were observed with an MRC 1000 Laser Scanning Confocal Microscope (Bio-Rad, Hercules, CA) equipped with a krypton/argon laser and attached to a Zeiss Axiovert microscope. A Zeiss Neofluor ×100 oil-immersion objective with a numerical aperture of 1.3 (0.17°) was used, and images were collected using an aperture of 3–4 mm and a zoom of 2–3. Typically, 10–20 optical sections were taken at 0.5 μm intervals through the cells. The resolution of the confocal microscope in the *x–y*-axis was 170–200 nm, and in the *z*-axis was 230–400 nm. Images of 768 × 520 pixels were obtained. Images were processed using Adobe Photoshop 3.0 (Adobe Systems, Mountain View, CA) and printed using a Techtronix Printer. In experiments in which markers were colocalized, colocalization was confirmed by examining individual organelles at higher magnification. In addition, we ensured that colocalization was eliminated by merging overlaying but noncoincident sections. The images shown correspond to optical sections through cells at mid-height.

To count TrkA-positive vesicles near the surface of cells, images of individual optical sections were examined. The edge of the cell was defined as the outermost limit of staining for the clathrin heavy chain, and this was marked with a line. A second line was drawn 0.5 μm interior to the first, and all bright, punctate TrkA-positive vesicles between the lines were counted. The number of these vesicles was then divided by the perimeter of the surface of the cell to yield a value for the number per micrometer cell perimeter.

Cell fractionation. Figure 4 depicts the cell fractionation strategy. Cells grown and harvested as above were first incubated with or without NGF (1 nM) in binding buffer at 4°C for 1 hr. They were then either washed briefly in binding buffer, or not washed and warmed in binding buffer for 10 min at 37°C. Cells were then chilled (4°C) and washed in PBS with 1 mM EDTA and 1 mM EGTA, and then in a cytoplasm-like buffer (buffer B; 38 mM each of the potassium salts of aspartic, gluconic, and glutamic acids, 20 mM MOPS, 5 mM reduced glutathione, 10 mM potassium bicarbonate, 0.5 mM magnesium carbonate, 1 mM EGTA, 1 mM EDTA, pH-adjusted to 7.1 at 37°C with potassium hydroxide). Cells were resuspended in 0.5 ml of buffer B containing proteinase inhibitors (as for lysis buffer #1). Sodium orthovanadate (1 mM) was included to inhibit phosphatase activity for the experiments shown in Figures 8–10. To permeabilize cells, we used a ball homogenizer obtained from the European Molecular Biology Laboratory (Heidelberg, Germany) and tungsten carbide grade-25 balls obtained from Industrial Tectonics (Ann Arbor, MI). The cell suspension was passed through the homogenizer (8.020 mm cylinder with an 8.0186 mm ball). More than 98% of the cells stained with trypan blue after this procedure. By centrifuging at 1000 × *g* for 10 min, cell ghosts (P1) were pelleted, thus separating them from the cytosol and the released vesicles. Membranes in the supernatant were isolated using two different protocols. In the first, they were layered over a 0.4 ml pad of 10% sucrose in buffer B (with inclusions, as above) and then centrifuged at 100,000 × *g* for 1 hr in a Beckman Ti SW50.1 rotor. The membrane pellet was referred to as P2′, and the supernatant (S2′) was cytosol. In the second protocol, the 1000 × *g* supernatant was centrifuged at 8000 × *g* for 35 min in a TiSW50.1 rotor. The pellet (P2) contained large organelles. The supernatant (S2) was layered over a 0.4 ml pad of 10% sucrose in buffer B (with inclusions, as above) and centrifuged at 100,000 × *g* for 1 hr to produce the pellet, P3. S3 was the cytosol.

To determine whether fragments of plasma membrane contaminated released intracellular organelles, we biotinylated cell surface proteins and determined whether amyloid precursor protein (APP), a relatively abundant protein (Haass et al., 1992), was detected in P2 and P3. PC12 cells were harvested, and cell surface proteins were biotinylated, as indicated above, for 20 min at 4°C. Cells were washed three times with 1 mM lysine in PBS and then permeabilized and fractionated. P1, P2, and P3 were lysed in lysis buffer #2 and immunoprecipitated with α-C7, an antibody to the C terminus of APP (Podlisny et al., 1991). Immunoprecipitates were submitted to 7.5% SDS-PAGE and blotted. [^{125}I]streptavidin was used to probe the blots, and the PhosphorImager was used for detection.

Electron microscopy of cell fractionations. PC12 cells treated with NGF, as just described, were permeabilized and fractionated. The P1, P2, and P3 pellets were fixed in 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4, for 90 min at room temperature. Each pellet was washed sequentially in the sodium cacodylate buffer, pH 7.4, and in 50 mM veronyl acetate buffer, pH 7.4, before fixation in 1% osmium tetroxide in the veronyl acetate buffer at 0°C for 45 min. After a final wash in the same veronyl acetate buffer, the pellets were dehydrated and embedded in Epon–Araldite. Thin sections were cut and examined in a Zeiss EM 10CA microscope. Micrographs were taken at ~20,000×. Several areas, each 20.5 μm^2, were chosen for vesicle measurements.

Fractionation of internalized NGF. To quantify [^{125}I]NGF in intracellular organelles, cells incubated with [^{125}I]NGF (1 nM) for 1 hr were washed, warmed at 37°C for 10 min, chilled, permeabilized, and fractionated. Fractions were assayed as above for [^{125}I]NGF. In some cases, we determined the amount of [^{125}I]NGF associated with the cytoskeleton, which was defined as the NP-40-insoluble pellet of P1 (Vale and Shooter, 1985). For these studies, P1 was resuspended in 0.45 ml of PBS, 1 mM EGTA, 1 mM EDTA containing the protease inhibitors listed above, NP-40 (1%) was then added, and the suspension was incubated on ice for 1 hr before centrifuging for 10 min at 10,000 × *g* in a microcentrifuge. In some experiments, acid washing was used to measure surface-bound [^{125}I]NGF (Bernd and Greene, 1984). To examine the distribution of [^{125}I]NGF in intracellular organelles, P2′ was resuspended in 10% sucrose in buffer B (with inclusions) using a 25 G needle and then applied to a 10–40% (w/w) sucrose gradient with a 0.4 ml 50% sucrose pad. The gradient was centrifuged at 100,000 × *g* for 1 hr in a Beckman Ti SW50.1 rotor. Gradient fractions (4 drops each) were collected from the bottom of the tube. Each fraction was surveyed for radiolabeled NGF using the gamma counter.

NGF cross-linking to intracellular TrkA. Cells incubated with [^{125}I]NGF (1 nM) in binding buffer for 1 hr at 4°C were washed and then warmed for 10 min at 37°C before permeabilization, as above. Unlabeled NGF (1 μM) was added to control for nonspecific binding and cross-linking. DSS (2 mM) was added to the permeabilized cells and released membranes, and the mixture was incubated while rotating for 30 min (4°C). The reaction was quenched with lysine (10 mM) for 10 min. P1, P2, and P3 were then prepared, as above. P1 was solubilized by extracting in 1% NP-40 in buffer B for 1 hr (4°C). After centrifuging at 1000 × *g* (10 min), SDS was added to bring the final concentration to 0.5%. P2 and P3 were resuspended in H_2O with 0.5% SDS. S3 was brought to the same SDS concentration. All samples were boiled for 5 min, chilled (4°C), and then brought to a volume of 1 ml and a final concentration of 0.1% SDS by diluting with immunoprecipitation (IP) buffer (20 mM HEPES, pH 7.4, 0.15 M NaCl, 1% NP-40, 0.5% DOC, 1 mM EDTA). To this was added 1088 (17 μg/ml). After incubating at 4°C overnight, one-tenth of the volume of 20% protein A–Sepharose beads (Pierce) was added for 1 hr, with rotation. The beads were washed twice with IP buffer and once with water before resuspending in 7 M urea SDS-PAGE sample buffer with 20 mM DTT. Samples were heated to 65°C for 10 min and run on a 5–12% SDS-PAGE. Dried gels were exposed to XAR film for 1–3 weeks.

Immunoprecipitation and blotting. Immunoprecipitation of cell fractions was performed by dissolving P1, P2, P3, or S3 in 1 ml of lysis buffer #1 with 1 mM Na-orthovanadate. To this was added 12 μg of 1088, 12 μg of RTA, or 5 μg of anti-PLCγ1. The mixture was incubated overnight at 4°C, and one-tenth of the volume of protein A– or protein A/G–Sepharose beads was added for 2 hr at 4°C. Sepharose beads were washed three times in lysis buffer #1 and once with water, then treated with 50 μl of 7 M urea sample buffer and heated (55°C, 15–30 min). Samples were loaded on 7.5% SDS-PAGE. After transferring to nitrocellulose, blots were

probed with anti-phosphotyrosine (4G10), RTA, or anti-PLCγ1. Immune complexes were detected with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG and chemiluminescence (Amersham) or with ^{125}I-labeled goat anti-mouse IgG. Data were quantified from multiple chemiluminescent exposures using National Institutes of Health Image or using a Molecular Dynamics PhosphorImager.

RESULTS

TrkA was internalized in response to NGF

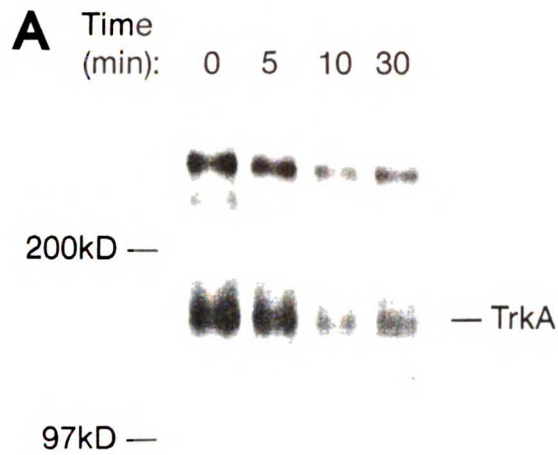
Two studies were used to define how NGF binding regulates trafficking of TrkA receptors. In the first, TrkA at the surface of PC12 cells was marked by cross-linking to radiolabeled NGF using BS^3, a membrane-impermeable cross-linker (Hartman et al., 1992). PC12 cells were incubated with [^{125}I]NGF (1 nM) at 4°C to allow for binding at a temperature that inhibits membrane traffic. The mixture was then warmed at 37°C for 0, 5, 10, or 30 min to permit endocytosis. After chilling (4°C) and cross-linking, immunoprecipitated TrkA was analyzed by SDS-PAGE. Two major radiolabeled cross-linked species were found (Fig. 1*A*). Each was specific for [^{125}I]NGF, because cross-linking done in the presence of excess unlabeled NGF eliminated the bands (data not shown). One band migrated at ~150 kDa, the position expected for a TrkA monomer–NGF monomer complex. A more slowly migrating band represents NGF bound to TrkA in an undefined oligomeric complex. There was a marked decrease in the amount of TrkA that could be cross-linked to NGF after warming. Relative to that present without warming, the level of the 150 kDa complex decreased by 15 ± 4% (SEM; n = 2) at 5 min and by 45 ± 3% (n = 2) at 30 min. Similar decreases were seen in the more slowly migrating species. We reproducibly detected a small increase in surface cross-linking between 10 and 30 min, which may be caused by receptors recycling back to the plasma membrane. These data indicate that NGF caused rapid downregulation of surface TrkA.

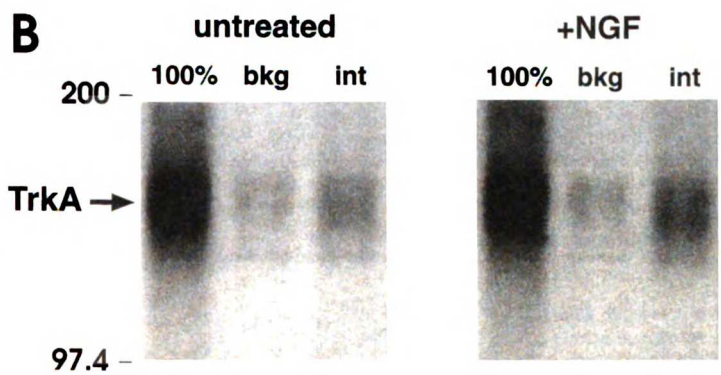
We next determined whether NGF downregulation of surface TrkA was caused by enhanced endocytosis of these receptors. Surface biotinylation has been used to examine trafficking of membrane proteins (Schmid and Carter, 1990). Using this method, constitutive and NGF-induced TrkA internalization were evaluated. Cells were incubated at 4°C either in the presence of NGF (1 nM) or in its absence. Surface proteins were then biotinylated using NHS-SS-biotin. After removing unreacted NHS-SS-biotin, cells were warmed to 37°C for 10 min to allow endocytosis, or not warmed. Samples were then chilled (4°C), and glutathione was added to release biotin on cell surface proteins (Schmid and Carter, 1990). TrkA immunoprecipitated from cell lysates was submitted to SDS-PAGE and blotting; [^{125}I]streptavidin was used to detect biotinylated TrkA. In Figure 1*B*, the lanes labeled (100%) show TrkA labeling under conditions in which there was neither warming nor addition of glutathione. This corresponds to TrkA present at the surface of cells before initiation of membrane traffic. The signals were large for both NGF-treated and untreated cells. The lanes labeled bkg (for background) show the extent to which the biotin label resisted reduction with glutathione when cells were not warmed. The signals were small for both NGF-treated and untreated cells. The TrkA available for internalization was 100% − bkg. The lanes labeled int (for internalized) show the signal when cells were warmed and then treated with glutathione. Whether or not cells were treated with NGF, the amount of labeled TrkA, representing internalized receptors, was increased by warming. In the absence of NGF, 6% of surface TrkA was internalized after 10 min. This finding points to constitutive endocytosis of TrkA receptors. Remarkably, ~37% of TrkA was internalized after 10 min warming with NGF. More than 66% of labeled TrkA was internalized after 20 min warming with NGF (Fig. 1*C*). These data show that NGF induced extensive and rapid internalization of TrkA receptors present at the cell surface.

TrkA was present in intracellular organelles

Internalization of TrkA by NGF suggested that it would be possible to detect TrkA in the endocytic pathway. Immunofluorescence and confocal microscopy were used to localize TrkA. In the first series of experiments, PC12 cells were incubated with or without NGF (2 nM) at 37°C. Cells were then fixed, permeabilized, and incubated with sc11, an antibody to the C terminus of Trk that binds to full-length receptors. TrkA antibody binding was detected using fluoresceinated goat anti-rabbit IgG. The confocal micrographs of cells not exposed to NGF showed that TrkA staining was predominantly intracellular (Fig. 2*A*). Some immunopositivity was found near the nucleus; much was present as small punctate accumulations distributed throughout the cytoplasm. Surprisingly little staining was noted at the cell surface. NGF treatment induced marked changes. At early times, there was dispersion of staining such that the boundaries of cells were better defined (Fig. 2*B–D*). There was also the appearance of densely stained punctate structures, many of which were present near the cell surface. The number of bright punctate organelles located within 0.5 μm of the cell surface was counted. After 30 sec NGF treatment, the number increased threefold from 0.08 ± 0.01 per μm of cell perimeter (n = 12 cells) in untreated cells to 0.21 ± 0.02 per μm (n = 12) in NGF-treated cells (Student's t test, $p <$ 0.05). At later times (Fig. 2*D*), there was marked redistribution of TrkA staining to the perinuclear region. In part, this staining corresponded to TrkA in lysosomes, because a lysosomal marker (GM10) colocalized with some of the TrkA staining (data not shown). The presence of TrkA immunostaining in lysosomes at 60 min suggests that endocytosed TrkA was destined for degradation, a finding consistent with earlier observations (Zhou et al., 1995). Thus, by confocal microscopy, NGF treatment markedly influenced TrkA trafficking.

To characterize further the punctate TrkA-positive organelles produced with NGF treatment, we attempted to colocalize TrkA with markers of the clathrin-coated pit endocytic pathway. Cells were first incubated with or without NGF at 37°C for 30 sec and then chilled and fixed. Clathrin vesicles were visualized with antibodies to the clathrin heavy chain (X22) and to α-adaptin (AP.6). Figure 3 shows that in the absence of NGF there was a small amount of TrkA and clathrin colocalization in an area near the nucleus (Fig. 3*D–F*). The perinuclear colocalization of TrkA and clathrin was probably attributable to the presence of these proteins in the biosynthetic pathway. Only rarely were TrkA and clathrin colocalized near the plasma membrane. In the presence of NGF, an increased number of clathrin and TrkA-positive organelles were located near the cell surface (Fig. 3*A,B*). Significantly, TrkA and clathrin could be colocalized in these organelles (Fig. 3*C,G,H*). Figure 3 (*A–C, G, H*) demonstrates a cell in which TrkA and clathrin colocalization was particularly marked. To quantitate the change with NGF, we determined the number of puncta showing colocalization in randomly selected control and NGF-treated cells. Puncta were counted if they fell within 0.5 μm of the plasma membrane. In control cells, such puncta ranged from 0 to 2 per section; they averaged 0.68 ± 0.10 puncta /section (n = 40 cells). With NGF, the number increased nearly fivefold. It ranged from 1 to 18 per section and averaged 3.25 ± 0.56 puncta/section (n = 31). The difference between NGF-treated and control cells was highly significant ($p <$ 0.001). TrkA could also be colocalized with α-adaptin in NGF-treated cells (data





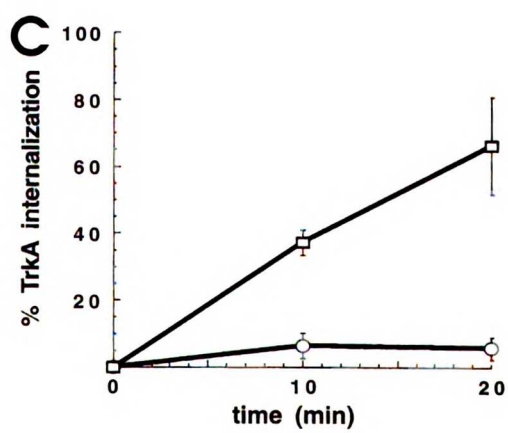


Figure 1. NGF induced trkA internalization. *A*, NGF treatment decreased cross-linking to surface TrkA receptors. PC12 cells, an equal number for each condition tested, were incubated with [^{125}I]NGF (1 nM) at 4°C for 2 hr and then warmed to 37°C for 0, 5, 10, or 30 min. Cells were chilled, and the membrane-impermeant cross-linker BS^3 was added for 30 min at 4°C. Cell lysates were immunoprecipitated with 1088, an anti-Trk C-terminal antibody, before SDS-PAGE. The dried gel was exposed to x-ray film. The positions of molecular weight markers are indicated, as is a band corresponding to a complex containing an [^{125}I]NGF monomer cross-linked to a TrkA monomer. The more slowly migrating band marks a higher-molecular-weight complex containing [^{125}I]NGF and TrkA. There was no [^{125}I]NGF cross-linking in experiments in which unlabeled NGF (1 μM) was present during binding and cross-linking. *B*, Constitutive and NGF-induced internalization of TrkA: internalization of TrkA increased after warming in the presence of NGF (*+NGF*). Cells were incubated with unlabeled NGF (1 nM) for 30 min at 4°C (*untreated*). Control samples were handled identically except that no NGF was present. NHS-SS-biotin was added at 4°C to biotinylate cell surface proteins; cells were then either kept on ice or warmed 10 min at 37°C to allow for endocytosis. Glutathione was added to some samples to remove biotin on cell surface proteins. After samples were lysed and boiled in 0.5% SDS, TrkA was immunoprecipitated with 1088. After SDS-PAGE, proteins were transferred to nitrocellulose and blotted with [^{125}I]streptavidin. TrkA is marked by an *arrow*. Biotinylated TrkA was analyzed without warming and without glutathione (*100%*), without warming but with glutathione (*bkg*), or with warming and with glutathione (*int*). Biotin on TrkA receptors internalized during warming was protected from glutathione reduction. *C*, Quantitation of TrkA internalization. Experiments were performed as in *B*. The signals for biotinylated TrkA were normalized for TrkA protein. The percent internalization of TrkA was computed as described in Materials and Methods. Values are mean ± SEM from three separate experiments at 10 min and two at 20 min (*open circles*, untreated; *open squares*, NGF-treated). There was a low level of constitutive internalization. NGF markedly increased TrkA internalization.

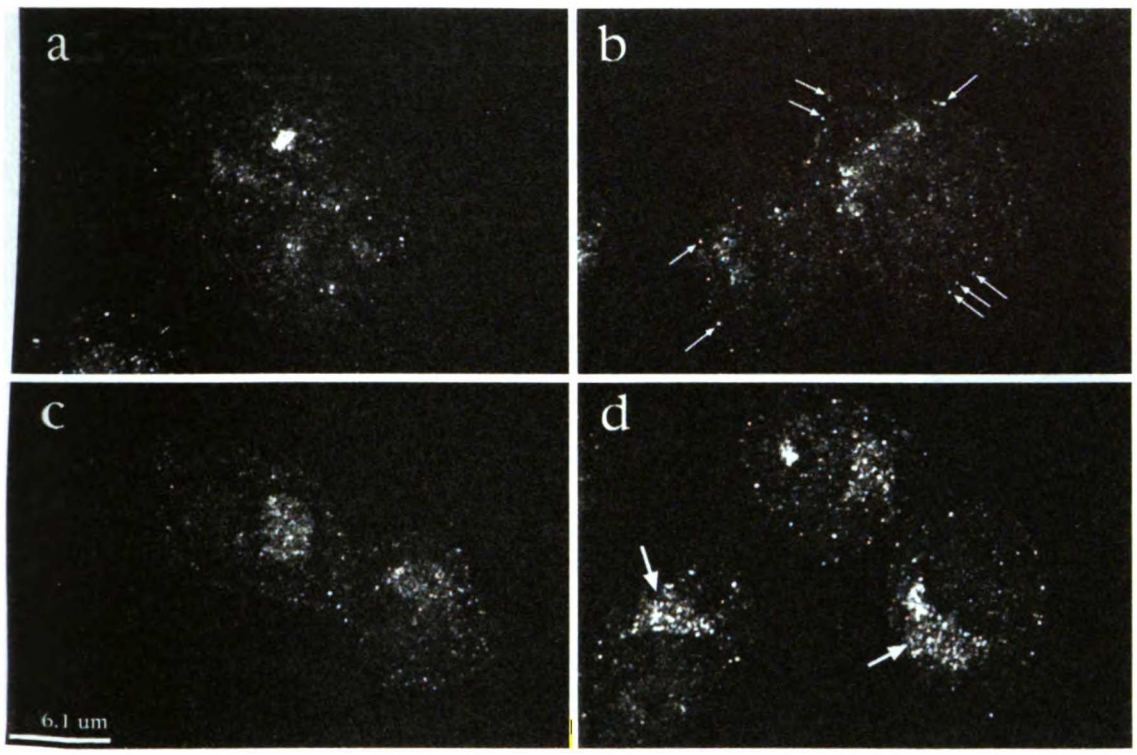


Figure 2. NGF changed the distribution of TrkA immunostaining in PC12 cells. Cells were exposed to media at 37°C without NGF (*a*) or with NGF (2 nM) for 30 sec (*b*), 2 min (*c*), and 60 min (*d*). A Trk-specific antibody, sc11, was used to examine the distribution of TrkA. Most TrkA staining was intracellular. With NGF treatment there was an increase in bright punctate staining near the plasma membrane (e.g., *small arrows* in *b*). Note the marked increase with NGF treatment of TrkA staining in the juxtanuclear region at 60 min (*large arrows* in *d*).

not shown). These data show that TrkA is found in endocytic vesicles produced via the clathrin-coated pit pathway.

Large and small endocytic organelles contained NGF

To characterize further the endocytic organelles that contain TrkA, we first examined those that could be labeled with [^{125}I]NGF. This approach was suggested by studies showing that TrkA expression confers the ability to internalize NGF on mutant PC12 cells that lack TrkA (Loeb et al., 1992). To prepare intracellular organelles, we took advantage of the fact that mechanically permeabilized cells release untethered organelles (Grimes and Kelly, 1992a,b). PC12 cells were permeabilized by passage through a Balch homogenizer (Balch and Rothman, 1985; Martin and Walent, 1989), and released membranes were harvested. PC12 cells incubated with NGF (1 nM) at 4°C for 1 hr were washed briefly to enrich for binding to slowly dissociating (i.e., high-affinity) receptors (Zhou et al., 1995) and to minimize fluid-phase endocytosis of free ligand. Cells were then warmed for 10 min, chilled (4°C), and permeabilized. Cytosol and released organelles were separated from the cell ghosts by centrifugation at 1000 × *g* (10 min). Organelles were then separated from cytosol using one of two alternative fractionation strategies (Fig. 4).

Electron microscopic examination of PC12 cells before and after permeabilization revealed several alterations (Fig. 5*A*,*B*). The cytoplasm of intact cells was typically dense with a normal complement of subcellular organelles, including ribosomes, endoplasmic reticulum, and mitochondria. The plasma membrane and nuclear envelope were intact. Although many of the same organelles could be identified in the permeabilized cells, they were less frequent and were dispersed in a less electron-dense cytoplasm. More significantly, the plasma membrane of permeabilized cells was interrupted (*arrow*). In the P2 fraction, the organelles were heterogeneous (Fig. 5*C*). P2 contained mitochondria, dense bodies, ribosomes, and many clear uncoated vesicles with a mean diameter of 180 ± 71 nm (SD; n = 57). In contrast, the P3 fraction contained a more homogeneous population of organelles (Fig. 5*D*). Most of the organelles were small vesicles of one of three types. Small, uncoated vesicles with a dense core were the most frequent; they were 86 ± 15 nm in diameter on average (n = 56). A second class consisted of clear, coated vesicles that were 63 ± 15 nm in diameter on average (n = 22). Infrequently, we found clear, uncoated vesicles that were variable in diameter (mean 94 ± 24 nm; n = 8) and small mitochondria and dense bodies.

The electron microscopy studies suggested that this method of very gentle homogenization tears the plasma membrane and allows some intracellular organelles to leak out of the cells. Two additional observations showed that intracellular organelles could be separated from plasma membrane using this fractionation scheme. First, we carried out experiments at 4°C in which cells

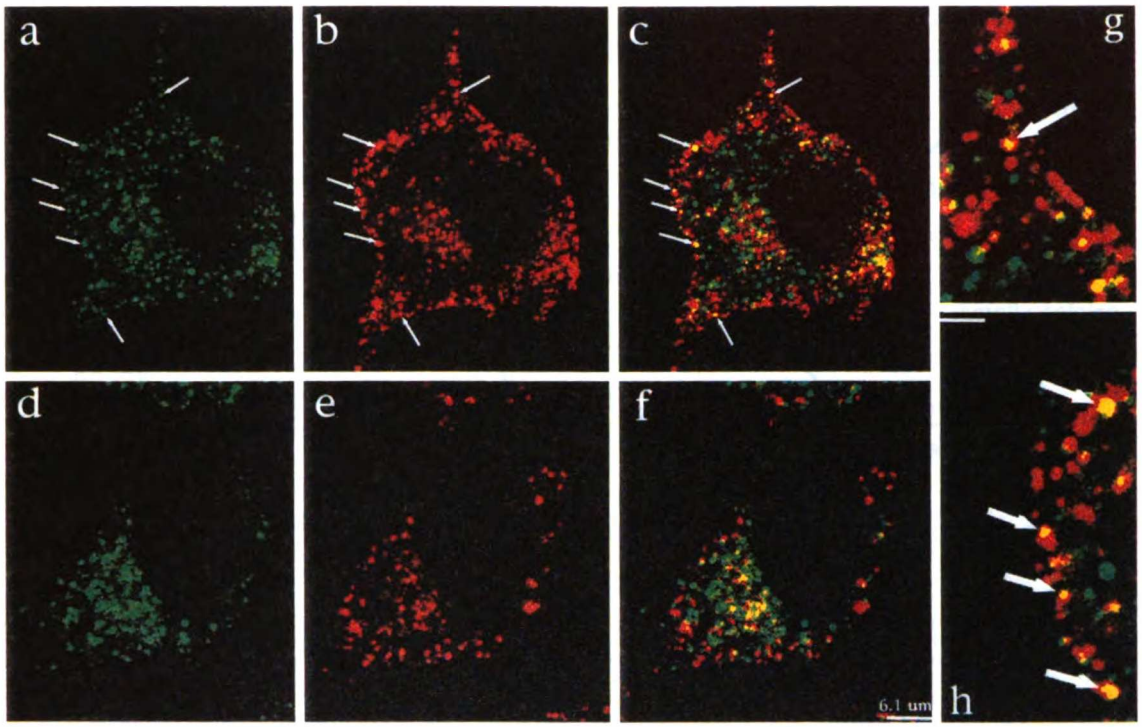


Figure 3. NGF treatment resulted in TrkA and clathrin colocalization. PC12 cells were treated with NGF for 30 sec (*A–C, G, H*) or with vehicle alone for the same interval (*D–F*). After fixation and permeabilization, cells were immunostained using antibodies to Trk (sc11) and the clathrin heavy chain (X22). TrkA staining is shown in *green*; clathrin heavy chain staining is in *red*. In the absence of NGF, TrkA (*D*) and clathrin (*E*) staining is present diffusely in the cytosol and in the juxtanuclear region. There is little overlap in their distribution (*F*) except in the juxtanuclear region. In the presence of NGF, TrkA (*A*) and clathrin (*B*) staining is widely distributed in the cytosol; some TrkA staining is seen near the plasma membrane. Clathrin staining appears to be concentrated at the plasma membrane. *C* (at lower power) and *G* and *H* (at higher power; scale bar, 2 μm) show colocalization of TrkA and clathrin staining near the plasma membrane (*arrows*; *yellow* denotes colocalization). The organelles showing colocalization had the same distribution and size as those seen with increased frequency after NGF treatment (Fig. 2).

were incubated with [^{125}I]NGF and washed, and the cross-linker BS^3 was added before permeabilization and fractionation. Under these conditions, the NGF–TrkA complex was not detected in either P2 or P3. Second, we asked whether surface APP could be detected in P2 or P3. At 4°C, APP was biotinylated at the cell surface and cells were then permeabilized and fractionated. Surface-labeled APP in P2 was 2.9% (n = 2) of that in P1; the corresponding value for P3 was 0.6% (n = 2) and for S3 was 0.1% (n = 2). Thus, although many intracellular organelles remained with the cell ghosts (Fig. 5*B*), those that emerged were virtually free of plasma membrane.

We quantified the amount of internalized [^{125}I]NGF that was released and recovered in membrane fractions. When cells were washed and then warmed 10 min at 37°C, 6.9 ± 0.6% (SEM; n = 9) of the total counts were recovered in P2 and 3.1 ± 0.4% (n = 9) were present in P3 (Student's t test, $p < 0.05$). Interestingly, a substantial fraction (32 ± 4%, n = 9) of the labeled NGF that remained associated with the cell ghosts was in the detergent-insoluble fraction, which was shown to be the cytoskeleton (Vale et al., 1985). Acid washing experiments (Bernd and Greene, 1984) indicated that 49.8 ± 4.9% (n = 3) of the total bound [^{125}I]NGF was on the cell surface under these conditions. This means that P2 + P3 together contained about one-fifth (20%) of total intracellular [^{125}I]NGF and about half of that which was not bound to the cytoskeleton. These studies show that the fractionation scheme used allowed us to recover a substantial fraction of intracellular organelles that contain internalized NGF.

Sucrose velocity gradients were used to analyze the intracellular organelles that emerged from permeabilized cells (Fig. 6). In this case, all organelles were concentrated and applied to the gradients (*P2′*, see Fig. 4). [^{125}I]NGF was found in organelles that migrated near the bottom of the gradient (fractions 2–9) as well as in lighter vesicles that were heterogeneous. Thus, NGF was present in both large vesicle- and small vesicle-containing fractions.

TrkA in intracellular organelles was bound to NGF and tyrosine-phosphorylated

Our data indicated that after binding, both NGF and TrkA were internalized. We next asked whether Trk was bound to NGF in intracellular organelles. Cells were incubated with [^{125}I]NGF (1 nM) at 4°C, washed, warmed 10 min as above, then chilled. After permeabilization, DSS, a membrane-permeable cross-linking reagent that has been used to cross-link NGF to TrkA (Radeke and Feinstein, 1991), was added to the cell suspension before fractionation. TrkA was immunoprecipitated from cell fraction lysates before SDS-PAGE and autoradiography. A radiolabeled band

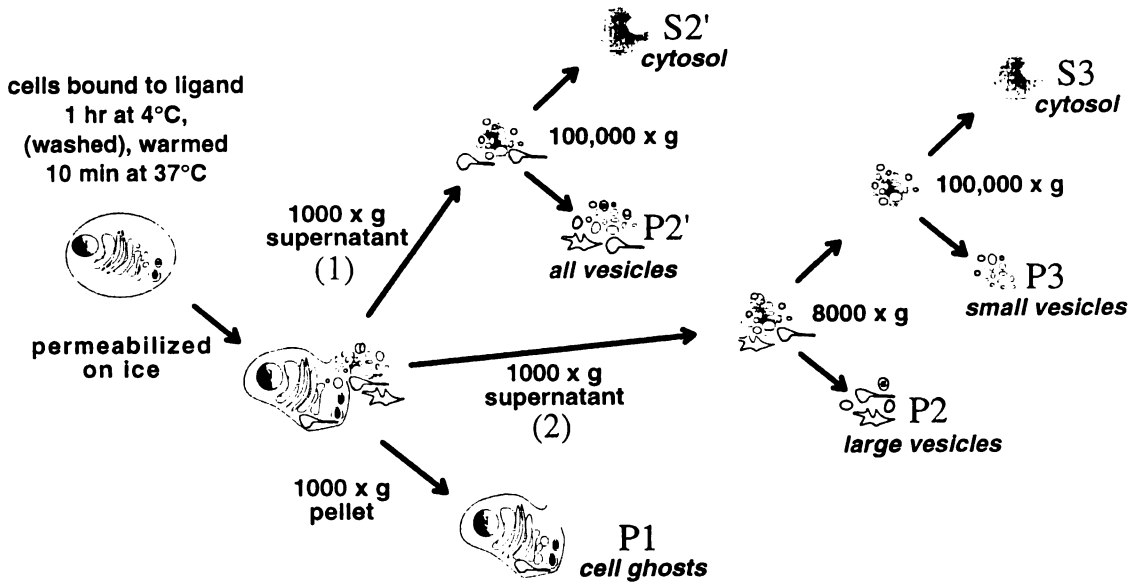


Figure 4. Strategy for cell fractionation experiments. NGF (1 nM) was bound to PC12 cells at 4°C for 1 hr. Cells were then either briefly washed in binding buffer at 4°C or not washed, and warmed at 37°C for 10 min. Cells were then chilled (4°C), washed, resuspended in a cytoplasm-like buffer, and permeabilized by passage through a ball homogenizer. The cell ghosts (*P1*) were separated from cytosol and organelles released from the cells by centrifugation at 1000 × *g*. Two alternative strategies were used to fractionate the membranes in the supernatant of the 1000 × *g* centrifugation. (1) They were layered over a 0.4 ml pad of 10% buffered sucrose and centrifuged at 100,000 × *g* (1 hr), forming a pellet (*P2'*) and the cytosol (*S2'*). (2) To separate large and small vesicles, membranes were centrifuged at 8000 × *g* for 35 min to pellet large vesicles (*P2*). The supernatant of the 8000 × *g* spin was then layered over a 0.4 ml pad of 10% buffered sucrose and centrifuged at 100,000 × *g* for 1 hr, which separated small vesicles (*P3*) from cytosol (*S3*).

that migrated at the position expected for a complex containing [^{125}I]NGF cross-linked to TrkA was seen in the membrane fractions (*P1*, *P2*, and *P3*) but not the cytosol (*S3*, Fig. 7). P2 contained 8 ± 2% (n = 3) of the total TrkA cross-linked to [^{125}I]NGF and P3 contained 10 ± 2% (n = 3; $p > 0.05$). These data roughly correlate with the amount of [^{125}I]NGF in these fractions in the experiments above. However, more [^{125}I]NGF was found in the P2. Either a smaller fraction of NGF was bound to TrkA in P2, or the cross-linking efficiency was lower in this fraction (or both). NGF cross-linked to TrkA was also seen when the cross-linker was added after fractionation (data not shown). These data are evidence that (1) Trk and NGF were present together in the same compartments after internalization and (2) NGF remained bound to TrkA after endocytosis.

We next sought to determine whether organelles derived from endocytosis contained activated TrkA. Cells were warmed for 10 min in the presence or absence of bound NGF and then submitted to fractionation. The phosphatase inhibitor sodium orthovanadate was added to the cell suspension during permeabilization at 4°C. The presence of TrkA was assessed by immunoprecipitation, followed by Western blotting, with RTA (Clary et al., 1994). Two proteins were identified, gp140^{TrkA} and gp110^{TrkA} (Fig. 8, *top*). The latter is a high-mannose precursor to the mature form, gp140^{TrkA}, which acts as a cell surface receptor (Martin-Zanca et al., 1989; Zhou et al., 1995). Neither form of TrkA was detected in the 100,000 × *g* supernatant (*S3*, Fig. 8). The amount of gp140^{TrkA} in P2 and P3 fractions together increased from 12% of the total in control cells to 16% after NGF treatment. The increase in gp140^{TrkA} was mostly in the P2 fraction (Fig. 9*A*). The amount of gp110^{TrkA} was ~20% of total, without or with NGF (Figs. 8, 9*A*). The presence of gp110^{TrkA} suggests that up to one-fifth of the endoplasmic reticulum, or vesicles derived from it, emerge upon permeabilization.

Other vesicles that emerge from permeabilized cells include regulated and constitutive secretory vesicles (Grimes and Kelly, 1992a,b). Newly synthesized TrkA should be present in these. Receptors in the biosynthetic pathway have no direct access to NGF, and it can be assumed that they have not been activated. The presence of phosphotyrosine on TrkA is a measure of its activation (Kaplan et al., 1991; Klein et al., 1991). Thus, tyrosine-phosphorylated TrkA should comprise plasma membrane and internalized TrkA, but not that in the biosynthetic pathway. Activated TrkA was detected in P2 and P3 fractions from NGF-treated cells by blotting TrkA immunoprecipitates with an anti-phosphotyrosine antibody (Fig. 8, *bottom*). Total gp140^{TrkA} was greater in P3 than P2; the reverse was true for tyrosine-phosphorylated TrkA (Figs. 8, 9). Fractions prepared from cells not exposed to NGF contained very little tyrosine-phosphorylated TrkA (Fig. 9*B*). In contrast, in the NGF-treated cells there was an ~17-fold increase overall in tyrosine-phosphorylated TrkA. Seventeen percent of tyrosine-phosphorylated TrkA was recovered in the P2 and P3 fractions (Fig. 9*B*). Comparing these data to those from Figure 1*C*, it appears that almost half of internalized TrkA was recovered in organelles that emerged from permeabilized cells. The data for TrkA and tyrosine-phosphorylated TrkA were used to calculate a "specific activity" for the receptor: the ratio of tyrosine-phosphorylated TrkA to total TrkA (Fig. 9*C*). The spe-

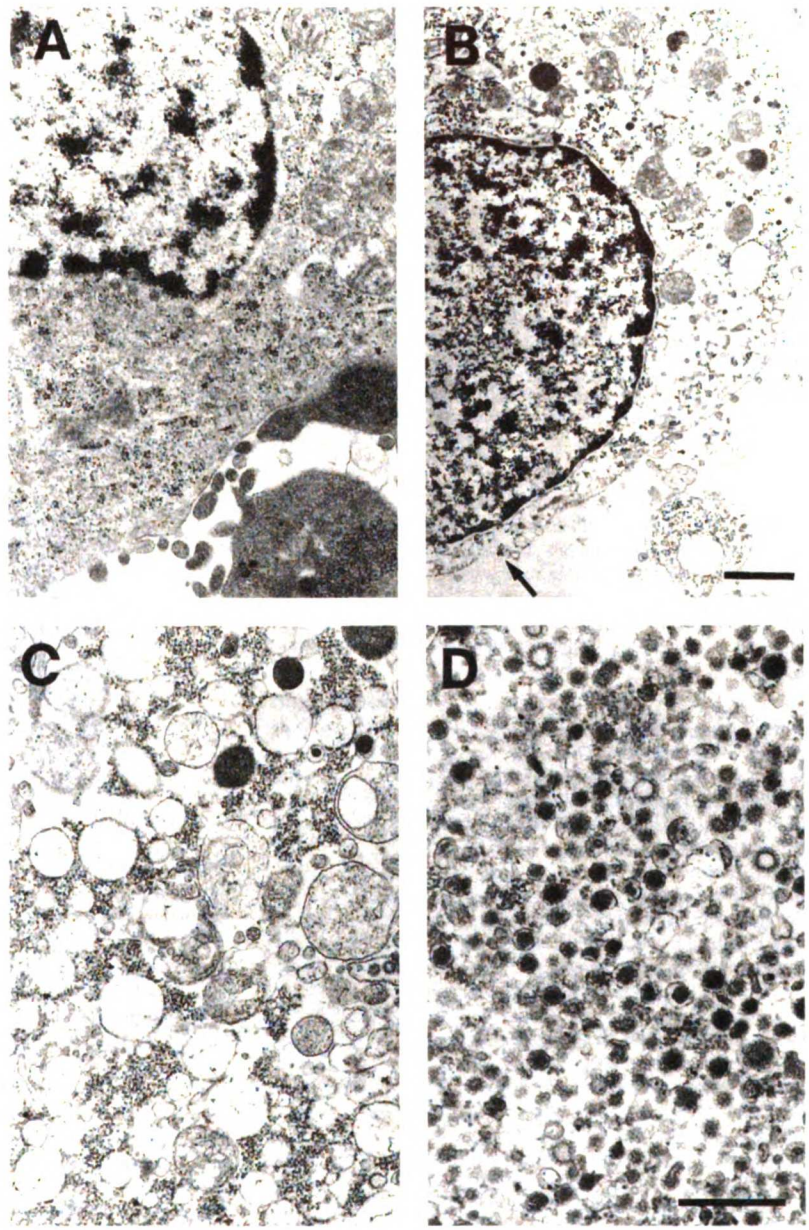


Figure 5. Electron micrographs of PC12 cells and vesicles released from these cells. Cell fractions were processed as indicated in Materials and Methods. *A*, A cell not permeabilized. *B*, P1: a cell after permeabilization. Note the marked decrease in the electron density of the cytoplasm. Numerous discontinuities were seen in the plasma membrane (*arrow*). *C*, Organelles in the P2 fraction. *D*, Organelles in the P3 fraction. Scale bars: *A*, *B*, 0.5 μm; *C*, *D*, 0.4 μm.

cific activity was about the same in intracellular organelles as in the cell ghost fraction.

TrkA in intracellular organelles was bound to PLC-γ1

Another measure of TrkA activation, one that contributes to TrkA signaling leading to differentiation, is binding of PLC-γ1 (Stephens et al., 1994). To determine whether PLC-γ1 was bound to internalized TrkA receptors, we examined immunoprecipitates from cells that were incubated with NGF, or left untreated, at 4°C and then warmed for 10 min at 37°C before chilling (4°C). After permeabilization, cells were fractionated into P1, P2′, and S2′. For these experiments, lysates were immunoprecipitated with antibodies to PLC-γ1 (Fig. 10, *lanes 1, 2, 5, 6, 9,* and *10*), with anti-Trk (1088; *lanes 4* and *8*), or with anti-Trk followed by anti-PLC-γ1 (*lanes 3* and *7*). In P1, P2′, and S2′, there was a marked increase in tyrosine-phosphorylated PLC-γ1 with NGF

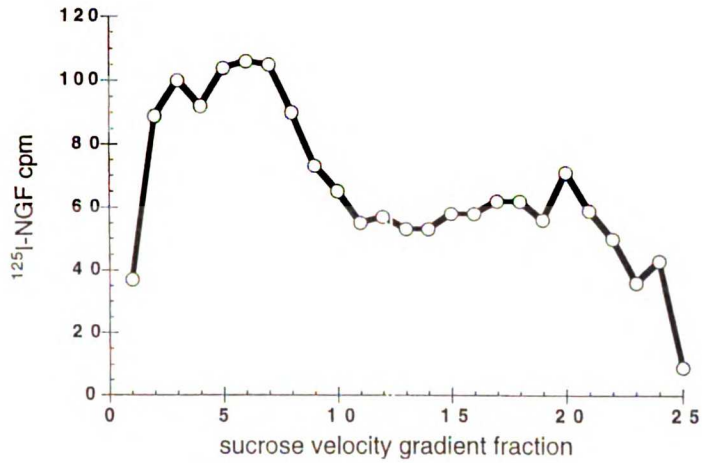


Figure 6. Sucrose gradient fractionation of internalized NGF. Cells incubated with [^{125}I]NGF (1 nM) for 1 hr at 4°C were washed, warmed 10 min, chilled (4°C), and then permeabilized. P2′ was applied to 10–40% sucrose gradients over a 50% sucrose pad and centrifuged at 100,000 × *g* for 1 hr. Gradient fractions were collected from the bottom of the tube. [^{125}I]NGF was quantified in each fraction. Data are representative of two experiments.

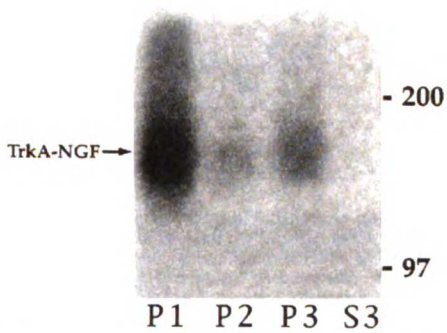


Figure 7. TrkA was cross-linked to NGF in intracellular organelles. Cells were incubated with [^{125}I]NGF (1 nM), washed, warmed 10 min (37°C), chilled (4°C), and then permeabilized and fractionated as in Figure 4. The membrane-permeable cross-linking reagent DSS was added before fractionation. One-fifth of the cell ghost membranes (*P1*), the entire 8000 × *g* pellet (*P2*), the entire 100,000 × *g* pellet (*P3*), and one-tenth of the 100,000 × *g* supernatant (*S3*) were immunoprecipitated with 1088 and analyzed by SDS-PAGE and autoradiography. The *arrow* marks the cross-linked complex containing TrkA and [^{125}I]NGF in P1, P2, and P3. There was no cross-linking when [^{125}I]NGF binding was carried out in the presence of unlabeled NGF (1 μM). The amount of [^{125}I]NGF cross-linked to TrkA was quantified by PhosphorImager.

treatment (*A*, *lane 1* vs *2*, *5* vs *6*, and *9* vs *10*). In the membrane fraction, there was a corresponding band for PLC-γ1 protein, and the amount of this was slightly increased with NGF treatment (*B*, *lane 1* vs *2* and *5* vs *6*). In the P1 and P2′ fractions, there was an additional tyrosine-phosphorylated band that migrated below PLC-γ1, whose amount was increased by NGF treatment. Evidence that this band corresponded to tyrosine-phosphorylated TrkA is as follows. (1) Tyrosine-phosphorylated TrkA was present in these fractions after NGF treatment [Figs. 8, 10*A* (*lanes 4* and *8*)]. (2) The tyrosine-phosphorylated band below PLC-γ1 migrated at the position expected for TrkA (Fig. 10*A*, *lanes 2* and *6*). (3) When cells were treated with NGF, TrkA was present in the PLC-γ1 immunoprecipitates formed with antibodies to PLC-γ1 (Fig. 10*C*, *lane 1* vs *2* and *5* vs *6*). (4) When lysates were first cleared by immunoprecipitating with anti-Trk, in the subsequent PLC-γ1 immunoprecipitate there was a marked decrease in the amount of tyrosine-phosphorylated PLC-γ1 (Fig. 10*A*, *lane 2* vs *3* and *6* vs *7*). In the P2′ fraction, the amount of PLC-γ1 was also decreased after preclearing with anti-Trk (Fig. 10*B*, *lane 6* vs *7*). These data show that TrkA in intracellular organelles is associated with PLC-γ1 and are further evidence for the ability of activated TrkA in intracellular vesicles to signal.

DISCUSSION

NGF signaling must be communicated from the target of responsive neurons to their cell bodies. Our studies were aimed at exploring the hypothesis that endocytosed activated TrkA receptors serve as the retrograde message (Grimes et al., 1993). We discovered that (1) NGF induced rapid internalization of TrkA in PC12 cells, (2) NGF and TrkA were both found in intracellular vesicles, (3) NGF was bound to TrkA in these vesicles, and (4) TrkA receptors in vesicles were activated, as assessed by tyrosine phosphorylation and association with PLC-γ1. Our findings raise the possibility that it is through the creation of signaling endosomes containing activated TrkA that NGF signals retrogradely to regulate neuronal survival and differentiation.

Target-derived NGF is critical for the normal survival and differentiation of several populations of PNS and CNS neurons (Levi-Montalcini, 1987; Longo et al., 1993; Crowley et al., 1994; Li et al., 1995). Given that NGF gene expression is localized to target tissues (Longo et al., 1993), a mechanism must exist to carry the NGF signal retrogradely from the processes of neurons to their cell bodies. Studies characterizing retrograde NGF signaling showed that although NGF and the signal were similar with respect to both the time course for retrograde transport and the requirement for microtubules (Hendry et al., 1974b; Paravicini et al., 1975; Hendry and Bonyhady, 1980), NGF itself was not the signal (Heumann et al., 1984). One possibility for the NGF signal is an intracellular signaling intermediate that is distinct from the receptor. Another is an activated NGF receptor or NGF–NGF receptor complex that continues to signal after endocytosis. Whether $p75^{NTR}$ signals, and if so, whether it could serve as a retrograde signal for NGF, is an active area of investigation (Bothwell, 1996; Carter et al., 1996). Significantly, $p75^{NTR}$ is retrogradely transported in NGF-responsive CNS and PNS neurons (Taniuchi and Johnson, 1985; Johnson et al., 1987; Raivich et

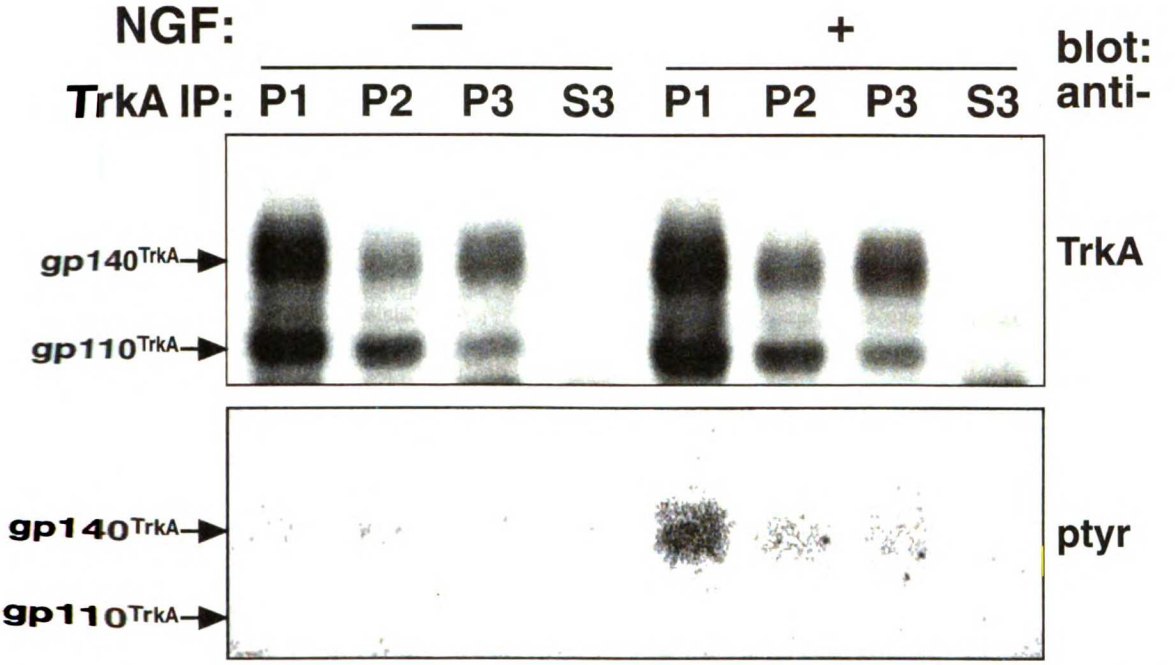


Figure 8. TrkA and tyrosine-phosphorylated TrkA were detected in intracellular organelles. *Top*, PC12 cells incubated with or without NGF (1 nM) at 4°C were washed, warmed 10 min (37°C), chilled (4°C), permeabilized, and fractionated as in Figure 4. Equal amounts of cells were used to compare conditions. TrkA was immunoprecipitated with RTA from one-fifth of *P1*, one-half of *P2*, one-half of *P3*, and one-tenth of *S3*. Shown is a Western blot of immunoprecipitates probed with RTA followed by HRP-conjugated anti-rabbit IgG. Chemiluminescence was used for detection. The bands for $gp140^{TrkA}$ and $gp110^{TrkA}$ are noted. *Bottom*, TrkA immunoprecipitates (as above) were Western-blotted and probed with anti-phosphotyrosine antibody (4G10) followed by ^{125}I-labeled goat anti-mouse IgG. Data were taken directly from the PhosphorImager. The position of the tyrosine-phosphorylated 140 kDa band comigrated exactly with TrkA. Tyrosine-phosphorylated TrkA was present in *P1*, *P2*, and *P3* in NGF-treated cells.

al., 1991; Kiss et al., 1993). However, there is no evidence that NGF induces p75 internalization and endocytosis. Indeed, in earlier studies using PC12 cells, there was little change in the amount of $p75^{NTR}$ at the surface of cells incubated with NGF for up to 5 hr at 37°C (Hosang and Shooter, 1987). Furthermore, Curtis et al. (1995) have shown recently that disrupting $p75^{NTR}$ function had little effect on the retrograde transport of NGF in sensory and sympathetic neurons. Thus, current data provide little support that the NGF retrograde signal is carried by $p75^{NTR}$.

Internalized, activated TrkA is an attractive candidate for the NGF retrograde signal. Ehlers et al. (1995) have shown recently that NGF induced an increased accumulation of tyrosine-phosphorylated TrkA distal to a ligature on the sciatic nerve. The studies reported herein suggest that NGF-mediated induction of rapid, extensive endocytosis of TrkA in the distal processes of DRG neurons was responsible for increased retrograde transport of activated TrkA. Using two different methods to assess the disposition of surface receptors on PC12 cells, the internalization of TrkA was increased significantly after NGF addition; indeed, surface-biotinylated TrkA was decreased by >60% after 20 min. These data are consistent with earlier studies showing that NGF downregulated surface TrkA receptors (Hosang and Shooter, 1987; Zhou et al., 1995) and extends them by demonstrating that endocytosed receptors are intact, at least at the treatment times assayed. These biochemical observations were complemented by confocal microscopy studies that showed an increase in TrkA in bright, punctate structures near the cell surface after NGF addition. These organelles were evident soon after NGF treatment and persisted through 60 min. Using Sc11, an antibody to the C terminus of TrkA, there was comparatively little TrkA staining at the surface of cells. However, we know that TrkA is present at the cell surface because of our biotinylation and cross-linking studies (Zhou et al., 1995). Also, we were able to stain the surface of live cells with RTA, a TrkA extracellular domain antibody (Clary et al., 1994) (D. Hall and W. Mobley, unpublished observations), which suggests that certain epitopes are more easily detected than others at the cell surface. In some TrkA-positive organelles, staining with antibodies to TrkA colocalized with staining for the clathrin heavy chain and for α-adaptin. Colocalization of TrkA with these markers indicates that TrkA internalization is mediated, at least in part, through clathrin-coated pit-mediated endocytosis. Many of the TrkA-positive organelles of the same size and distribution that failed to stain with antibodies to clathrin and α-adaptin may also have been derived from this pathway. Taken together, our findings suggest that activation of TrkA enhances recruitment of the receptor into clathrin-coated pits. In this respect, TrkA may behave as do other receptor tyrosine kinases (Lamaze and Schmid, 1995).

Surface downregulation targets other receptor tyrosine kinases to lysosomes and is believed to serve an important role in regu-

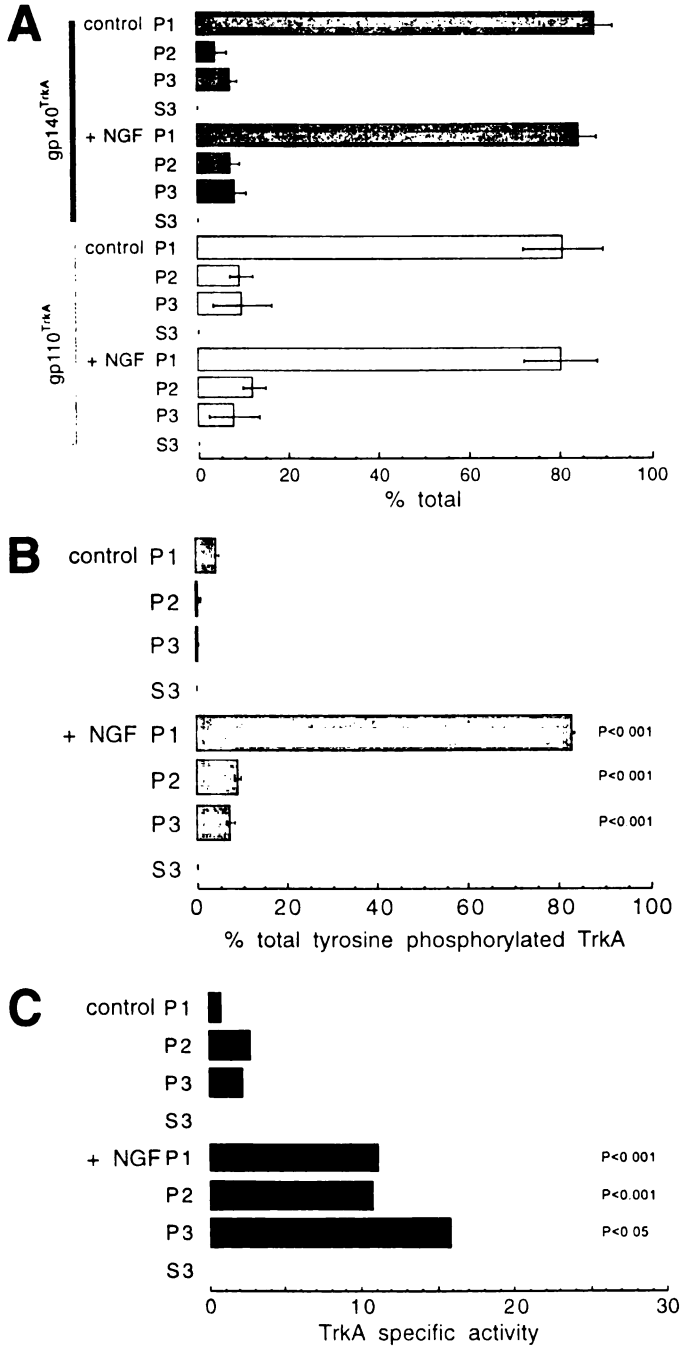


Figure 9. The "specific activity" of TrkA tyrosine phosphorylation increased after NGF treatment. *A*, Data for $gp140^{TrkA}$ and $gp110^{TrkA}$ from three experiments as in the top of Figure 8 were quantified by densitometry and plotted with error bars (±SEM). The proteins, conditions, and fractions are labeled at the *left*. *B*, Data for tyrosine-phosphorylated TrkA from three experiments as in the bottom of Figure 8 were quantified by PhosphorImaging or densitometry and plotted as in *A*. The values are reported as a percentage of total tyrosine-phosphorylated TrkA in NGF-treated cells. *C*, The specific activity of TrkA tyrosine phosphorylation was the ratio of the amount of tyrosine-phosphorylated TrkA to the amount of $gp140^{TrkA}$, plotted in arbitrary units. The average specific activity was calculated using data from four individual experiments. Differences between the *P2* and *P3* fractions within a treatment group were not significant. When comparing fractions from control and NGF-treated cells (for example, P1-control vs P1-NGF-treated), significant differences, calculated using Student's *t* test, are indicated by the probability value (*P*).

lating signaling (van der Geer et al., 1994). There was evidence in our studies that endocytosed TrkA was also targeted to lysosomes. In confocal microscopy, we noted marked redistribution of TrkA to the juxtanuclear region in NGF-treated cells. By 60 min, staining for TrkA at this site was quite intense. Using confocal microscopy, some juxtanuclear TrkA staining was colocalized with GM10, a lysosomal marker. This finding suggests that these TrkA molecules were destined for degradation, a view consistent with

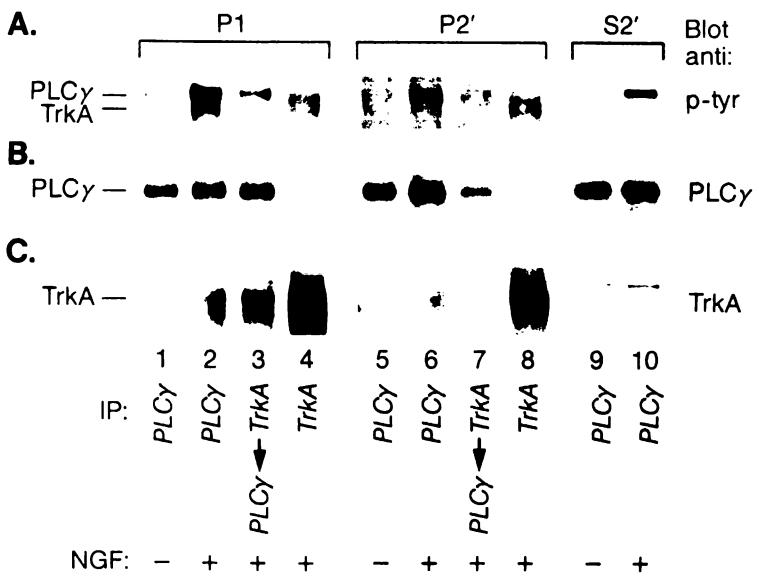


Figure 10. PLC-γ1 was bound to TrkA in intracellular organelles. PC12 cells incubated at 4°C for 1 hr with NGF (1 nM; *lanes 2–4, 6–8, 10*) or without NGF (*lanes 1, 5, 9*) were warmed 10 min at 37°C. Cells were chilled (4°C), permeabilized, and fractionated as in Figure 4. *P1*, *P2'*, and *S2'* were lysed and immunoprecipitated with anti-PLC-γ-1 (*lanes 1, 2, 5, 6, 9, 10*) or anti-TrkA (1088; *lanes 4, 8*). In *lanes 3* and 7, TrkA-immunoprecipitated lysates were subsequently immunoprecipitated with anti-PLC-γ1. Immunoprecipitates were Western-blotted with anti-phosphotyrosine antibody (4G10; *A*) and then stripped and reprobed with anti-PLC-γ1 (*B*) and, finally, with anti-TrkA (RTA; *C*). Chemiluminescence was used for detection: *P1* and *S2'*, 1 min exposure; *P2'*, 2 hr exposure. The positions for PLC-γ1 and TrkA are indicated. NGF treatment resulted in association of tyrosine-phosphorylated TrkA and tyrosine-phosphorylated PLC-γ1 in *P1* and *P2'* (see text). Although anti-PLC-γ1 brought down TrkA in *P1* and *P2'* after NGF treatment (*A*, *C*, *lanes 2, 6*), PLC-γ1 was not reproducibly found on blots of TrkA immunoprecipitates (*lanes 4, 8*, *A*, *B*). Because TrkA immunoprecipitation clearly brought down PLC-γ1 in both *P1* and *P2'*, the complexes present in the TrkA immunoprecipitates may have been unstable.

earlier studies in which NGF treatment for 60 min markedly decreased total cellular TrkA levels (Zhou et al., 1995). The significance of the perinuclear TrkA staining not present in lysosomes is uncertain. It is likely these receptors are also destined for degradation. However, earlier studies suggest an additional possibility. Using EM, Schwab (1977) detected an NGF–HRP conjugate in nonlysosomal smooth vesicles in the perinuclear cytoplasm of adrenergic neurons. Bernd and Greene (1983), using EM autoradiography to detect labeled NGF, found grains well above background at the nuclear membrane. It is possible, although not proven, that the TrkA-immunopositive organelles present in the juxtanuclear region also contained NGF. If so, they would be ideally positioned to initiate NGF signaling leading to changes in gene expression.

It is possible that activated TrkA receptors internalized at the tips of axons would be targeted to lysosomes. However, if activated TrkA is the retrograde NGF signal, it must avoid degradation in the axon. Current evidence suggests that it would. Studies on the endosomal–lysosomal pathway in neurons (Parton et al., 1992; Hollenbeck, 1993; Parton and Dotti, 1993; Nixon and Cataldo, 1995) suggest that late endosomes and lysosomes are located predominantly in the cell body and proximal dendrites. Indeed, there was no evidence for these organelles in the axons or presynaptic terminals of cultured hippocampal neurons (Parton et al., 1992). Further evidence to suggest that degradative activity in axons is limited is that a minority of endocytic organelles in axons are acidic, and those that are present have an average pH of 5.4, a value consistent with that for late endosomes (Overly et al., 1995). These observations suggest that endocytosed NGF and TrkA would remain intact during retrograde transport. Retrogradely transported NGF was shown to be intact in one study (Claude et al., 1982), and in another, using EM, an NGF–HRP conjugate was found in multivesicular bodies in cell bodies and dendrites but not in axons (Schwab, 1977). Moreover, TrkA retrogradely transported in the sciatic nerve was apparently intact (Ehlers et al., 1995). These data suggest that NGF, and the TrkA receptors endocytosed in response to NGF, resist degradation during retrograde transport.

Cell fractionation studies were used to characterize the organelles that contained NGF and TrkA. A very gentle method of homogenization was chosen in order to avoid contaminating intracellular organelles with bits of plasma membrane (Martin and Walent, 1989; Grimes and Kelly, 1992b). Based on data for the kinetics of NGF and TrkA internalization and degradation in PC12 cells (Bernd and Greene, 1983, 1984; Layer and Shooter, 1983; Hosang and Shooter, 1987; Eveleth and Bradshaw, 1988; Zhou et al., 1995), we examined organelles produced after a brief period of internalization (10 min) so as to restrict our attention to primary endocytic vesicles, endosomes, and the vesicles derived from them. NGF and TrkA were both present in the large and small vesicle fractions. Indeed, using a membrane-permeable cross-linking reagent, we found that NGF was bound to TrkA in these fractions. Given the need for receptor dimerization to induce TrkA activation (Jing et al., 1992), it is possible that persistent NGF binding to TrkA may be required to maintain TrkA kinase activation. In recent studies using 3T3 cells expressing TrkA, we have found that most NGF bound to surface receptors at pH 7.4 remains bound at pH 5.5 (J. Zhou and W. Mobley, unpublished observations), i.e., near the average pH for acidified endocytic organelles in axons (Overly et al., 1995). Thus, some NGF could remain bound to TrkA during retrograde transport. It will be important to define further the organelles that contain NGF and TrkA, those in which NGF is bound to TrkA, and those that carry activated TrkA retrogradely. In earlier studies of NGF retrograde transport in axons, NGF was found in smooth-walled tubules (45–60 nm) and in clear vesicles ranging in diameter from 50 to 150 nm (Schwab, 1977; Claude et al., 1982). Recent data (M. Grimes, E. Beattie, and W. Mobley, unpublished observations) in which *in vitro* reactions were used to characterize organelles that emerged from permeabilized cells suggest that P2

contains clathrin-coated vesicles and that P3 contains uncoated primary endocytic vesicles as well as transport vesicles (Grimes and Kelly, 1992b). We do not know which organelle(s) carries the NGF signal, but small transport vesicles are an attractive possibility (Grimes et al., 1993).

NGF binding to TrkA in intracellular vesicles suggested that activation of TrkA receptors persisted after endocytosis. Evidence for this was the presence of tyrosine-phosphorylated TrkA in both P2 and P3 after NGF treatment. NGF markedly increased the specific activity of tyrosine-phosphorylated TrkA in all cell fractions. TrkA signaling is communicated through the activation of signaling intermediates, including PLC-γ1. Immunoprecipitation of TrkA in intracellular organelles of NGF-treated cells showed that activated TrkA formed a complex with tyrosine-phosphorylated PLC-γ1. In preliminary studies, we have shown that tyrosine-phosphorylated SHC is also associated with activated TrkA in intracellular organelles (J. Zhou and W. Mobley, unpublished observations). These data link activated intracellular TrkA to important signaling cascades (Stephens et al., 1994) and thereby suggest strongly that internalized activated TrkA receptors are capable of signaling. Together with the data of Ehlers et al. (1995), our observations support the hypothesis that through endocytosis of activated TrkA, NGF creates signaling endosomes that convey its retrograde signal. To test this idea, studies must be done to determine whether activated TrkA in endocytic vesicles can initiate NGF signal transduction in the neuron cell body.

REFERENCES

Balch WE, Rothman JE (1985) Characterization of protein transport between successive compartments of the Golgi apparatus: asymmetric properties of donor and acceptor activities in a cell-free system. Arch Biochem Biophys 240:413–425.

Barker PA, Shooter EM (1994) Disruption of NGF binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to trkA on PC12 cells. Neuron 13:203–215.

Bernd P, Greene LA (1983) Electron microscopic radioautographic localization of iodinated nerve growth factor bound to and internalized by PC12 cells. J Neurosci 3:631–643.

Bernd P, Greene LA (1984) Association of ^{125}I-nerve growth factor with PC12 pheochromocytoma cells: evidence for internalization via high-affinity receptors only and for long-term regulation by nerve growth factor of both high- and low-affinity receptors. J Biol Chem 259:15509–15516.

Bothwell M (1995) Functional interactions of neurotrophins and neurotrophin receptors. Annu Rev Neurosci 18:223–253.

Bothwell M (1996) p75NTR: a receptor after all. Science 272:506–507.

Brodsky FM (1985) Clathrin structure characterized with monoclonal antibodies. I. Analysis of multiple antigenic sites. J Cell Biol 101:2047–2054.

Campenot RB (1977) Local control of neurite development by nerve growth factor. Proc Natl Acad Sci USA 74:4516–4519.

Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhauser N, Bohm-Matthaei R, Baeuerle PA, Barde Y-A (1996) Selective activation of NF-kappaB by nerve growth factor through the neurotrophin receptor p75. Science 272:542–545.

Chin DJ, Straubinger RM, Acton S, Nathke I, Brodsky FM (1989) 100 kDa polypeptides in peripheral clathrin-coated vesicles are required for receptor-mediated endocytosis. Proc Natl Acad Sci USA 86:9289–9293.

Clary DO, Weskamp G, Austin LR, Reichardt LF (1994) TrkA cross-linking mimics neuronal responses to nerve growth factor. Mol Biol Cell 5:549–563.

Claude P, Hawrot E, Dunis DA, Campenot RB (1982) Binding, internalization, and retrograde transport of ^{125}I-nerve growth factor in cultured rat sympathetic neurons. J Neurosci 2:431–442.

Crowley C, Spencer SD, Nishimura MC, Chen KS, Pitts MS, Armanini MP, Ling LH, MacMahon SB, Shelton DL, Levinson AD, et al (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell 76:1001–1011.

Curtis R, Adryan KM, Stark JL (1995) Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins. Neuron 14:1201–1211.

Davies AM, Lee K-F, Jaenisch R (1993) p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. Neuron 11:565–574.

Ehlers MD, Kaplan DR, Price DL, Koliatsos VE (1995) NGF-stimulated retrograde transport of TrkA in the mammalian nervous system. J Cell Biol 130:1–8.

Eveleth DD, Bradshaw RA (1988) Internalization and cycling of nerve growth factor in PC12 cells: interconversion of type II (fast) and type I (slow) nerve growth factor receptors. Neuron 1:929–936.

Grady EF, Garland AM, Gamp PD, Lovett M, Payan DG, Bunnett NW (1995) Delineation of the endocytic pathway of substance P and its seven-transmembrane domain NK1 receptor. Mol Biol Cell 6:509–524.

Grimaldi KA, Hutton JC, Siddle K (1987) Production and characterization of monoclonal antibodies to insulin secretory granule membranes. Biochem J 245:557–566.

Grimes ML, Kelly RB (1992a) Sorting of chromogranin B into immature secretory granules in pheochromocytoma (PC12) cells. Ann NY Acad Sci 674:38–52.

Grimes ML, Kelly RB (1992b) Intermediates in the constitutive and regulated secretory pathways released in vitro from semi-intact cells. J Cell Biol 117:539–549.

Grimes ML, Zhou J, Li Y, Holtzman D, Mobley WC (1993) Neurotrophin signalling in the nervous system. Semin Neurosci 5:239–247.

Haass C, Koo EH, Mellon A, Hung AY, Selkoe DJ (1992) Targeting of cell-surface beta-amyloid precursor protein to lysosomes: alternative processing into amyloid-bearing fragments. Nature 357:500–503.

Hantzopoulos PA, Suri C, Glass DJ, Goldfarb MP, Yancopoulos GD (1994) The low affinity NGF receptor, p75, can collaborate with each of the trks to potentiate functional responses to the neurotrophins. Neuron 13:187–201.

Hartman DS, McCormack M, Schubenel R, Hertel C (1992) Multiple trkA proteins in PC12 cells bind NGF with a slow association rate. J Biol Chem 267:24516–24522.

Hendry IA (1977) The effect of the retrograde axonal transport of nerve growth factor on the morphology of adrenergic neurones. Brain Res 134:213–223.

Hendry I, Bonyhady R (1980) Retrogradely transported nerve growth factor increases ornithine decarboxylase activity in rat superior cervical ganglia. Brain Res 200:39–45.

Hendry IA, Stach R, Herrup K (1974a) Characteristics of the retrograde axonal transport system for nerve growth factor in the sympathetic nervous system. Brain Res 82:117–128.

Hendry IA, Stockel K, Thoenen H, Iversen LL (1974b) The retrograde axonal transport of nerve growth factor. Brain Res 68:103–121.

Heumann R, Schwab M, Merkl R, Thoenen H (1984) Nerve growth factor-mediated induction of choline acetyltransferase in PC12 cells: evaluation of the site of action of nerve growth factor and the involvement of lysosomal degradation products of nerve growth factor. J Neurosci 4:3039–3050.

Hollenbeck PJ (1993) Products of endocytosis and autophagy are retrieved from axons by regulated retrograde organelle transport. J Cell Biol 121:305–315.

Holtzman DM, Kilbridge J, Li Y, Cunningham ET, Lenn NJ, Clary DO, Reichardt LF, Mobley WC (1995) TrkA expression in the CNS: evidence for the existence of several novel NGF-responsive CNS neurons. J Neurosci 15:1567–1576.

Hosang M, Shooter EM (1987) The internalization of nerve growth factor by high-affinity receptors on pheochromocytoma PC12 cells. EMBO J 6:1197–1202.

Jing S, Tapley P, Barbacid M (1992) Nerve growth factor mediates signal transduction through trk homodimer receptors. Neuron 9:1067–1079.

Johnson EMJ, Taniuchi M, Clark HB, Springer JE, Koh S, Tayrien MW, Loy R (1987) Demonstration of the retrograde transport of nerve growth factor receptor in the peripheral and central nervous system. J Neurosci 7:923–929.

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science 252:554–558.

Kaplan DR, Stephens RM (1994) Neurotrophin signal transduction by the Trk receptor. J Neurobiol 25:1404–1417.

Kiss J, Shooter EM, Patel AJ (1993) A low-affinity nerve growth factor receptor antibody is internalized and retrogradely transported selec-

tively into cholinergic neurons of the rat basal forebrain. Neuroscience 57:297–305.

Klein R, Jing SQ, Nanduri V, O'Rourke E, Barbacid M (1991) The trk proto-oncogene encodes a receptor for nerve growth factor. Cell 65:189–197.

Knusel B, Rabin S, Hefti F, Kaplan D (1994) Regulated neurotrophin receptor responsiveness during neuronal migration and early differentiation. J Neurosci 14:1542–1554.

Lamaze C, Schmid SL (1995) Recruitment of epidermal growth factor receptors into coated pits requires their activated tyrosine kinase. J Cell Biol 129:47–54.

Layer PG, Shooter EM (1983) Binding and degradation of nerve growth factor by PC12 pheochromocytoma cells. J Biol Chem 258:3012–3018.

Lee K-F, Bachman K, Landis S, Jaenisch R (1994) Dependence on p75 for innervation of some sympathetic targets. Science 263:1447–1449.

Levi-Montalcini R (1987) The nerve growth factor 35 years later. Science 237:1154–1161.

Li Y, Holtzman DM, Kromer LF, Kaplan DR, Chua-Couzens J, Clary DO, Knusel B, Mobley WC (1995) Regulation of TrkA and ChAT expression in developing rat basal forebrain: evidence that both exogenous and endogenous NGF regulate differentiation of cholinergic neurons. J Neurosci 15:2888–2905.

Loeb DM, Greene LA (1993) Transfection with trk restores "slow" NGF binding, efficient NGF uptake, and multiple NGF responses to NGF-nonresponsive PC12 cell mutants. J Neurosci 13:2919–2929.

Loeb DM, Tsao H, Cobb MH, Greene LA (1992) NGF and other growth factors induce an association between ERK1 and the NGF receptor, gp140protoTrk. Neuron 9:1053–1065.

Longo FM, Holtzman DM, Grimes ML, Mobley WC (1993) Nerve growth factor: actions in the peripheral and central nervous systems. In: Neurotrophic factors (Fallon J, Loughlin S, eds), pp 209–256. New York: Academic.

Lucidi-Phillipi CA, Clary DO, Reichardt LF, Gage FH (1996) TrkA activation is sufficient to rescue axotomized cholinergic neurons. Neuron 16:653–663.

Mahadeo D, Kaplan L, Chao MV, Hempstead BL (1994) High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. J Biol Chem 269:6884–6891.

Martin TF, Walent JH (1989) A new method for cell permeabilization reveals a cytosolic protein requirement for Ca^{2+}-activated secretion in GH3 pituitary cells. J Biol Chem 264:10299–10308.

Martin-Zanca D, Oskam R, Mitra G, Copeland T, Barbacid M (1989) Molecular and biochemical characterization of the human *trk* proto-oncogene. Mol Cell Biol 9:24–33.

Meakin SO, Shooter EM (1991) Tyrosine kinase activity coupled to the high-affinity nerve growth factor-receptor complex. Proc Natl Acad Sci USA 88:5862–5866.

Meakin SO, Shooter EM (1992) The nerve growth factor family of receptors. Trends Neurosci 15:323–331.

Mobley WC, Rutkowski JL, Tennekoon GI, Gemski J, Buchanan K, Johnston MV (1986) Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. Mol Brain Res 387:53–62.

Nixon RA, Cataldo AM (1995) The endosomal-lysosomal system of neurons: new roles. Trends Neurosci 18:489–496.

Overly CC, Lee K-D, Berthiaume E, Hollenbeck PJ (1995) Quantitative measurement of intraorganelle pH in the endosomal-lysosomal pathway in neurons by using ratiometric imaging with pyranine. Proc Natl Acad Sci USA 92:3156–3160.

Paravicini U, Stoeckel K, Thoenen H (1975) Biological importance of retrograde axonal transport of nerve growth factor in adrenergic neurons. Brain Res 84:279–291.

Parton RG, Dotti CG (1993) Cell biology of neuronal endocytosis. J Neurosci Res 36:1–9.

Parton RG, Simons K, Dotti CG (1992) Axonal and dendritic endocytic pathways in cultured neurons. J Cell Biol 119:123–137.

Podlisny MB, Tolan DR, Selkoe DJ (1991) Homology of the amyloid beta protein precursor in monkey and human supports a primate model for beta amyloidosis in Alzheimer's disease. Am J Pathol 138:1423–1435.

Radeke MJ, Feinstein SC (1991) Analytical purification of the slow, high affinity NGF receptor: identification of a novel 135 kd polypeptide. Neuron 7:141–150.

Raivich G, Hellweg R, Kreutzberg GW (1991) NGF receptor-mediated reduction in axonal NGF uptake and retrograde transport following sciatic nerve injury and during regeneration. Neuron 7:151–164.

Schmid SL, Carter LL (1990) ATP is required for receptor-mediated endocytosis in intact cells. J Cell Biol 111:2307–2318.

Schwab ME (1977) Ultrastructural localization of a nerve growth factor-horseradish peroxidase (NGF-HRP) coupling product after retrograde axonal transport in adrenergic neurons. Brain Res 130:190–196.

Smeyne RJ, Klein R, Schnapp A, Long LK, Bryant S, Lewin A, Lira SA, Barbacid M (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature 368:246–249.

Stephens RM, Loeb DM, Copeland TD, Pawson T, Greene LA, Kaplan DR (1994) Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ1 to mediate NGF responses. Neuron 12:691–705.

Taniuchi M, Johnson EMJ (1985) Characterization of the binding properties and retrograde axonal transport of a monoclonal antibody directed against the rat nerve growth factor receptor. J Cell Biol 101:1100–1106.

Tischler AS, Greene LA (1975) Nerve growth factor-induced process formation by cultured rat pheochromocytoma cells. Nature 258:341–342.

Vale RD, Shooter EM (1985) Assaying binding of nerve growth factor to cell surface receptors. Methods Enzymol 109:21–39.

Vale RD, Ignatius MJ, Shooter EM (1985) Association of nerve growth factor receptors with the Triton X-100 cytoskeleton of PC12 cells. J Neurosci 5:2762–2770.

van der Geer P, Hunter T, Lindberg RA (1994) Receptor protein-tyrosine kinases and their signal transduction pathways. Annu Rev Cell Biol 10:251–337.

Verdi JM, Birren SJ, Ibanez CF, Persson H, Kaplan DR, Benedetti M, Chao MV, Anderson DJ (1994) $p75^{LNGFR}$ regulates trk signal transduction and NGF-induced neuronal differentiation in MAH cells. Neuron 12:733–745.

Yuen EC, Mobley WC (1995) Therapeutic applications of neurotrophic factors in disorders of motor neurons and peripheral nerves. Mol Med Today 1:278–286.

Zhou J, Valletta JS, Grimes ML, Mobley WC (1995) Multiple levels for regulation of TrkA in PC12 cells by nerve growth factor. J Neurochem 65:1146–1156.

Chapter Three

A signaling endosome hypothesis to explain NGF actions. Potential implications for neurodegeneration.

Introduction

This chapter is a summary of our work investigating TrkA as the best candidate for the retrogradely trafficked trophic signal initiated by NGF binding. In PC12 studies we sought to determine by confocal microscopy whether TrkA and clathrin were colocalized and what trafficking responses TrkA made after NGF treatment. In response to NGF, cells displayed a rapid increase in the colocalization of clathrin heavy chain and TrkA staining at the plasma membrane.

Biochemical studies detailing TrkA and NGF association in PC12 endosomal fractions showed further evidence that TrkA is capable of signaling after endocytosis. New data not published prior to this chapter include: 1) confocal micrographs showing colocalization of markers of lysosomal and TrkA proteins after 20 minutes of NGF application to PC12 cells; 2) a new application of the method of TrkA and NGF crosslinking, confirming that NGF and TrkA are associated in endosome fractions from treated PC12 cells; and 3) Shc and TrkA association after NGF application in PC12 cells.

A Signaling Endosome Hypothesis to Explain NGF Actions: Potential Implications for Neurodegeneration

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Nerve growth factor (NGF), a polypeptide neurotrophic factor of the neurotrophin (NT) gene family, acts to enhance the survival and differentiation of specific populations of neurons in the central (CNS) and peripheral (PNS) nervous systems (Levi-Montalcini 1987; Yuen and Mobley 1995). NGF acts by binding to both high- and low-affinity receptors. Its receptors are $p75^{NGFR}$ and TrkA. $p75^{NGFR}$ is a receptor for all the neurotrophins, exhibiting binding affinities in the nanomolar range (i.e., low-affinity binding) (Meakin and Shooter 1992; Bothwell 1995). The role that $p75^{NGFR}$ plays in NGF signaling is not well defined; however, in addition to a direct signaling function (Bothwell 1996; Carter et al. 1996; Taglialatela et al. 1996), $p75^{NGFR}$ has been shown to modulate NGF binding and activation of TrkA (Meakin and Shooter 1992; Davies et al. 1993; Barker and Shooter 1994; Hantzopoulos et al. 1994; Mahadeo et al. 1994; Verdi et al. 1994; Bothwell 1995). TrkA is a receptor tyrosine kinase (Kaplan et al. 1991; Klein et al. 1991; Meakin and Shooter 1991). Although the exact composition of high-affinity receptors is controversial (Hempstead et al. 1991; Meakin and Shooter 1992; Mahadeo et al. 1994; Bothwell 1995), TrkA alone has been shown to create both high- and low-affinity NGF-binding sites (Jing et al. 1992). It is also the receptor responsible for the slowly dissociating component of NGF binding that characterizes high-affinity receptors (Meakin et al. 1992). NGF binding causes TrkA dimerization with resulting activation of its kinase domain (Kaplan et al. 1991; Klein et al. 1991; Meakin and Shooter 1991; Jing et al. 1992). Autophosphorylation of certain tyrosine residues in the intracellular domain of the receptor creates sites for binding and activation of signaling intermediates which continue the signal transduction cascade (Stephens et al. 1994). Phospholipase C-γ (PLC-γ), SHC (an adapter protein containing sequences to the Src domain 2), and phosphatidylinositol 3 kinase are known intermediaries in this cascade (Stephens et al. 1994). The significance of TrkA activation for NGF signaling has been tested in vitro and in vivo. In cultured cells, TrkA activation induces many of the typical neuronal responses to NGF (Loeb and Greene 1993). TrkA also plays an important role in NGF signaling in vivo. Animals in which the gene for TrkA was disrupted showed marked abnormalities in the development of NGF-responsive neurons in the CNS and PNS (Smeyne et al. 1994).

In earlier studies, we defined NGF-responsive CNS neurons by localizing TrkA gene expression and by demonstrating that all *TrkA*-expressing populations responded to NGF with hypertrophy of the cell body (Holtzman et al. 1992, 1995). Significantly, some of these populations do not express $p75^{NGFR}$. These data suggest that in these neurons TrkA is able to transduce the NGF signal. To further characterize NGF actions, we examined gene expression in the NGF-responsive cholinergic neurons of the basal forebrain (BFCN) and caudate-putamen (CPCN). Consistent with findings in earlier studies (Johnston et al. 1987; Fusco et al. 1989; Higgins et al. 1989), NGF treatment induced an increase in mRNA for choline acetyltransferase (ChAT) in both populations (Holtzman et al. 1992). Remarkably, *TrkA* expression was also increased by NGF. Intracerebroventricular infusion of NGF into adult rats for 2 weeks led to approximately a 60% increase in TrkA mRNA in both BFCNs and CPCNs (Holtzman et al. 1992). These data show that NGF signaling is positively linked to expression of *TrkA*. Such a relationship also appears to exist under physiological conditions. Studies in developing animals show that *TrkA* expression in BFCNs parallels that for *NGF* expression in the hippocampus and neocortex, the target fields of BFCN axons, and that *TrkA* expression is suppressed by infusion of specific NGF antibodies (Li et al. 1995). It is often the case that ligands negatively regulate their receptors (Collins et al. 1992). Thus, the NGF effect on *TrkA* was surprising and unexplained.

To pursue further the mechanism and significance of NGF actions on *TrkA* expression, we carried out studies in PC12 cells, a neuron-like cell line that has been used extensively to characterize NGF signaling (Tischler and Greene 1975; Stephens et al. 1994; Zhou et al. 1995). Our findings demonstrate that NGF has widespread effects on TrkA, regulating activation, trafficking, degradation, and synthesis. How these actions are integrated was suggested by noting distinct phases in the NGF response: an early phase characterized by down-regulation of TrkA and a later phase that featured enhanced *TrkA* expression. Dramatic down-regulation of surface TrkA within minutes of NGF treatment, with subsequent degradation of TrkA

[5]These authors contributed equally to the work.

raised the possibility that NGF induces internalization of activated TrkA receptors and targets them to lysosomes. We gathered evidence to show that this is the case. Our findings suggest that it is through the formation and retrograde transport of activated TrkA-containing signaling endosomes that NGF released in the target of responsive neurons acts to regulate gene expression to support survival, differentiation, and maintenance. NGF-induced increases in *TrkA* expression may serve to replace receptors degraded after carrying the retrograde signal. Certain aspects of these studies have been reported previously (Zhou et al. 1995; Grimes et al. 1996).

MATERIALS AND METHODS

Materials

Bis(sulfosuccinimidyl)suberate (BS^3), disuccinimidyl suberate (DSS), and NHS-SS-biotin were obtained from Pierce (Rockford, Illinois). Normal goat serum and Vectashield mounting medium were from Vector Laboratories, Inc. (Burlingame, California). Except as noted herein, or as indicated (M.L. Grimes et al., in prep.), Sigma (St. Louis, Missouri) was the source of all other reagents and chemicals. NGF was prepared as described previously (Mobley et al. 1986) and was labeled with ^{125}I (Amersham, Arlington Heights, Illinois) using lactoperoxidase, as modified from Vale and Shooter (Vale and Shooter 1985; M.L. Grimes et al., in prep.). 1088 is a rabbit antibody against the carboxyl terminus of human TrkA. It was purified using protein A-Sepharose (Pierce) and has been characterized previously (Zhou et al. 1995). Sc11 is another rabbit antibody to the carboxyl terminus of human TrkA (Santa Cruz Biotechnology, Santa Cruz, California). RTA is a rabbit antibody against the extracellular domain of rat TrkA (Clary et al. 1994; Lucidi-Phillipi et al. 1996). X22 is a mouse monoclonal antibody to the clathrin heavy chain (Brodsky 1985). AP-6 is a mouse monoclonal antibody to the α-adaptin 100-kD subunits (Chin et al. 1989). GM10 is a mouse monoclonal antibody that stains lysosomes (Grimaldi et al. 1987; Grady et al. 1995). The antibody to PLC-γ1 was a mixed monoclonal antibody; 4G10 is a mouse monoclonal antibody to phosphorylated tyrosine; the antibody to SHC is a rabbit polyclonal antibody (all from Upstate Biotechnology Inc., Lake Placid, New York). ^{125}I-labeled goat anti-mouse was prepared using $Na^{125}I$ (Amersham), iodobeads (Pierce), and goat-anti-mouse (Pierce) according to the manufacturer's instructions, and desalted on a PD-10 column (Pharmacia Biotech, Inc., Piscataway, New Jersey).

Methods

Cell culture procedures for biochemical examinations. PC12 cells were maintained in Dulbecco's modified Eagle's (DME H-21) medium supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. PC12 cells not treated with NGF are also referred to as "naive" cells. PC12 cells were "primed" by incubating with mouse NGF (50 ng/ml = 1.9 nM) for at least 1 week (Zhou et al. 1995). For binding and surface cross-linking experiments, culture plates (Falcon) were precoated with rat tail collagen (Collaborative Biomedical, Bedford, Massachusetts). Immediately before each experiment on primed cells, the cells were washed three times with NGF-free, fresh tissue culture medium for 2 hours at 37°C.

RNA isolation and Northern blot analysis. The procedures used were essentially those described previously (Holtzman et al. 1992). PC12 cells grown to 30% confluence were treated with mouse NGF (50 ng/ml) at 37°C for the times indicated. Cells were harvested and total RNA was prepared using guanidinium isothiocyanate extraction. For Northern blots, 20 μg of total RNA was electrophoresed on 1% agarose-formaldehyde gels and blotted to Genescreen (Dupont, Boston, Massachusetts). Blots were probed with ^{32}P-labeled cDNAs for rat *trkA*, rat $p75^{NGFR}$, or 18S rRNA. After washing at high stringency, blots were exposed to X-ray films and quantitated as described previously (Zhou et al. 1995).

Immunoprecipitation and Western blotting of whole-cell lysates. Immunoprecipitation and Western blotting were performed as described previously (Zhou et al. 1995). PC12 cells were lysed at 4°C in lysis buffer #1 (20 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, and a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride 10 mg/ml benzamidine, 1 mg/ml *o*-phenanthroline, 0.1 mg/ml each of pepstatin, chymostatin, leupeptin, and aprotinin). Lysates (1 ml) were immunoprecipitated while rocking overnight at 4°C with antibodies to TrkA (1088) (10 mg), PLC-γ1 (5 mg), or $p75^{NGFR}$ (7 mg) (REX), and immunoprecipitates were collected and processed according to the method of Zhou et al. (1995). After boiling for 5 minutes in 50 μl of 2x SDS-PAGE sample buffer (2% SDS, 10% glycerol, 0.1 M DTT, 125 mM Tris, pH 6.95, 0.1% bromophenol blue), they were subjected to 7.5% SDS-PAGE and transferred to nitrocellulose. For examining TrkA, blots probed with 1088 or RTA were incubated with 1 μCi ^{125}I-labeled protein A (Amersham). For measuring phosphotyrosine, blots were incubated with 4G10, then washed and incubated with goat anti-mouse IgG antibodies (Boehringer Mannheim Corporation, Indianapolis, Indiana) prior to using ^{125}I-labeled protein A or the ECL chemiluminescence system (Amersham) for detection. All blots were exposed to X-ray film, and band intensities were quantified on a densitometer (LKB Ultroscan, Gaithersberg, Maryland) (Zhou et al. 1995).

NGF binding and cross-linking to surface receptors. PC12 cells were maintained as indicated. Naive and primed cells were seeded in six-well collagen-coated plates at a density of 5×10^5 cells per well 2 days before assay. Naive cells and primed cells (washed free of NGF for 2 hours at 37°C with culture medium) were washed with binding buffer at 4°C for 10 minutes and then incubated in binding buffer containing ^{125}I-labeled NGF at either 100 pM or 2 nM for 2 hours at 4°C. To measure binding to slowly dissociating receptors, cells were washed in NGF-free binding buffer at 4°C for 1 hour. For internalization studies, cells bound to NGF at steady state were incubated at 37°C for various time periods and chilled to 4°C. Internalized NGF was then measured, as indicated previously (Zhou et al. 1995). To measure down-regulation of surface gp140^{TrkA}, cells (10^7/15-cm dish), plated 2 days before the experiment, were washed and then incubated with ^{125}I-labeled NGF, as above. They were then warmed to 37°C for 1 hour, and chilled (4°C) before cross-linking. To measure cross-linking to slowly dissociating receptors, cells were washed in ligand-free binding buffer at 4°C for 1 hour prior to cross-linking.

To cross-link NGF to gp140^{TrkA}, BS^3 was added to a final concentration of 0.8 mM in binding buffer. Samples were incubated at 4°C for 30 minutes, washed, and lysed in lysis buffer, as above. ^{125}I-labeled NGF/gp140^{TrkA} complexes were immunoprecipitated with 1088. The immunoprecipitates were boiled in the SDS-PAGE sample buffer for 5 minutes and subjected to SDS-PAGE using a gradient resolving gel (5.0% acrylamide with 0.1% bisacrylamide to 12.0% acrylamide with 0.5% bisacrylamide). Gels were fixed in 20% methanol with 10% isopropanol and 10% acetic acid, dried, and exposed to X-ray film or to a phosphorimager screen. Quantitation was via a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, California).

Cell surface biotinylation. Cells were incubated with or without NGF (1 nM) for 30 minutes at 4°C in PBS with 1 mg/ml glucose, and NHS-SS-biotin (0.5 mg/ml) was then added. The mixture was incubated with gentle rocking for 90 minutes at 4°C. Cells were pelleted (1000 rpm for 5 min) and then washed 3 times in cold PBS containing 1 mM lysine. Samples were then processed as indicated previously (M.L. Grimes et al., in prep.) producing three equal aliquots. Aliquot #1 was held at 4°C. Aliquot #2 was treated at 4°C with 50 mM reduced glutathione in 50 mM Tris, pH 8.6, 100 mM NaCl 1 mg/ml glucose, and 1 mg/ml BSA for 30 minutes. This treatment was repeated twice. Aliquot #3 was warmed to 37°C for either 10 or 20 minutes to allow endocytosis, and then treated with glutathione as above. All aliquots were then incubated 1 hour on ice in 0.2 ml lysis buffer #2 (20 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing 1% BSA and 1 mg/ml iodoacetamide. The lysates were centrifuged 10 minutes at 10,000*g*. SDS (final concentration 0.5%) was added to the supernatant, and the lysates were boiled for 5 minutes. Lysis buffer #2 (0.8 ml) was then added, and TrkA was immunoprecipitated with 1088 (12 μg). Each lysate was divided into two parts. The first was used to detect the amount of biotinylated TrkA. Proteins were separated on non-reducing 7.5% SDS-PAGE, transferred to nitrocellulose (Hoefer Pharmacia Biotech Inc., San Francisco, California), and blotted with ^{125}I-labeled streptavidin (Amersham). Biotinylated TrkA was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, California). The second part was used to quantitate TrkA protein. It was submitted to 7.5% SDS-PAGE in a reducing environment, transferred to nitrocellulose, and blotted with 1088 (1:500). The signal was developed using ^{125}I-protein A (Amersham) and quantified using the PhosphorImager. The signal for biotinylated TrkA was normalized to the amount of TrkA protein. The TrkA available for internalization was calculated as follows: aliquot #1 – aliquot #2. TrkA internalized during warming was not susceptible to reduction with glutathione. The amount of TrkA internalized was calculated: aliquot #3 – aliquot #2. The percentage of TrkA internalized was calculated as follows: aliquot #3 – aliquot #2) (aliquot #1 – aliquot #2) x 100.

Immunostaining and confocal microscopy. Cells were plated on 8-well chamber slides (Nunc Inc., Naperville, Illinois) that had been coated with Matrigel (Collaborative Biomedical Products) using a 1:200 dilution in PBS (Ca^{++}- and Mg^{++}-free) overnight at 4°C. Wells were washed 3 times with cold PBS. PC12 cells were plated in DME H-21 medium with 10% horse serum and 5% fetal calf serum 1–2 days before experiments. After aspirating the medium, NGF (2 nM) was added to cells for 30 seconds, 2 minutes, or 60 minutes in 300 μl of DME H-21 containing 0.5 mg/ml bovine serum albumin, 10 mM HEPES at 37°C. This medium, without NGF, was added to controls.

Cells were fixed on ice with 1% paraformaldehyde in PBS for 15 minutes. Cells were permeabilized in PSS (PBS with 10% normal goat serum and saponin [1 mg/ml]) at room temperature for 30 minutes, changing the solution every 10 minutes. Primary antibodies were diluted in PSS (sc11 at 1 μg/ml; X22 at 3.1 μg/ml; GM10 at 1:6000; AP.6 at 8 μg/ml) and incubated with cells overnight at 4°C. After three 10-minute washes with PSS, secondary antibodies diluted in PSS (FITC-conjugated goat-anti-rabbit IgG at 1:100; Texas Red-conjugated goat-anti-mouse immunoglobulins 1:200; both from Cappel Research Products, Durham, North Carolina) were applied for 45 minutes at room temperature. After 3 washes in PBS, coverslips were mounted using Vectashield mounting solution. No staining was evident when primary antibodies were excluded. For Sc11, preliminary incubation (overnight, 4°C) with the peptide immunogen (10 μg/ml) eliminated staining.

Cells were observed with an MRC 1000 Laser Scanning Confocal Microscope (Biorad Laboratories, Inc.,

Hercules, California) equipped with a krypton/argon laser and attached to a Zeiss Axiovert microscope. A Zeiss Neofluor x 100 oil-immersion objective with a numerical aperture of 1.3 (∞0.17) was used, and images were collected using an aperture of 3–4 mm and a zoom of 2–3. Typically, 10–20 optical sections were taken at 0.5-μm intervals through the cells. The resolution of the confocal microscope in the X-Y axis was 170–200 nm, and in the Z axis was 230–400 nm. Images of 768 x 520 pixels were obtained. Images were processed using Adobe Photoshop 3.0 (Adobe Systems Inc., Mountain View, California) and printed using a Techtronix Printer. In experiments in which markers were colocalized, colocalization was confirmed by examining individual organelles at higher magnification. In addition, we ensured that colocalization was eliminated by merging overlaying, but noncoincident, sections. The images shown correspond to optical sections through cells at mid-height.

To count TrkA-positive vesicles or TrkA/clathrin colocalizations near the surface of cells, images of individual optical sections were examined. The edge of the cell was defined as the outermost limit of staining for the clathrin heavy chain, and this was marked with a line. A second line was drawn 0.5 μm interior to the first, and all bright, punctate TrkA-positive vesicles between the lines were counted. The number of these vesicles was then recorded per cell or was divided by the perimeter of the surface of the cell to yield a value for the number per micron of cell perimeter.

Cell fractionation. In some experiments, cells were first incubated with or without NGF (1 nM) in binding buffer at 4°C for 1 hour. They were then either washed briefly in binding buffer, or not washed, and warmed in binding buffer for 10 min at 37°C. In other experiments, NGF was added to cells at 37°C. Cells were then chilled (4°C) and washed in PBS with 1 mM EDTA and 1 mM EGTA, and then in a cytoplasm-like buffer (buffer B) (38 mM each of the potassium salts of aspartic, gluconic, and glutamic acids; 20 mM MOPS; 5 mM reduced glutathione; 10 mM potassium bicarbonate; 0.5 mM magnesium carbonate; 1 mM EGTA; 1 mM EDTA; pH adjusted to 7.1 at 37°C with potassium hydroxide). Cells were resuspended in 0.5 ml buffer B containing proteinase inhibitors (as for lysis buffer #1). Sodium orthovanadate (1 mM) was included to inhibit phosphatase activity for the experiments shown in Figures 11 and 12. To permeabilize cells, we used a ball homogenizer obtained from the European Molecular Biology Laboratory (Heidelberg, Germany) and tungsten carbide grade 25 balls obtained from Industrial Tectonics, Inc. (Ann Arbor, Michigan). The cell suspension was passed through the homogenizer (8.020 mm cylinder with an 8.0186 mm ball). Greater than 98% of the cells stained with trypan blue after this procedure. By centrifuging at 1000*g* for 10 minutes, cell ghosts (P1) were pelleted, thus separating them from the cytosol and the released vesicles. The 1000*g* supernatant was centrifuged at 8000*g* for 35 minutes in a TiSW50.1 rotor. The pellet (P2) contained large organelles. The supernatant (S2) was layered over a 0.4-ml pad of 10% sucrose in buffer B (with inclusions, as above) and centrifuged at 100,000*g* for 1 hour to produce the pellet, P3. S3 was the cytosol.

To determine whether fragments of plasma membrane contaminated released intracellular organelles, we biotinylated cell-surface proteins and determined whether amyloid precursor protein (APP), a relatively abundant protein (Haass et al. 1992), was detected in P2 and P3. PC12 cells were harvested and cell-surface proteins were biotinylated, as indicated above, for 20 minutes at 4°C. Cells were washed 3 times with 1 mM lysine in PBS and then permeabilized and fractionated. P1, P2, and P3 were lysed in lysis buffer #2 and immunoprecipitated with α-C7, an antibody to the carboxyl terminus of APP (Podlisny et al. 1991). Immunoprecipitates were submitted to 7.5% SDS-PAGE and blotted. ^{125}I-labeled streptavidin was used to probe the blots, and the Phosphorimager was used for detection. To quantify ^{125}I-labeled NGF in intracellular organelles, fractions were assayed for ^{125}I-labeled NGF. In some experiments, acid-washing (Bernd and Greene 1984) was used to measure surface-bound ^{125}I-labeled NGF.

NGF cross-linking to intracellular TrkA. Cells incubated with ^{125}I-labeled NGF (1 nM) in binding buffer for 1 hour at 4°C were washed and then warmed for 10 minutes at 37°C prior to permeabilization, as above. Unlabeled NGF (1 mM) was added to control for nonspecific binding and cross-linking. DSS (2 mM) was added to the permeabilized cells and released membranes and the mixture was incubated while rotating for 30 minutes (4°C). The reaction was quenched with lysine (10 mM) for 10 minutes. P1, P2, and P3 were then prepared, as above. P1 was solubilized by extracting in 1% NP-40 in buffer B for 1 hour (4°C). After centrifuging at 1000*g* (10 min), SDS was added to bring the final concentration to 0.5%. P2 and P3 were resuspended in H_2O with 0.5% SDS. S3 was brought to the same SDS concentration. All samples were boiled for 5 minutes, chilled (4°C), and then brought to a volume of 1 ml and a final concentration of 0.1% SDS by diluting with immunoprecipitation (IP) buffer (20 mM HEPES, pH 7.4, 0.15 M NaCl, 1% NP-40, 0.5% DOC, 1 mM EDTA). To this was added 1088 (17 μg/ml). After incubating at 4°C overnight 1/10 volume of 20% protein A–Sepharose beads (Pierce) was added for 1 hour, with rotation. The beads were washed twice with IP buffer and once with water before resuspending in 7 M urea SDS-PAGE sample buffer with 20 mM DTT. Samples were heated to 65°C for 10 minutes and run on a 5–12% SDS-PAGE. Dried gels were exposed to XAR film for 1–3 weeks.

Immunoprecipitation and blotting of cellular fractions. PC12 cells incubated without NGF, or with NGF

(2 nm), at 37°C were chilled (4°C), mechanically permeabilized, and fractionated as above. Immunoprecipitation of cell fractions was performed by dissolving P1, P2, P3, or S3 in 1 ml of lysis buffer #1. To this was added 12 μg of 1088, or 5 μg of anti-SHC. The mixture was incubated overnight at 4°C and 1/10 volume of protein A or protein-A/G Sepharose beads was added for 2 hours at 4°C. Sepharose beads were washed 3 times in lysis buffer #1 and once with water, then treated with 50 μl of 7 M urea SDS-PAGE sample buffer (125 mM Tris, pH 6.95, 7 M urea, 2% SDS, 1 mM EDTA, 0.1% Bromphenol Blue) with 100 mM DTT and heated (55°C, 15–30 min). Samples were loaded on 7.5% SDS-PAGE. After transferring to nitrocellulose, blots were probed with antiphosphotyrosine (4G10), RTA, or anti-PLC-γ1. Immune complexes were detected with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG and chemiluminescence (Amersham).

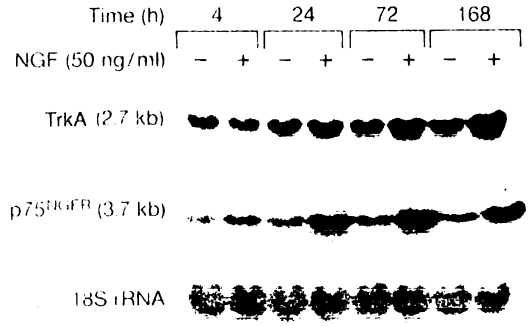

Figure 1. NGF induces gene expression for *TrkA* and $p75^{NGFR}$ in PC12 cells. PC12 cells were treated with NGF (50 ng/ml = 1.9 nM) for 4 hr, 24 hr, 72 hr, and 168 hr. Cells were harvested and total RNA from untreated (−) or NGF-treated (+) cells was prepared and analyzed on Northern blot; 20 μg was loaded in each lane. Blots were hybridized with rat *TrkA* cDNA or rat $p75^{NGFR}$ cDNA. The 18S rRNA probe was used to control for RNA loading. (Reprinted, with permission, from Zhou et al. 1995.)

RESULTS

NGF Increased TrkA mRNA in PC12 Cells

In vivo studies showed that NGF infusion into the CNS induced an increase in TrkA mRNA levels in forebrain cholinergic neurons of both developing and adult rats (Holtzman et al. 1992; Li et al. 1995). Earlier studies documenting NGF-induced increases in TrkA mRNA in PC12 cells (Holtzman et al. 1992; Meakin et al. 1992) encouraged use of these cells to examine further NGF regulation of *TrkA*. Northern blotting was used to examine total RNA isolated from naive (i.e., untreated) PC12 cells and from PC12 cells treated with NGF (50 ng/ml = 1.9 nM) for 4 hours, 24 hours, 3 days, 7 days, or 14 days. TrkA mRNA was decreased about 40% (n = 4) at 4 hours following NGF addition (Fig. 1). It was increased to 1.5-fold (n = 4) the untreated level at 2 days and reached a maximum (3.7-fold, n = 4) at day 7 (Fig. 1), remaining at this level for up to 14 days (data not shown). NGF is also known to increase $p75^{NGFR}$ expression in PC12 cells (Miller et al. 1991; Meakin et al. 1992). A different time course was seen for NGF effects on $p75^{NGFR}$ mRNA. It was increased to 2.0-fold (n = 4) the untreated level by 8 hours (data not shown), 3.4-fold (n = 4) at 24 hours, and remained at about this level through 7 days (Fig. 1). For both genes, the effect of NGF was transient. After NGF removal, mRNA levels for TrkA and $p75^{NGFR}$ fell to control levels within 48 hours (data not shown).

TrkA Protein Levels: Distinct Early and Late Responses to NGF

To ask whether the NGF-induced increase in *TrkA* expression would result in increased protein levels, we measured TrkA protein following NGF addition. The time points chosen (5 minutes, 30 minutes, 60 minutes, 2 days, and 7 days) allowed us to evaluate both immediate and long-term NGF effects. TrkA proteins were immunoprecipitated from cell lysates using 1088, an antibody directed at the carboxyl terminus of human TrkA (Zhou et al. 1995). Immunoprecipitates were submitted to SDS-PAGE and analyzed after immunoblotting with 1088. Proteins of 140 kD and 110 kD were detected. The former corresponds to the mature form of the receptor (Martin-Zanca et al. 1989; Kaplan et al. 1991; Klein et al. 1991; Meakin and Shooter 1991) and will be referred to herein as $gp140^{TrkA}$. The 110-kD species is a precursor (Martin-Zanca et al. 1989) and is referred to as $gp110^{TrkA}$. Figure 2 shows that NGF markedly influenced the levels of both $gp140^{TrkA}$ and $gp110^{TrkA}$ and that the early response was distinct from that which occurred after prolonged treatment. Soon after NGF addition, there was a marked decrease in $gp140^{TrkA}$. Although there was little change at 5 minutes, $gp140^{TrkA}$ was decreased at 30 minutes, and by 60 minutes the level was only 33% of that present in untreated cells (Fig. 2). The persistence of $gp110^{TrkA}$ at levels equal to those in untreated cells suggested that increased degradation of $gp140^{TrkA}$, and not a decrease in synthesis, was responsible.

With prolonged NGF treatment there were increases in both $gp110^{TrkA}$ and $gp140^{TrkA}$ (Fig. 2). On a per-cell basis, $gp110^{TrkA}$ was increased to 3.4-fold (n = 2) at 2 days, and at day 7 it was increased to 6.7-fold (n = 5). At day 2 of treatment, $gp140^{TrkA}$ was increased slightly relative to the level at 60 minutes. At day 7, it was increased to 3.0-fold (n = 5) that present in untreated cells. Although the increase in $gp140^{TrkA}$ was less pronounced than for $gp110^{TrkA}$, it was marked. One suggestion for the relatively smaller increase in $gp140^{TrkA}$ is that with continued NGF treatment, $gp140^{TrkA}$ continued to be targeted for degradation.

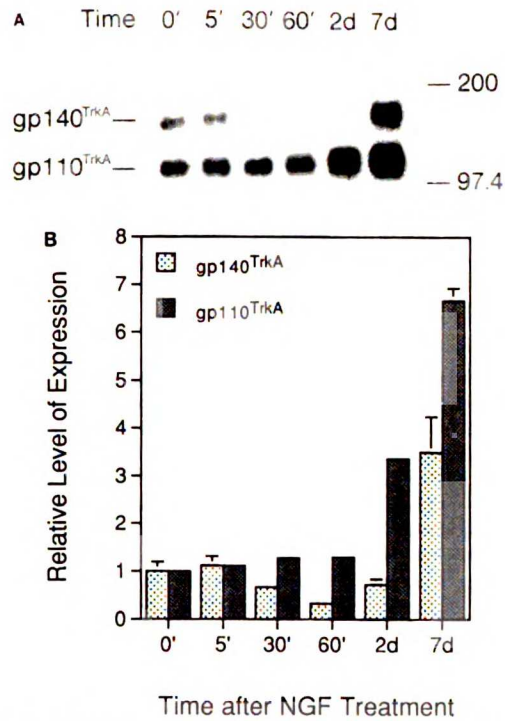

Figure 2. NGF regulates the level of gp140^{TrkA} and gp110^{TrkA}. (*A*) TrkA proteins were immunoprecipitated with 1088 from lysates of untreated PC12 cells (0') or cells treated with NGF for 5 min, 30 min, 60 min, 2 days, and 7 days. Immunoprecipitates were analyzed on SDS-PAGE and immunoblotted with 1088. An equal number of cells was used for each lane. (*B*) gp140^{TrkA} and gp110^{TrkA} bands were quantitated by densitometry. The level of expression relative to untreated cells is plotted (n = 2 for each point). (Reprinted, with permission, from Zhou et al. 1995.)

Total protein increased in NGF-treated cells by 28% at 2 days and 60% at 7 days. Therefore, whether expressed on a per-cell or per-milligram of protein basis, TrkA protein increased with prolonged NGF treatment. Increased TrkA protein levels were correlated temporally with increased mRNA levels, suggesting that the increase in TrkA mRNA was responsible for the increase in TrkA protein. The same may be true of NGF effects on p75^{NGFR}; the increase in mRNA at day 7 (Fig. 1) was associated with a p75^{NGFR} protein level that was 2.4-fold (n = 3) that in untreated cells.

Increased gp140^{TrkA} Resulted in Increased NGF Binding and Signaling

The biphasic response of gp140^{TrkA} levels to NGF treatment suggested that NGF induces both the degradation and the synthesis of its receptors. We reasoned that both may mark important aspects of NGF signaling. To explore the significance of increased gp140^{TrkA}, we asked whether NGF treatment resulted in increased gp140^{TrkA} receptors at the cell surface. This possibility was suggested by earlier studies on PC12 cells showing that NGF produced an increase in both high-affinity and low-affinity binding (Bernd and Greene 1984). We investigated ^{125}I-labeled NGF binding to naive (i.e., untreated) and primed (i.e., NGF-treated for 7 days) cells, carrying out these experiments at 4°C to inhibit membrane traffic and endocytosis. Two NGF concentrations were used. At 100 pM NGF, one is below the ED_{50} for the neurite outgrowth response (ED_{50} ~ 400 pM), and at a level that is subsaturating for high-affinity receptors (K_D ~ 400 pM) (Bernd and Greene 1984). At 2 nM, NGF occupies most of these receptors and produces maximal neurite outgrowth. We found that ^{125}I-labeled NGF binding was increased on primed cells, when expressed on a per-cell basis. Relative to naive cells, binding to primed cells was increased to 1.8 (± 0.2)-fold (S.E.M.) (n = 6) at 100 pM and 1.6 (± 0.1)-fold (n = 3) at 2 nM. NGF bound to gp140^{TrkA} receptors dissociates slowly (Meakin et al. 1992). At 2 nM, the ^{125}I-labeled NGF bound to slowly dissociating surface receptors on primed cells was increased 1.6 (± 0.1)-fold (n = 3); there was a corresponding increase in fast-dissociating binding (i.e., binding to low-affinity receptors) to 1.5 (± 0.1)-fold on these cells. ^{125}I-labeled NGF internalization is known to be mediated by gp140^{TrkA} (Jing et al. 1992; Loeb and Greene 1993). After a 60-minute treatment, ^{125}I-labeled NGF internalization in primed cells was 1.8 (± 0.1)-fold (n = 3) that in naive cells. To show directly whether increased TrkA receptors contributed to increased NGF binding, we performed cross-linking studies. Naive and primed cells were incubated with ^{125}I-labeled NGF at 4°C for 2 hours, and then a membrane-impermeant cross-linking reagent was added. Following immunoprecipitation with 1088, the immunoprecipitates were analyzed by SDS-PAGE. For primed cells, ^{125}I-labeled NGF cross-linked to gp140^{TrkA} was increased 44% (n = 2) at 100 pM (Fig. 3) and 100% at 2 nM (n = 2). There was a comparable increase in ^{125}I-labeled NGF cross-linking to gp140^{TrkA} in slowly dissociating receptors on primed cells (data not shown). These data are evidence that increased gene expression for TrkA results in an increase in the number of cell-surface gp140^{TrkA} receptors on individual PC12 cells.

The increase in gp140^{TrkA} receptors suggested there may be increased NGF signaling in primed cells. NGF binding to gp140^{TrkA} induces receptor dimerization and autophosphorylation (Jing et al. 1992; Kaplan and Stephens 1994; Stephens et al. 1994). A pronounced increase in tyrosine-phosphorylated gp140^{TrkA} is registered within minutes of NGF treatment (Stephens et al. 1994). To ask whether NGF signaling was increased in primed cells, we examined tyrosine phosphorylation of gp140^{TrkA} in naive cells and in primed cells washed free of NGF for 2 hours before assay. After treatment for 5, 30, or 120 minutes at 37°C, cell lysates were immunoprecipitated with 1088 and probed with an antiphosphotyrosine antibody (4G10). As shown in Figure 4, a relatively high level of tyrosine phos-

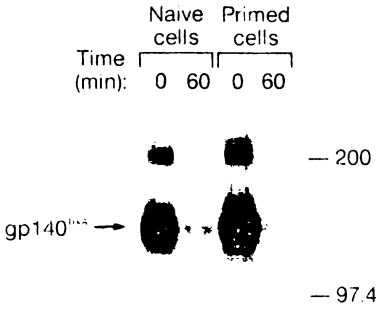

Figure 3. Cross-linking of ^{125}I-labeled NGF to surface gp140^{TrkA} in PC 12 cells. PC12 cells, naive or NGF-primed, were incubated with 100 pM ^{125}I-labeled NGF at 4°C for 2 hr and then warmed to 37°C for 0 or 60 min. Cells were then chilled and cross-linking was carried out as described in Materials and Methods. Cells were lysed and lysates were immunoprecipitated with 1088. The resulting immunoprecipitates were analyzed by SDS-PAGE. The gel was dried and exposed to X-ray film. After 60 min at 37°C, the amount of gp140^{TrkA} cross-linked to ^{125}I-labeled NGF dropped to the same level in naive and primed cells. There is also a band at 200 kD; a band of similar molecular mass has been seen in prior studies (Hartman et al. 1992). In this band, NGF specifically cross-linked to TrkA appears to be present in a higher-order complex. Each lane represents protein from an equal number of cells. The experiment was done twice with the same results. (Reprinted, with permission, from Zhou et al. 1995.)

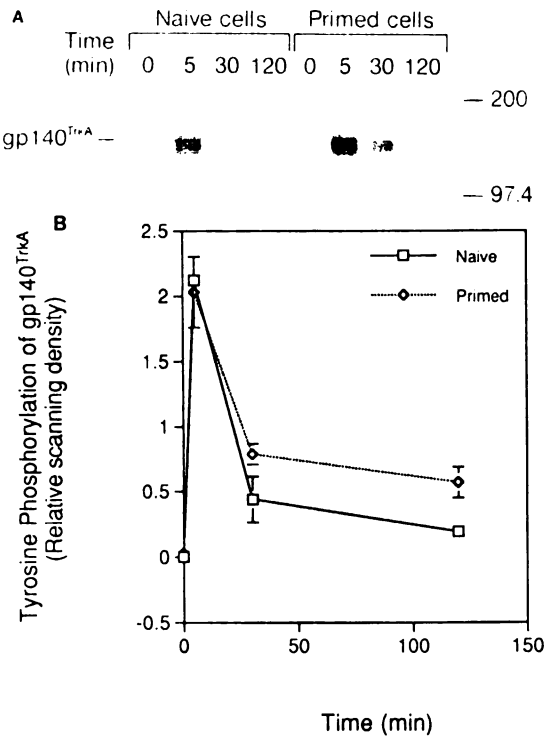

Figure 4. NGF-mediated tyrosine phosphorylation of TrkA was increased in primed cells. (*A*) Naive and primed PC12 cells were untreated (0) or treated with NGF (50 ng/ml) for 5 min, 30 min, or 120 min. Cell lysates were immunoprecipitated with 1088, subjected to SDS-PAGE, immunoblotted and the blots probed with anti-phosphotyrosine antibody (4G10). Lanes contain protein from an equal number of cells. (*B*) Tyrosine-phosphorylated TrkA was quantitated by densitometry. The data are corrected for the increase in protein that results from NGF priming. (Reprinted, with permission, from Zhou et al. 1995.)

phorylated gp140^{TrkA} was present at 5 minutes in both naive and primed cells. Tyrosine-phosphorylated gp140^{TrkA} decreased thereafter in both naive and primed cells, and the decreases followed a similar time course. However, the level of tyrosine-phosphorylated gp140^{TrkA} was greater in primed cells at each time point tested. As noted above, primed cells contain more protein. To normalize for this, the results were expressed per milligram of protein (Fig. 4B). The ratio of the signal in primed to that in naive cells was 0.9-fold (n = 3) at 5 minutes, 1.8-fold (n = 3) at 30 minutes, and 2.9-fold (n = 3) at 2 hours.

PLC-γ1 is a target of NGF-induced tyrosine phosphorylation and activation (Kim et al. 1991; Ohmichi et al. 1991; Vetter et al. 1991; Stephens et al. 1994). We investigated the level of tyrosine phosphorylation of PLC-γ1 in naive and primed PC12 cells. Tyrosine phosphorylation of PLC-γ1 was demonstrable at 5 minutes and was sustained at lower levels through 7 days of NGF treatment (Fig. 5, first five lanes). In primed cells washed free of NGF, tyrosine-phosphorylated PLC-γ1 was barely detected (Fig. 5, lane 6). When primed cells were then reexposed to NGF for 5 minutes, the level of tyrosine phosphorylation of PLC-γ1 was 1.7-fold (n = 3) that in naive cells on a per-milligram-of-protein basis (Fig. 5, lane 7). An increased ratio was also present at later time points. The data for tyrosine phosphorylation of gp140^{TrkA} and PLC-γ1 are evidence for a modest increase in NGF signaling through gp140^{TrkA} in primed cells.

NGF Induced Endocytosis of gp140^{TrkA}

Quite distinct from the increase in gp140^{TrkA} seen after prolonged NGF treatment, the early response to NGF was characterized by a rather dramatic decrease in gp140^{TrkA} (Fig. 2). Given that gp110^{TrkA} was not affected, this finding was most consistent with an NGF-mediated increase in the degradation of gp140^{TrkA} receptors. There are relatively few data for NGF effects on the cellular disposition of gp140^{TrkA}. However, ligand-mediated cell-surface down-regulation of other receptor tyrosine kinases is linked to lysosomal degradation of these receptors (Beguinot et al. 1984; Stoscheck and Carpenter 1984; Sorkin and Waters 1993), and in earlier studies it was shown that NGF treatment induced down-regulation of surface

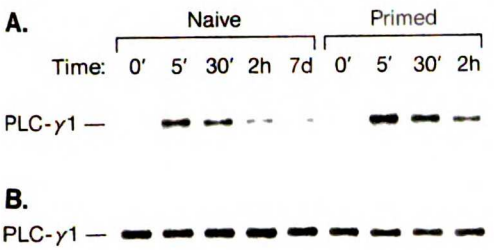

Figure 5. NGF-mediated tyrosine phosphorylation of PLC-γ1 was increased in primed cells. Naive and primed PC12 cells were untreated (0') or treated with NGF (50 ng/ml) for 5 min, 30 min, 2 hr, or 7 days. Cells were lysed, and equal amounts of protein were immunoprecipitated with anti-PLC-γ1 antibody. Immunoprecipitated proteins were analyzed on SDS-PAGE and probed first with anti-phosphotyrosine antibody (*A*) and then with anti-PLC-γ1 antibody (*B*). (Reprinted, with permission, from Zhou et al. 1995.)

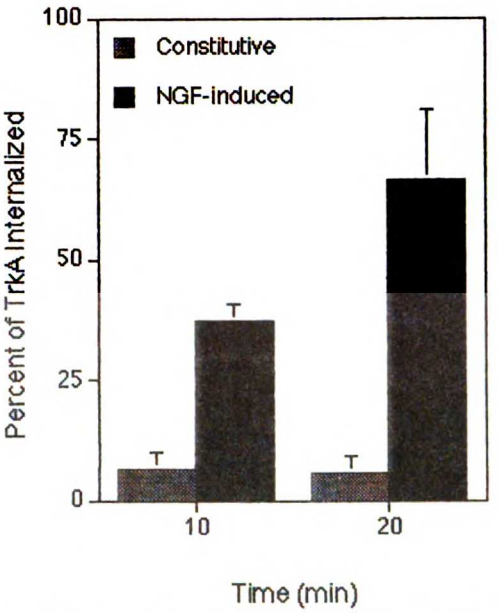

Figure 6. Constitutive and NGF-induced TrkA internalization. Experiments were performed as described in Materials and Methods, and the percent internalization of TrkA was computed as described. Values are mean (± S.E.M) from three separate experiments at 10 min and two at 20 min. There was a low level of constitutive internalization. NGF markedly increased TrkA internalization.

$gp140^{TrkA}$ in PC12 cells (Hosang and Shooter 1987). To ask if trafficking of $gp140^{TrkA}$ is similar to that of other receptor tyrosine kinases, we attempted first to confirm that NGF decreased surface $gp140^{TrkA}$. For these experiments, ^{125}I-labeled NGF (100 pM) was bound to its receptors on PC12 cells at 4°C to inhibit membrane traffic. In the continued presence of ^{125}I-labeled NGF, the cells were then either warmed to 37°C for 60 minutes or kept at 4°C. ^{125}I-labeled NGF was then cross-linked to $gp140^{TrkA}$ at 4°C, and TrkA immunoprecipitates were analyzed by SDS-PAGE. There was a marked decrease in cross-linking to $gp140^{TrkA}$ under these conditions; indeed, in naive cells only 16% of the amount detected in cells held at 4°C was present after warming (Fig. 3). Surface $gp140^{TrkA}$ was also down-regulated in primed cells.

To show that NGF-mediated down-regulation of surface TrkA was due to enhanced endocytosis of these receptors, we used surface biotinylation (Schmid and Carter 1990) to examine trafficking of $gp140^{TrkA}$. Cells were incubated either in the presence of NGF (1 nM) or in its absence at 4°C. NHS-SS-biotin was used to biotinylate surface proteins. After removing unreacted NHS-SS-biotin, cells were warmed to 37°C for 10 minutes to allow endocytosis, or not warmed. After chilling (4°C) the samples, glutathione was added to release biotin only on cell-surface proteins (Schmid and Carter 1990). TrkA immunoprecipitates were submitted to SDS-PAGE and blotting; ^{125}I-labeled streptavidin was used to detect biotinylated TrkA. Endocytosed $gp140^{TrkA}$ molecules would be expected to retain their biotin; the extent of $gp140^{TrkA}$ biotinylation was therefore taken as a measure of $gp140^{TrkA}$ endocytosis. Figure 6 shows the results of these studies. Whether or not cells were treated with NGF, endocytosis of $gp140^{TrkA}$ was increased by warming. In the absence of NGF, 6% of surface $gp140^{TrkA}$ was internalized after 10 minutes. This finding points to constitutive endocytosis of TrkA receptors. Remarkably, after 10 minutes warming with NGF, approximately 37% of TrkA was internalized. More than 66% of labeled TrkA was internalized after 20 minutes warming with NGF (Fig. 6). Thus, NGF induced extensive, rapid endocytosis of cell-surface $gp140^{TrkA}$.

Evidence that Endocytosis of $gp140^{TrkA}$ Creates a Signaling Endosome

Endocytosis of activated receptor tyrosine kinases targets these receptors to lysosomes where degradation prevents the possibility of further signaling (Beguinot et al. 1984; van der Geer et al. 1994). Some have suggested that activated receptors in endosomes could have a role in signaling (Baass et al. 1995), but the physiological significance of such signaling is uncertain. We entertained the intriguing possibility that via endocytosis of activated $gp140^{TrkA}$ receptors, NGF signaling could be communicated from axon tips to neuronal cell bodies. This arrangement was suggested by noting that NGF is a target-derived factor. For many NGF-responsive neurons, the target field of innervation provides the principal source of NGF (Longo et al. 1993). Thus, NGF is available to bind and activate its receptors on distal axons. Prior studies have made clear the importance of signaling through receptors on axons (Campenot 1977), but they did not elucidate how this signaling is communicated down the

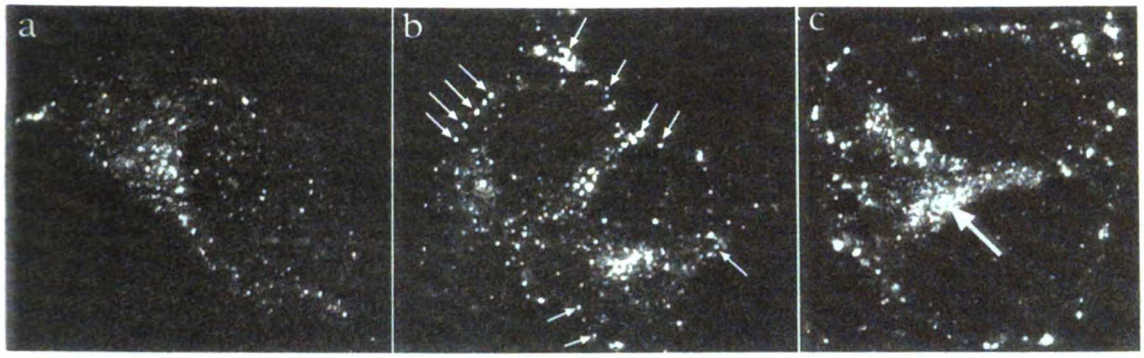

Figure 7. NGF changed the distribution of TrkA immunostaining in PC12 cells. Cells were exposed to media at 37°C without NGF (*a*), or with NGF (2 nM) for 2 min (*b*) or 60 min (*c*). A Trk-specific antibody, sc11, was used to examine the distribution of TrkA. Most TrkA staining was intracellular. With NGF treatment there was an increase in bright punctate staining near the plasma membrane (e.g., *small arrows* in *b*). Note the marked increase with NGF treatment of TrkA staining in the juxtanuclear region at 60 min (*large arrow* in *c*).

axon to the cell body. Evidence that NGF induced rapid, robust endocytosis of $gp140^{TrkA}$ suggested that retrograde transport of activated $gp140^{TrkA}$ receptors in endocytic vesicles could serve as the signal.

TrkA was present in endocytic vesicles. The signaling endosome hypothesis predicts that activated $gp140^{TrkA}$ would be present in endocytic vesicles. To test this we used immunofluorescence and confocal mi-

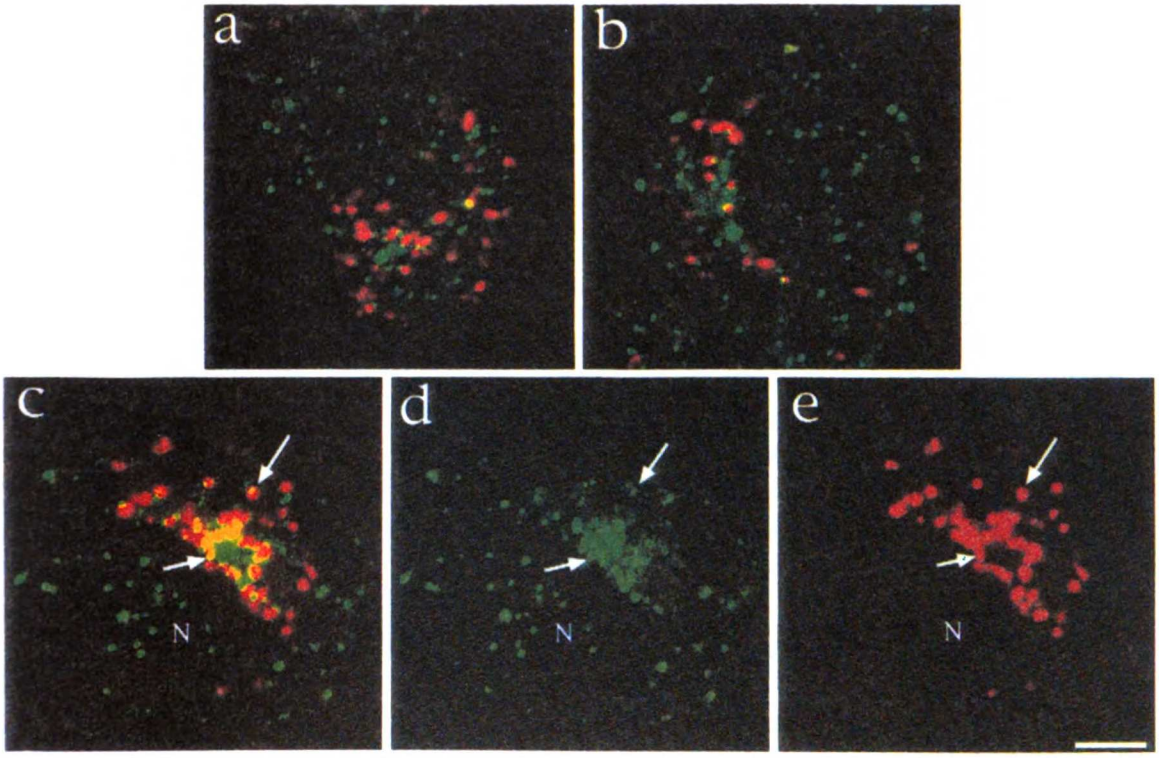

Figure 8. TrkA staining was colocalized with a lysosomal marker after 60 min of NGF treatment. Cells were exposed to media at 37°C without NGF (*a*), or with NGF (2 nM) for 2 min (*b*) or 60 min (*c, d,* and *e*). A Trk-specific antibody, sc11, was used to examine the distribution of TrkA. TrkA immunoreactivity was detected using an FITC-conjugated goat anti-rabbit IgG. GM-10 was used to visualize lysosomal staining. GM-10 immunoreactivity was detected with a Texas-Red-bound goat anti-mouse IgG. Panels *a, b,* and *c* show the labeling patterns for both markers. The immunostaining patterns after 60 min NGF treatment for TrkA (*d*) and GM10 (*e*) are also shown. N denotes the nucleus. The bar in panel *e* is 4 μM.

croscopy to localize TrkA proteins. PC12 cells were incubated with or without NGF (2 nM) at 37°C. Cells were then fixed, permeabilized, and incubated with sc11, an antibody to the carboxyl terminus of human TrkA. Fluoresceinated goat anti-rabbit IgG was used to detect antibody binding. In cells not exposed to NGF, TrkA staining was predominantly intracellular (Fig. 7a). Some immunoreactivity was found near the

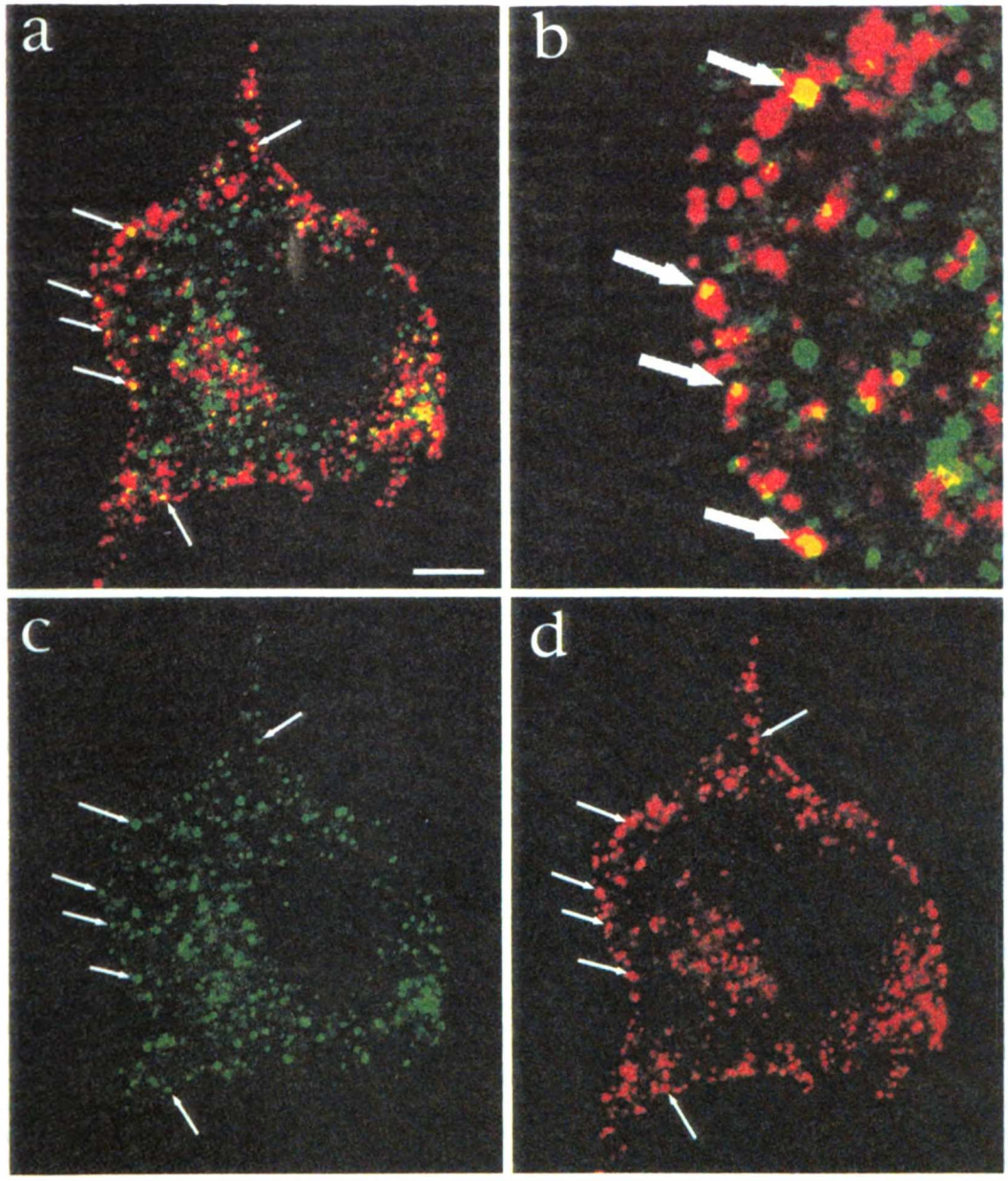

Figure 9. NGF treatment resulted in TrkA and clathrin colocalization. PC12 cells were treated with NGF for 30 sec. After fixation and treatment with saponin to induce permeabilization, cells were immunostained using antibodies to Trk (sc11) and to the clathrin heavy chain (X22). TrkA immunoreactivity, detected using FITC-conjugated goat anti-rabbit IgG, is shown in green. Clathrin heavy chain immunoreactivity, detected using Texas-Red-conjugated goat anti-mouse IgG, is in red. In the absence of NGF, there was little overlap in TrkA and clathrin staining except in the juxtanuclear region (data not shown). In the presence of NGF, TrkA (*c*) and clathrin (*d*) staining were widely distributed in the cytosol; some staining was seen for both near the plasma membrane. Panel *a* shows colocalization of TrkA and clathrin staining near the plasma membrane (*arrows*) (yellow denotes colocalization). The organelles showing colocalization had the same distribution and size as those seen with increased frequency following NGF treatment (Fig. 7). Panel *b* shows a 3-fold magnification of panel *a* to provide a better view of the colocalizations on the left side of the cell. Bar in panel *a* is 6 μm. (Modified, with permission, from Grimes et al. 1996.)

nucleus; most was present as small punctate accumulations distributed throughout the cytoplasm. With NGF treatment there was within minutes the appearance of many densely stained punctate structures, most of which were present near the cell surface (Fig. 7b). The number of bright punctate organelles located within 0.5 μm of the cell surface was quantified. After 30-second treatment, the number increased approximately 3-fold from 0.08 (± 0.01) puncta/μm of cell perimeter in untreated cells (n = 12) to 0.21 (± 0.02) puncta/μm in NGF-treated cells (n = 12) (student's t-test, p <0.05). At later times (Fig. 7c), TrkA staining was most prominent in the perinuclear region. To examine the possibility that this perinuclear staining was due to the presence of TrkA in lysosomes, we looked at the subcellular localization of immunostaining for TrkA and the lysosomal marker, GM10. In the absence of NGF, there was little colocalization (Fig. 8a). The same was true after brief NGF treatment (Fig. 8b). However, after NGF treatment for 60 minutes, TrkA present in the perinuclear region was colocalized, in part, with GM10 (Fig. 8c). The presence of TrkA immunostaining in lysosomes at 60 minutes, and the observations on gp140^{TrkA} cited above, suggest that endocytosed TrkA was destined for degradation.

To characterize further the immunostained puncta seen near the plasma membrane with NGF treatment, we attempted to colocalize TrkA with markers of the clathrin-coated pit endocytic pathway. Cells incubated with or without NGF at 37°C for 30 seconds were chilled and fixed. Clathrin was visualized with antibodies to the clathrin heavy chain (X22) and to α-adaptin (AP.6). Without NGF treatment there was a small amount of TrkA and clathrin colocalization near the nucleus (data not shown). This was probably due to the presence of these proteins in the biosynthetic pathway. Only rarely were TrkA and clathrin colocalized in puncta near the cell surface. In the presence of NGF, however, TrkA and clathrin could be colocalized in many more puncta near the cell surface. The result shown in Figure 9 demonstrates a particularly marked example. The number of organelles in which TrkA and clathrin were colocalized within 0.5 mM of the plasma membrane was quantified. In cells not treated with NGF there was a range of 0 to 2 puncta/cell with a mean of 0.68 (± 0.10) (n = 40 cells). With NGF the range was 1 to 18 puncta per cell, and the mean increased nearly 5-fold to 3.25 (± 0.56) (n = 31 cells) (student's t-test, p<0.001). TrkA was also colocalized with α-adaptin in NGF-treated cells (data not shown). These data are evidence that TrkA is found in endocytic vesicles produced via the clathrin-coated pit pathway.

NGF was bound to gp140^{TrkA} in endocytic vesicles. TrkA expression confers on cells the ability to internalize NGF (Jing et al. 1992). To characterize further the endocytic organelles that contain TrkA, we examined those that could be labeled with ^{125}I-labeled NGF. After incubating PC12 cells with ^{125}I-labeled NGF (1 nM) for 1 hour at 4°C, we washed them briefly to enrich for binding to slowly dissociating receptors (Zhou et al. 1995) and to minimize fluid-phase endocytosis of free ligand. Cells were then warmed for 10 minutes and chilled (4°C). To prepare intracellular organelles, we took advantage of the fact that mechanically permeabilized cells release untethered organelles (Grimes and Kelly 1992a,b). A Balch homogenizer (Balch and Rothman 1985; Martin and Walent 1989) was used to permeabilize cells, and released membranes were then harvested. Cytosol and released organelles were separated from the cell ghosts (P1) by centrifugation at 1000g (10 minutes). Differential centrifugation was used to separate large vesicle-containing fractions (P2) and small vesicle-containing fractions (P3) from cytosol (S3) (see Methods). Using these methods, we showed that endocytic vesicles could be isolated free of plasma membrane. In one series of experiments, cells incubated with ^{125}I-labeled NGF at 4°C were washed, and the cross-linker BS^3 was added prior to permeabilization and fractionation. Under these conditions, we did not detect a cross-linked NGF/gp140^{TrkA} complex in either P2 or P3. In a second series, we asked whether surface amyloid precursor protein (APP) could be detected in P2 or P3. Cells were incubated with NHS-SS-biotin at 4°C to biotinylate surface APP; they were then permeabilized and fractionated. The amount of surface-labeled APP detected in P2 was 2.9% (n = 2), of that in P1; the corresponding value for P3 was 0.6% (n = 2), and for S3 it was 0.1% (n = 2). Thus, the intracellular organelles that emerged from permeabilized cells were virtually free of plasma membrane. We quantified the amount of internalized ^{125}I-labeled NGF that was recovered in membrane fractions: 6.9% (± 0.6; n = 9) of total cell-associated ^{125}I-labeled NGF was recovered in P2 and 3.1% (± 0.4; n = 9) was present in P3. Using acid washing, we found that 49.8% (± 4.9%, n = 3) of the total bound ^{125}I-labeled NGF was on the cell surface under these conditions. Therefore, P2 and P3 together contained about one-fifth of total intracellular ^{125}I-labeled NGF. These studies showed that the fractionation scheme allowed us to recover a substantial fraction of intracellular organelles that contained internalized NGF, and that ^{125}I-labeled NGF was present in both the large and small vesicle-containing fractions.

To ask whether gp140^{TrkA} was bound to NGF in intracellular organelles, PC12 cells were incubated with ^{125}I-labeled NGF (1 nM) at 4°C, washed, warmed 10 minutes as above, then chilled. Following permeabilization, DSS, a membrane-permeable cross-linking reagent, was added to the cell suspension prior to fractionation. gp140^{TrkA} was immunoprecipitated from lysates prior to SDS-PAGE. A radiolabeled band that migrated at the position expected for a complex containing ^{125}I-labeled NGF cross-linked to TrkA was seen in P1, P2, and P3 but not in the cytosol (S3) (Fig. 10). These data are evidence that gp140^{TrkA} and NGF

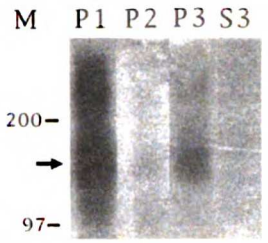

Figure 10. TrkA was cross-linked to NGF in intracellular organelles. Cells were incubated with ^{125}I-labeled NGF (1 nM), washed, warmed 10 min (37°C), chilled (4°C), and then permeabilized and fractionated. The membrane-permeable cross-linking reagent DSS was added before fractionation. One-fifth of the cell ghost membranes (P1), the entire 8000*g* pellet (P2), the entire 100,000*g* pellet (P3), and one-tenth of the 100,000*g* supernatant (S3) were immunoprecipitated with 1088 and analyzed by SDS-PAGE and autoradiography. The arrow marks the 150-kD cross-linked complex containing TrkA and ^{125}I-labeled NGF in P1, P2, and P3. The more slowly migrating complex may correspond to that seen in Fig. 3. There was no cross-linking when the experiment was carried out in the presence of unlabeled NGF (1 μM). The amount of ^{125}I-labeled NGF cross-linked to TrkA was quantified by Phosphorimager.

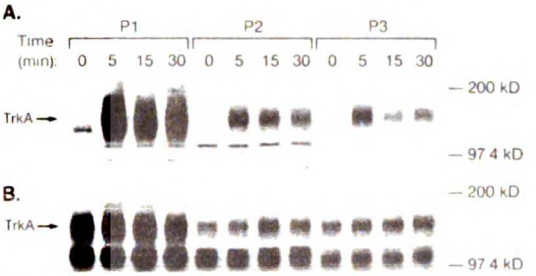

Figure 11. TrkA and tyrosine-phosphorylated TrkA were detected in intracellular organelles. PC12 cells were incubated without NGF (0) or with NGF (2 nM) for 5, 15, or 30 min at 37°C. Cells were then chilled (4°C), permeabilized, and fractionated. Using 1088, TrkA was immunoprecipitated from 1/3 of P1, or from the entire P2 or P3 fractions. (*A*) A Western blot of immunoprecipitates probed with antiphosphotyrosine antibody (4G10) followed by HRP-conjugated anti-mouse IgG. Chemiluminescence was used for detection. (*B*) The blot in *A* was stripped and probed with RTA followed by HRP-conjugated anti-rabbit IgG. Chemiluminescence was used for detection. In P1, there was a modest (9%) but significant decrease at 5 min in gp140^{TrkA} ($p<0.04$); although gp140^{TrkA} was increased at 5 min in P2 and P3 in the experiment shown, the increases varied across experiments and the changes were not significant.

were present together in the same compartments after internalization, and that NGF remained bound to gp140^{TrkA} after endocytosis.

Endocytic vesicles contained activated gp140^{TrkA}. To determine whether organelles derived from endocytosis contained activated TrkA, cells were cultured at 37°C in the absence of NGF, or with NGF (2 nM) for 5, 15, or 30 minutes. They were then chilled and submitted to permeabilization and fractionation, as above. TrkA present in lysates was immunoprecipitated and submitted to SDS-PAGE and blotting. Both gp140^{TrkA} and gp110^{TrkA} were detected in P1, P2, and P3 (Fig. 11, panel B). Neither was detected in S3 (data not shown). The presence of gp110^{TrkA} suggests that the endoplasmic reticulum, or vesicles derived from it, emerge upon permeabilization. Regulated and constitutive secretory vesicles also emerge from permeabilized cells (Grimes and Kelly 1992a,b). Newly synthesized TrkA should be present in these. Since receptors in the biosynthetic pathway have no direct access to NGF, it can be assumed that they have not been activated. The presence of phosphotyrosine on TrkA is a measure of its activation (Stephens et al. 1994). Thus, tyrosine-phosphorylated TrkA should comprise plasma membrane and internalized TrkA. Activated TrkA was detected by blotting TrkA immunoprecipitates with an anti-phosphotyrosine antibody. Fractions prepared from cells not exposed to NGF contained very little tyrosine-phosphorylated TrkA (Fig. 11A). In the NGF-treated cells, there was a marked increase in tyrosine-phosphorylated TrkA in P1, P2 and P3. Only gp140^{TrkA} was tyrosine-phosphorylated. The amount of tyrosine-phosphorylated gp140^{TrkA} was greatest at 5 minutes in all three fractions. Although the amount decreased at later time points, it was present through 30 minutes. These data are evidence that activated gp140^{TrkA} is present in endocytic vesicles following NGF treatment.

Another measure of TrkA activation, one that contributes to TrkA signaling leading to differentiation, is binding and tyrosine phosphorylation of SHC (Stephens et al. 1994). To ask whether SHC was bound to internalized TrkA receptors, we examined immunoprecipitates from cells that were incubated without NGF, or with NGF (2 nM), for 5 or 15 minutes at 37°C prior to chilling (4°C). After permeabilization, cells were fractionated. Lysates from P2, P3, and S3 were immunoprecipitated with antibodies to SHC. SHC immunoprecipitates were submitted to SDS-PAGE, and blotted proteins were probed with antibodies to phosphotyrosine (Fig. 12, panel A), TrkA (panel B), or SHC (panel C). As reported previously (Stephens et al. 1994), three SHC proteins were detected in PC12 cell immunoprecipitates: p68SHC, p53SHC, and p48SHC. Although the amount of each was greatest in S3, each SHC protein was also present in P2 and P3. With NGF treatment there was no apparent increase in SHC in P2 or P3, but two changes were evident. First, all three SHC species were more heavily tyrosine-phosphorylated (panel A); the change was most apparent in P2 and P3. Second, NGF treatment induced a shift in the electrophoretic mobility of p68SHC, a finding consistent with earlier studies (Stephens et al. 1994). Importantly, gp140^{TrkA} was

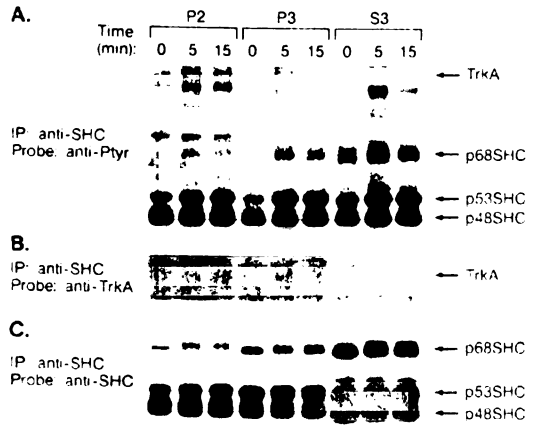

Figure 12. SHC was bound to TrkA in intracellular organelles. PC12 cells were incubated without NGF (0) or with NGF (2 nM) for 5 or 15 min at 37°C. Cells were chilled (4°C), permeabilized, and fractionated. P2, P3, and S3 were lysed and immunoprecipitated with anti-SHC antibodies. Immunoprecipitates were Western blotted with antiphosphotyrosine antibody (4G10) (*A*), then stripped and reprobed with anti-TrkA (RTA) (*B*), and finally, with anti-SHC (*C*). Chemiluminescence was used for detection. NGF increased the amount of tyrosine-phosphorylated SHC and its association with $gp140^{TrkA}$ in P2 and P3. NGF treatment also increased the amount of a tyrosine-phosphorylated band that migrated slightly faster than $gp140^{TrkA}$. Unlike $gp140^{TrkA}$, it was also present in S3; its identity is unknown.

present in the SHC immunoprecipitates of NGF-treated cells. Panel A shows a tyrosine-phosphorylated band in the P2 and P3 fractions at the position for TrkA; the amount was increased by NGF treatment and was greatest at 5 minutes. That this band is $gp140^{TrkA}$ is indicated by immunostaining for TrkA in panel B. Here again, the amount of $gp140^{TrkA}$ was increased with NGF treatment. These data show that activated $gp140^{TrkA}$ in intracellular organelles is associated with tyrosine-phosphorylated SHC. In other studies, we discovered that activated PLC-γ1 is also associated with tyrosine-phosphorylated $gp140^{TrkA}$ in the P2 and P3 fractions (Grimes et al. 1996). These findings are strong evidence for the ability of activated $gp140^{TrkA}$ in endocytic vesicles to signal.

DISCUSSION

NGF acts to evoke the responses required for maintenance of neuronal viability and enhanced neuronal differentiation. Defining the biochemical and cellular events that characterize these responses is an important objective. The discovery that TrkA is a receptor tyrosine kinase for NGF (Kaplan et al. 1991; Klein et al. 1991; Meakin and Shooter 1991) and that it has an essential role in NGF signaling (Loeb and Greene 1993) has greatly facilitated these studies. We discovered that NGF regulated TrkA in PC12 cells in distinct early and late phases. Within minutes of NGF treatment there was activation of TrkA and marked changes in TrkA trafficking, with resulting down-regulation of surface and cellular TrkA. After prolonged NGF treatment there was increased *TrkA* expression, and this was associated with a modest enhancement of NGF signaling. To understand how these responses are integrated in vivo, we entertained the hypothesis that endocytosis of activated TrkA at axon tips provides a means for retrogradely transporting the NGF signal to cell bodies, where it influences gene expression prior to its degradation in lysosomes. In testing this hypothesis, we discovered in PC12 cells that: (1) NGF induced rapid internalization of TrkA; (2) NGF and TrkA were both found in intracellular vesicles; (3) NGF was bound to TrkA in these vesicles; (4) TrkA receptors in vesicles were activated, as assessed by tyrosine-phosphorylation and association with activated SHC and PLC-γ1; and (5) TrkA receptors were targeted to lysosomes in NGF-treated cells. Our findings raise the possibility that it is through the creation of signaling endosomes containing activated TrkA that NGF signals retrogradely to regulate neuronal survival and differentiation. Since continuous NGF signaling may be required to maintain the trophic effect (Zhou et al. 1995), NGF induction of *TrkA* expression may be necessary for replacing degraded receptors. Thus, NGF may increase *TrkA* expression to both maintain and enhance signaling through this receptor.

In earlier work, it was apparent that NGF induces expression of TrkA (Holtzman et al. 1992; Meakin et al. 1992). To explore the biological significance of this finding, we examined NGF signaling in PC12 cells. We discovered that after prolonged NGF exposure there was an increase in gene expression for both $gp140^{TrkA}$ and $p75^{NGFR}$. Increased receptor gene expression was correlated with increased receptor binding. In cross-linking studies, NGF induction of *TrkA* expression was shown to result in increased cell-surface $gp140^{TrkA}$ receptors. Interestingly, the increase in NGF binding to $gp140^{TrkA}$ was smaller than the increase in $gp140^{TrkA}$ protein. When expressed on a per-cell basis, a greater than 200% increase in total $gp140^{TrkA}$ protein was associated with only a 60% increase in NGF binding to slowly dissociating receptors and a 100% increase in cross-linking to $gp140^{TrkA}$ at the cell surface. When these data are expressed on a per-milligram of protein basis, $gp140^{TrkA}$ was nearly doubled, while there was little, if any, increase in binding. These observations suggest that the number of surface $gp140^{TrkA}$ receptors is not simply a function of the total protein level. Although further studies are required, our data suggest that trafficking of $gp140^{TrkA}$ is complex, even in the absence of NGF. For example, we noted that much immunostaining for TrkA was intracellular, pointing to possible controls on exocytosis. There was also constitutive endocytosis of $gp140^{TrkA}$. Regulation of the extent to which newly synthesized receptors are inserted at the cell surface and on the recycling of recep-

tors to the plasma membrane could have an important influence on the number of surface receptors. Our data raise the possibility that the number of cell-surface TrkA receptors is regulated at several levels.

There was enhanced NGF signaling in primed cells, as evidenced by increased tyrosine phosphorylation of gp140^{TrkA} and PLC-γ. These experiments link increased *TrkA* expression to increased signaling and suggest that the increase in gp140^{TrkA} played a role. Of note, the difference in signaling between primed and naive cells was greatest 2 hours after NGF addition, at which time it more closely approximated the increase in total cellular gp140^{TrkA} than the increase in receptor binding. It is tempting to speculate that increased signaling in primed cells was due, in part, to increased trafficking of gp140^{TrkA} to the plasma membrane. If so, NGF may have influenced its own signaling through increasing the number of receptors as well as through enhancing their movement to the plasma membrane. It will be important to more carefully examine TrkA trafficking in primed cells and to show whether there is a link between increased gp140^{TrkA} and increased NGF signaling in vivo. It is known that early exposure to exogenous NGF enhances the response to later treatment (Johnston et al. 1987), but it is not known that increased signaling through TrkA is responsible.

Whereas up-regulation of gp140^{TrkA} marked the response to prolonged NGF treatment, a very different response was seen within the first 60 minutes, during which time there was a marked decrease in gp140^{TrkA}. Two studies gave direct evidence that NGF treatment targeted gp140^{TrkA} for degradation. First, NGF was shown to down-regulate gp140^{TrkA} surface receptors through enhanced endocytosis. This was apparent in both biochemical and confocal microscopy studies. In the latter, the increase in TrkA-positive puncta near the cell surface and the increase in colocalization of TrkA and clathrin were consistent with increased TrkA endocytosis. Second, NGF treatment increased colocalization of TrkA with a lysosomal marker, suggesting that gp140^{TrkA} in endocytic vesicles was delivered to lysosomes. Surface down-regulation targets other receptor tyrosine kinases to lysosomes and is believed to serve an important role in signal up-regulation (van der Geer et al. 1994). For example, in the case of the receptor for the epidermal growth factor (EGF), EGF addition results in a pronounced and rapid decrease in surface receptors and accelerated receptor degradation (Beguinot et al. 1984, Stoscheck and Carpenter 1984; Murthy et al. 1986). In the case of EGF and EGFR, it has been presumed that endocytosis of receptors serves simply to turn off the signal. However, some have suggested that since EGFR is still activated following endocytosis, these receptors could have a role in intracellular signaling (Baass et al. 1995). Most TrkA signaling in vivo is likely to be initiated by NGF in contact with receptors on axons (Longo et al. 1993); we were intrigued by the possibility that, if these receptors continue to signal after endocytosis, trafficking them retrogradely would provide a means to convey the NGF signal to the cell body. In studies to address this hypothesis, we examined organelles and found continued NGF binding to gp140^{TrkA}. Moreover, we detected tyrosine-phosphorylated TrkA in intracellular organelles following NGF treatment. In studies similar to those reported here, we showed that NGF markedly increased the specific activity of tyrosine-phosphorylated TrkA in intracellular organelles (Grimes et al. 1996). Finally, we discovered that internalized activated TrkA formed complexes with tyrosine-phosphorylated SHC and with activated PLC-γ1 (Grimes et al. 1996). Taken together, our data suggest strongly that endocytosis of activated TrkA receptors has two consequences for NGF signaling. One is the creation of a signaling endosome. The second is the eventual degradation of this signal in lysosomes.

NGF is critical for the normal survival and differentiation of several populations of PNS and CNS neurons (Levi-Montalcini 1987; Longo et al. 1993; Crowley et al. 1994; Li et al. 1995). Since NGF gene expression is localized to target tissues (Longo et al. 1993), a mechanism must exist to carry the NGF signal retrogradely from the processes of neurons to their cell bodies. Earlier studies characterizing retrograde NGF signaling showed that, although NGF and the signaling process were similar with respect to both the time course for transport and the requirement for microtubules (Hendry et al. 1974a,b; Paravicini et al. 1975; Hendry and Bonyhady 1980), NGF itself was not the signal (Heumann et al. 1984). Internalized, activated TrkA is an attractive candidate for the NGF retrograde signal. Recently, Ehlers et al. (1995) showed that NGF infusion into the rat foot-pad, the target of sciatic nerve sensory neurons, induced an increased accumulation of tyrosine-phosphorylated TrkA distal to a ligature on the sciatic nerve. Their experiments showed that target-derived NGF regulates retrograde transport of TrkA. However, they did not examine the mechanism by which NGF accomplishes this effect or demonstrate its significance for NGF signaling. The studies reported herein suggest that the increased retrograde transport of activated TrkA seen in their studies followed induction of rapid, extensive endocytosis of these receptors in the distal processes of DRG neurons.

Current evidence suggests that activated TrkA in axonal endosomes would be protected from degradation. Studies on the endosomal-lysosomal pathway in neurons (Parton et al. 1992; Hollenbeck 1993; Parton and Dotti 1993; Nixon and Cataldo 1995) indicate that late endosomes and lysosomes are located predominantly in the cell body and proximal dendrites. Indeed, there was no evidence for these organelles in the axons or presynaptic terminals of cultured hippocampal neurons (Parton et al. 1992). Recent evidence shows that only a few acidified organelles can be found in distal axons (Overly et al. 1995). There are few observations that address directly axonal transit of endocytosed NGF and TrkA. As just indicated, TrkA retrogradely

transported in the sciatic nerve was apparently intact (Ehlers et al. 1995). In earlier studies, retrogradely transported NGF was also shown to be intact (Claude et al. 1982), and, using electron microscopy (EM), an NGF-HRP conjugate was not found in degradative organelles in axons (Schwab 1977). Indeed, it is possible that NGF would remain bound to $gp140^{TrkA}$ during axonal transport since, even at a pH characteristic of acidified endocytic organelles in axons (pH 5.5) (Overly et al. 1995), most NGF remains bound to $gp140^{TrkA}$ (J. Zhou et al., unpubl.). These data combine to suggest that NGF, and the TrkA receptors endocytosed in response to NGF binding, would resist degradation during retrograde transport.

Our observations support the hypothesis that through endocytosis of activated TrkA, NGF creates signaling endosomes that convey its retrograde signal and that, following delivery to the cell body, activated receptors are degraded. It is reasonable to suggest that because there is degradation of activated TrkA receptors, NGF induction of *TrkA* gene expression is required for NGF signaling to continue. How it is that retrograde TrkA signaling is linked to new TrkA synthesis is uncertain, but a direct effect of TrkA signaling on *TrkA* gene expression is possible. This arrangement creates some interesting consequences, as well as problems. One consequence would be that the levels of TrkA receptors are buffered. For example, although increased NGF levels would result in a rapid increase in endocytosis and degradation, the resulting increase in TrkA synthesis would act over a longer interval to blunt the decrease. Thus, the trophic state of NGF-responsive neurons would be driven by NGF levels in the postsynaptic target without major changes in the number of cell-surface TrkA receptors. This situation would ensure that even with marked increases or decreases in NGF levels, responsive neurons would be able to continue to respond to NGF in their target. However, this same arrangement predisposes to potentially serious problems if there is uncoupling of degradation and synthesis. For example, one can envision a situation in which there is degradation of activated receptors without the benefit of enhanced receptor synthesis. In this case, there would be a progressive decrease in the number of NGF receptors and NGF signaling would fail, possibly with neuronal atrophy and death as consequences. It is interesting to consider disease states in which NGF-responsive neurons are affected. BFCNs are known to atrophy and die in Alzheimer's disease (AD), and their loss may contribute to cognitive decline (Coyle 1983). One aspect of AD neuropathology, highlighted by the studies of Nixon and colleagues (Cataldo et al. 1996), is a marked increase in the number of late endosomes and lysosomes in AD neurons. Cataldo et al. (1996) recently reported that pyramidal neurons of the cortex and hippocampus of the AD brain showed a 2- to 8-fold increase in the number of such structures. Lysosomal structures have also been shown to be increased in the axons and dendrites of AD patients. In early EM studies, Suzuki and Terry (1967) showed a marked increase in dense bodies in dystrophic neurites in the AD cortex. These dense bodies were acid phosphatase-positive, signifying their lysosomal character (Suzuki and Terry 1967). These observations may have relevance to NGF signaling. If endocytic vesicles containing activated TrkA are trafficked to lysosomes in BFCN axons, rather than to their cell bodies, the NGF signal would be destroyed prematurely. There are data to suggest this scenario. Whereas NGF levels in the cortex and hippocampus of AD patients are increased 2-fold relative to normal, the NGF level in the basal forebrain is only one-half normal (Scott et al. 1995). These data suggest a failure of NGF retrograde transport. Indeed, Mufson et al. (1995) have used immunocytochemical studies to suggest that less NGF is present in individual BFCNs in the AD brain. Although a number of alternatives can be envisioned to explain these results, the most attractive possibility is that retrograde transport of NGF and of activated TrkA are decreased in AD. Studies to test these ideas in animal models will help to define whether activation of the endosomal-lysosomal system can interrupt retrograde NGF signaling and, if so, what significance this has for the death of BFCNs in AD.

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REFERENCES

Baass, P.C., G.M. Di Guglielmo, F. Authier, B.I. Posner, and J.J.M. Bergeron. 1995. Compartmentalized signal transduction by receptor tyrosine kinases. *Trends Cell Biol.* **5:** 465.

Balch, W.E. and J.E. Rothman. 1985. Characterization of protein transport between successive compartments of the Golgi apparatus: Asymmetric properties of donor and acceptor activities in a cell-free system. *Arch. Biochem. Biophys.* **240:** 413.

Barker, P.A. and E.M. Shooter. 1994. Disruption of NGF binding to the low affinity neurotrophin receptor

p75LNTR reduces NGF binding to trkA on PC12 cells. *Neuron* **13:** 203.

Beguinot, L., R.M. Lyall, M.C. Willingham, and I. Pastan. 1984. Down-regulation of the epidermal growth factor receptor in KB cells is due to receptor internalization and subsequent degradation in lysosomes. *Proc. Natl. Acad. Sci.* **81:** 2384.

Bernd, P. and L.A. Greene. 1984. Association of ^{125}I-nerve growth factor with PC12 pheochromocytoma cells: Evidence for internalization via high-affinity receptors only and for long-term regulation by nerve growth factor of both high- and low-affinity receptors. *J. Biol. Chem.* **259:** 15509.

Bothwell, M. 1995. Functional interactions of neurotrophins and neurotrophin receptors. *Annu. Rev. Neurosci.* **18:** 223.

———. 1996. p75NTR: A receptor after all. *Science* **272:** 506.

Brodsky, F.M. 1985. Clathrin structure characterized with monoclonal antibodies. I. Analysis of multiple antigenic sites. *J. Cell Biol.* **101:** 2047.

Campenot, R.B. 1977. Local control of neurite development by nerve growth factor. *Proc. Natl. Acad. Sci.* **74:** 4516.

Carter, B.D., C. Kaltschmidt, B. Kaltschmidt, N. Offenhäuser, R. Böhm-Matthaei, P.A. Baeuerle, and Y.-A. Barde. 1996. Selective activation of NF-κB by nerve growth factor through the neurotrophin receptor p75. *Science* **272:** 542.

Cataldo, A.M., D.J. Hamilton, J.L. Barnett, P.A. Paskevich, and R.A. Nixon. 1996. Properties of the endosomal-lysosomal system in the human central nervous system: Disturbances mark most neurons in populations at risk to degenerate in Alzheimer's disease. *J. Neurosci.* **16:** 186.

Chin, D.J., R.M. Straubinger, S. Acton, I. Nathke, and F.M. Brodsky. 1989. 100-kDa polypeptides in peripheral clathrin-coated vesicles are required for receptor-mediated endocytosis. *Proc. Natl. Acad. Sci.* **86:** 9289.

Clary, D.O., G. Weskamp, L.R. Austin, and L.F. Reichardt. 1994. TrkA cross-linking mimics neuronal responses to nerve growth factor. *Mol. Biol. Cell* **5:** 549.

Claude, P., E. Hawrot, D.A. Dunis, and R.B. Campenot. 1982. Binding, internalization, and retrograde transport of ^{125}I nerve growth factor in cultured rat sympathetic neurons. *J. Neurosci.* **2:** 431.

Collins, S., M.G. Caron, and R.J. Lefkowitz. 1992. From ligand binding to gene expression: New insights into the regulation of G-protein-coupled receptors. *Trends Biochem Sci.* **17:** 37.

Coyle, J.T., D.L. Price, and M.R. DeLong. 1983. Alzheimer's disease: A disorder of cortical cholinergic innervation. *Science* **219:** 1184.

Crowley, C., S.D. Spencer, M.C. Nishimura, K.S. Chen, M.S. Pitts-Meek, M.P. Armanini, L.H. Ling, S.B. McMahon, D.L. Shelton, A.D. Levinson, and H.S. Phillips. 1994. Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* **76:** 1001.

Davies, A.M., K.-F. Lee, and R. Jaenisch. 1993. p75-deficient trigeminal sensory neurons have an altered response to NGF but not to other neurotrophins. *Neuron* **11:** 565.

Ehlers, M.D., D.R. Kaplan, D.L. Price, and V.E. Koliatsos. 1995. NGF-stimulated retrograde transport of TrkA in the mammalian nervous system. *J. Cell Biol.* **130:** 149.

Fusco, M., B. Olderfeld-Nowak, G. Vantini, N. Schiavo, M. Gradkowska, M. Zaremba, and A. Leon. 1989. Nerve growth factor affects uninjured, adult rat septohippocampal cholinergic neurons. *Neuroscience* **33:** 47.

Grady, E.F., A.M. Garland, P.D. Gamp, M. Lovett, D.G. Payan, and N.W. Bunnett. 1995. Delineation of the endocytic pathway of substance P and its seven-transmembrane domain NK1 receptor. *Mol. Biol. Cell* **6:** 509.

Grimaldi, K.A., J.C. Hutton, and K. Siddle. 1987. Production and characterization of monoclonal antibodies to insulin secretory granule membranes. *Biochem. J.* **245:** 557.

Grimes, M.L. and R.B. Kelly. 1992a. Sorting of chromogranin B into immature secretory granules in pheochromocytoma (PC12) cells. *Ann. N.Y. Acad. Sci.* **674:** 38.

———. 1992b. Intermediates in the constitutive and regulated secretory pathways released in vitro from semi-intact cells. *J. Cell Biol.* **117:** 539.

Grimes, M.L., J. Zhou, E.C. Beattie, E.C. Yuen, D.E. Hall, J.S. Valletta, K.S. Topp, J.H. LaVail, N.W. Bunnett, and W.C. Mobley. 1996. Endocytosis of activated TrkA: Evidence that nerve growth factor induces formation of signaling endosomes. *J. Neurosci.* **16:** 7950.

Haass, C., E.H. Koo, A. Mellon, A.Y. Hung, and D.J. Selkoe. 1992. Targeting of cell-surface β-amyloid precursor protein to lysosomes: Alternative processing into amyloid-bearing fragments. *Nature* **357:** 500.

Hantzopoulos, P.A., C. Suri, D.J. Glass, M.P. Goldfarb, and G.D. Yancopoulos. 1994. The low affinity NGF receptor, p75, can collaborate with each of the Trks to potentiate functional responses to the neurotrophins. *Neuron* **13:** 187.

Hartman, D.S., M. McCormack, R. Schubenel, and C. Hertel. 1992. Multiple trkA proteins in PC12 cells bind NGF with a slow association rate. *J. Biol. Chem.* **267:** 24516.

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

Hendry, I. and R. Bonyhady. 1980. Retrogradely transported nerve growth factor increases ornithine decarboxylase activity in rat superior cervical ganglia. *Brain Res.* **200:** 39.

Hendry, I.A., R. Stach, and K. Herrup. 1974a. Characteristics of the retrograde axonal transport system for nerve growth factor in the sympathetic nervous system. *Brain Res.* **82:** 117.

Hendry, I.A., K. Stockel, H. Thoenen, and L.L. Iversen. 1974b. The retrograde axonal transport of nerve growth factor. *Brain Res.* **68:** 103.

Heumann, R., M. Schwab, R. Merkl, and H. Thoenen. 1984. Nerve growth factor-mediated induction of choline acetyltransferase in PC12 cells: Evaluation of the site of action of nerve growth factor and the involvement of lysosomal degradation products of nerve growth factor. *J. Neurosci.* **4:** 3039.

Higgins, G.A., S. Koh, K.S. Chen, and F.H. Gage. 1989. NGF induction of NGF receptor gene expression and cholinergic neuronal hypertrophy within the basal forebrain of the adult rat. *Neuron* **3:** 247.

Hollenbeck, P.J. 1993. Products of endocytosis and autophagy are retrieved from axons by regulated retrograde organelle transport. *J. Cell Biol.* **121:** 305.

Holtzman, D.M., J. Kilbridge, Y. Li, E.T. Cunningham, N.J. Lenn, D.O. Clary, L.F. Reichardt, and W.C. Mobley. 1995. *TrkA* expression in the CNS: Evidence for the existence of several novel NGF-responsive CNS neurons. *J. Neurosci.* **15:** 1567.

Holtzman, D.M., Y. Li, L.F. Parada, S. Kinsman, C.K. Chen, J.S. Valletta, J. Zhou, J. Long, and W.C. Mobley. 1992. p140trk mRNA marks NGF-responsive forebrain neurons: Evidence that *trk* gene expression is induced by NGF.

Neuron **9:** 465.

Hosang, M. and E.M. Shooter. 1987. The internalization of nerve growth factor by high-affinity receptors on pheochromocytoma PC12 cells. *EMBO J.* **6:** 1197.

Jing, S., P. Tapley, and M. Barbacid. 1992. Nerve growth factor mediates signal transduction through trk homodimer receptors. *Neuron* **9:** 1067.

Johnston, M.V., J.L. Rutkowski, B.H. Wainer, J.B. Long, and W.C. Mobley. 1987. NGF effects on developing forebrain cholinergic neurons are regionally specific. *Neurochem. Res.* **12:** 985.

Kaplan, D.R. and R.M. Stephens. 1994. Neurotrophin signal transduction by the Trk receptor. *J. Neurobiol.* **25:** 1404.

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

Kim, U.H., D.J. Fink, H.S. Kim, D.J. Park, M.L. Contreras, G. Guroff, and S.G. Rhee. 1991. Nerve growth factor stimulates phosphorylation of phospholipase C-γ in PC12 cells. *J. Biol. Chem.* **266:** 1359.

Klein, R., S.Q. Jing, V. Nanduri, E. O'Rourke, and M. Barbacid. 1991. The *trk* proto-oncogene encodes a receptor for nerve growth factor. *Cell* **65:** 189.

Levi-Montalcini, R. 1987. The nerve growth factor 35 years later. *Science* **237:** 1154.

Li, Y., D.M. Holtzman, L.F. Kromer, D.R. Kaplan, J. Chua-Couzens, D.O. Clary, B. Knusel, and W.C. Mobley. 1995. Regulation of TrkA and ChAT expression in developing rat basal forebrain: Evidence that both exogenous and endogenous NGF regulate differentiation of cholinergic neurons. *J. Neurosci.* **15:** 2888.

Loeb, D.M. and L.A. Greene. 1993. Transfection with *trk* restores "slow" NGF binding, efficient NGF uptake, and multiple NGF responses to NGF-nonresponsive PC12 cell mutants. *J. Neurosci.* **13:** 2919.

Longo, F.M., D.M. Holtzman, M.L. Grimes, and W.C. Mobley. 1993. Nerve growth factor: Actions in the peripheral and central nervous systems. In *Neurotrophic factors* (ed. J. Fallon and S. Loughlin), p. 209. Academic Press, New York.

Lucidi-Phillipi, C.A., D.O. Clary, L.F. Reichardt, and F.H. Gage. 1996. TrkA activation is sufficient to rescue axotomized cholinergic neurons. *Neuron* **16:** 653.

Mahadeo, D., L. Kaplan, M.V. Chao, and B.L. Hempstead. 1994. High affinity nerve growth factor binding displays a faster rate of association than $p140^{trk}$ binding. *J. Biol. Chem.* **269:** 6884.

Martin, T.F. and J.H. Walent. 1989. A new method for cell permeabilization reveals a cytosolic protein requirement for Ca^{2+}-activated secretion in GH3 pituitary cells. *J. Biol. Chem.* **264:** 10299.

Martin-Zanca, D., R. Oskam, G. Mitra, T. Copeland, and M. Barbacid. 1989. Molecular and biochemical characterization of the human *trk* proto-oncogene. *Mol. Cell. Biol.* **9:** 24.

Meakin, S.O. and E.M. Shooter. 1991. Tyrosine kinase activity coupled to the high-affinity nerve growth factor-receptor complex. *Proc. Natl. Acad. Sci.* **88:** 5862.

———. 1992. The nerve growth factor family of receptors. *Trends Neurosci.* **15:** 323.

Meakin, S.O., U. Suter, C.C. Drinkwater, A.A. Welcher, and E.M. Shooter. 1992. The rat trk protooncogene product exhibits properties characteristic of the slow nerve growth factor receptor. *Proc. Natl. Acad. Sci.* **89:** 2374.

Miller, T., T.C. Mathew, and J.G. Toma. 1991. Regulation of nerve growth factor receptor gene expression by nerve growth factor in the developing peripheral nervous system. *J. Cell Biol.* **112:** 303.

Mobley, W.C., J.L. Rutkowski, G.I. Tennekoon, J. Gemski, K. Buchanan, and M.V. Johnston. 1986. Nerve growth factor increases choline acetyltransferase activity in developing basal forebrain neurons. *Mol. Brain Res.* **387:** 53.

Mufson, E.J., J.M. Conner, and J.H. Kordower. 1995. Nerve growth factor in Alzheimer's disease: Defective retrograde transport to nucleus basalis. *NeuroReport* **6:** 1063.

Murthy, U., M. Basu, A. Sen-Majumdar, and M. Das. 1986. Perinuclear location and recycling of epidermal growth factor receptor kinase: Immunofluorescent visualization using antibodies directed to kinase and extracellular domains. *J. Cell Biol.* **103:** 333.

Nixon, R.A., and A.M. Cataldo. 1995. The endosomal-lysosomal system of neurons: New roles. *Trends Neurosci.* **18:** 489.

Ohmichi, M., S.J. Decker, L. Pang, and A.R. Saltiel. 1991. Nerve growth factor binds to the 140 kd *trk* proto-oncogene product and stimulates its association with the *src* homology domain of phospholipase C γ1. *Biochem. Biophys. Res. Commun.* **179:** 217.

Overly, C.C., K.-D. Lee, E. Berthiaume, and P.J. Hollenbeck. 1995. Quantitative measurement of intraorganelle pH in the endosomal-lysosomal pathway in neurons by using ratiometric imaging with pyranine. *Proc. Natl. Acad. Sci.* **92:** 3156.

Paravicini, U., K. Stoeckel, and H. Thoenen. 1975. Biological importance of retrograde axonal transport of nerve growth factor in adrenergic neurons. *Brain Res.* **84:** 279.

Parton, R.G. and C.G. Dotti. 1993. Cell biology of neuronal endocytosis. *J. Neurosci. Res.* **36:** 1.

Parton, R.G., K. Simons, and C.G. Dotti. 1992. Axonal and dendritic endocytic pathways in cultured neurons. *J. Cell Biol.* **119:** 123.

Podlisny, M.B., D.R. Tolan, and D.J. Selkoe. 1991. Homology of the amyloid beta protein precursor in monkey and human supports a primate model for beta amyloidosis in Alzheimer's disease. *Am. J. Pathol.* **138:** 1423.

Schmid, S.L. and L.L. Carter. 1990. ATP is required for receptor-mediated endocytosis in intact cells. *J. Cell Biol.* **111:** 2307.

Schwab, M.E. 1977. Ultrastructural localization of a nerve growth factor-horseradish peroxidase (NGF-HRP) coupling product after retrograde axonal transport in adrenergic neurons. *Brain Res.* **130:** 190.

Scott, S.A., E.J. Mufson, J.A. Weingartner, K.A. Skau, and K.A. Crutcher. 1995. Nerve growth factor in Alzheimer's disease: Increased levels throughout the brain coupled with declines in nucleus basalis. *J. Neurosci.* **15:** 6213.

Smeyne, R.J., R. Klein, A. Schnapp, L.K. Long, S. Bryant, A. Lewin, S.A. Lira, and M. Barbacid. 1994. Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. *Nature* **368:** 246.

Sorkin, A. and C.M. Waters. 1993. Endocytosis of growth factor receptors. *BioEssays* **15:** 375.

Stephens, R.M., D.M. Loeb, T.D. Copeland, T. Pawson, L.A. Greene, and D.R. Kaplan. 1994. Trk receptors use redundant signal transduction pathways involving SHC and PLC-γ1 to mediate NGF responses. *Neuron* **12:** 691.

Stoscheck, C.M. and G. Carpenter. 1984. Down regulation of epidermal growth factor receptors: Direct demonstration of receptor degradation in human fibroblasts. *J. Cell Biol.*

98: 1048.

Suzuki, K. and R.D. Terry. 1967. Fine structural localization of acid phosphatase in senile plaques in Alzheimer's presenile dementia. *Acta Neuropathol.* **8:** 276.

Taglialatela, G., C.J. Hibbert, L.A. Hutton, K. Werrbach-Perez, and J.R. Perez-Polo. 1996. Suppression of $p140^{trkA}$ does not abolish nerve growth factor-mediated rescue of serum-free PC12 cells. *J. Neurochem.* **66:** 1826.

Tischler, A.S. and L.A. Greene. 1975. Nerve growth factor-induced process formation by cultured rat pheochromocytoma cells. *Nature* **258:** 341.

Vale, R.D. and E.M. Shooter. 1985. Assaying binding of nerve growth factor to cell surface receptors. *Methods Enzymol.* **109:** 21.

van der Geer, P., T. Hunter, and R.A. Lindberg. 1994. Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu. Rev. Cell Biol.* **10:** 251.

Verdi, J.M., S.J. Birren, C.F. Ibanez, H. Persson, D.R. Kaplan, M. Benedetti, M.V. Chao, and D.J. Anderson. 1994. $p75^{LNGFR}$ regulates trk signal transduction and NGF-induced neuronal differentiation in MAH cells. *Neuron* **12:** 733.

Vetter, M.L., D. Martin-Zanca, L.F. Parada, J.M. Bishop, and D.R. Kaplan. 1991. Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-γ 1 by a kinase activity associated with the product of the *trk* protooncogene. *Proc. Natl. Acad. Sci.* **88:** 5650.

Yuen, E.C. and W.C. Mobley. 1995. Therapeutic applications of neurotrophic factors in disorders of motor neurons and peripheral nerves. *Mol. Med. Today* **1:** 278.

Zhou, J., J.S. Valletta, M.L. Grimes, and W.C. Mobley. 1995. Multiple levels for regulation of TrkA in PC12 cells by nerve growth factor. *J. Neurochem.* **65:** 1146.

Chapter Four

A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA

Introduction

We sought to test the hypothesis that endosomal fractions from PC12 cells containing active TrkA were capable of giving rise to TrkA-containing transport vesicles. Retrograde movement of transport vesicles along neuronal processes is believed to be a major mode of protein delivery from axon terminals to the soma (Overly, et al., 1996). We reasoned if signaling TrkA could be found in these structures, this would provide further evidence for the retrograde transport of TrkA in signaling vesicles.

We also wished to determine if any population of these endosomal fractions were clathrin coated as this would confirm our confocal findings detailed in Chapters 2 and 3 that TrkA enters the cell via a clathrin coated pit pathway. This chapter characterizes further the endosomal fractions harvested from PC12 cells and introduces an in vitro budding reaction to show that these endosomal fractions are capable of producing transport-vesicle-like membrane structures with the following characteristics: 1) NGF is bound to TrkA; 2) TrkA is phosphorylated and bound to PLC-γ; and 3) the endosomal fractions that give rise to transport vesicle-like membranes and contain TrkA and NGF also contain clathrin and the AP2 subunit, α-adaptin. This last point supports data described in chapters 2, 3, and 5 that clathrin and AP2 are involved in an early stage of TrkA endocytosis, and that TrkA may signal to recruit such proteins.

A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA

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ABSTRACT **The topology of signal transduction is particularly important for neurons. Neurotrophic factors such as nerve growth factor (NGF) interact with receptors at distal axons and a signal is transduced by retrograde transport to the cell body to ensure survival of the neuron. We have discovered an organelle that may account for the retrograde transport of the neurotrophin signal. This organelle is derived from endocytosis of the receptor tyrosine kinase for NGF, TrkA. *In vitro* reactions containing semi-intact PC12 cells and ATP were used to enhance recovery of a novel organelle: small vesicles containing internalized NGF bound to activated TrkA. These vesicles were distinct from clathrin coated vesicles, uncoated primary endocytic vesicles, and synaptic vesicles, and resembled transport vesicles in their sedimentation velocity. They contained 10% of the total bound NGF and almost one-third of the total tyrosine phosphorylated TrkA. These small vesicles are compelling candidates for the organelles through which the neurotrophin signal is conveyed down the axon.**

A long-standing hypothesis predicts that neurons exploit retrograde transport of "signaling vesicles" to convey the nerve growth factor (NGF) signal from the distal axon, where receptors bind NGF, to the cell body to evoke changes in gene expression (1–3). Putative signaling vesicles may arise after endocytosis of neurotrophin receptors (4). It is known that endocytosis occurs at neurite tips (5, 6). TrkA, the receptor tyrosine kinase for the neurotrophin NGF, follows the pattern of other receptor tyrosine kinases, which dimerize upon ligand binding, with resulting activation of the intracellular kinase domain and rapid internalization (7). It is known that tyrosine phosphorylated TrkA is retrogradely transported in axons and that the amount increases in response to NGF treatment (8). Significantly, however, the organelles containing TrkA that convey signal transduction down the axon have not been isolated. We set out to capture such an organelle.

PC12 cells have been used as a model for signal transduction events that occur at neurite tips (9). PC12 cells mimic quite closely signal transduction events that occur in sympathetic neurons; in their undifferentiated state they express TrkA and respond to NGF by inducing expression of neuronal proteins and adopting a neuronal morphology (10, 11). Prolonged NGF treatment, followed by its withdrawal, induces these cells to undergo programmed cell death closely resembling that seen in sympathetic neurons (12–15). Undifferentiated PC12 cells also contain synaptic vesicles (16, 17) whose biogenesis and trafficking are controlled by regulated secretory processes similar to those in nerve terminals (18, 19). These observations suggest that the membrane traffic that occurs at axon tips, including formation of other organelles that are specific to neurons, may be modeled in undifferentiated PC12 cells. We recently examined TrkA receptors in intracellular organelles after a brief period of internalization in PC12 cells (20). These organelles contained tyrosine phosphorylated TrkA, bound to both NGF and to phospholipase C-γ (PLC-γ) (20). These findings are evidence that internalized TrkA receptors continue to be activated after endocytosis.

A number of organelles are involved in trafficking proteins to and from endosomes (21, 22), and any one of these could in principle be used to convey a signal. Activated TrkA is internalized via clathrin-mediated endocytosis (20) from the plasma membrane into primary endocytic vesicles, which might in theory be retrogradely transported. However, primary endocytic vesicles also contain synaptic vesicle and housekeeping proteins. Sorting of receptors away from these proteins at the plasma membrane could be invoked to explain their specific retrograde transport, but the existence of a distinct class of primary endocytic vesicles for receptors has not been established. The endosome, in contrast, is known to be a sorting organelle (21, 22). For example, synaptic vesicle proteins are delivered by clathrin-mediated endocytosis to the endosome. From there they are sorted into synaptic vesicles, away from other proteins destined for recycling as well as degradation (23). We hypothesized that neurons evolved a mechanism for sending a signal to the cell body by building on existing sorting machinery to create a novel class of transport vesicles (3). Transport vesicles would be ideal for retrograde signaling, having a high surface to volume ratio, and a topology that would allow the cytoplasmic tail of receptors to easily interact with the appropriate proteins. Small transport vesicles derived from endosomes have not yet been isolated. However, at least two different types of coats have recently been shown to be associated with the sorting endosome (24–27). Distinct coats are presumably required for the formation of different types of vesicles. Neurons express more types of coat proteins than other cells (28), and it is possible that these are used to form different types of transport vesicles.

In vitro reconstitution of membrane traffic has been used to characterize short-lived intermediates, such as transport vesicles, that convey proteins from one compartment to another (29–32), as well as formation of synaptic vesicles (33). *In vitro* reactions were used here to identify and characterize membrane traffic intermediates formed through endocytosis, which contained NGF and TrkA. The data suggest that a unique transport vesicle derived from endosomes containing activated neurotrophin receptors may well serve the purpose of conveying signal transduction down the axon.

MATERIALS AND METHODS

PC12 cells were obtained from Lloyd Greene (Columbia University, New York). They were grown on collagen-coated plates in RPMI 1640 medium, 10% horse serum, 5% fetal calf serum exactly as described (9). NGF or ^{125}I-NGF (1 nM) was

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Abbreviations: NGF, nerve growth factor; TrkA, the receptor tyrosine kinase for NGF; PLC-γ, phospholipase-C-γ1.

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bound to PC12 cells at 4°C as described (20). Cells were then warmed to 37°C for 10 min (warmed *in vivo*) to initiate membrane traffic, then chilled, washed, and permeabilized by passage through a Balch homogenizer in a cytoplasm-like buffer containing 38 mM each of the potassium salts of aspartic, gluconic and glutamic acids, 20 mM Mops, 5 mM reduced glutathione, 10 mM potassium bicarbonate, 0.5 mM magnesium carbonate, 1 mM EGTA, 1 mM EDTA; pH adjusted to 7.1 at 37°C with potassium hydroxide (31, 34, 35). *In vitro* reactions were performed with hexokinase and glucose (−ATP) or creatine phosphate, creatine kinase, and ATP (+ATP) for 15 min at 37°C as described (31). Where indicated (see Fig. 3*F*), 1 mM sodium orthovanadate was added to *in vitro* reactions. Cytosol and organelles released from the cells were separated from the cell ghosts by centrifugation at 1,000 × *g*. Organelles that emerged from permeabilized cells (the supernatant of the 1,000 × *g* spin) were separated from the cytosol and free NGF by layering over a 0.4 ml pad of 10% sucrose and centrifuging 100,000 × *g* for 1 hr (Fig. 1 *A* and *B*). Alternatively, an 8,000 × *g* centrifugation (P2, Fig. 1 *C–E*) preceded the one at 100,000 × *g* (P3, Fig. 2). Vesicles in the pellets were resuspended and applied to 10–40% (wt/wt, for velocity sedimentation) or 15–50% (for equilibrium density) sucrose gradients with a 60% sucrose pad and centrifuged at 100,000 × *g* for 1 hr (velocity) or 16 hr (equilibrium) in a Beckman TiSW50.1 rotor. For Fig. 2 *A* and *B* the P3 was applied to a 5–25% glycerol gradient in buffer B with a 50% glycerol pad (16, 31). Gradient fractions were trichloroacetic acid-precipitated and counted and/or submitted to SDS/PAGE and Western blotting (20). NGF crosslinking to TrkA was carried out with disuccinimidyl suberate (Pierce), as described (20). In some experiments intact ^{125}I-NGF (14 kDa) was quantified by SDS/PAGE and a Molecular Dynamics PhosphorImager (data not shown). The profile of total ^{125}I cpm in gradients corresponded to that of intact NGF, indicating that there was no degradation of NGF. For equilibrium gradients, the density of each fraction was calculated from refractometry measurements.

Immunoprecipitation of cell fractions was performed on 100 μl P1M, P2, P3 or S3 fractions by the addition of 0.5 ml IP buffer (150 mM NaCl/1% Nonidet P-40/0.5% deoxycholate/20 mM Tris, pH 8.0/1 mM EDTA/1 mM sodium orthovanadate), 100 μl 2% BSA, and 100 μl 10% glycerol. Then 10 μg 1088 (IgG), 10 μg anti-rat TrkA (RTA; ref. 72; IgG) or 5 μg anti PLC-γ mixed monoclonal (Upstate Biotechnology, Lake Placid, NY) was added, incubated overnight and recovered with protein-A- or protein-A/G Sepharose (Pierce). Sepharose beads were washed twice in IP buffer and once with water, then resuspended in 50 μl 7 M urea sample buffer (7 M urea/2% SDS/125 mM Tris, pH 6.95/20 mM DTT/0.1% Bromophenol blue) and heated (55°C for 15–30 min). Gradient gels (from 5% acrylamide, 0.1% bisacrylamide to 12%

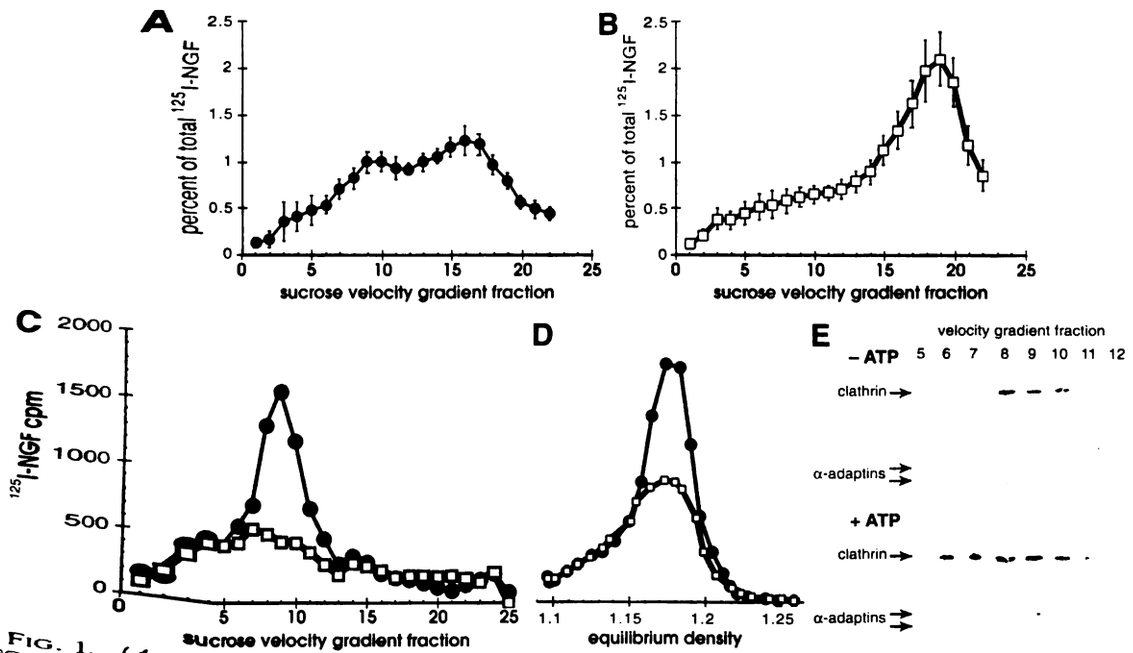

FIG. 1. (*A* and *B*) Sucrose velocity sedimentation resolved different classes of vesicles containing ^{125}I-NGF. Cells bound to 1 nM ^{125}I-NGF at 4°C were washed, warmed 10 min *in vivo*, chilled, permeabilized, then subjected to *in vitro* reactions by warming for 15 min either in the presence of (*A*) an ATP depleting system (−ATP, ●) or (*B*) an ATP regenerating system (+ATP, □). Released vesicles were separated from cell ghosts, concentrated by 100,000 × *g* centrifugation, and applied to sucrose velocity gradients. Gradient fractions were collected from the bottom of the tube, so that the largest organelles were in the lowest numbered fractions. Shown is the amount of ^{125}I-NGF in gradient fractions expressed as a percent of the total from all cell fractions (20). Error bars indicate the SD of measurements from four (*A*) or six (*B*) experiments. (*C* and *D*) Large vesicles containing internalized NGF are clathrin-coated primary endocytic vesicles. Vesicles that emerged from permeabilized cells were fractionated by centrifuging 8,000 × *g* for 35 min (P2), either directly after warming 10 min *in vivo*, (●), or after warming and an *in vitro* reaction with ATP (+ATP, □). Pellets were resuspended and applied to sucrose velocity (*C*) and equilibrium (*D*) gradients. Representative experiments are plotted on the same *y*-axis scale. (*E*) Clathrin and α-adaptins were localized in the P2 in velocity gradient fractions 6–11 with or without *in vitro* reactions under all conditions. Shown is a Western blot of positive gradient fractions from *in vitro* reactions with an ATP-depleting system (−ATP) and ATP regenerating system (+ATP). Monoclonal antibodies TD.1 (70) and AP.6 (71) (a gift of F. Brodsky, University of California, San Francisco) detected clathrin (180 kDa) and two α-adaptins (a doublet centered around 100 kDa), respectively, using enhanced chemiluminescence (Amersham) for detection (1 hr exposure).

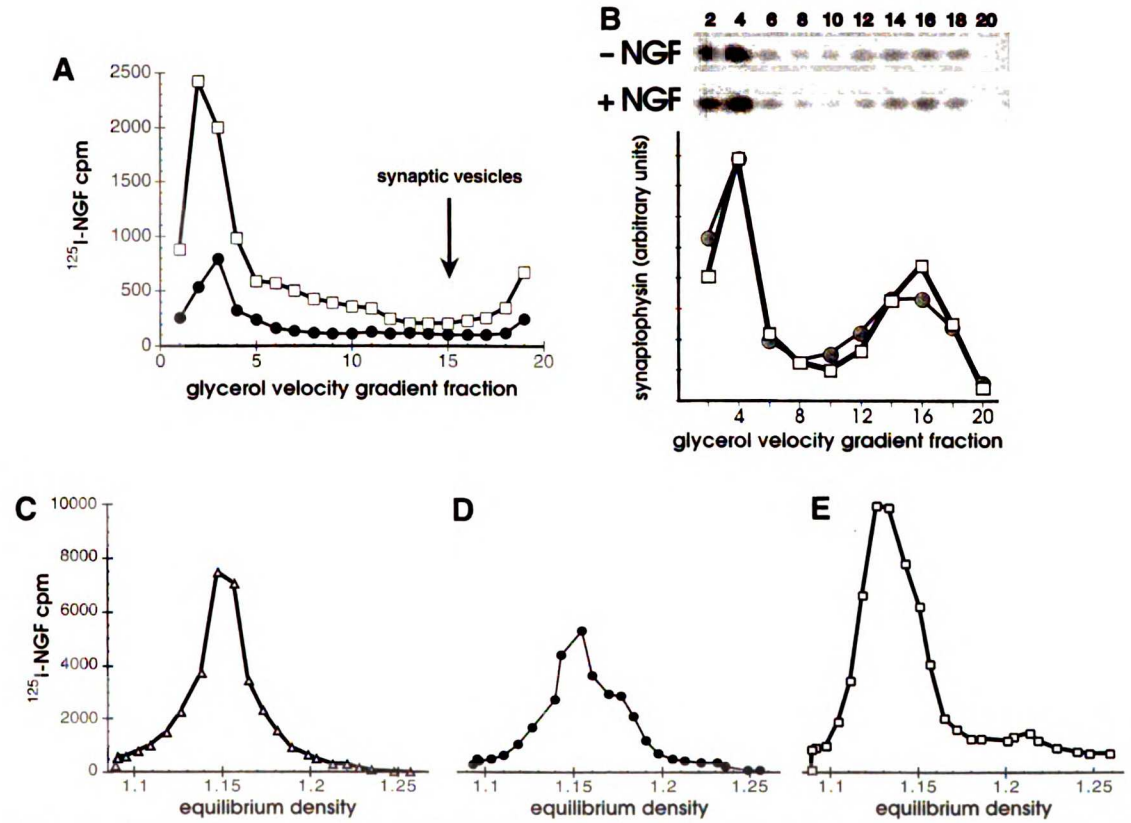

FIG. 2. (*A* and *B*) Synaptic vesicles did not contain NGF. (*A*) Cells bound to ^{125}I-NGF were washed, warmed 10 min *in vivo*, chilled, washed, then permeabilized. Cells were fractionated by successive 1,000 × *g* (P1), 8,000 × *g* (P2), and 100,000 × *g* (P3) centrifugations before or after *in vitro* reactions with ATP. Vesicles in the P3 without an *in vitro* reaction, (●) or after an *in vitro* reaction with ATP (+ATP, □) were applied to 5–25% glycerol velocity gradients and centrifuged 200,000 × *g* for 1 hr. (*B*) Synaptophysin in cell fractions was quantified by Western blotting. After incubating with 1 nM NGF or the vehicle alone at 4°C for 1 hr, PC12 cells were warmed 10 min at 37°C, chilled, washed, and permeabilized without *in vitro* reactions. Glycerol velocity gradient fractions (as in *A* except that fractions were pooled in pairs) were analyzed by SDS/PAGE, proteins were transferred to nitrocellulose and probed with anti-synaptophysin (SY38), followed by goat anti-mouse and ^{125}I-protein A. Autoradiograms of Western blots (above, 3 day exposure) and PhosphorImager quantification (below) show the amount of synaptophysin present in fractions in the absence of NGF (−NGF, ●) or its presence (+NGF, □). The distribution of rab3A (a gift of L. Elferink and R. Scheller, Stanford University, Stanford; not shown) and synaptophysin identified synaptic vesicles in a peak at fractions 14–16. (*C–E*) *In vitro* reactions capture intermediates at different stages of endocytosis. Equilibrium density of ^{125}I-NGF-containing vesicles in the 100,000 × *g* P3 (plotted on the same *y*-axis scale) after 2 min internalization *in vivo* plus a 15 min *in vitro* reaction with ATP (*C*, +ATP, △), or after 10 min internalization *in vivo*, plus a 15 min *in vitro* reaction without ATP (*D*, −ATP, ●), or after 10 min internalization *in vivo*, plus a 15 min *in vitro* reaction with ATP (*E*, +ATP, □).

acrylamide, 0.5% bisacrylamide, with a 4% acrylamide, 0.1% bisacrylamide stacking gel) were run (36) and gels were dried for autoradiography or proteins were transferred to nitrocellulose. For blots probed with monoclonal antibodies against synaptophysin (SY38, Boehringer Mannheim), incubation with goat anti-mouse IgG preceded ^{125}I-protein A. Blots probed with anti-phosphotyrosine (4G10) were detected with ^{125}I-goat anti-mouse IgG that was prepared with Iodobeads according to the commercial protocol (Pierce). Blots probed with anti-PLC-γ or anti-TrkA antibodies were detected with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG and chemiluminescence (Amersham).

RESULTS AND DISCUSSION

We used mechanical permeabilization, which allows isolation of intracellular organelles free from plasma membrane, to examine NGF and TrkA-containing endocytic organelles in PC12 cells (20). In vitro reconstitution of membrane traffic in mechanically permeabilized PC12 cells (31) was used to enhance recovery of short-lived organelles, such as transport vesicles (37), that contained internalized NGF and TrkA. ^{125}I-NGF was bound to cells at 4°C. Warming was used to allow internalization of bound NGF. After a short period of internalization (10 min at 37°C) about 10% of the total bound NGF was released from permeabilized cells in the form of intracellular organelles (20). *In vitro* reactions were then performed for 15 min at 37°C with permeabilized cells in a cytoplasm-like buffer containing either hexokinase plus glucose, which depletes ATP, or creatine phosphate, creatine kinase and ATP, which regenerates ATP. The amount of bound NGF in released organelles increased to 16–18% after *in vitro* reactions.

Organelles that emerged from permeabilized cells were separated from cell ghosts, concentrated by 100,000 × *g* centrifugation, and applied to sucrose velocity gradients (Fig. 1 *A* and *B*). After an *in vitro* reaction without ATP (Fig. 1*A*) organelles that contained internalized NGF were heterogeneous and mostly in two peaks at fractions 9 and 16. When reactions were performed in the presence of ATP, the amount of ^{125}I-NGF in the larger vesicles (fraction 9) decreased, and that in small vesicles increased dramatically (Fig. 1*B*, +ATP).

The new peak did not reflect organelle lysis during the *in vitro* reaction because free NGF had been removed in an earlier step. The small vesicles whose release was ATP-dependent formed a peak around fraction 19, migrating more slowly than those present without ATP. The increase in ^{125}I-NGF in the small vesicles was greater than the decrease in the large ones, which suggests that donor compartment(s) in the cell ghosts contributed to their formation.

We expected to find NGF in clathrin-coated vesicles, since NGF induced colocalization of intracellular clathrin and TrkA in PC12 cells (20). To separate different vesicle species, released organelles were centrifuged at 8,000 × *g* prior to centrifugation at 100,000 × *g*. The 8,000 × *g* pellet (P2) contained the large vesicles that peaked at fraction 9 on sucrose velocity gradients (Fig. 1*C*). After an *in vitro* reaction with ATP, NGF was depleted from large vesicles that were present before the reaction (Fig. 1*C*). The large vesicles in the pellet of an 8,000 × *g* centrifugation had an density of 1.18 g/ml on sucrose equilibrium gradients under all conditions (Fig. 1*D*). Both clathrin and α-adaptins were found in the P2 fraction, peaking at fractions 8–9 on sucrose velocity gradients (Fig. 1*E*) and at 1.18 g/ml on equilibrium gradients (not shown). α-adaptins were depleted from this peak after *in vitro* reactions with ATP (Fig. 1*E*). We conclude that the vesicles containing NGF in the 8,000 × *g* P2 represent clathrin-coated vesicles derived from the plasma membrane (28).

The small vesicles released with or without ATP *in vitro* were found in the 100,000 × *g* pellet (P3). We asked if they were synaptic vesicles, a neuronal organelle whose composition is relatively well defined (38). The P3 was subjected to glycerol velocity sedimentation (16) because synaptic vesicles are not well resolved from transport vesicles on sucrose velocity gradients (31). The small vesicles containing internalized NGF migrated to the bottom of glycerol velocity gradients (fractions 1–5, Fig. 2*A*). As expected, an *in vitro* reaction with ATP increased the amount of NGF in the vesicles that migrated to the bottom of the glycerol gradient (Fig. 2*A*). In contrast, synaptic vesicles formed a peak around fraction 15; they were identified by Western blots of glycerol gradient fractions with antibodies to the synaptic vesicle antigens synaptophysin (Fig. 2*B*) and rab3A (not shown). Both markers also colocalized with vesicles at the bottom of the glycerol gradient (fractions 1–4), but it is only the lighter peak at fraction 15 that corresponds to synaptic vesicles (16, 17, 39). Furthermore, under no conditions was a peak of ^{125}I-NGF (or TrkA, not shown) observed in synaptic vesicle fractions.

The data suggested that the *in vitro* reactions could be used to capture intermediates at different stages of endocytosis. One such intermediate is clathrin-coated vesicles, as characterized above. Uncoated primary endocytic vesicles are derived from clathrin-coated vesicles (28). Formation of these vesicles should be favored after a very short internalization *in vivo*, followed by an *in vitro* reaction with ATP to activate the uncoating ATPase (28). If internalization of NGF *in vivo* was allowed to proceed only 2 min instead of 10 min, the vesicles released following *in vitro* reactions with ATP had an equilibrium density of 1.15 g/ml (Fig. 2*C*). Vesicles of this same density were produced after 10 min internalization and *in vitro* reactions in the absence of ATP (Fig. 2*D*). Significantly, none of these vesicles in the P3 contained clathrin or α-adaptins (data not shown). The data support the view that NGF and TrkA are internalized via clathrin coated vesicles (20), which are subsequently uncoated to form vesicles that migrate to fraction 16 on velocity gradients (Fig. 1*A*) and peak at 1.15 g/ml on sucrose equilibrium gradients.

It is possible that vesicles derived from endosomes serve to transport the neurotrophin signal (3). One would expect these vesicles to have a lower sedimentation velocity and equilibrium density than primary endocytic vesicles (21, 22). Vesicles produced *in vitro* with ATP after a 10 min internalization may correspond to vesicles derived from endosomes. Under these conditions, when the 100,000 × *g* P3 was centrifuged to equilibrium on sucrose gradients the NGF peak had an equilibrium density of 1.13 g/ml (Fig. 2*E*). This value is different from that for vesicles derived in the absence of ATP (Fig. 2*D*), and only slightly less than that for constitutive secretory vesicles, which in PC12 cells have a similar sedimentation velocity (31, 40). Thus, we appear to have detected a novel class of small endocytic vesicles that are downstream from both clathrin-coated and uncoated primary endocytic vesicles, and are distinct from synaptic vesicles.

The "signaling vesicle" hypothesis (1–3) motivated analysis of TrkA receptors in these organelles. We examined TrkA in the following cell fractions after *in vitro* reactions in the presence of ATP: 1) the NP40-soluble cell ghost membranes (P1), containing plasma membrane; 2) the 8,000 × *g* pellet (P2), containing clathrin-coated vesicles; 3) the 100,000 × *g* pellet (P3), containing small vesicles, and 4) supernatant (S3) containing cytosol (20). We asked if NGF was bound to TrkA in these fractions and if TrkA was activated (i.e., tyrosine phosphorylated). A membrane permeable crosslinking reagent, disuccinimidyl suberate, crosslinked ^{125}I-NGF to TrkA in the small vesicle (P3) fraction (Fig. 3*A*). The amount of NGF crosslinked to TrkA in the small vesicles was 10% of total crosslinked NGF; this is in good agreement with the amount of NGF in these vesicles (10.1% ± 1 of total bound NGF, $n = 10$), suggesting that NGF in the small vesicles remained bound to TrkA.

Continued receptor occupancy suggested that TrkA would be activated in the small vesicles. TrkA was immunoprecipitated after a 10 min internalization in the absence and presence of NGF, followed by *in vitro* reactions with ATP (Fig. 3*B*). Under these conditions about a third of the mature form of the receptor, gp140^{TrkA}, was recovered in the P3 (Fig. 3*C*). This represents TrkA in both the biosynthetic and endocytic pathways. The P2 and P3 fractions together contained 10–15% of the immature form of the receptor, gp110^{TrkA} (41, 42), and the amount changed little with NGF (Fig. 3 *B* and *D*). Activated TrkA, which must be derived from the plasma membrane where it was exposed to NGF, was assessed by blotting immunoprecipitates with anti-phosphotyrosine (Fig. 3*B Right*). Fractions prepared from cells not exposed to NGF contained very little tyrosine phosphorylated TrkA, indicating that *in vitro* reactions did not artificially activate the receptor (Fig. 3 *B* and *E*). The small vesicle (P3) fraction contained almost one-third (28%) of the total tyrosine phosphorylated TrkA after NGF treatment (Fig. 3*E*). TrkA activation was persistent in these vesicles since it was detected 25 min after initiating NGF treatment (i.e., 10 min *in vivo*, 15 min *in vitro*).

Since the reactions above were performed in the absence of phophatase inhibitors, it is possible that tyrosine phosphatases were acting on TrkA. Without *in vitro* reactions, NGF induced a 17-fold increase in total cellular tyrosine phosphorylated TrkA (20). After *in vitro* reactions with ATP, stimulation was reduced to 14-fold. If the phosphatase inhibitor orthovanadate was added to *in vitro* reactions, a large increase in background tyrosine phosphorylation of TrkA was observed in cells not treated with NGF, and more tyrosine phosphorylated TrkA was detected in the P2 than in the P3, with or without NGF (Fig. 3*F* and data not shown). In spite of the increase in background, NGF induced a 5-fold increase in total tyrosine phosphorylated TrkA (Fig. 3*F Right*).

PLC-γ has been shown to bind to TrkA after NGF activation (43, 44). We have shown previously that PLC-γ was bound to TrkA in cell ghosts and intracellular organelles after NGF treatment (20). We asked whether this binding persisted in small vesicles after *in vitro* reactions. PLC-γ was immunoprecipitated from the small vesicle (P3) fractions after a 10 min internalization followed by *in vitro* reactions in the presence of ATP and orthovanadate (Fig. 3*F*). Under these conditions tyrosine phosphorylated PLC-γ was detected in the small vesicle fraction only

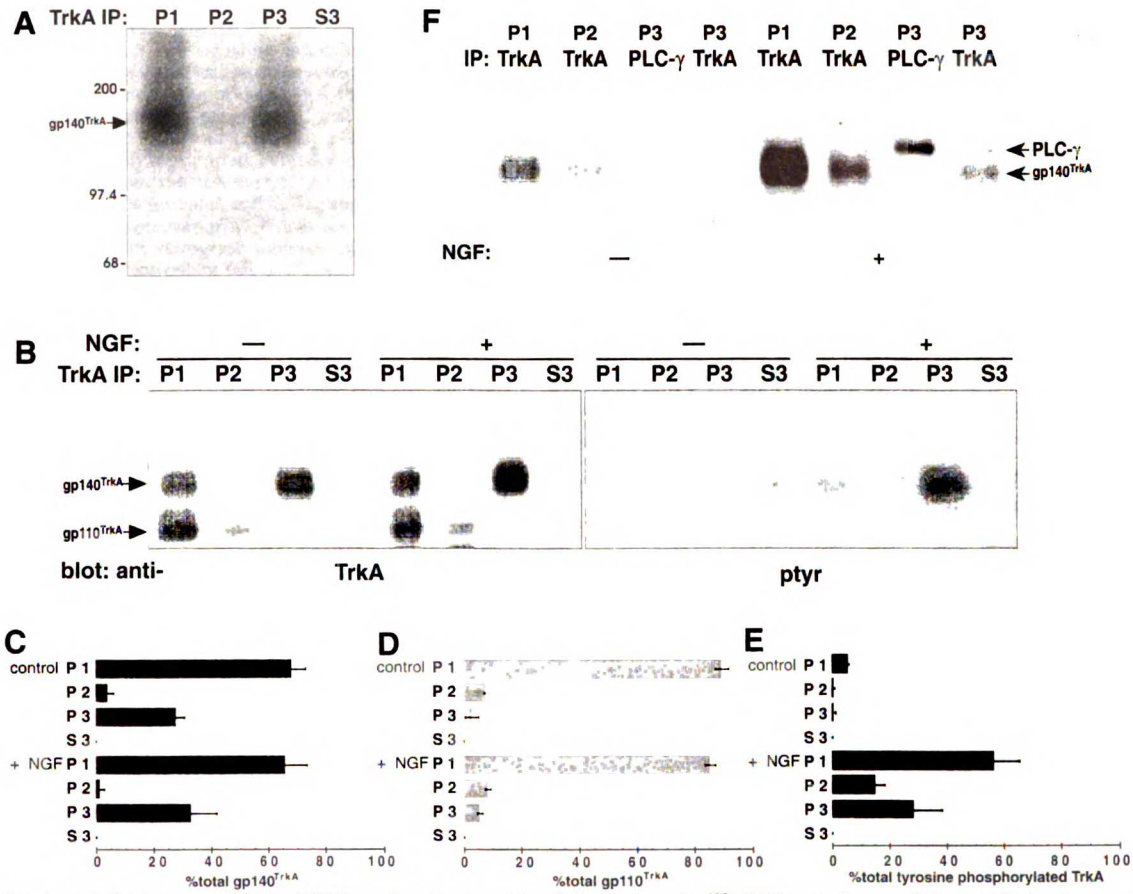

FIG. 3. (*A*) TrkA was crosslinked to NGF in small endocytic vesicles. Cells were bound to ^{125}I-NGF, washed, warmed 10 min, chilled and analyzed after an *in vitro* reaction with ATP. The membrane permeable crosslinking reagent disuccinimidyl suberate was added (2 mM, 4°C, 30 min) to the permeabilized cell suspension before fractionation of membranes. One-fifth of the cell ghost membranes (1,000 × *g* pellet, P1), one-half of the 8,000 × *g* pellet (P2) one-half of the 100,000 × *g* pellet (P3), and one-tenth of the 100,000 × *g* supernatant (S3) were immunoprecipitated from samples with anti-TrkA (1088) (20, 42) and analyzed by SDS/PAGE and autoradiography (22 day exposure). The position of molecular weight markers (kDa) is indicated. (*B*) TrkA is still activated in intracellular organelles after *in vitro* reactions. Untreated PC12 cells or PC12 cells bound to NGF (1 nM, 4°C) were warmed 10 min and fractionated and immunoprecipitated as in *A* after *in vitro* incubation with ATP. The presence of TrkA was assessed by immunoprecipitation, followed by Western blotting, with anti-TrkA and anti-phosphotyrosine (indicated). RTA (a gift of D. Clary, ref. 72) was used for immunoprecipitations. Western blots were probed with RTA followed by horseradish peroxidase-conjugated anti-rabbit IgG and ECL (*Left*, 1 min exposure). Two proteins were identified, gp140^{TrkA} and gp110^{TrkA} (indicated). The latter is a precursor to gp140^{TrkA} (41, 42), and remained mostly with the cell ghosts after *in vitro* reactions with ATP. TrkA immunoprecipitates were also probed with anti-phosphotyrosine (4G10, a gift of S. Robbins and M. Bishop, University of California, San Francisco) followed by ^{125}I-goat anti-mouse IgG (*Right*, 34 day exposure). Mature 140-kDa TrkA was tyrosine phosphorylated, while the 110-kDa immature glycosylated TrkA was not. Equal amounts of cells were used to compare conditions. The top and bottom edges of the panels mark the position of the 200- and 97.4-kDa molecular weight markers, respectively. (*C–E*) Quantification of TrkA and tyrosine phosphorylated TrkA in intracellular organelles. Data for gp140^{TrkA} (*C*) and gp110^{TrkA} (*D*) from 4 experiments as in *B* (*Left*) were quantified by densitometry and plotted as a percent of the total in all cell fractions with error bars showing standard deviations. The conditions and fractions are labeled at the left. Data for tyrosine phosphorylated TrkA (*E*) from four experiments as in (*B Right*) were quantified by phosphorimaging or densitometry and plotted in reference to total tyrosine phosphorylated TrkA following NGF treatment with error bars as in *C*. (*F*) Sodium orthovanadate was added to *in vitro* incubations with ATP. Untreated cells (*Left*) and NGF-treated cells (*Right*) were permeabilized and incubated *in vitro* with ATP in the presence of 1 mM sodium orthovanadate. TrkA was immunoprecipitated as in *A* with anti-TrkA (1,088) (20, 42). In the P3 fraction, PLC-γ, then TrkA was immunoprecipitated from the P3 fractions (indicated by IP). Proteins were Western blotted with antiphosphotyrosine followed by horseradish peroxidase-conjugated antimouse and detected using ECL (20 sec exposure). Equal amounts of cells were used to compare conditions. The top and bottom edges of the panels mark the position of the 200- and 97.4-kDa molecular weight markers, respectively.

in NGF treated cells (Fig. 3*F*, PLC-γ immunoprecipitation) and some was precipitated by anti-TrkA (P3, TrkA immunoprecipitation). The presence of activated TrkA and TrkA bound to PLC-γ in small vesicles suggests that signal transduction could be initiated from receptors in these organelles.

Our studies have identified one possible organelle that may convey the NGF signal down axons. Important signaling events may be mediated through TrkA in other organelles, through p75^{NTR}, or through signal transduction mediators downstream from receptors (45). Because it was the first NGF receptor to be discovered, p75^{NTR} was invoked in earlier versions of the "signaling vesicle" hypothesis (1, 2). The role of p75^{NTR}, which binds other neurotrophins as well as NGF, has been controversial (46–49). The importance of this receptor is demonstrated by the fact that mice in whom the gene has been disrupted show abnormalities in both the peripheral and

central nervous system (50). $p75^{NTR}$ has been shown to enhance NGF binding and signaling through TrkA (51–54). $p75^{NTR}$ also initiates signal transduction separately from TrkA (55–57). There is increasing evidence that the signals generated by $p75^{NTR}$ promote programmed cell death (58–61) rather than prevent it, as does TrkA (62). $p75^{NTR}$ is internalized and retrogradely transported, but it does not appear to be down-regulated from the cell surface in response to NGF (M.G., D. Hall, A. Schroepfel, and W.M., unpublished observations) (63–65). Moreover, $p75^{NTR}$ does not appear to play a significant role in the retrograde transport of NGF, but does transport other neurotrophins (66, 67). These findings suggest that $p75^{NTR}$ does not play a role in survival-promoting retrograde signaling by NGF.

In previous work (20) on NGF-treated PC12 cells, we showed that NGF was internalized bound to TrkA. Different endocytic organelles were separated by differential sedimentation and both large and small organelles were found to contain activated TrkA. In the present study we used *in vitro* reactions containing semi-intact PC12 cells and ATP to examine the kinetics of formation and physical characteristics of a novel organelle: small vesicles containing internalized NGF bound to activated TrkA. These vesicles were distinct from clathrin-coated vesicles, uncoated primary endocytic vesicles, and synaptic vesicles, and resembled transport vesicles in their sedimentation velocity and equilibrium density. They contained at least 10% of the total bound NGF and almost one-third of the total tyrosine phosphorylated TrkA. Because not all of the organelles may have emerged from permeabilized cells, we regard these values as a lower limit for the content of NGF and activated TrkA. Receptor activation was persistent in the vesicles. Sustained activation of receptors in intracellular organelles may explain prolonged activation of downstream kinases by TrkA (4, 68, 69). These findings provide strong evidence for the existence of a distinct class of vesicles derived from endosomes. Furthermore, the small vesicles are a compelling candidate for retrograde NGF signaling. They support the hypothesis that internalized, activated TrkA receptors could transduce the NGF signal from the platform of a small vesicle (1–3). It remains to be seen if these organelles are used to retrogradely transport activated TrkA from distal axons to neuron cell bodies (8).

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1. Misko, T. P., Radeke, M. J. & Shooter, E. M. (1987) *J. Exp. Biol.* **132,** 177–190.
2. Halegoua, S., Armstrong, R. C. & Kremer, N. E. (1991) *Curr. Top. Microbiol. Immunol.* **165,** 119–170.
3. Grimes, M., Zhou, J., Li, Y., Holtzman, D. & Mobley, W. C. (1993) *Semin. Neurosci.* **5,** 239–247.
4. Baass, P. C., Di Guglielmo, B. M., Authier, F., Posner, B. I. & Bergeron, J. J. (1995) *Trends Cell Biol.* **5,** 465–475.
5. Tooze, J., Hollinshead, M., Fuller, S. D., Tooze, S. A. & Huttner, W. B. (1989) *Eur. J. Cell Biol.* **49,** 259–273.
6. Sulzer, D. & Holtzman, E. (1989) *J. Neurocytol.* **18,** 529–540.
7. van der Geer, P., Hunter, T. & Lindberg, R. A. (1994) *Annu. Rev. Cell. Biol.* **10,** 251–337.
8. Ehlers, M. D., Kaplan, D. R., Price, D. L. & Koliatsos, V. E. (1995) *J. Cell Biol.* **130,** 149–156.
9. Greene, L. A. & Tischler, A. S. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 2424–2428.
10. Kaplan, D. R. & Stephens, R. M. (1994) *J. Neurobiol.* **25,** 1404–1417.
11. Levi, A., Biocca, S., Cattaneo, A. & Calissano, P. (1988) *Mol. Neurobiol.* **2,** 201–226.
12. Mills, J. C., Wang, S., Erecinska, M. & Pittman, R. N. (1995) *Methods Cell Biol.* **46,** 217–242.
13. Messner, P. W., Winters, T. R. & Green, S. H. (1992) *J. Cell Biol.* **119,** 1669–1680.
14. Batistatou, A. & Greene, L. A. (1993) *J. Cell Biol.* **122,** 523–532.
15. Edwards, S. N. & Tolkovsky, A. M. (1994) *J. Cell Biol.* **124,** 537–546.
16. Clift-O'Grady, L., Linstedt, A. D., Lowe, A. W., Grote, E. & Kelly, R. B. (1990) *J. Cell Biol.* **110,** 1693–1703.
17. Cameron, P. L., Sudhof, T. C., Jahn, R. & De Camilli, P. (1991) *J. Cell Biol.* **115,** 151–164.
18. Kelly, R. B. (1993) *Cell/Neuron* **72/10,** 43–53.
19. Bean, A. J., Seifert, R., Chen, Y. A., Sacks, R. & Scheller, R. H. (1997) *Nature (London)* **385,** 826–829.
20. Grimes, M. L., Zhou, J., Beattie, E., Yuen, E. C., Hall, D. E., Valletta, J. S., Topp, K. S., LaVail, J. H., Bunnett, N. W. & Mobley, W. C. (1996) *J. Neurosci.* **16,** 7950–7964.
21. Trowbridge, I. S., Collawn, J. F. & Hopkins, C. R. (1993) *Annu. Rev. Cell Biol.* **9,** 129–161.
22. Gruenberg, J. & Maxfield, F. R. (1995) *Curr. Opin. Cell Biol.* **7,** 552–563.
23. Sudhof, T. C. (1995) *Nature (London)* **375,** 645–653.
24. Takei, K., Mundigl, O., Daniell, L. & De Camilli, P. (1996) *J. Cell Biol.* **133,** 1237–1250.
25. Whitney, J. A., Gomez, M., Sheff, D., Kreis, T. E. & Mellman, I. (1995) *Cell* **83,** 703–713.
26. Aniento, F., Gu, F., Parton, R. G. & Gruenberg, J. (1996) *J. Cell Biol.* **133,** 29–41.
27. Stoorvogel, W., Oorschot, V. & Geuze, H. J. (1996) *J. Cell Biol.* **132,** 21–33.
28. Schmid, S. L. & Damke, H. (1995) *FASEB J.* **9,** 1445–1453.
29. Podbilewicz, B. & Mellman, I. (1990) *EMBO J.* **9,** 3477–3487.
30. Bennett, M. K., Wandinger-Ness, A. & Simons, K. (1988) *EMBO J.* **7,** 4075–4085.
31. Grimes, M. & Kelly, R. B. (1992) *J. Cell Biol.* **117,** 539–549.
32. de Curtis, I. & Simons, K. (1989) *Cell* **58,** 719–727.
33. Desnos, C., Clift, O. G. L. & Kelly, R. B. (1995) *J. Cell Biol.* **130,** 1041–1049.
34. Martin, T. F. (1989) *Methods Enzymol.* **168,** 225–233.
35. Balch, W. E. & Rothman, J. E. (1985) *Arch. Biochem. Biophys.* **240,** 413–425.
36. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
37. Rothman, J. E. (1994) *Nature (London)* **372,** 55–63.
38. Calakos, N. & Scheller, R. H. (1996) *Physiol. Rev.* **76,** 1–29.
39. Linstedt, A. D. & Kelly, R. B. (1991) *Neuron* **7,** 309–317.
40. Grimes, M. & Kelly, R. B. (1992) *Ann. N.Y. Acad. Sci.* **674,** 38–52.
41. Martin-Zanca, D., Oskam, R., Mitra, G., Copeland, T. & Barbacid, M. (1989) *Mol. Cell. Biol.* **9,** 24–33.
42. Zhou, J., Valletta, J. S., Grimes, M. L. & Mobley, W. C. (1995) *J. Neurochem.* **65,** 1146–1156.
43. Stephens, R. M., Loeb, D. M., Copeland, T. D., Pawson, T., Greene, L. A. & Kaplan, D. R. (1994) *Neuron* **12,** 691–705.
44. Obermeier, A., Bradshaw, R. A., Seedorf, K., Choidas, A., Schlessinger, J. & Ullrich, A. (1994) *EMBO J.* **13,** 1585–1590.
45. Crouch, M. F., Heydon, K., Garnaut, S. M., Milburn, P. J. & Hendry, I. A. (1994) *Eur. J. Neurosci.* **6,** 626–631.
46. Chao, M. V. (1994) *J. Neurobiol.* **25,** 1373–1385.
47. Greene, L. A. & Kaplan, D. R. (1995) *Curr. Opin. Neurobiol.* **5,** 579–587.
48. Bothwell, M. (1995) *Annu. Rev. Neurosci.* **18,** 223–253.
49. Bothwell, M. (1996) *Science* **272,** 506–507.
50. Lee, K. F., Li, E., Huber, L. J., Landis, S. C., Sharpe, A. H., Chao, M. V. & Jaenisch, R. (1992) *Cell* **69,** 737–749.
51. Barker, P. A. & Shooter, E. M. (1994) *Neuron* **13,** 203–215.
52. Hantzopoulos, P. A., Suri, C., Glass, D. J., Goldfarb, M. P. & Yancopoulos, G. D. (1994) *Neuron* **13,** 187–201.
53. Benedetti, M., Levi, A. & Chao, M. V. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7859–7863.
54. Hempstead, B. L., Martin, Z. D., Kaplan, D. R., Parada, L. F. & Chao, M. V. (1991) *Nature (London)* **350,** 678–683.
55. Carter, B. D., Kaltschmidt, C., Kaltschmidt, B., Offenhauser, N., Bohm-Matthaei, R., Baeuerle, P. A. & Barde, Y. A. (1996) *Science* **272,** 542–545.
56. Dobrowsky, R. T., Werner, M. H., Castellino, A. M., Chao, M. V. & Hannun, Y. A. (1994) *Science* **265,** 1596–1599.
57. Canossa, M., Twiss, J. L., Verity, A. N. & Shooter, E. M. (1996) *EMBO J.* **15,** 3369–3376.
58. Casaccia-Bonnefil, P., Carter, B. D., Dobrowsky, R. T. & Chao, M. V. (1996) *Nature (London)* **383,** 716–719.
59. Frade, J. M., Rodriguez-Tebar, A. & Barde, Y. A. (1996) *Nature (London)* **383,** 166–168.
60. Van der Zee, C. E., Ross, G. M., Riopelle, R. J. & Hagg, T. (1996) *Science* **274,** 1729–1732.
61. Carter, B. D. & Lewin, G. R. (1997) *Neuron* **18,** 187–190.
62. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J. & Greenberg, M. E. (1995) *Science* **270,** 1326–1331.
63. Johnson, E. M. J., Andres, R. Y. & Bradshaw, R. A. (1978) *Brain Res.* **150,** 319–331.
64. Hosang, M. & Shooter, E. M. (1987) *EMBO J.* **6,** 1197–202.
65. Eveleth, D. D. & Bradshaw, R. A. (1992) *J. Cell Biol.* **117,** 291–299.
66. Curtis, R., Adryan, K. M., Stark, J. L., Park, J. S., Compton, D. L., Weskamp, G., Huber, L. J., Chao, M. V., Jaenisch, R. & Lee, K. F. (1995) *Neuron* **14,** 1201–1211.
67. von Bartheld, C. S., Williams, R., Lefcort, F., Clary, D. O., Reichardt, L. F. & Bothwell, M. (1996) *J. Neurosci.* **16,** 2995–3008.
68. Marshall, C. J. (1995) *Cell* **80,** 179–185.
69. Qui, M. S. & Green, S. H. (1992) *Neuron* **9,** 705–717.
70. Nathke, I. S., Heuser, J., Lupas, A., Stock, J., Turck, C. W. & Brodsky, F. M. (1992) *Cell* **68,** 899–910.
71. Chin, D. J., Straubinger, R. M., Acton, S., Nathke, I. & Brodsky, F. M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9289–9293.
72. Clary, D. O., Weskamp, G., Austin, L. R. & Reichardt, L. F. (1994) *Mol. Biol. Cell* **5,** 549–563.

Chapter Five

NGF-induced TrkA signaling induces recruitment and phosphorylation of coated pit proteins

Introduction

Having seen the NGF-induced effects on clathrin redistribution that were described in previous Chapters, we asked if TrkA signaling was responsible. In the course of our studies we gathered data supporting several interesting TrkA associations with clathrin, α-adaptin, and $pp60^{src}$ that were increased as a function of TrkA kinase activity.

Coated pits, clathrin, and endocytosis of receptors

Clathrin, described in detail below, is an important component in coated pit endocytosis of receptors that are internalized constitutively (e.g.- Transferrin receptor) or in response to ligand binding (e.g.- EGF-R, insulin receptor, and TrkA). Clathrin is also involved in synaptic vesicle production and recycling, and in transport vesicle budding from the trans-golgi network (Pearse and Robinson, 1990; Schmid, 1997).

Though the body of literature regarding coated pit formation is large, little is known about the control of this process. A careful survey of earlier research provides the interesting clue that clathrin coated pit-endocytosis is regulated by activation of RTKs. A

study conducted by Connolly and Green in the 1980's examined the plasma membrane of PC12 cells treated with NGF. This electron microscopy (EM) study showed radioactive NGF positioned over what are now considered classic coated pit plasma membrane densities. Interestingly, the number of these densities increased two to threefold within minutes of NGF addition, while cell surface area remained relatively constant (Connolly et al., 1981). Similar results were seen in PC12 cells treated with EGF (Connolly et al., 1984). The authors concluded that NGF and EGF regulated the number of clathrin coated pits, and argued that this event may represent a fundamental characteristic of the response to polypeptide growth factors. However, the mechanism for this response and its biological significance were uncertain.

In related studies, there was a six-fold increase in plasma membrane-associated coated pits in bovine chromaffin cells 1 to 2 minutes after adding carbachol, a cholinergic agonist. The coat for these new pits appeared to be recruited from a cellular pool not detected by EM. (Geisow et al., 1985). Further evidence that receptor activation influences the distribution of clathrin was provided by studies demonstrating that insulin treatment of adipocytes caused a doubling in the amount of clathrin in plasma membrane (Corvera, 1990). The authors used biochemistry and sub-cellular fractionation to document their findings. The reason for increased membrane association of clathrin was suggested to be an increase at the cell surface of the insulin receptor in response to insulin treatment.

These studies along with our independent observations that NGF elicited a marked and rapid increase in plasma membrane-associated clathrin (Chapters 2-3) prompted us to investigate the effect of NGF on clathrin redistribution. The goal was to

confirm that NGF does regulate the association of clathrin with surface membrane and to define the mechanism for the effect. We discovered a robust effect for NGF signaling in redistributing clathrin. This was due to activation of TrkA and was shown to be associated with the creation of complexes containing TrkA, AP2, clathrin, and $pp60^{src}$. Significantly, we gathered evidence to indicate that the clathrin heavy chain (CHC) was phosphorylated in response to NGF treatment and that $pp60^{src}$ was responsible. These data raise the possibility that NGF signaling regulates clathrin coated pit formation through the binding and phosphorylation of CHC to plasma membrane TrkA receptors.

Basic components involved in coated pit endocytosis

Clathrin is a complex composed of three clathrin heavy chain (CHC) subunits and three clathrin light chains that come together to form a triskelion shaped multimer forming the basic structural unit of clathrin cages (see model, figure 22). During endocytosis, single clathrin triskelions and other coated pit molecules bind and assemble to form a hexagonal clathrin lattice over the cytosolic surface of the plasma membrane. These lattices then deflect inward and produce the classic coated pits that carry cargo (e.g.- receptors and their ligands) into the cell. Endocytosis then occurs through the pinching off of these coated pits to form coated vesicles. Coated vesicle proteins are quickly shed from these vesicles and are then available to engage in another round of endocytosis.

Adaptor proteins (AP) are bridging proteins that connect the carboxyl-terminus of receptors to the CHC lattice. Different isotypes of APs have been identified that are specific to plasma membrane endocytosis (AP2) and TGN budding (AP1). Both adaptor family members are complexes of four subunits; two of 100kD, one of 50 kD, and one of

17 kD. AC1M11, an antibody specific to the 100kD AP2-specific subunit, α-adaptin, was used in this study to mark this plasma membrane-specific coated pit protein. Another molecule that has been found to be vital to coated pit formation and function is dynamin. Dynamin is a protein localized to the necks of newly formed coated pits and is thought to be involved in the membrane fusion or "pinching-off" step of coated vesicle formation (Hinshaw and Schmid, 1995).

In constitutive endocytosis of cargo-carrying receptors, like the transferrin receptor, this process occurs in the presence or the absence of the ligand. In the ligand-induced endocytosis seen with receptors like EGF-R and the insulin-receptor, the presence of growth factor causes an increase in coated pit formation (Connolly et al., 1981; Connolly et al., 1984; Corvera et al., 1989), (Geisow et al., 1985). In these cases, ligand application is believed to expose sorting motifs within the C-termini of receptors that promote binding with AP2 (Schmid, 1997).

A few distinct models of ligand-induced receptor entry into coated pits exist. One of these involves the activation-induced exposure of receptor carboxy-terminal AP motifs that allow entry into preformed coated pits (Santini and Keen, 1996). Another calls for the formation of coated pit proteins around an activated receptor (Kirchhausen et al., 1997). Though our studies may not distinguish between these possibilities, receptor activation of clathrin recruitment can be applied to both of these models.

This chapter describes experiments using cells expressing the three permutations of NGF receptor expression. Initial experiments were performed with cells expressing endogenous levels of both wild type TrkA and p75 (PC12 KB), and with cells expressing either p75 (Schwann) or TrkA (TrkA 3T3 fibroblasts and D283 meduloblastomas). TrkA

is a member of the Trk family of receptors; TrkA, TrkB, and TrkC, which bind the neurotrophins NGF, BDNF, and NT3 respectively. These receptors are homologous and elicit very similar signal cascades (Yuen et al., 1996). 3T3 fibroblasts expressing TrkA or TrkB were used to assess Trk receptor signaling effects on clathrin redistribution and the AP2 subunit α-adaptin. Confocal studies with these cells yielded NGF-induced redistribution results for both of these coated pit proteins.

Cells expressing both wild-type p75 and TrkA receptors (TrkA PC12 nnR5) or expressing mutant TrkA receptors with defective signaling capacity (TrkA PC12 nnR5 22.7 and M1 lines) were compared for their NGF-induced clathrin responses. The results from these experiments support the theory that TrkA signaling is responsible for the clathrin redistribution since only cells with functional TrkA receptors redistributed clathrin to the plasma membrane.

Phosphorylation of coated pit proteins

In searching for a possible mechanism for clathrin redistribution we became interested in coated pit protein phosphorylation. Though no previous data shows that ligand-induced RTK signaling leads to changes in CHC phosphorylation, there is ample precedent for coated pit protein phosphorylation (Lamaze and Schmid, 1995); (Fallon et al., 1994; Holen et al., 1995a). Brodsky and Wilde showed that in constitutive endocytosis, the β subunits of adaptins are phosphorylated on serine residues located in a CHC binding site (Wilde and Brodsky, 1996). Phosphorylation of AP2 is thought to negatively affect CHC-AP2 binding and may aid in the clathrin uncoating process that occurs rapidly after endosome formation. Interestingly, these same authors noted that CHC was tyrosine phosphorylated in unstimulated MDBK cells. But as the focus of the

study was the phophorylation state of AP2 in these cells, effects on CHC phosphorylation elicited by RTK stimulation were not examined.

The clathrin light chain, of which there are two subtypes in mammals (LCa and LCb), has also been reported to be phosphorylated. In vivo studies showed that clathrin light chain was phosphorylated on serines in reticulocytes (Bar-Zvi et al., 1988).

One kinase that could play a role in phosphorylation of clathrin is the non-receptor kinase pp60^{src}. This protein is the best studied member of the SRC family of kinases. It regulates a variety of cellular responses to extracellular stimuli (Brown and Cooper, 1996). Prior studies have localized pp60^{src} to the plasma membrane and to endosomes, moreover there is evidence that pp60^{src} binds to and phosphorylates EGF-R (Sato et al., 1995a; Sato et al., 1995b). Therefore, pp60^{src} is likely to be in close proximity to coat proteins and is a candidate for a kinase that could act on them. A clue that CHC might be a target of a SRC family kinase can be found in the following study. Increased CHC phosphorylation and redistribution to the plasma membrane was seen in Rous Sarcoma virus transformed fibroblasts when compared to CHC from control fibroblasts (Martin-Perez et al., 1989). Recent papers showing that EGF-R and TrkA activation lead to activation of SRC family kinases suggest that pp60^{src} could link signaling that begins with RTK activation, leading to CHC phosphorylation (Hilborn et al., 1998); (Kremer et al., 1991). Finally, our collaborative studies with Wilde and Brodsky (Appendix) provide strong evidence that SRC kinase activity initiated by EGF-R does cause phosphorylation of CHC. Herein we show pp60^{src} associates with TrkA after NGF treatment.

Methods

Reagents

Antibodies to clathrin and α-adaptin were provided by the laboratory of Frances Brodsky. X22, a mouse monoclonal for the clathrin heavy chain, was used for immunoprecipitation and immunostaining at 10 ug/ml. TD.1, also a mouse monoclonal specific for clathrin heavy chain, was used for probing western blots at 3 ug/ml. AC1M11, a monoclonal specific to α-adaptin, was used at 1:500 for immunoblotting while the monoclonal AP.6 was used to mark AP-2 in immunohistochemistry. The antibody specific to $pp60^{src}$, Src327 (mouse monoclonal) used for immunoblotting at a dilution of 1:1000 was provided by the laboratory of Michael Bishop. Immunoprecipitation of phosphotyrosine proteins was accomplished using mouse IgG2bk, an agarose conjugated anti-phosphotyrosine antibody from UBI. Blotting for phosphotyrosine proteins was accomplished with the mouse monoclonal antiphosphotyrosine antibody, 4G10 (UBI). Immunoprecipitation of TrkA was performed using a polyclonal antibody from UBI specific to the amino-terminus of TrkA (06574) at a final concentration of 10 ug/ml. Secondary goat-anti-mouse antibodies for immunohistochemical clathrin distribution analysis were obtained from Cappel. These were conjugated with FITC or rhodamine for visualization.

PC12 nnR5 cells transfected with TrkA or the TrkA mutants, 22.7 (activation loop mutant, YY674/675FF) and M1 (kinase dead, K538N), were obtained from Robert Kupta and the Reichardt laboratory and were originally made by David Kaplan. Schwann cells were supplied kindly supplied by Gihan Tennekoon. TrkA expressing D283 cells were produced by Janice Valletta in the Mobley laboratory. KB PC12 cells were originally

cloned by Kevin Tommaselli from a PC12 line supplied by Lloyd Green. TrkA and TrkB expressing 3T3 cells were supplied by Regeneron, Inc.

NGF was isolated by the method of Mobley et al. (Mobley et al., 1976).

Cell Culture

PC12 cells were maintained as described in Chapters 2-4. All cells were maintained at 37°C with 5% CO_2 . PC12 cells were cultured in DME-H21, 10% horse serum, and 5% fetal calf serum. All tissue culture media and media additions were supplied by the UCSF Cell Culture Facility. Priming of PC12 cells was accomplished by addition of 2nM NGF to cells for seven days prior to the time of the experiment. Cells were washed with three media changes at 30 minute intervals to remove NGF before the initiation of the experiment.

PC12 nnR5 TrkA and mutant cell lines were cultured on collagen coated plates in the media described above with the addition of 100 ug/ml Geneticin.

D283 cells were grown on plastic and fed with MEM Earl's BSS containing 10% horse serum, Na pyruvate, nonessential amino acids, pen/strep, and 600ug/ml G418. Schwann cells were maintained in high glucose DMEM containing 10% fetal calf serum, pen/strep, and amphoterasin B (4ng/ml). 3T3 TrkA and TrkB cells were grown on plastic and maintained in H-21 DMEM containing 10% horse serum and 200ug/ml G418.

Immunohistochemistry and Microscopy

Preparation of cells for immunohistochemistry, immunohistochemical staining, and confocal microscopy was done as described in Chapters 2 and 3. Cells were treated with NGF as described in Chapters 2 and 3 with the exception of PC12 cells, which were chilled to 4°C on Chamberslides, given 2nM NGF in serum free media for 1 hour, and then warmed to 37°C for 2 minutes. After chilling cells quickly to 4°C, the cells were fixed with 4% paraformaldehyde for 20 minutes at 4°C, and blocked with a PBS/1ug/ml saponen/ 10% goat serum solution. Clathrin distribution was visualized using the monoclonal anti-clathrin heavy chain antibody, X22, in conjunction with a goat-anti-mouse FITC-conjugated secondary antibody. Confocal microscopic analysis of clathrin distribution was accomplished using a MRC 1000 Laser Scanning Confocal Microscope (Bio-Rad, Hercules, CA) equipped with a krypton/argon laser and attached to a Zeiss Axiovert microscope. Care was taken to ensure data was collected a point midway between the substrate-attached plasma membrane level and the top of the cell.

Clathrin Redistribution and Assembly Assays

KB PC12 cells were assayed for clathrin assembly. Cells were grown to 70% confluency and media was changed to 1% horse serum 12 hours prior to the experiment. One 15cm plate of cells per condition was harvested with calcium and magnesium free PBS, and resuspended in 5 mls of cold DME/25mM HEPES buffer with or without 2nM NGF. The cell suspensions, in 15 ml conical tubes, were rotated for 1 hour at 4 °C, then warmed for 2 minutes in a 37°C water bath with periodic gentle mixing. Samples were chilled in an ice bath for 3 minutes to stop endocytosis, spun at 1000x g for 5 minutes,

washed once with cold PBS, and resuspended in 1 ml cold MES buffer (100 mM MES, pH 6.8, 0.5 mM $MgCl_2$, 0.2 mM DTT, 1 mM PMSF, 0.1 ug/ml each leupeptin and aprotinin). Membranes were disrupted by three cycles of freeze/thawing and pellet disruption with a 25-gauge needle. Samples were spun at 1000x g for 5 minutes to remove nuclei and intact cells and the supernatant was centrifuged at 100,000x g for 40 minutes. The resulting pellet (assembled and membrane associated clathrin triskelion fraction) was resuspended in lysis buffer (see Chapter 2 for lysis buffer contents). The supernatant (unassembled clathrin triskelions) was diluted 1:2 in a solution of 0.5 M Tris buffer and 1% TX-100. Both lysed fractions were processed and subjected to SDS-PAGE as described in Chapters 2 and 3.

Redistribution of clathrin to membrane fractions in PC12 nnR5 TrkA and TrkA mutant cells lines was assayed in the same manner as the assembly assay described above except for the following exceptions in protocol. After membrane disruption by freeze/thawing in MES buffer, samples were spun at 10,000x g for 10 minutes at 4°C. The supernatant (cytosolic fraction) and the pellet (membrane fraction) were lysed and processed as described above.

KB PC12 cells were used in the TrkA/clathrin association NGF time-course assay. Here cells were grown to 70% confluency in the media described above in 10 cm plates and given vehicle or 2nM NGF at 37°C for 1, 2, 5,15, or 60 minutes. At the end of each time-point, media was aspirated off and cold PBS was used to wash cells while the plates were transferred onto ice. Cells were then lysed and immunoprecipitated for TrkA with the anti-TrkA antibody 06574 (UBI). Samples were subjected to SDS-PAGE, transfer, and immunoblotting as described in Chapters 2 and 3. Antibodies against

clathrin heavy chain (TD.1), phosphotyrosine (4G10), and α-adaptin were used to sequentially probe the resulting blot. The blot was acid stripped using pH2.0 TBS for 30 minutes 25°C to prepare for subsequent antibody probing.

MHC Class I Biotinylation

One 15 cm plate of PC12 cells per condition were grown to 70 % confluency before treating in suspension with vehicle or NGF (2nM) as described previously. After this treatment, cells were kept at 4° C for 30 minutes in the presence of 0.5 mg/ml sulfo-NHS-biotin. Cells were then spun down and resuspended three times in cold 5mM glycine/ 5mM lysine / 50 mM Tris in PBS. Cells were then lysed as described above and immunoprecipitated with the anti-MHC Class I antibody OX-18 (1:1000) (Pharmingen). SDS-PAGE and blotting to nitrocellulose was followed by probing with HRP-conjugated streptavidin. Visualization of bands was accomplished as described above.

Results

Cells expressing a Trk family kinase or co-expressing Trk and p75 redistribute clathrin upon NGF treatment. A qualitative assessment of clathrin redistribution by confocal microscopy.

Our initial observation was that NGF treatment increased staining for clathrin near the plasma membrane in rat pheochromocytoma (PC12) cells (Grimes et al., 1996b). Of note, PC12 cells express both TrkA and p75. As a first test of the mechanistic basis for clathrin redistribution, we asked which NGF receptor was responsible. Cell lines co-expressing p75 and TrkA receptors, and cells expressing only one receptor were used. Cultures were maintained and treated as described in Methods and were fixed and prepared to show clathrin distribution before and after neurotrophin application. Fixed cells were incubated with the CHC-specific mouse monoclonal antibody X22 followed by a goat anti-mouse FITC conjugated secondary for visualization of distribution.

In these experiments, to confirm that the outer limit of clathrin staining near the edge of the cell observed to increase with NGF was indeed located at the plasma membrane, lipophilic carbocyanine dye, DiI (Molecular Probes), was used. DiI marks plasma membrane lipids. We asked whether DiI staining would colocalize with staining using the CHC-specific antibody X22. Figure 1 shows that warming cells in the absence of NGF is associated with very little CHC staining near the plasma membrane. This is evident in the relatively small amount of CHC staining at the apparent edge of the cell

(green channel), and in the very modest degree of yellow staining in the overlap panel. After 2 minutes of warming in the presence of 2 nM NGF there was a marked increase in clathrin near the plasma membrane (green channel) and many points of overlap were apparent between staining for CHC and DiI. These clathrin-positive structures may represent flat triskelion lattices on the plasma membrane or clathrin coated pits.

The NGF effect on clathrin redistribution was also seen in PC12 cells treated with NGF for 7 days before the experiment. Such treatment has been shown to enhance neuronal differentiation in PC12 cells and to increase expression of both TrkA and p75. (Zhou et al., 1995). Figure 2 shows that after washing primed cells, treatment with 2 nM NGF for 2 min at 37°C resulted in increased clathrin recruitment to the plasma membrane. Indeed, the difference between NGF and vehicle treated cells is striking. These findings suggest that enhanced neuronal differentiation does not block the NGF effect on clathrin.

Figures 3 & 4 show confocal micrographs of Schwann cells treated with NGF for either 0, 1, or 2 minutes. These cells endogenously express p75 at high levels but lack TrkA. We saw no evidence for clathrin redistribution even with NGF treatments of up to 20 minutes at 37°C (data not shown).

D283 human meduloblastoma cells transfected with TrkA (D283-TrkA) cells were used to show whether or not clathrin redistribution could be seen in cells expressing TrkA but lacking p75. The bottom panel of Figure 5 shows a confocal micrograph of clathrin staining in TrkA meduloblastoma cells given NGF (2nM) for 2 minutes at 37°C. The arrows highlight an outer boundary of the cell with intensified clathrin redistribution to the plasma membrane. The upper panel shows a representative cell not treated with

NGF. Cells not treated with NGF failed to show any of this characteristically distinctive clathrin staining at the plasma membrane.

Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family, that binds and specifically activates the TrkB receptor. BDNF signaling through TrkB is very similar to signaling through TrkA (Yuen et al., 1996). Figure 6 shows confocal micrographs of clathrin redistribution in 3T3 TrkB cells treated with BDNF (2 nM). The cells in the top panel have diffuse clathrin staining and lack the marked pattern for clathrin edging at the plasma membrane seen following BDNF treatment for 2, 10, and 60 minutes. Similar results were obtained with NGF treatment of 3T3 TrkA cells (data not shown).

To see if AP2 was also redistributed to plasma membrane, TrkB 3T3 cells were examined with and without BDNF application (Figure 7). Two minutes of BDNF treatment caused a dramatic redistribution of α-adaptin staining. The diffuse distribution seen in the cytosol of untreated cells was converted to a pattern suggesting plasma membrane localization (arrow, bottom panel). These results show that the clathrin redistribution seen in Figure 6 is accompanied by the redistribution of AP2. This observation is consistent with a model of RTK signaling in which several components of clathrin coated pits are moved to the plasma membrane, possibly to a location on activated RTKs (see Figure 22 model).

In summary, using confocal microscopy we gathered evidence indicating that NGF and BDNF induced a marked, robust redistribution of clathrin and AP2 to the plasma membrane. Our findings suggest that signaling from the TrkA and TrkB receptor is responsible.

Quantitative measurements of clathrin redistribution

Confocal microscopy provided data for a qualitative Trk-induced increase in plasma membrane clathrin. We next sought to quantitatively examine redistribution. We used the 2 minute timepoint because our original observations of the redistribution were from cells with 2 minutes of NGF application at 37°C, and studies of Connolly and Corvera showed a peak in the increase of plasma membrane coated pits at this timepoint (Connolly et al., 1981) (Corvera, 1990). In Figure 8, the clathrin assembly assay developed by Wilde and Brodsky was used to measure conversion of unassembled clathrin to assembled clathrin. The assay is similar to previously published assays and is described in detail in the methods section of this chapter (Corvera, 1990); (Jackson, 1993). This assay measures: 1) the amount of pelletable assembled clathrin triskelions which are found mainly associated with membrane; and 2) the amount of unassembled triskelions, representing the soluble, cytosolic fraction. PC12 cells were treated with or without NGF for 2 minutes at 37°C and fractionated in MES buffer at pH 6.8. This pH prevents disassembly of clathrin lattices during sample processing and fractionation. After disruption of membranes by repeated freeze/thawing, the suspension was centrifuged to separate the unassembled and assembled clathrin fractions. Fractions were lysed with standard lysis buffer and equalized for protein content before processing by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to nitrocellulose. TD.1, a CHC specific monoclonal antibody was used to probe the blot. An HRP-conjugated secondary was used to visualize the signal using ECL-hyperfilm exposure to

ECL phosphorescence. After scanning the exposed gel images into a digitized format, band intensities were determined by measuring the density of the hyperfilm band exposure per unit area with the gel plotting macro of NIH Image. In a representative experiment shown in Figures 8 and 9, there was a doubling of assembled clathrin with NGF treatment and a concomitant decrease in unassembled clathrin fraction. Four separate repetitions of this experiment yielded an average NGF-induced increase in the assembled fraction of 256% (standard error = 90%) above the vehicle treatment condition. The average decrease in the unassembled fraction upon NGF treatment was 7.59% (standard error = 2.30%) below the vehicle treatment condition.

Previous studies have shown no measurable increase in plasma membrane surface area after two minutes NGF exposure to PC12 cells (Connolly et al., 1981). To confirm these results and to ensure that the increase in clathrin assembly was not due to a general increase in plasma membrane surface area, we measured plasma membrane surface area using the cell surface MHC class I molecule as a marker. Figure 10 shows results from 3 experiments in which biotinylated MHC class I was measured before or after 2 minutes of NGF application. PC12 cells were treated with or without NGF and then chilled. To biotinylate surface proteins, sulfo-NHS-biotin was added to the cells at a final concentration of 0.5 mg/ml and incubated for 30 minutes at 4°C. The reaction was quenched with Tris buffer containing 5mM lysine and glycine. Cells were lysed with lysis buffer and immunoprecipitated with the anti-MHC class I antibody, OX-18 (Pharmingen). After SDS-PAGE and blotting to nitrocellulose, HRP-streptavidin was used to probe the blot. The signal was detected with the ECL chemiluminescence system described above, and bands were quantified using NIH Image. No statistically significant

changes in biotinylated MHC class I were found, confirming that an increase in plasma membrane surface area does not account for the increase in NGF-induced clathrin assembly.

To ask whether, as expected, clathrin redistribution was receptor mediated, we examined the NGF dose-response. Figure 11 shows the NGF dose-response curve for an assembly assay experiment similar to that in Figure 8. The NGF concentration ranged from 20 pM to 20 nM. The results show at the lowest concentration tested, clathrin assembly occurs. At this low concentration, only a small percentage of low-affinity receptors are occupied while a large proportion of high-affinity receptors are bound (Bernd and Greene, 1983). This data suggest a role for high-affinity receptors in clathrin redistribution and supports the involvement of TrkA in this process.

A timecourse of NGF-induced TrkA signaling effects on clathrin assembly is shown in Figure 12. The clathrin assembly assay described above was used to measure the response of NGF (2nM) at 37°C over 10 minutes. The lower panel shows a blot probed with the CHC-specific antibody, TD.1. The quantification of these data is displayed in the top panel. The data show that activation of TrkA causes a maximal clathrin assembly at 1 minute following ligand application. A commensurate decrease in unassembled clathrin was seen extending to at least 10 minutes. Warming in the absence of NGF resulted in no increase in assembly. These results indicated that TrkA signaling affects clathrin assembly. Since clathrin assembly is mainly membrane-based, these data are consistent with those from confocal microscopy in suggesting that NGF causes clathrin redistribution to the plasma membrane.

NGF-induced clathrin recruitment is blocked by TrkA kinase domain mutants

Since NGF binding to TrkA alone caused clathrin assembly, experiments were carried out to determine if redistribution and assembly of clathrin was due to activation of the TrkA kinase domain. TrkA signaling was disrupted through mutation of the TrkA kinase activation site (K538N) or through mutation of the TrkA activation loop (YY674/675FF). PC12 nnR5 is a line of cells derived from PC12 cells in which TrkA expression is depressed to very low levels compared to wild type. These cells show little or no activation of TrkA in response to NGF. P75 is present in these cells. We examined PC12 nnR5 cells that expressed mutant or wild type TrkA introduced via stable transfection. The kinase domain mutants, designated M1 (kinase dead, K538N) and 22.7 (activation loop point mutant, YY674/675FF), both prevent normal kinase activation and thus normal signaling of the receptor (Cunningham et al., 1997; Ferrari et al., 1995).

Confocal microscopy (Figure 13) was carried out with PC12 nnR5 cells expressing wild type or mutant TrkA. Cells in the right panels were exposed for 2 minutes to NGF (2nM) at 37°C; in the left panels, cells were treated in the same manner except that no NGF was added. The large arrowheads in the top left panel point to the relatively obscure cell boundary, as marked by clathrin staining, in cells not treated with NGF. The small arrows in the top right panel point to the more distinct clathrin edging found in cells after NGF treatment. This increase in clathrin near the plasma membrane was not seen in the M1 or 22.7 mutants suggesting that disruption of TrkA signaling also disrupts CHC distribution to the plasma membrane.

Results of a clathrin assembly assay using these cells are shown in Figure 14. The studies were similar to those shown in Figure 8. The graph shows the percent increase in band intensity of membrane fractions from 4°C in the presence of NGF (2 nM) to 2 minutes at 37°C with NGF. Wild type Trk-containing cells showed a significant increase in membrane-associated clathrin with warming in the presence of NGF; this treatment had no effect in cells expressing the activation loop mutant and actually reduced the clathrin content of membrane fractions in cells expressing the TrkA kinase domain mutants (n=2). These results confirm that TrkA is the NGF receptor responsible for redistribution and show that Trk kinase activation is responsible. The decrease in clathrin assembly in the kinase mutant raises the possibility that NGF binding to p75 suppresses clathrin assembly on membranes.

NGF induced an increase in phosphorylation of CHC in cells expressing TrkA

The requirement for TrkA kinase activation suggested the possibility that NGF acted through phosphorylation of one or more of the proteins in clathrin coated pits. The CHC was a logical candidate. To ask whether NGF signaling caused CHC phosphorylation, PC12 cells and Schwann cells were treated with or without NGF (2nM) for 2 minutes at 37°C. They were then chilled and lysed. Lysate aliquots containing an equal amount of protein were immunoprecipitated with the CHC-specific antibody X22, followed by SDS-PAGE and blotted to nitrocellulose. The resulting western was probed for phosphotyrosine with the antibody 4G10, and this was visualized with ECL phosphorescence using an HRP-conjugated anti-mouse secondary antibody (Figure 15).

Identical X22 immunoprecipitations were submitted to PAGE and blotted for TD1 to ensure equal protein loading. Band intensities were quantified by NIH Image and the signal for phosphotyrosine (bands in left blot) were divided by the signals for CHC (right blot) to obtain a value for the relative level of tyrosine phosphorylation. The percent change of this signal with NGF treatment is graphed at the bottom of Figure 15. In PC12 cells, NGF treatment caused a 67% increase in CHC phosphorylation. Three separate experiments using KB PC12 cells (expressing wild type TrkA and p75), produced an average increase in CHC phosphorylation of 86% ± 28% (SE). In contrast, Schwann cells showed an 8% decrease in CHC phosphorylation when treated with NGF. The band below CHC here could be EPS 15 as this protein, found at the necks of coated pits, runs below 183kD (Schmidt, 1997). The data point to a NGF effect on CHC phosphorylation and suggest that signaling through TrkA is responsible.

NGF-induced CHC phosphorylation is blocked by TrkA kinase domain mutants

To ask whether TrkA kinase activation is responsible for the increase in CHC phosphorylation, we compared the response to NGF of PC12 nnR5 cells expressing wild type and mutant TrkA. Figure 16 shows the results from an experiment done similarly to that in Figure 15, but here a specific SRC kinase inhibitor, PP1, was used in one condition (Hanke et al., 1996). Cells were held at 4°C or were treated with NGF (2nM) for 2 minutes at 37°C . They were then lysed, immunoprecipitated with the CHC-specific antibody, X22 and the immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose for blotting with 4G10, an anti-phosphotyrosine antibody. The secondary

antibody was visualized as described in Figure 15. The data show that NGF induced an increase in CHC phosphorylation of 85% and 200% at 4°C and 37°C, respectively, in cells expressing wild type TrkA (Fig 16, top bar graph). The SRC kinase inhibitor PP1 all but abolished this effect in the wild type TrkA cells. NGF failed to increase phosphorylation of CHC in the TrkA activation loop mutant, 22.7. In fact, in comparing the warmed and unwarmed cells, NGF addition to this mutant caused a 25% decrease in CHC phosphorylation (Fig. 16 bottom bar graph). These data confirm that TrkA kinase activation is responsible for CHC phosphorylation and suggest that a kinase inhibited by PP1 (e.g.- a SRC family kinase) plays a role. Surprisingly, NGF addition to the mutant in the presence of PP1 increased CHC phosphorylation. Possible explanations for this unexpected result are put forth in the discussion.

To further confirm these results, the above experiment with PC12 nnR5 TrkA and TrkA mutant cells was repeated using an anti-phosphotyrosine antibody for immunoprecipitation in place of CHC immunoprecipitation. The resulting western blot was sequentially probed for TrkA and clathrin with signal visualized as described previously with ECL phosphorescence and an HRP-conjugated secondary antibody. The results in Figure 17 show blots of PC12 nnR5 wild type TrkA along with the TrkA M1 and TrkA 22.7 mutants probed for TrkA (top panel) with an anti-N-terminal TrkA antibody (06574, UBI) and probed for CHC (bottom panel) with the TD1 antibody. The bar graph in the middle shows the results of the NIH-Image quantification of the blots. Mutant cell lines show little or no TrkA kinase activity and essentially no CHC phosphorylation with or without NGF. In the wild type TrkA cells there was some TrkA activation in the absence of NGF but there was a clear-cut increase in this activation in

the presence of ligand. This increase was associated with an increase in CHC phosphorylation. Thus TrkA kinase activity appears to be needed for the increased phosphorylation by NGF.

TrkA association with CHC, tyrosine phosphorylated CHC, α-adaptin, and pp60^{src} increases with NGF application

The data suggested a model in which activated TrkA is required for NGF-induced increases in CHC tyrosine phosphorylation and redistribution and assembly of clathrin at plasma membrane. We asked if CHC (as an indicator of the presence of clathrin triskelions) and other coated pit proteins could be found associated with TrkA. PC12 cells were treated with NGF for 0, 1,2,5,15, and 60 minutes, chilled and lysed with standard lysis buffer. The lysates were equalized for protein and immunoprecipitated with an anti-amino-terminal TrkA antibody (06574, UBI). Immunoprecipitations were subjected to SDS-PAGE and Western blotted to nitrocellulose. Blots were sequentially probed with TD1 (anti-CHC), 4G10 (anti-phosphotyrosine antibodies), and AC1M11 (anti-α-adaptin). These blots are shown in this order, top to bottom, in Figure 18 accompanied by a bar graph showing the NIH Image quantification of the band intensities. CHC was associated with Trk even without NGF application. The timecourse shows a ~20% increase in TrkA-associated CHC at 1 and 2 minutes after NGF application that falls off gradually over 60 minutes. This increase is measured from the no NGF condition and is similar to the increases in CHC redistribution seen in earlier experiments.

The phosphotyrosine blot in Figure 18 (middle blot) shows an increase in TrkA-associated phospho-CHC (arrow) that peaks during the 5 minute timepoint at 90% above the no NGF condition and persists through 60 minutes. In this blot, the larger band below the phospho-CHC signal is phospho-TrkA . The increase in TrkA phosphorylation at 1 minute correlated with the appearance of associated phospho-CHC, but the two peak at different times. The peak of TrkA phosphorylation is 2 minutes while the peak of CHC phosphorylation is at 5 minutes. This result raises the possibility that though TrkA and CHC are found in a complex, Trk kinase association is not linked directly to phosphorylation of CHC.

The lower blot of Figure 18 shows that there is a rapid 60% increase of α-adaptin associated with TrkA at the 1 minute time point, which gradually falls off over 60 minutes. The association of α-adaptin with TrkA was confirmed in a similar experiment examining wild type and mutant cell lines expressing Trk. A clear increase in TrkA/α-adaptin association is seen with NGF treatment for 2 minutes of both PC12 cells and PC12 nnR5 cells expressing wild type TrkA. The increase was not found in the TrkA 22.7 mutant. In fact, there appeared to be background association of α-adaptin in the mutant cell line without NGF, which was decreased by 50% after treating with NGF. There is a doublet of α-adaptin visible in these immunoprecipitations which has been observed previously (Wilde et al., 1996).

To show that the association of TrkA and CHC was due to kinase activation, we compared PC12 cells with PC12 nnR5 cells expressing either wild type or mutant Trk. There was a robust increase in CHC in TrkA immunoprecipitates from cells expressing

wild type TrkA. The TrkA kinase mutant failed to show an increase in TrkA/CHC association following NGF treatment. As for α-adaptin, there was a higher background of CHC association with TrkA without NGF which decreases with NGF application. More background binding of CHC and α-adaptin to TrkA kinase mutants may be caused by conformational changes in the C-terminus of the receptor caused by the mutation. These data also raise the possibility that NGF-induced signaling activity of p75 leads to reduced TrkA/ clathrin association. In this scenario, TrkA and p75 would have competing and inverse effects on clathrin redistribution, phosphorylation, and receptor association. Other data that show aspects of TrkA and p75 signaling to work antagonistically with regards to apoptosis adds credence to this possibly (Bredesen and Rabizadeh, 1997).

NGF application induces TrkA and pp60^{src} association in PC12 cells

Data outlined in the Appendix shows a requirement for SRC kinase activity in CHC phosphorylation via activation of EGF-R signaling. Since TrkA activates pp60^{src} (Hilborn et al., 1998; Kremer et al., 1991), and since pp60^{src} has been shown to be activated and bound by EGF-R (Appendix), we asked if pp60^{src} was associated with activated TrkA. PC12 cells were treated with or without NGF (2nM) for 2 minutes and processed (up to the western blot probing step) as in the experiments shown in Figures 19 and 20. In short, cells were lysed and immunoprecipitated with an anti-TrkA antibody and submitted to SDS-PAGE and western blotting. The resulting blot was probed with an anti- pp60^{src} antibody obtained from the lab of Michael Bishop (Ab. 327) and the signal was visualized as described above. The band marked by the arrow denotes pp60^{src}'s

molecular weight, at 60 kD, and the band below it is the TrkA antibody band. Virtually no pp60^{src} was found associated with TrkA without NGF, but with NGF application, there was a dramatic increase in pp60^{src} in TrkA immunoprecipitates.

Models for coated pit machinery interaction

The above data adds to the old model of coated pit machinery interaction in that we can propose new roles and new physical associations for the proteins involved. The model in Figure 23 shows one possible scenario. The data that helped construct the old model, depicted in Figure 22 featured; 1) TrkA localized to coated pits (Beattie et al., 1996; Connolly et al., 1981; Grimes et al., 1996b) ; 2) pp60^{src} localized in plasma membrane and demonstrated its ability to be activated and to bind RTKs (EGF-R) ((Brown and Cooper, 1996), Appendix); and 3) demonstrated binding of CHC to AP2 and AP2 to sorting motifs on RTKs (Schmid, 1997).

Our data suggest that TrkA activation causes the recruitment of AP2, clathrin, and pp60^{src} to TrkA receptors in plasma membrane. With the data showing EGF-R activation of CHC phosphorylation by pp60^{src} (Appendix) it is tempting to speculate that activated TrkA is able to bring pp60^{src} and CHC together and thereby stimulate pp60^{src} –mediated tyrosine phosphorylation of CHC. This event may increase the ability of clathrin triskelions to assemble, thus enhancing the speed and efficiency of TrkA endocytosis.

Discussion

Cells expressing a Trk family kinase or co-expressing Trk and p75 redistribute clathrin upon NGF treatment. Qualitative assessment of clathrin redistribution by confocal microscopy.

Initial confocal observations that NGF induces rapid clathrin redistribution to the plasma membrane in KB PC12 cells (Chapter 2) compelled us to seek confirmation in other cells lines. Two minutes of NGF application was chosen as an optimal time to look for the redistribution effect because: 1) our initial observations were at this timepoint; and 2) Connolly et al. reported this to be the peak of coated pit formation after NGF application to PC12 cells (Connolly et al., 1981). Our results show that this redistribution was robust, rapid, and placed clathrin at or near the plasma membrane. Importantly, using a number of different cell lines, it was shown that the TrkA receptor was responsible for the effect. Indeed, using PC12 cell lines transfected with either wild type or mutant TrkA, it was evident that clathrin redistribution requires an intact kinase domain. In Schwann cells, which express only the p75 neurotrophin receptor, NGF treatment did not result in clathrin redistribution. These findings suggest that activation of TrkA kinase results in the movement of clathrin to plasma membrane.

Clathrin coated pits contain other proteins integral to their structure and function. Neurotrophin-induced redistribution of the AP2 adaptor protein subunit, α-adaptin, was used to confirm that Trk activation was effecting the initiation of coated pit formation on the plasma membrane. AP2 is specific to plasma membrane coated pit formation (Page and Robinson, 1995). A related adaptor protein, AP1 is also involved in coated pit

formation but is specific to vesicle budding from the trans-golgi network. Previous work indicates that AP2 binds the carboxyl-terminus of receptors after ligand binding and activation (Glickman et al., 1989; Pearse, 1988). Among such receptors are the insulin and EGF receptors, both RTKs. It appears that AP2 forms the inner coat of the coated pit and acts as a bridge between receptors and clathrin (Schmid, 1997). Our results showing a redistribution of α-adaptin staining with 2 minutes of neurotrophin application indicates that neurotrophin binding to Trk is responsible for the recruitment of this coated pit protein to the plasma membrane. This also suggests that the entire coated pit is assembled in response to Trk activation. Our findings are consistent with earlier studies demonstrating an increase in coated pits following addition of NGF, EGF, and insulin. They enlarge upon the earlier studies by indicating that the effect is dependent upon RTK activation.

NGF induces an increase in assembled clathrin. Quantitative biochemical measurement of clathrin assembly in KB PC12 cells.

Clathrin in coated pits is assembled into large triskelion lattices. EM studies indicated that NGF caused redistribution of clathrin to plasma membrane, but they did not define the state of CHC assembly (Connolly et al., 1984). To address this question, we used a method to measure clathrin assembly. This protocol, which was similar to previously published methods, separates single triskelions from fractions with assembled triskelion lattices (Corvera, 1990) (Jackson, 1993). These lattices are found mainly on membrane. In PC12 cells, NGF induced an average increase of 256% ± 90% (S.E.) (n=4)

in assembled clathrin fractions compared with the controlled condition. This increase occurred rapidly and was quite reproducible. The increase detected was comparable to that seen by Corvera et al. These investigators showed a 200% increase in the concentration of assembled clathrin with 2 minutes of insulin treatment on adipocytes (Corvera, 1990). But, the increase is also comparable to the ~300% increase in coated pit formation seen by Connolly et al. in their EM studies of NGF treated PC12 cells. An important finding that links this study to that by Connolly et al. is that NGF did not increase the amount of plasma membrane. In earlier studies, brief (2 minutes) NGF treatment did not increase cell volume or surface area as measured by EM (Connolly et al., 1984). We confirmed this result in cell surface biotinylation experiments with NGF treatment showing that there was no significant change in PC12 cell-surface MHC class I molecule with NGF treatment. MHC class I molecule presents peptides to immune system cells that scan for non-self protein sequences and has been shown to be a good marker of plasma membrane surface area (Brodsky, et al., unpublished data). Thus, an increase in plasma membrane cannot account for the redistribution of clathrin.

To extend earlier findings, we attempted to define the receptor responsible for increased clathrin assembly. To characterize the dose-dependence of clathrin assembly, we examined NGF concentrations from 20 pM to 20 nM in studies on PC12 cells. Even at 20 pM, assembly of clathrin was robust, with essentially no further increase at higher concentrations. The sensitivity of the assay to the lowest concentrations of NGF supports a role for high-affinity receptors in effecting the redistribution. Since TrkA is a known component of such receptors, these data are consistent with a role for TrkA (Meakin and Shooter, 1992).

To confirm that TrkA is the mediator of NGF-induced clathrin assembly, we assayed this activity after disrupting normal TrkA kinase function. PC12 nnR5 cells transfected with either wild type TrkA or TrkA mutants were assayed biochemically for clathrin assembly and membrane association after NGF application. Our results showed that wild type TrkA cells responded well to NGF as evidenced by TrkA tyrosine phosphorylation. Both TrkA mutants, however, showed little or no increase in NGF-induced TrkA phosphorylation when compared to wild type expressing cells. PC12 nnR5 with reintroduced wild type TrkA showed an increase in clathrin redistribution with NGF application. In contrast, the PC12 nnR5 lines expressing the two TrkA mutants (M1 and 22.7) responded to NGF application with no increase or with a decrease in clathrin assembly. Compared to the pre-treatment condition, membrane bound clathrin levels reduce by 25% in M1 kinase domain mutants. These findings indicate that Trk is the receptor responsible for clathrin assembly and point to the need for Trk kinase activation. In showing that Trk kinase activation was needed for both clathrin redistribution and clathrin assembly, the data show that the two are mechanistically linked. Given earlier findings on the number of clathrin coated pits, our data argue that Trk activation initiates signaling events that lead to the recruitment of clathrin coated pit proteins to the plasma membrane.

The data from our timecourse experiments also support the view that a common mechanism is responsible for clathrin recruitment and clathrin assembly. Both occur rapidly and are present by 2 minutes. NGF's effect on clathrin assembly peaked at 1 minute and was decreased at 10 minutes. Interestingly, Connolly et al. found an early

peak for clathrin coated pits at 2 minutes and slow decrease of coated pits on plasma membrane by 15 min. This early peak could represent the cell's complement of TrkA receptors binding to NGF and becoming activated with the ready transmission of the signal causing clathrin redistribution and assembly. The decrease over the next 10 to 15 minutes is likely to reflect downregulation of activated TrkA receptors at the cell surface. Confocal data from Chapter 3 showing significant co-localization of TrkA and lysosomal markers after 20 min of NGF application suggests that Trk signaling may in fact be terminated in lysosomes soon after NGF binding and internalization. If much of a cell's signaling-competent Trk complement is degraded by NGF-induced endocytosis by 20 minutes, signaling effects including clathrin redistribution would be expected to fall until new Trk receptors are made.

Phosphorylation of clathrin increases after NGF application and this increase is blocked by TrkA mutants and inhibitors of SRC kinase activity.

Evidence that Trk kinase activation was required for clathrin redistribution and assembly, and previous evidence for phosphorylation of 1) clathrin light chain, 2) various coated pit proteins (Schmid and Damke, 1995) (Fallon et al., 1994) (Holen et al., 1995b), and 3) clathrin heavy chain itself (Wilde and Brodsky, 1996) encouraged us to examine the possible effects of NGF on clathrin heavy chain tyrosine phosphorylation. Using the same concentration and timepoint used in our previous experiments we discovered that NGF increases CHC phosphorylation in PC12 cells. No increase was seen in Schwann cells. Thus, as was the case with clathrin redistribution and assembly, NGF appeared to

increase CHC phosphorylation through TrkA. This was confirmed in studies of PC12 nnR5 cells expressing wild type and mutant TrkA receptors. It was only in cells expressing wild type TrkA receptors that NGF induced an increase in CHC phosphorylation. In collaborative studies with Brodsky and Wilde (see Appendix), $pp60^{src}$ is shown to be the agent responsible for this phosphorylation. Confirming this in thesis studies, the CHC phosphorylation increase was prevented if an inhibitor of SRC kinase activity was administered prior to NGF application. Thus, TrkA kinase activity was shown to be required for increased CHC phosphorylation, redistribution and assembly of clathrin.

Other receptors (e.g. the insulin receptor and the β2-adrenergic G-protein coupled receptor) have shown clathrin recruitment capacity. When activated by ligand, the β2-adrenergic G-protein coupled receptor becomes phosphorylated and binds β-arrestin 1 which in turn binds clathrin (Goodman et al., 1996). Could phosphorylation and assembly of clathrin be related? The collaborative studies looking at EGF-R's effects on clathrin with Brodsky and Wilde (Appendix) show that they are. Here, the blockage of EGF-induced CHC phosphorylation through SRC kinase activity prevented redistribution of clathrin to the plasma membrane in A431 cells. The redistribution and phosphorylation changes induced by EGF in A431 cells were more marked than those seen in the TrkA expressing cell lines. The differences could reflect differential receptor expression levels, or the presence of p75 negative modulatory effects in the TrkA cells. Studies outlined in the Appendix determined the site of CHC phosphorylation in response to EGF to be tyrosine 1477, which resides in the CHC's assembly domain near the vertex of the clathrin triskelion. This finding suggests that phosphorylation of CHC may modulate

contact points between clathrin triskelions. Based on the experiments showing that recruitment is prevented by blockage of CHC phosphorylation, it is likely that phosphorylation is important to clathrin assembly. One question raised by this suggestion is how clathrin and $pp60^{src}$ are brought together. Immunoprecipitation data described in this thesis points to TrkA as a organizing center for multiple signaling and endocytosis proteins.

NGF treatment induces increased TrkA association with clathrin, α-adaptin, and $pp60^{src}$

We asked whether TrkA could serve as signaling scaffold to bring together $pp60^{src}$ with endocytosis-promoting proteins to allow for CHC phosphorylation and to increase triskelion assembly and subsequent facilitation of receptor endocytosis (Figure 23). NGF timecourse experiments were performed in PC12 KB cells to assess possible TrkA associations. Anti-TrkA immunoprecipitations of cell lysates from these experiments show a correlation of wild type TrkA activation with TrkA-bound CHC phosphorylation. These experiments also correlate TrkA activation with increased clathrin/TrkA and α-adaptin/TrkA associations. At the 1 minute NGF application timepoint, TrkA/ α-adaptin and Trk/ clathrin associations peak at ~60% and 20% above control levels. CHC phosphorylation peaks at ~90% over background after 5 minutes NGF application.

Experiments in PC12 nnR5 cells with wild type and mutated TrkA cells demonstrated the need for wild type TrkA kinase activity for increased associations of

clathrin and α-adaptin with TrkA. In addition, pp60^{src} can be found associated with TrkA at 2 minutes of NGF treatment in PC12 KB cells. With the body of literature linking TrkA signaling to pp60^{src} activation (Hillborn et al., 1998; Kremer et al., 1991) and the precedent for pp60^{src} association with active EGF-R (Sato et al, 1995), this last piece of evidence is especially interesting.

There is a significant amount of clathrin associated with TrkA without NGF treatment. This may represent receptors associated with clathrin in the process of budding from the trans-golgi network or receptors awaiting ligand in cell surface coated pits. NGF application may allow more access of receptors to these coated pits or cause clathrin to assemble de novo around TrkA receptors.

The data presented here can be used to support the simple model presented in Figure 23. According to such a model, signaling and recruitment might proceed as follows. First, TrkA is activated by NGF, causing conformational changes in carboxyl-terminal AP2 binding motifs. The dileucine motif is an example of these AP2 binding sites which have been shown to be important in coated pit endocytosis (Hamer et al., 1997) and a few of these sites can be found on the carboxyl-terminus of TrkA . Second, these exposed binding motifs would increase AP2 association and allow for entry into formed coated pits or for binding of soluble clathrin. Concurrently, pp60^{src} could bind to sites directly on activated TrkA or to TrkA associated proteins. Clathrin would then be in close proximity to pp60^{src} for CHC phosphorylation, which would then increase triskelion assembly around the complex. The next result of this model is enhanced internalization of TrkA following NGF binding and activation. Our studies are the first of their kind to show the ligand-induced redistribution and phosphorylation of CHC as well as the

association of TrkA with CHC, α-adaptin, and pp60^{src}. These results provide a new and relatively simple model to test with future experiments.

How might p75 fit into this model? The suggestion that signaling from p75 may oppose Trk-induced trafficking and phosphorylation of clathrin is supported by studies demonstrating the antagonistic signaling effects of TrkA and p75 (Bredesen and Rabizadeh, 1997) as well as our CHC phosphorylation and clathrin redistribution data. First, p75-positive Schwann cells display a NGF-induced decrease in CHC phosphorylation. Second, TrkA kinase domain mutant cell lines show a NGF-induced reduction of assembled and membrane bound clathrin in biochemical studies. Finally, confocal micrographs of the TrkA kinase domain mutant, M1, seems to show less plasma membrane associated clathrin after NGF application.

As previously stated the effect of EGF on CHC phosphorylation and redistribution in A431 cells is more marked than is the effect of NGF on the same parameters in PC12 cells. P75 may serve to attenuate the clathrin modulating signaling of TrkA for the purpose of keeping the resulting effects localized around the TrkA receptor. TrkA and EGF-R elicit very similar signaling cascades (Stephens et al., 1994) This proposed difference in controlling cell-wide clathrin modulating signaling might contribute to the different mitogenic and differentiation effects of EGF and NGF.

The observed effects upon clathrin and associated endocytosis molecules induced by TrkA and EGF-R signaling give us a new view of the mechanistic control of RTK endocytosis. They also provide us with many possible avenues of investigation to pursue that stretch to other receptor systems utilizing coated pit endocytosis and beyond.

Which NGF receptor is responsible for clathrin redistribution?

Evidence that both TrkA and p75 elicit NGF-induced signal cascades exists (Bredesen and Rabizadeh, 1997). Thus, we asked which NGF receptor was responsible for the redistribution of CHC from the cytosol to the plasma membrane. Our data showing that only cell lines expressing wild type TrkA can redistribute clathrin to the plasma membrane with NGF treatment supported Trk's role. In addition, our immunoprecipitation data supporting the associations between NGF-activated TrkA and clathrin,α-adaptin, and $pp60^{src}$ suggest that at least a portion of the clathrin redistributed to plasma membrane resides on TrkA. These data paint for us a new and interesting mechanistic picture of how receptor tyrosine kinases may be endocytosed (see model, figure 23)

Review of results and a new coated pit protein interaction model

In searching for a mechanistic explanation for this action, it was observed that CHC tyrosine phosphorylation was increased when NGF was applied to cells with functional TrkA receptors. In addition, after NGF application to TrkA-positive cells, AP2, phosphorylated CHC, and the non-receptor kinase $pp60^{src}$ were found to increase association with TrkA. These exciting results hint at the possibility that TrkA may serve as a signaling scaffold upon which AP2, clathrin, and $pp60^{src}$ coalesce to promote coated pit endocytosis of TrkA. Future studies may involve a search for these interactions in other receptor systems that utilize the clathrin coated pit pathway. As has been outlined in

Chapters 2 and 3, internalization allows for the continued signaling of TrkA from endosomal structures. Since we no longer think of endocytosis as simply a means to an end of signaling but rather a transition to a new platform for continued signaling, endocytosis has come to be seen as a key control point for signaling modulation.

Our collaborators, Wilde and Brodsky, are studying clathrin and the effects that the epidermal growth factor receptor (EGF-R) has on its redistribution and phosphorylation. Recent results (Appendix) show that, like TrkA, EGF-R activation leads to redistribution and phosphorylation of clathrin. We have also shown that a SRC family kinase is responsible for CHC phosphorylation and pinpoint the tyrosine of CHC that is its target. These data, along with our data showing a NGF-induced TrkA/ $pp60^{src}$ association, supports this model.

Thus, TrkA's proposed role as a signaling scaffold may include bringing together $pp60^{src}$ and clathrin for the latter's tyrosine phosphorylation. How this phosphorylation contributes to the endocytosis process is yet unclear but some evidence suggests that it may aid in triskelion assembly over the c-terminus of TrkA (Pley et al., 1995). This enhancement of clathrin triskelion latticing would positively affect internalization of TrkA.

Chapter 6

Implications and Future Directions

The signaling TrkA endosome as a retrograde neurotrophic signal

Examination of the subcellular locations from which TrkA signals are required to fully understand growth and differentiation of NGF-responsive neurons. Early studies proposed that ligand-induced endocytosis of RTK's was simply a means for downregulation of the receptor and its signal (Khan et al., 1993). Other examinations of certain RTK's including the insulin receptor and the EGF-R along with our TrkA studies, suggests that endosomal RTK signaling occurs and may be important for the propagation of the trophic signal (Khan et al., 1989) (Bevan et al., 1995). Recent evidence supports the retrograde transport of NGF and Trk that initiates CREB translocation into the nucleus (Riccio et al., 1997b) The neuron's requirement for intact retrograde transport machinery has also been demonstrated (Johnson et al., 1980). This suggests the possibility that activated TrkA signals from the mobile, intracellular platform of an endosome. Our results detailing clathrin mediated endocytosis and the continued competence for signaling by endocytosed TrkA in PC12 cell fraction support this suggestion.

This evidence along with other laboratories' contributions (Riccio et al., 1997b) form a strong basis for the theory that we have proposed as to how some sets of neurons (e.g. basal forebrain cholinergics) survive with the help of distant target sources of NGF. Riccio et al. used chamber-separated cultures of sympathetic neurons to effectively

confirm our model in primary cultures. They have shown that if TrkA is allowed to signal at the tips of sympathetic neuron processes while NGF is covalently bound to an endocytosis-preventing matrix, evidence of TrkA signaling does not reach the nucleus. If, however, NGF is allowed to be taken up freely by the tips of processes (isolated in a separate media bath from their cell bodies by a silicon barrier), evidence of TrkA signaling is elicited in a time course consistent with that of the retrograde trafficking of transport vesicles (Overly et al., 1996).

The data suggest a model of survival in CNS neurons where a supply of NGF is accessible to the tips of basal forebrain cholinergic projections in cortex. They support the uptake of neurotrophin here by TrkA receptors and a subsequent retrograde trafficking of the actively signaling receptor-ligand complex to the soma with the transport vesicle as the platform. Once in close proximity to the nucleus this signaling endosome would then be able to elicit the translocation into the nucleus of transcription promoting factors supportive of the neuronal growth, differentiation, and maintenance.

Our data are derived from a most highly characterized tissue culture model of TrkA-containing CNS neurons, the PC12 cell. These cells have the great advantage of being amenable to subcellular fractionation, biochemical analysis, as well as to immunohistochemical evaluation. Their easy passaging, similarities to neurons after NGF application, and homogeneous purity made them our choice to establish the existence of TrkA-containing endosomes in neuron-like cells.

This model has implications for the development and maintenance of neuronal health in the subset of CNS and PNS TrkA positive neurons. It is interesting to note that in disease states such as Down's syndrome and Alzheimer's, TrkA expression is highly

correlated with CNS regions that are particularly affected by these maladies. Progressive neuronal death in these instances may involve the interruption of the NGF trophic signal, possibly by interfering with the formation or retrograde transport of the signaling vesicle. If this scenario continues to be born out, our best hope for successful intervention in these neurological diseases, demands understanding fully the processes that lead to the successful transport of this trophic signal.

Future directions for study regarding the retrogradely transported signaling vesicle include a more detailed characterization of resident vesicle proteins and the transport mechanisms involved in their transport. Implications for other receptor systems, which may not have remote trophic signal sources but which still may require the signaling from the endosome are also plentiful. Analysis of the importance of this mode of signaling not only for development and maintenance of neuronal health and prevention of apoptosis, but of modulation of synaptic strength, targeting, and plasticity are possible avenues of future study.

Receptor Signaling and Coated Pit Endocytosis

Our data are consistent with the first stage of the retrograde journey of the trophic signal involving the endocytosis of TrkA and NGF by clathrin coated pits. Thus endocytosis by this pathway is a possible trophic signal control point. This work detailed in this thesis has explored the mechanics of this process and presents data that support the theory that at least a proportion of TrkA is endocytosed by clathrin-coated pits (Chapters 2 and 3). We also present data for Trk and clathrin colocalization along with the presence of CHC and AP2 in endosomal fractions (Chapter 4). Furthermore, these studies

prompted us to investigate various NGF-induced clathrin redistribution, phosphorylation, and receptor association phenomena that have lead us to a new model for ligand-mediated coated pit endocytosis (Chapter 5). In this model (Figure 23) TrkA and EGF-R activation leads to increased α-adaptin, clathrin, and $pp60^{src}$ association with the receptor. Since our earlier studies helped to show that endocytosis is not just a means to a downregulation end, but the beginning of a new location of RTK signaling, this process of endocytosis can be seen as a key control point for receptor signaling.

Chapter 5 and the Appendix highlight similarities in ligand-induced clathrin responses between the EGF-R and TrkA systems. Therefore these data may have important implications for the endocytosis and signaling of other receptors thought to be endocytosed by clathrin coated pits. Our initial confocal studies suggested that the increase in clathrin at the plasma membrane is not simply utilized for TrkA endocytosis but likely is bringing other receptors into the cell upon NGF stimulation. This putative trans-activation of clathrin coated pits that increases the uptake of other receptors, and which is stimulated by NGF application, may be important to neurons differentiation and maintenance.

The ligand-induced increase in CHC phosphorylation, association with TrkA, and redistribution are reported here for the first time. The discovery of $pp60^{src}$ as the best candidate for CHC phosphorylation also outlined in the Appendix brings more detail to a putative endocytosis model that has $pp60^{src}$ and CHC brought together upon the signaling scaffold of TrkA. These ligand-induced responses may shed new light on the mechanisms of coated pit endocytosis for a wide variety of receptor systems and bring up many new questions to be asked in future studies.

For example, what other receptors that utilize CHC endocytosis effect the phosphorylation state of CHC? How exactly does this phosphorylation enhance endocytosis as the data outlined in the Appendix and previous studies of Pley et al. suggest?

Can we also see an effect on coated pit formation involved in outward cellular traffic (golgi)? If so what are the similarities and differences between the plasma membrane and golgi mechanisms? The biochemical data in this thesis examining the redistribution and assembly of CHC must be seen to be assessing both of these possibilities. Therefore, the sum of these two processes must equate to a positive ligand-increase in CHC membrane recruitment and assembly.

What types of receptors can be found in the increased number of coated pits brought about by TrkA activation? Will blockage of CHC-mediated endocytosis with CHC hub expression, mutant dynamin expression, high sucrose, etc. prevent TrkA endocytosis? Is caveolin involved in the process of TrkA endocytosis?

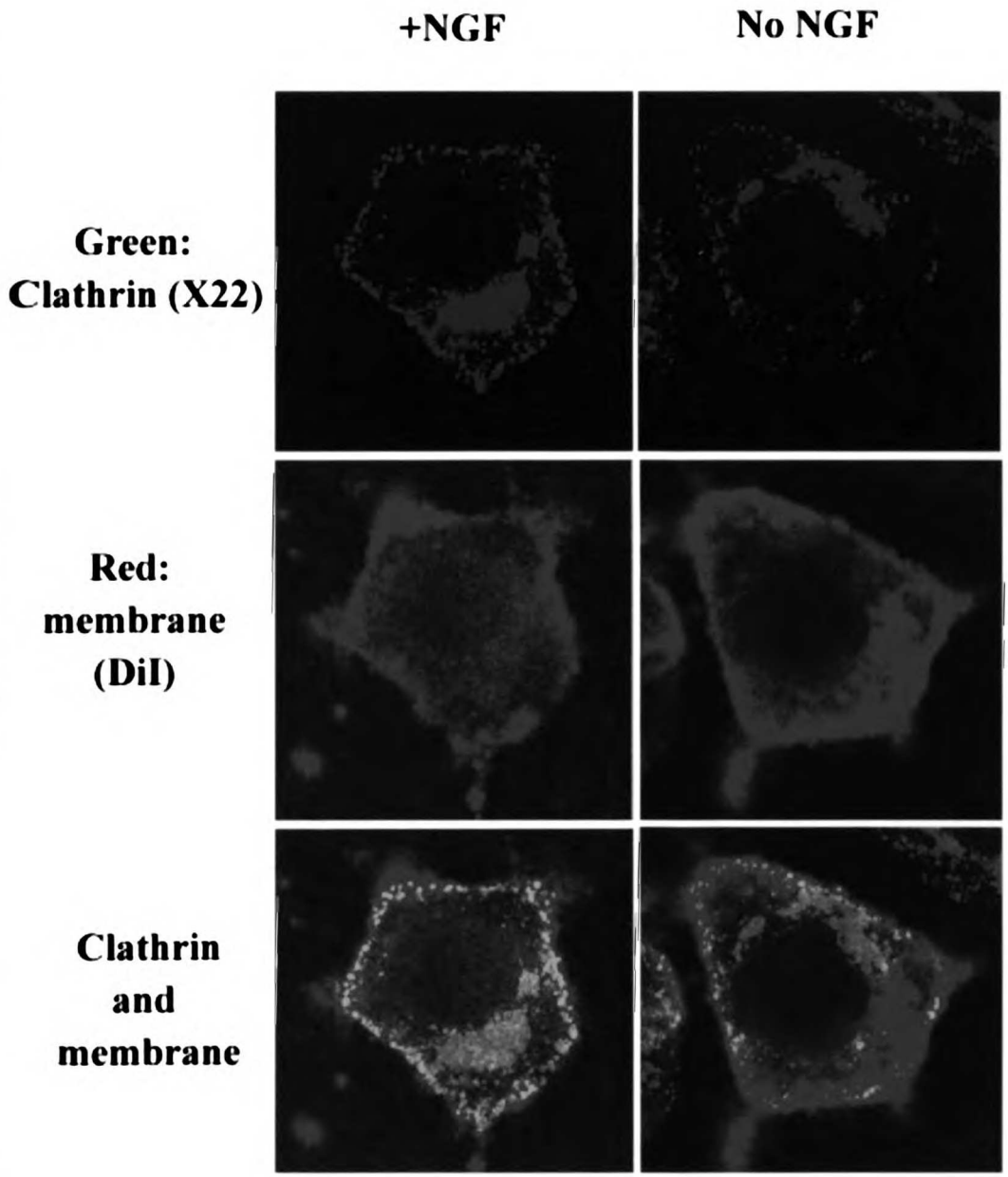
Though two new roles for TrkA have been found, and a possible new endocytosis control point has been uncovered in the NGF and EGF-induced phosphorylation of CHC, these studies have spawned more questions than they have answered. Future investigations into questions such as those posed above will undoubtedly further enlighten our understanding of endocytosis regulation.

Figure 1. Clathrin is recruited to PC12 cell plasma membrane upon NGF application.

The left column of panels shows confocal micrographs of clathrin (top row-green) and plasma membrane (middle row-red) staining in the same PC12 cell, treated for 2 minutes at 37°C in the presence of 2 nM NGF. The right column of panels shows a representative cell from the control experiment without NGF treatment. X22, a rabbit polyclonal antibody specific for clathrin heavy chain (CHC), and an FITC-conjugated goat-anti-rabbit secondary antibody were used to label clathrin distribution. The lipophilic dye, DiI was used to mark plasma membrane. The lower row of panels shows a double-channel image of both clathrin and plasma membrane where yellow marks colocalized red and green signal. This lower row of panels shows that the distinct outer boundary of clathrin staining observable in the NGF positive condition is located at the cell surface.

Figure 1

Clathrin Is Recruited To Membrane Upon NGF Application and 2 Min. Warming, 37 degrees C.



PC12 Cells

Figure 2. NGF-induced clathrin recruitment to plasma membrane (PM) in primed PC12 cells.

Both panels show confocals of primed PC12 cells, washed of NGF, and subsequently warmed in the absence (top) or presence (bottom) of 2 nM NGF. Staining represents clathrin visualized using X22 (CHC) as primary antibody and a goat-anti-rabbit FITC-conjugated secondary antibody. In cells treated with NGF, there is a marked redistribution of clathrin from a diffuse cytosolic staining pattern to a distinct plasma membrane pattern as seen in the lower panel.

Figure 2

NGF-Induced Clathrin Recruitment To PM In Primed PC12 Cells

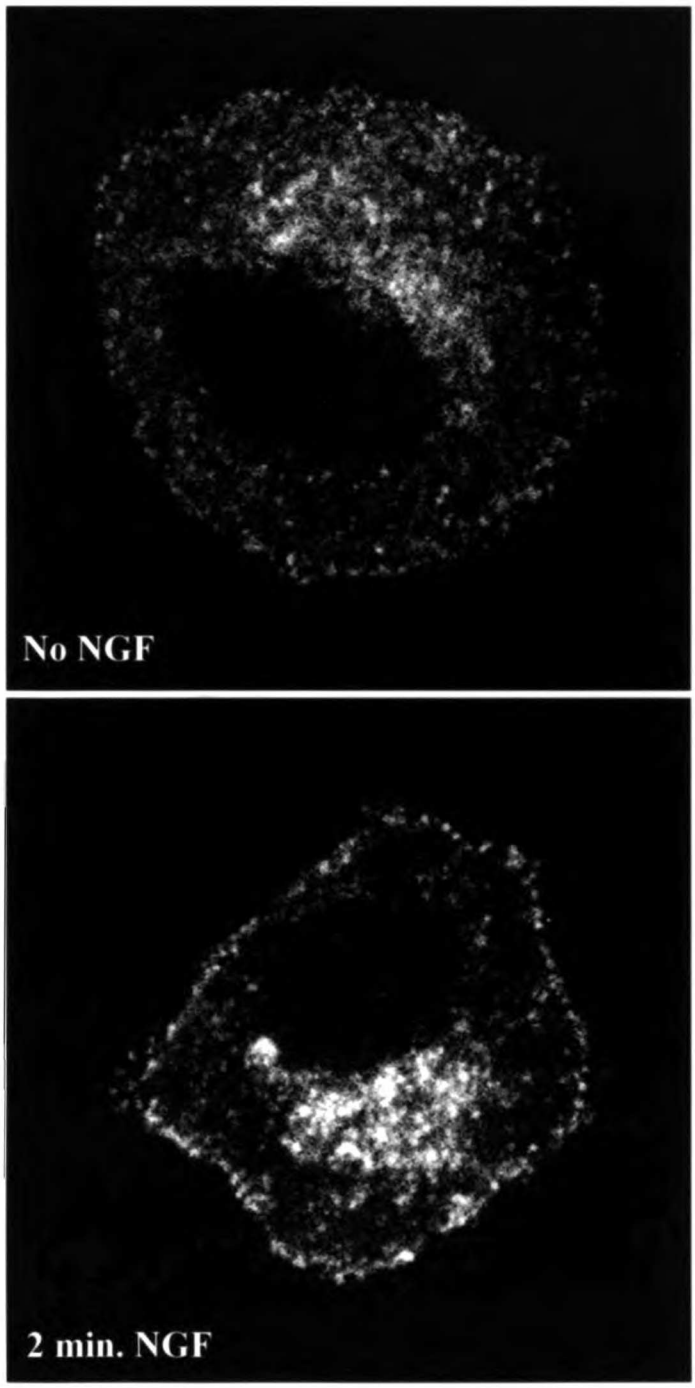


Figure 3 & 4. Schwann Cells (p75-positive, TrkA-negative) do not show NGF-induced membrane recruitment of clathrin.

Clathrin distribution is visualized as in Figs. 1 & 2. Cells treated with or without NGF show no redistribution of clathrin staining to the plasma membrane.

Figure 3

Schwann Cells (p75-positive, TrkA-negative) Do Not Show NGF-Induced Membrane Recruitment of Clathrin

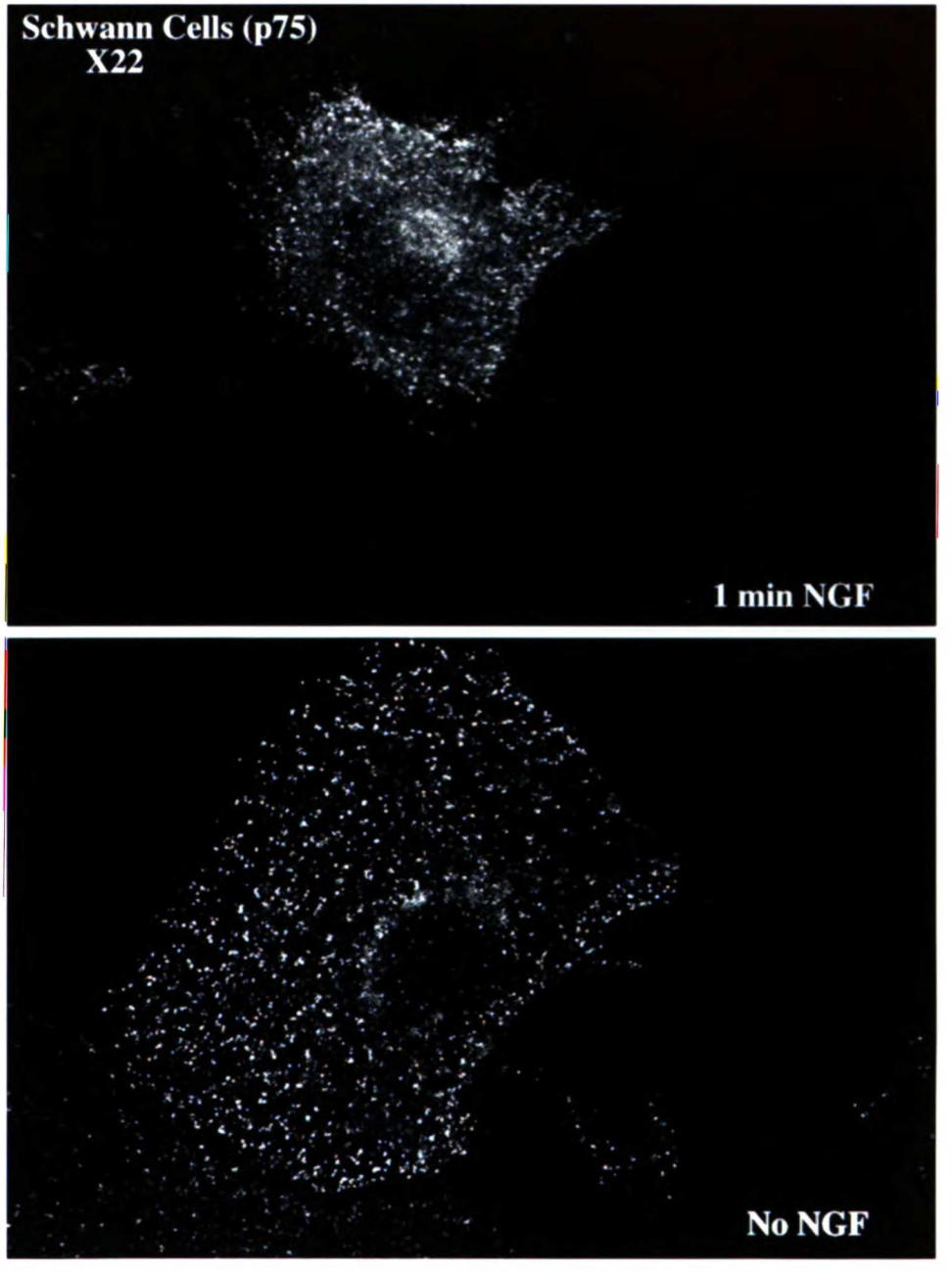


Figure 4

Schwann Cells (p75-positive, TrkA-negative) Do Not Show NGF-Induced Membrane Recruitment of Clathrin

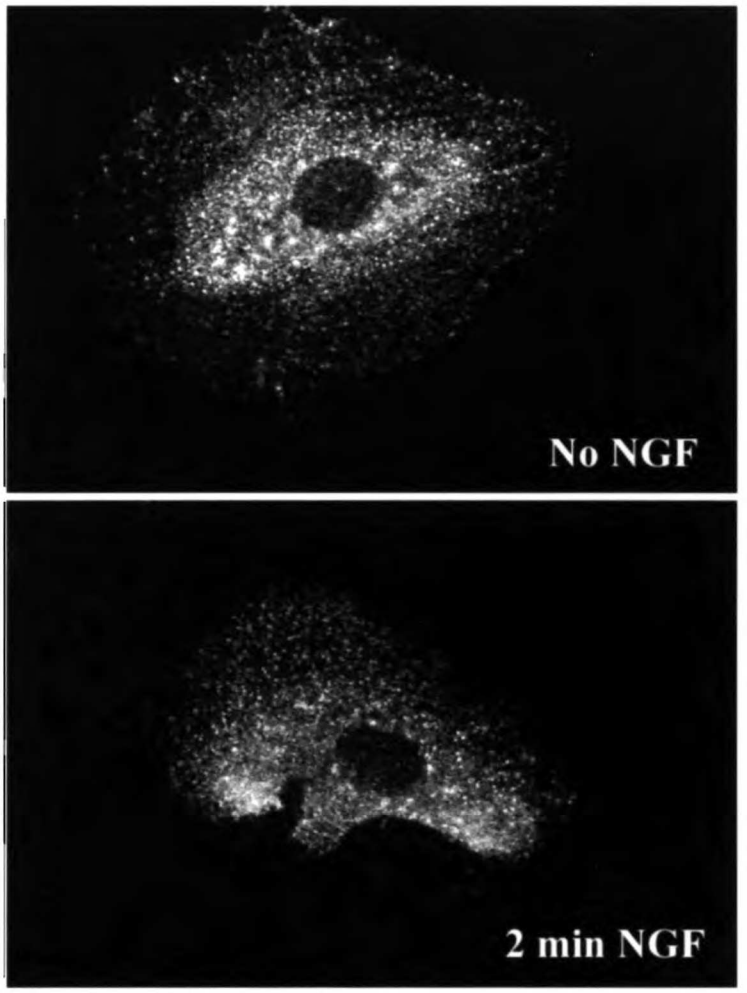


Figure 5. NGF-induced clathrin recruitment to the plasma membrane in D283 TrkA cells.

Clathrin is visualized as in Figs. 1-4. The bottom panel shows D283 cells (expressing TrkA but lacking p75) after 2 min of 2nM NGF application at 37°C. The arrow points to an example of the distinct line of clathrin staining at the plasma membrane often seen in cells with this treatment. The top panel shows a cell treated in the absence of NGF where there is lack of any well-defined cell border as marked by clathrin staining.

Figure 5

NGF-Induced Clathrin Recruitment To
The Plasma Membrane in D283 TrkA Cells

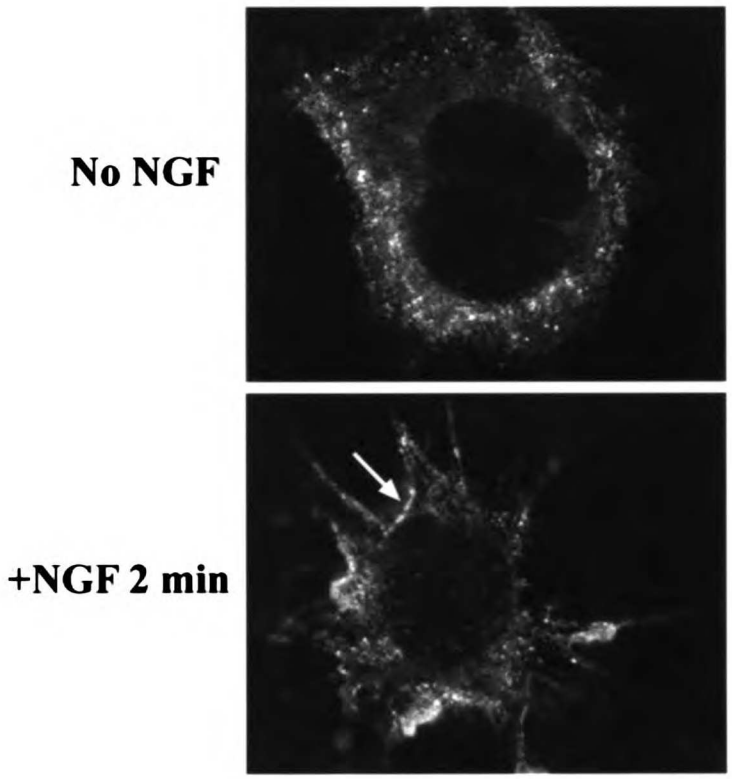


Figure 6. TrkB activation induces clathrin recruitment to membranes.

Clathrin staining was obtained as in Figs 1-5. TrkB transfected 3T3 mouse fibroblasts lacking p75 expression show increased plasma membrane-associated clathrin with BDNF applications of 2, 10, and 60 minutes in 37°C.

Figure 6

TrkB Activation Induces Clathrin Recruitment To Membranes

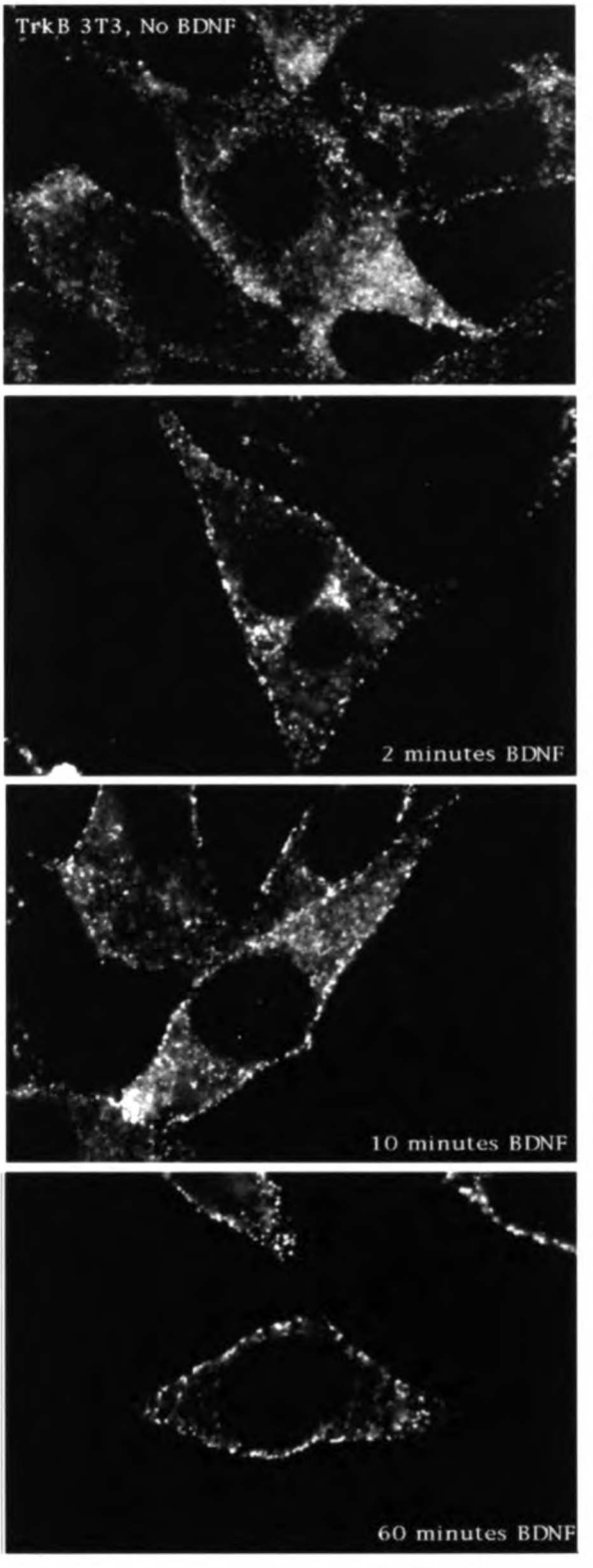


Figure 7. TrkB 3T3 fibroblasts redistribute AP2 to the plasma membrane with ligand treatment (stained for α-adaptin).

α-Adaptin staining was visualized in TrkB 3T3 cells using the monoclonal antibody specific to this endocytosis-specific adaptor protein, AP.6 (see methods). 2 minutes of BDNF application during warming redistributes α-adaptin staining from a diffuse, cytosolic pattern to the plasma membrane (bottom panel). Since α-adaptin is a member of the complex of subunits that constitute AP2, this is evidence that Trk kinase activation redistributes AP2 to this location.

TrkB 3T3 Cells Redistribute AP2 To The Plasma Membrane with Ligand Treatment (stained for alpha-adaptin) **Figure 7**

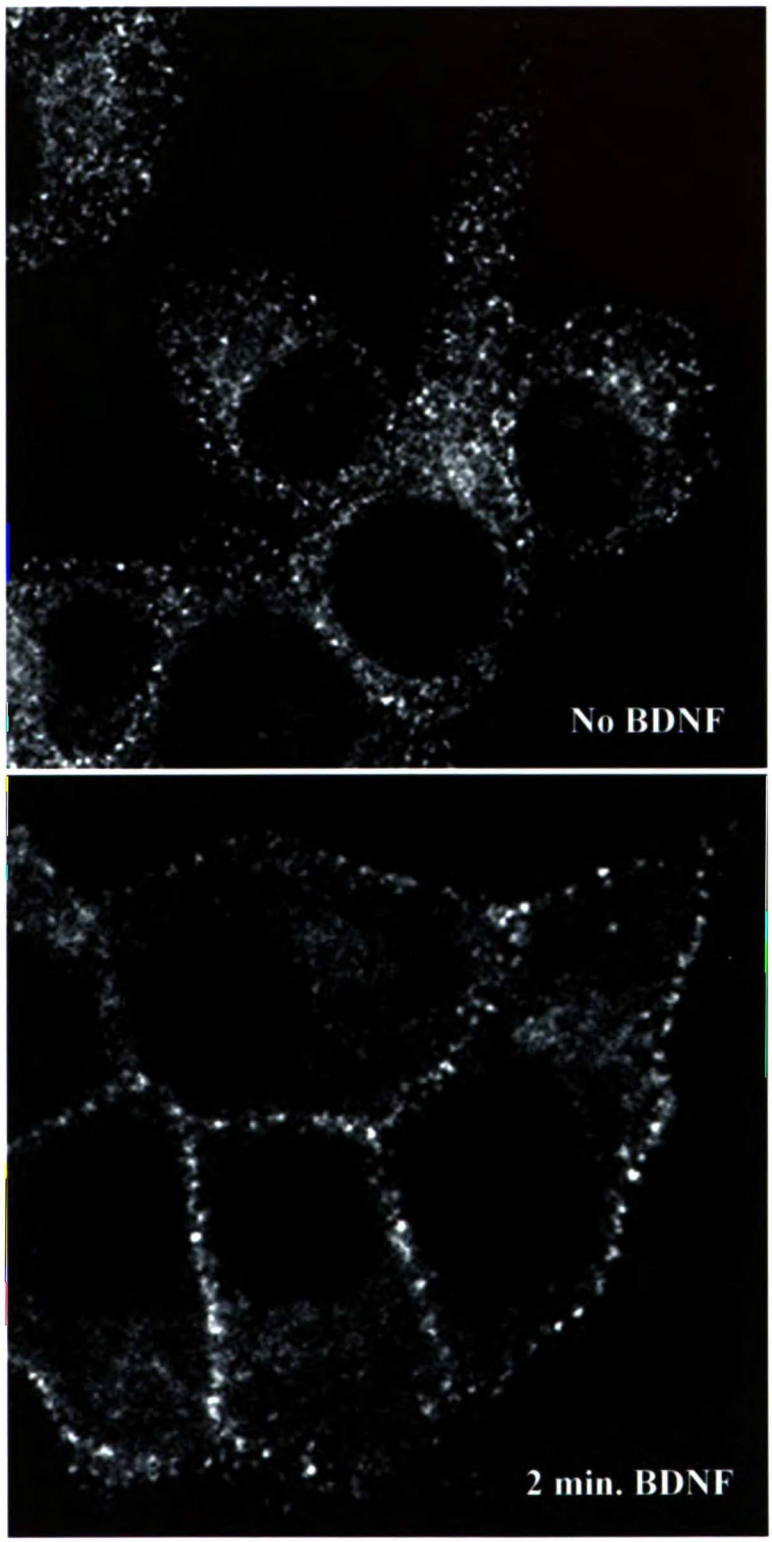


Figure 8. NGF redistributes clathrin from unassembled to assembled fractions.

Here biochemical measurements of assembled and unassembled clathrin fractions from PC12 cells before and after NGF application confirm our confocal findings and support a ligand-induced increase in assembly. PC12 were incubated at 4°C for 1 hour in the presence or absence of 2nM NGF as described in methods. Cells were then warmed for 2 minutes at 37°C and quickly chilled to stop endocytosis. Unassembled and assembled clathrin fractions were then harvested from each condition as described in methods and subjected to SDS-PAGE after normalization for protein content. The gel was transferred to nitrocellulose which was blotted for clathrin with a monoclonal antibody specific to CHC (TD.1). The signal was visualized by HRP-conjugated anti-mouse secondary and quantified using NIH Image.

Figure 8

NGF Redistributes Clathrin From Unassembled To Assembled Fractions.

NGF:	-	+	-	+
Assembled (A) and Unassembled (U) Clathrin:	A	A	U	U

Probed with the anti-clathrin antibody, TD.1

Figure 9. NGF redistributes clathrin from unassembled to assembled fractions. Band intensity quantification.

The graph shows the amount of redistribution in a representative experiment performed exactly as described in Fig. 8. The height of each bar represents the percent of clathrin in that fraction relative to the total (unassembled plus assembled) clathrin signal in each experimental condition. Results are representative of n=4 experiments with an average NGF-induced increase of unassembled CHC equal to 256% ± 90% (S.E.).

Figure 9: NGF Redistributes Clathrin From Unassembled to Assembled Fractions. 2 Minutes Warming

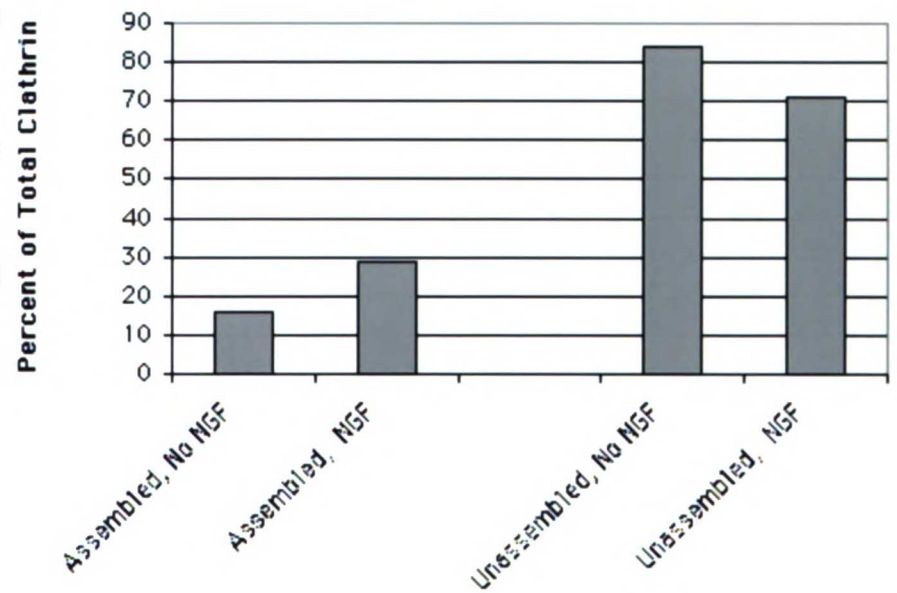


Figure 10. Cell-surface biotinylated MHC class I molecule is not significantly altered by NGF treatment.

This figure supports previously published data (Connolly, 1983) that NGF does not induce significant plasma membrane surface area changes at 2 minutes of NGF application. MHC Class I molecule cell surface content was measured by biotinylation as described in methods. Results averaged from three experiments fail to show a significant difference in this marker of plasma membrane area.

Figure 10: Cell-Surface Biotinyated MHC class I Is Not Significantly Altered By NGF Treatment

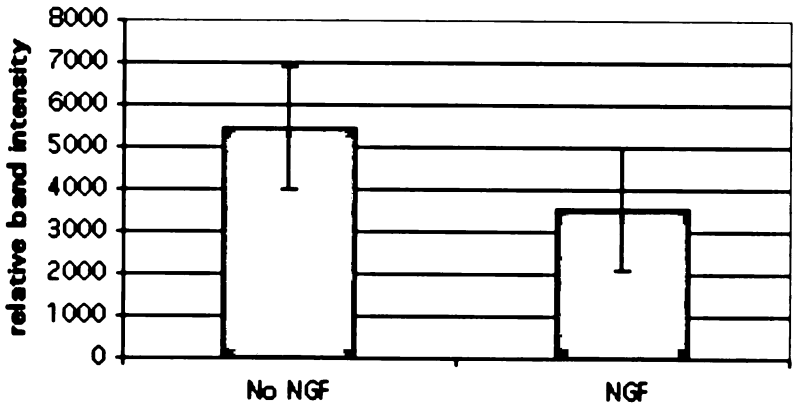


Figure 11. NGF dose-response curve of clathrin assembly after 2 minutes warming.

Low concentrations of NGF can affect CHC redistribution, suggesting that high affinity (TrkA mediated) binding supports this activity. KB PC12 cells were treated for 2 minutes, 37°C with 20pM, 200pM, 2nM, 20nM NGF, or vehicle as described in methods. Assembled CHC fractions were obtained as in figure 8 and subjected to SDS-PAGE, blotted and probed for CHC as described in methods. The bar graph represents the increase of assembled CHC after NGF application as a percent of total (assembled plus unassembled fractions).

Figure 11: NGF Redistribution Dose-Response Curve of CHC Assembly After 2 Minutes Warming

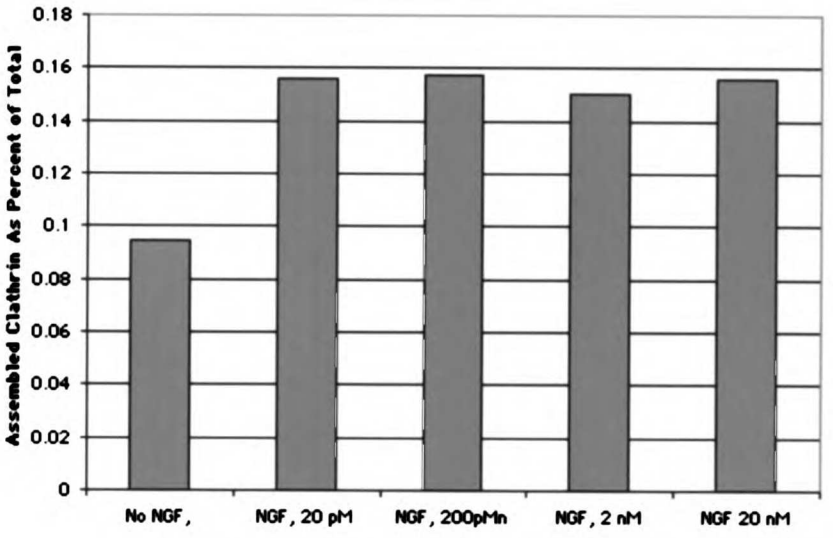
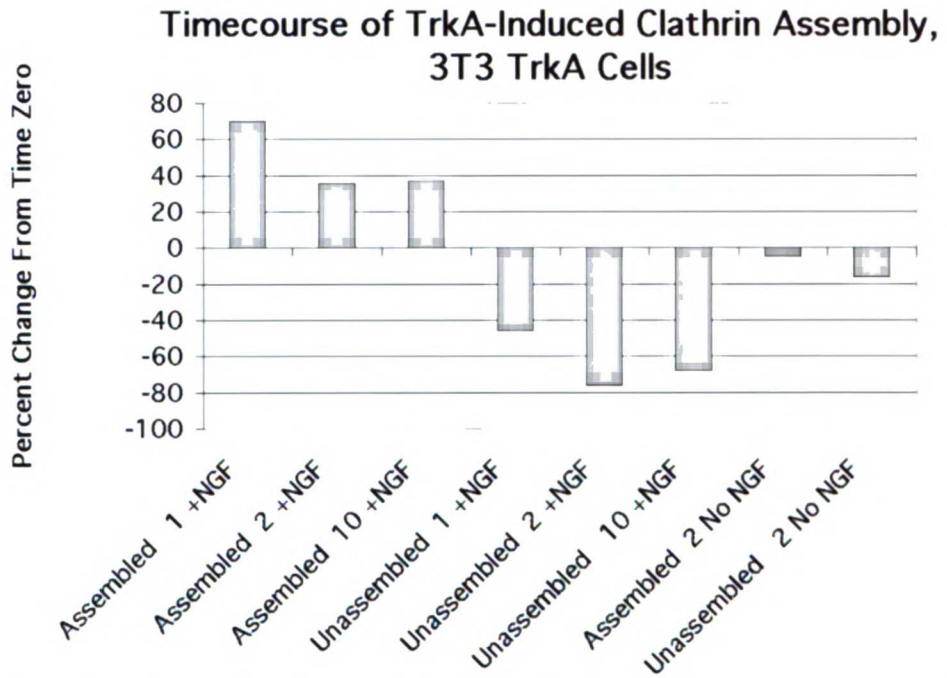


Figure 12. NGF time course; TrkA signaling is sufficient to initiate clathrin assembly in 3T3 fibroblasts.

The figure shows the ability of TrkA alone to allow for NGF-induced CHC assembly. TrkA transfected 3T3 cells were treated with NGF as described in methods and were either kept at 4°C or warmed to 37°C for 1, 2, and 10 minutes. Cells not treated with NGF and either kept at 4°C or warmed to 37°C for 2 minutes were used as controls. All cells were chilled to 4°C after treatment and processed to obtain assembled and monomer CHC fractions as described above and in methods. These fractions were assessed for their relative concentrations of CHC by quantifying gel band intensities as described above. The bottom panel shows the resulting blot probed with the anti-CHC antibody TD1. Assembled and unassembled CHC fractions from the 0, 1,2, and 10 minute + NGF time-points and assembled and unassembled NGF-minus controls for the 0 and 2 minute time-points are shown. The top panel shows a graph of the NIH-Image gel band intensity quantification and demonstrates the following NGF-induced increases in assembled CHC concentrations at the following timepoints relative to 0 minutes warming; 1 minute ~70%, 2 minutes ~40%, 10 minutes ~40%. The graph also shows a corresponding and NGF-induced decrease in CHC concentrations of unassembled fractions over 10 minutes of warming. The NGF-minus control shows a decrease in assembled CHC of ~10% at the 2 minute warming timepoint.

Figure 12


Timecourse of TrkA-Induced Clathrin Assembly,
3T3 TrkA Cells
Percent Change From Time Zero
80
60
40
20
0
-20
-40
-60
-80
-100
Assembled 1 +NGF
Assembled 2 +NGF
Assembled 10 +NGF
Unassembled 1 +NGF
Unassembled 2 +NGF
Unassembled 10 +NGF
Assembled 2 No NGF
Unassembled 2 No NGF

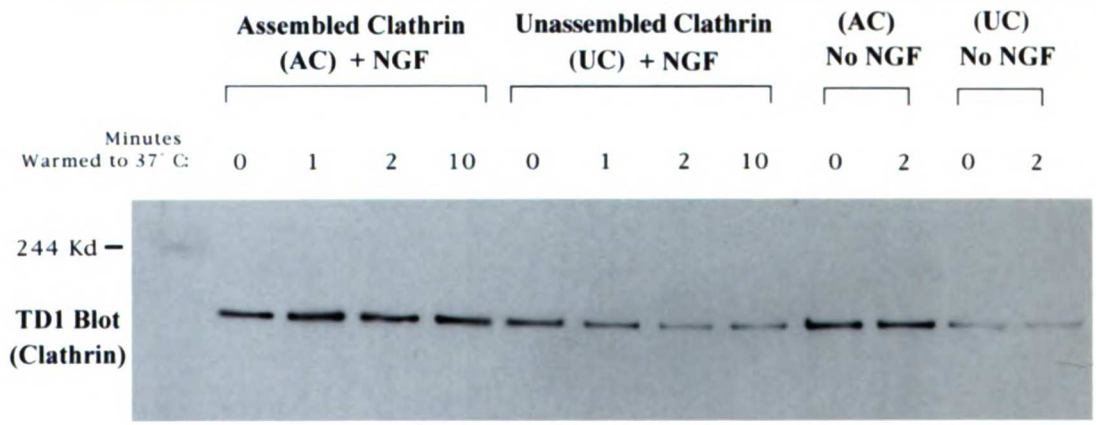

Assembled Clathrin
(AC) + NGF
Unassembled Clathrin
(UC) + NGF
(AC)
No NGF
(UC)
No NGF
Minutes
Warmed to 37° C:
0 1 2 10 0 1 2 10 0 2 0 2
244 Kd
TD1 Blot
(Clathrin)

Figure 13. NGF-induced clathrin redistribution in PC12 nnR5 TrkA cells is inhibited by TrkA mutants. Confocal micrographs.

PC12 nnR5 transfected with either wild type TrkA (top row of panels), or the kinase domain mutants TrkA M1 or TrkA 22.7 (middle and bottom row of panels) were grown on chamberslides and treated with NGF (right column of panels) or without NGF (left column of panels) for 2 minutes as described in methods. All cells had endogenous levels of p75. Cells were fixed and stained with the CHC specific antibody X22 as described. The wild type TrkA cells (top row of panels) shows an NGF-induced increase in the distinctiveness of plasma membrane as seen by clathrin staining (small arrows). Untreated cells do not show a distinctive plasma membrane border by this staining (large arrowheads). TrkA kinase domain mutants fail to show such an increase in plasma membrane-associated clathrin with NGF treatment.

Figure 13

NGF-Induced Clathrin Redistribution In PC12 nnR5 TrkA Cells Is Inhibited by Mutations Affecting TrkA's Kinase Domain

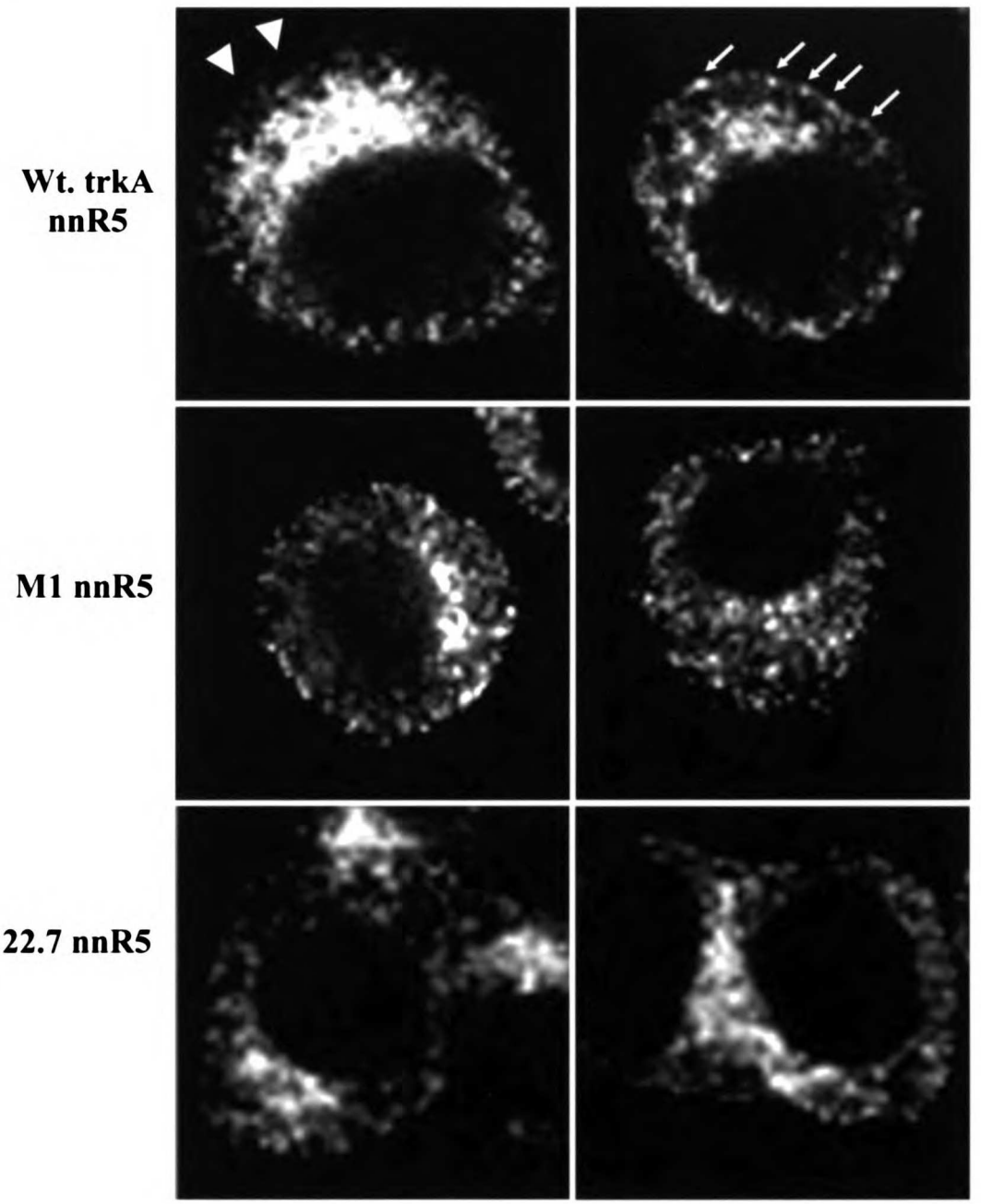


Figure 14. The NGF-induced clathrin plasma membrane recruitment seen in PC12nnR5 cells is blocked by TrkA mutants. Band intensity bar graph.

PC12 nnR5 transfected with either wild type TrkA, or the TrkA mutants TrkA M1 or TrkA 22.7 were treated with NGF as above and warmed for 2 minutes at 37°C. All cells had endogenous levels of p75. Cells were then chilled, lysed and processed as described in methods to produce membrane fractions. Lysates were equalized for protein content and were blotted onto nitrocellulose and probed for CHC with the monoclonal antibody,TD1. Band intensities were quantified as above using the ECL system and NIH Image. Membrane fractions from wild type TrkA transfected cells showed a greater than 10% increase in CHC content with NGF, while the M1 and 22.7 kinase domain mutants showed a ~25% and ~4% decrease in CHC concentration (n=2).

Figure 14: The NGF-Induced Clathrin Assembly Seen In PC12 nnR5 TrkA Cells Is Blocked By TrkA Kinase Domain Mutants. 2 min. NGF Application.

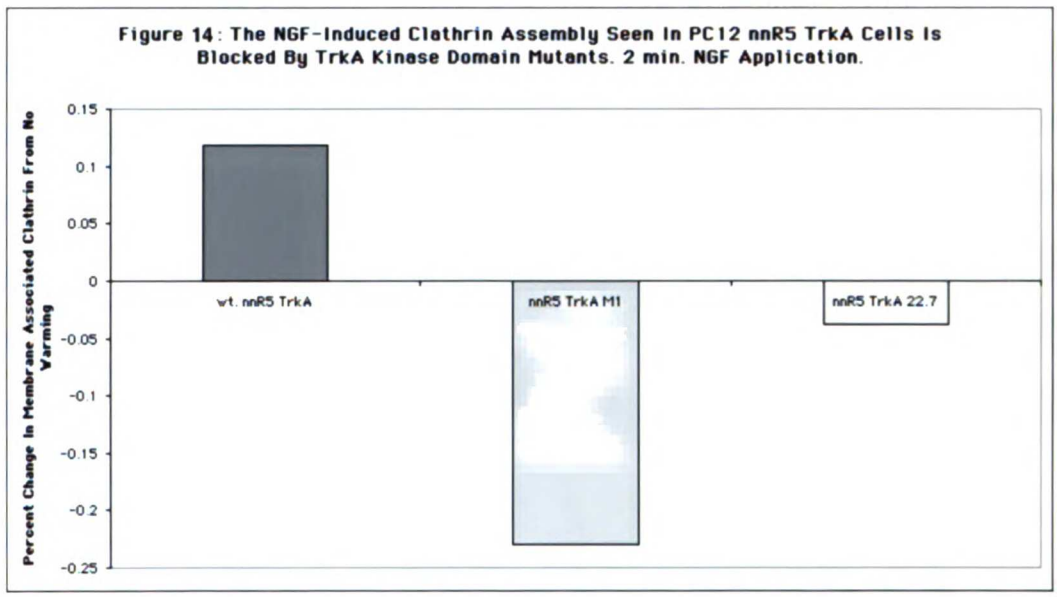
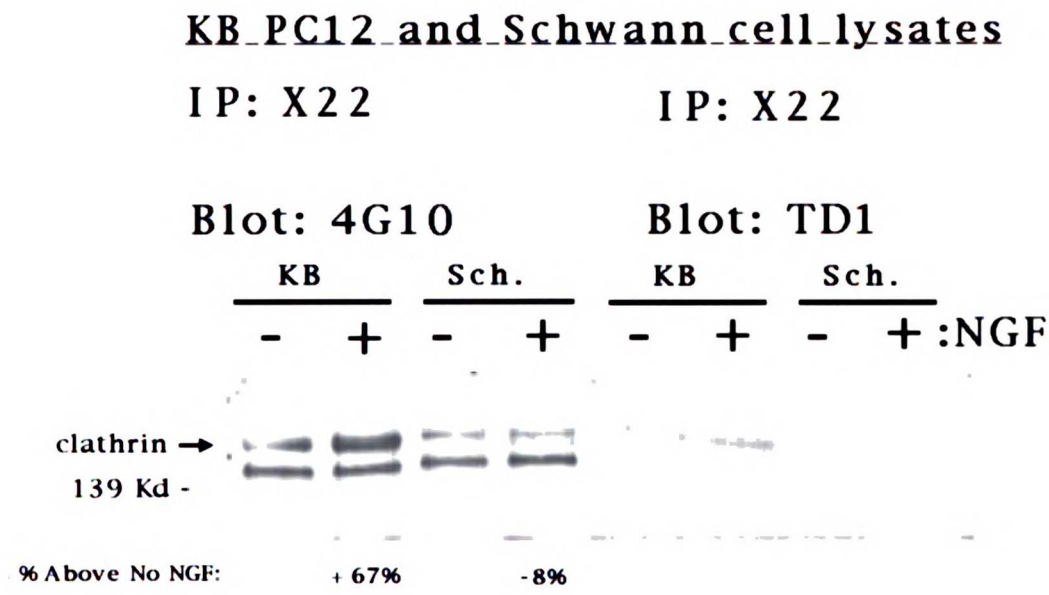


Figure 15. NGF-Induced increase in CHC phosphorylation in KB PC12 cells is absent in p75-positive Schwann cells lacking TrkA.

KB PC12 cells expressing endogenous levels of TrkA and p75, and Schwann cells (p75 only), were treated with or without NGF for 2 minutes at 37°C as described in methods. Cells were chilled, lysed, equalized for protein content, and immunoprecipitated with the CHC-specific antibody X22. Immunoprecipitates were split into two aliquots, both of which were subjected to PAGE and blotted to nitrocellulose. One set of aliquots, after western blotting, was probed for phosphotyrosine with the antibody 4G10 (top left panel) while the other set of aliquots was blotted for CHC with TD1 (top right panel). Signals were visualized by ECL as described and quantified with NIH-Image. Values for the phosphotyrosine signals were divided by values for CHC signals in each condition to give phosphorylation comparisons further normalization for CHC content. Quantification results are graphed in the lower panel and show a ~68% NGF-induced increase in CHC tyrosine phosphorylation in PC12 cells while Schwann cells show an 8% NGF-induced decrease in CHC phosphorylation.

Figure 15: NGF Increases CHC Tyrosine Phosphorylation of PC12 Cells



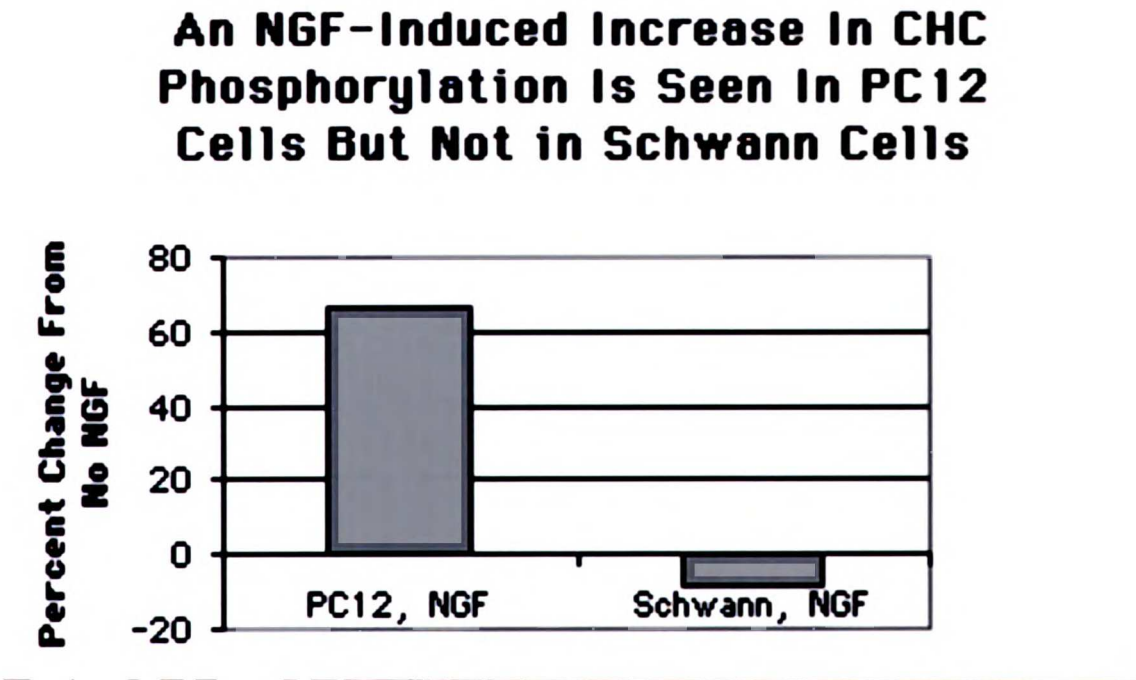
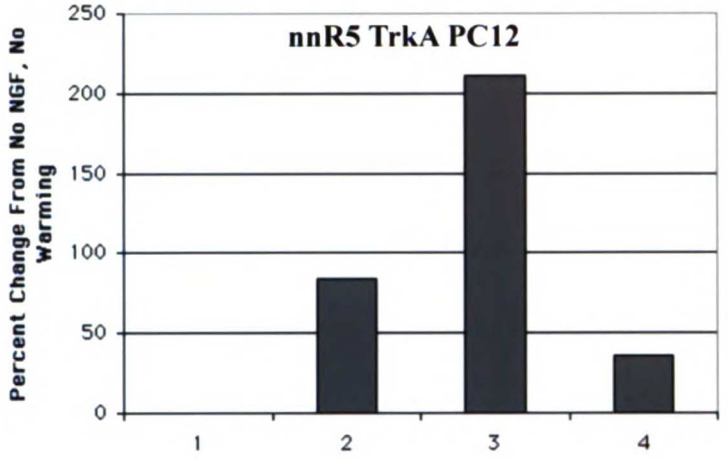


Figure 16. The NGF-induced CHC phosphorylation increase in PC12 nnR5 TrkA cells is inhibited by TrkA kinase domain mutants.

PC12 nnR5 cells transfected with either wild type TrkA or the TrkA kinase domain mutant 22.7 were treated with or without NGF and the specific SRC kinase inhibitor, PP1, at 4°C or 37°C for 10 minutes as described. Cells were chilled, lysed, equalized for protein content and immunoprecipitated with the CHC-specific antibody, X22, and subjected to PAGE. Blots were probed with the anti-phosphotyrosine antibody, 4G10. Signals were visualized by ECL and quantified by NIH-Image. The top panel shows a graphed representation of the increase in CHC phosphorylation from the 4°C, no NGF condition in wild type TrkA nnR5 PC12 cells. Here, NGF application alone, without warming caused a ~85% increase in CHC phosphorylation and NGF coupled with warming increased phosphorylation over 200% (top blot). This phosphorylation increase was held to less than 40% by the specific SRC kinase inhibitor PP1. In contrast, TrkA 22.7 kinase mutant CHC phosphorylation remained relatively constant or decreased with 4°C NGF or 37°C NGF treatment, respectively (bottom blot and bottom graph).

Figure 16

CHC Phosphorylation Increases With NGF Application In PC12 Cells Expressing TrkA and This Increase Is Substantially Blocked In TrkA Kinase Domain Mutants



PP1:	-	-	-	+
NGF:	-	+	+	+
Warmed, 10 min.:	-	-	+	+

nnR5 TrkA

nnR5 TrkA 22.7

IP: CHC
Probe: p-tyr

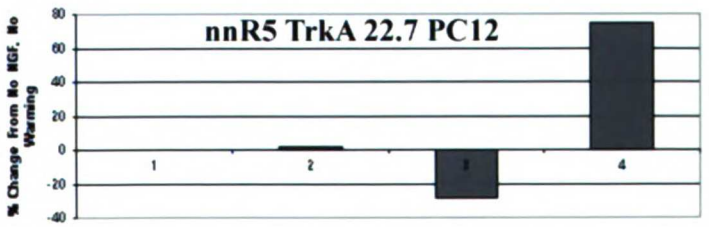


Figure 17. TrkA and CHC tyrosine phosphorylation is greatly reduced in cells expressing TrkA kinase domain mutants.

PC12 nnR5 transfected with either wild type TrkA or the TrkA mutants TrkA M1 or TrkA 22.7 were treated with NGF or without NGF for 10 minutes, 37°C as described in methods. Lysates were immunoprecipitated with an anti-phosphotyrosine antibody (UBI), subjected to PAGE, and the resulting western blot was probed sequentially for TrkA (top blot) and CHC (bottom blot). The measured band intensities, graphed in the center panel, show that NGF increases TrkA phosphorylation in wild type TrkA cells while TrkA phosphorylation with or without NGF is greatly reduced in mutant cell lines (light bar graphs and top blot). CHC phosphorylation doubles in the presence of NGF in the TrkA wild type cells while the mutant lines show little base line phosphorylation and no NGF induced increase (dark bar graphs and bottom panel).

Figure 17

TrkA and CHC Tyrosine Phosphorylation Is Greatly Reduced In Cells Expressing TrkA Kinase Domain Mutants

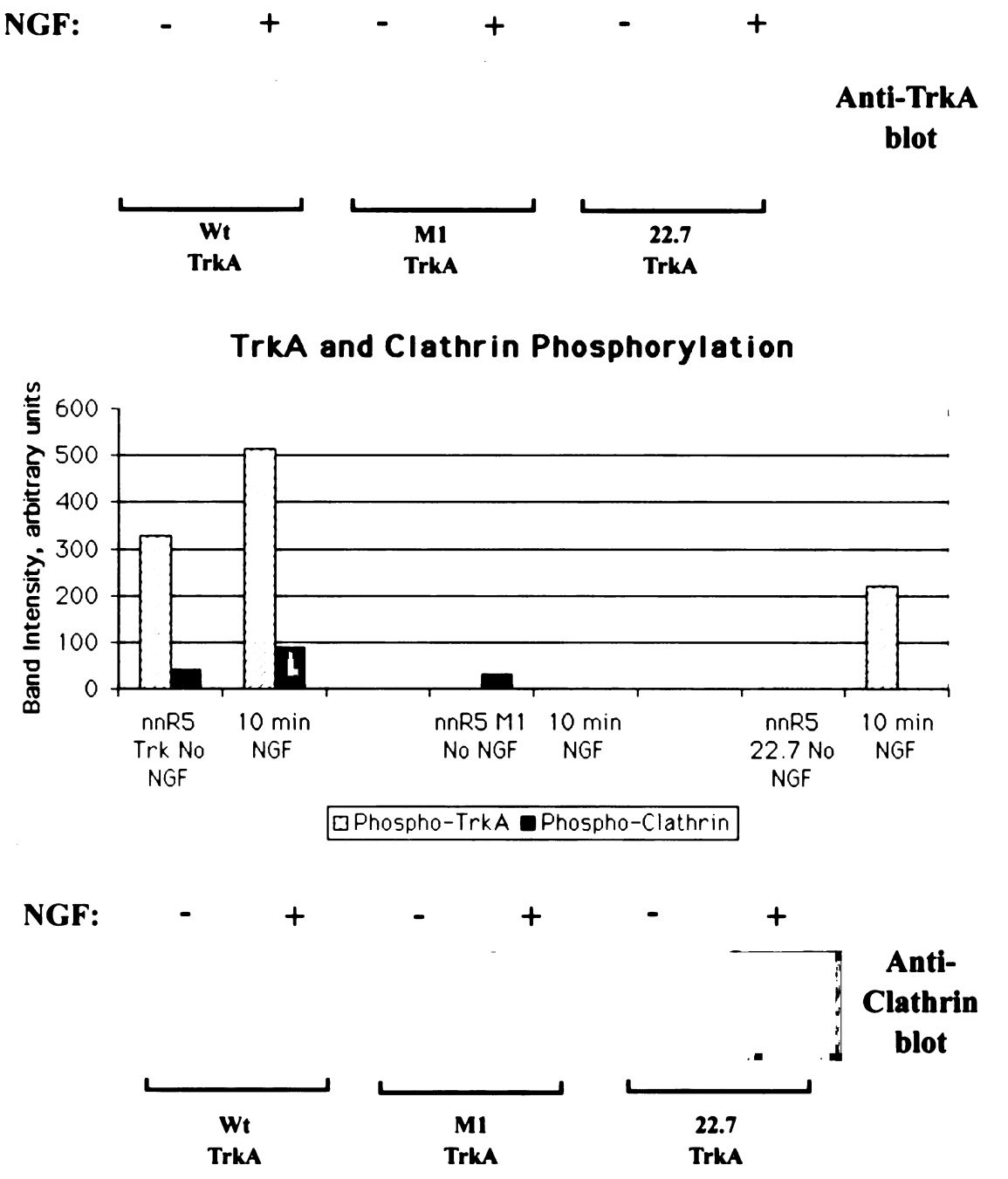
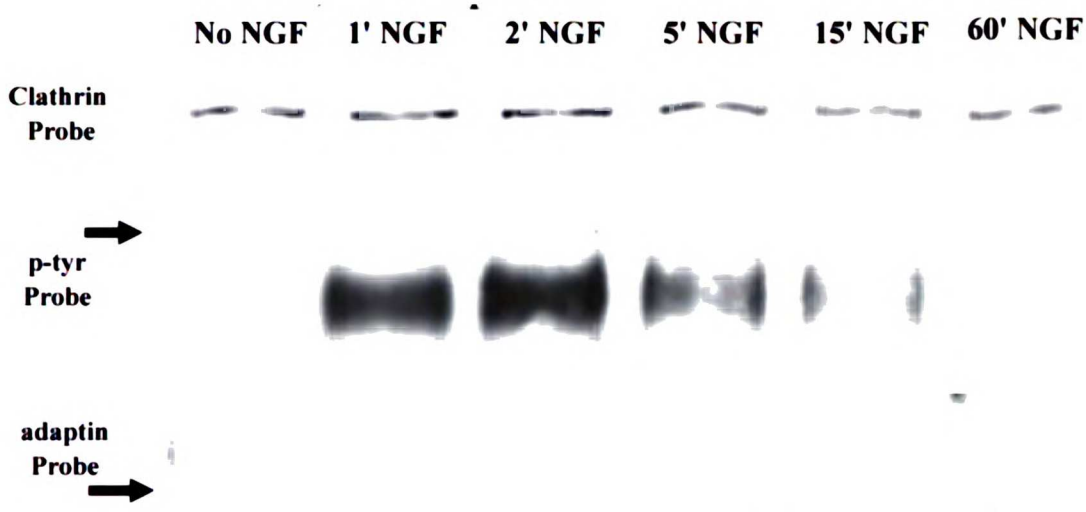


Figure 18. NGF timecourse of Trk/ clathrin /α-adaptin association.

KB PC12 cells were left untreated or were given NGF for 1, 2, 5, 15, or 60 minutes at 37°C, lysed, and immunoprecipitated for TrkA. After PAGE and transfer, the resulting blot was successively probed for CHC (TD1, top blot), phosphotyrosine (4G10, middle blot, arrow), and α-adaptin (AC1M11, bottom blot, arrow). The gel band intensities are graphed in the lower panel. A significant background of CHC-TrkA and α-adaptin-TrkA binding is observed, but a rapid increase in these associations is seen at 1 minute NGF treatment (20% and 60% respectively). The large band in the anti-phosphotyrosine blot (middle blot) represents phospho-TrkA and can be seen to increase dramatically at 1 and 2 minutes of NGF treatment, trailing off over 60 minutes. The phospho-CHC timecourse (middle blot, arrow and striped bar graph in lower panel) correlates with the phospho-TrkA increase.

Figure 18
NGF Timecourse; Trk Association with CHC, Phosphorylated CHC, and Adaptin Increases With NGF



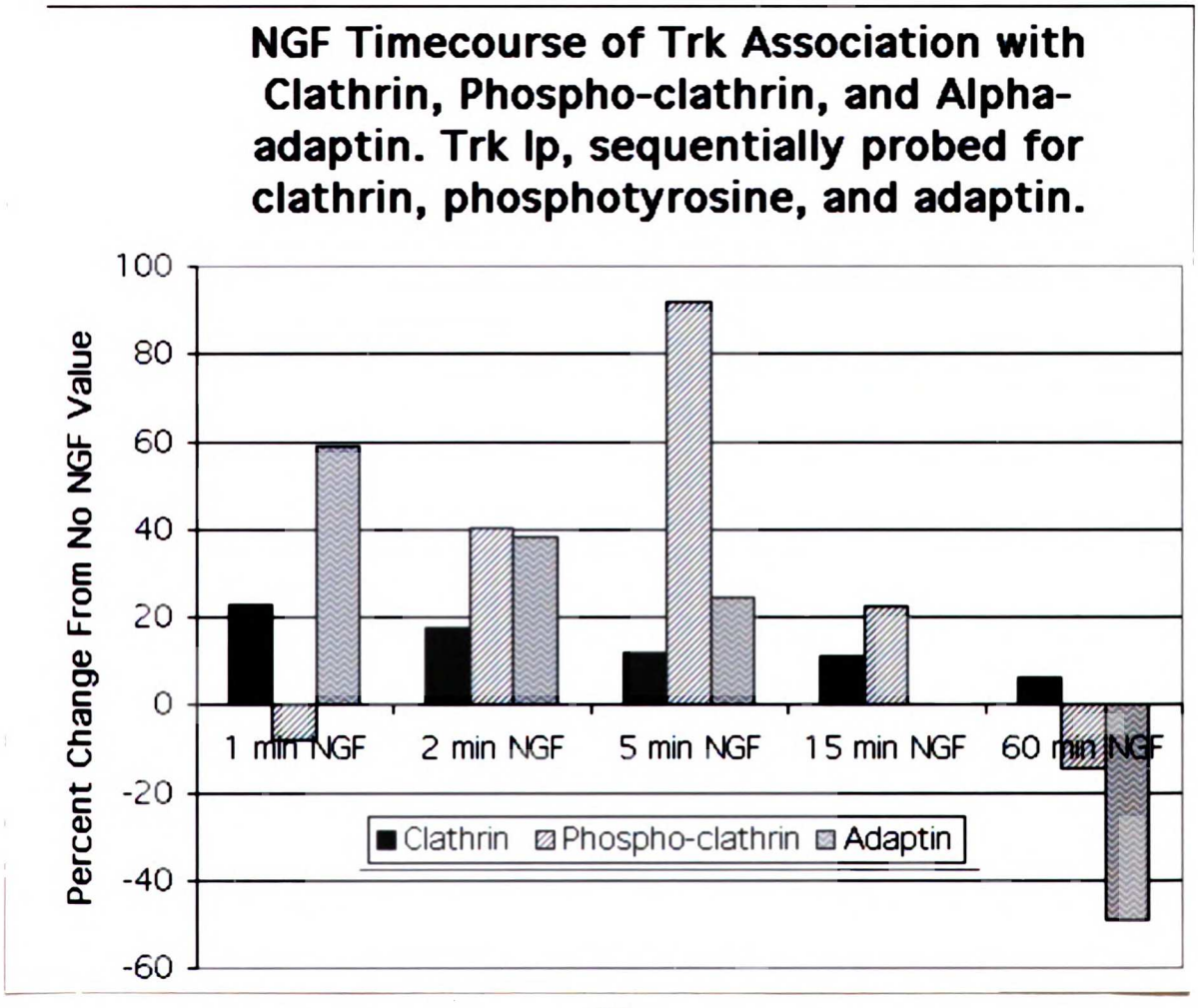
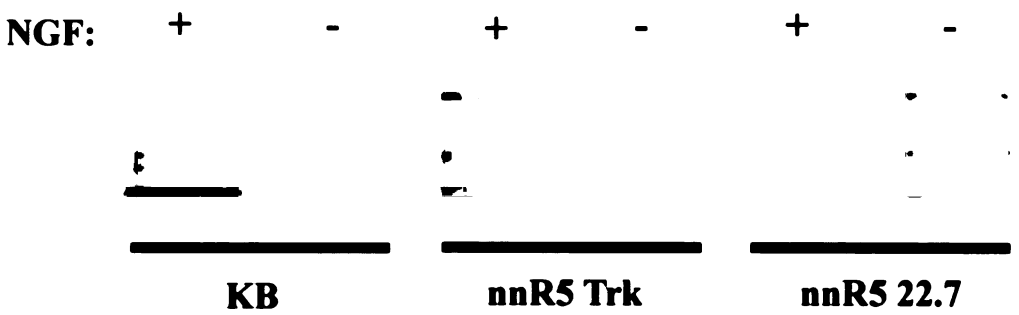


Figure 19. A NGF-induced increase in clathrin and TrkA association is blocked in TrkA kinase domain mutants.

KB PC12 cells and PC12 nnR5 re-transfected with either wild type TrkA or the kinase domain mutant TrkA 22.7 were treated with or without NGF for 2 minutes, 37°C as described in methods. Lysates were immunoprecipitated for TrkA, subjected to PAGE, and the resulting western blot was probed for CHC (TD1). The measured band intensities graphed in the bottom panel show that NGF increases TrkA-CHC association from the no-NGF conditions in wild type TrkA but not in the TrkA kinase domain mutant 22.7. The mutants have a higher background of TrkA-CHC association without NGF, but with NGF this association does not increase. The bar graphs express the percent change of TrkA-CHC association from the no-NGF condition to the 2 minute NGF treatment condition.

Figure 19

Clathrin and Trk Association Increases After NGF Application
IP: Trk
probe: clathrin



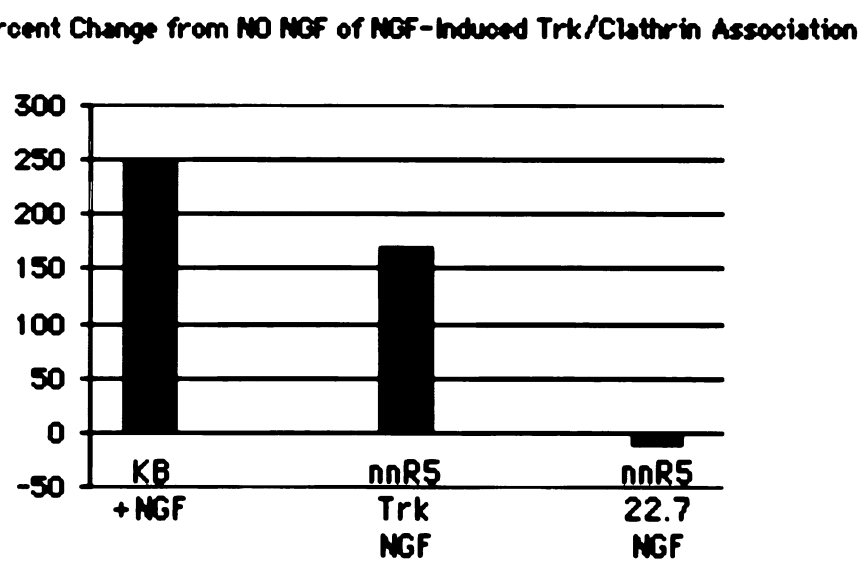


Figure 20. A NGF-induced increase in α-Adaptin and TrkA association is blocked in TrkA kinase domain mutants.

KB PC12 cells and PC12 nnR5 re-transfected with either wild type TrkA or the kinase domain mutant TrkA 22.7 were treated with or without NGF for 2 minutes, 37°C as described in methods. Lysates were immunoprecipitated for TrkA, subjected to PAGE, and the resulting western blot was probed for α-adaptin. The measured band intensities graphed in the bottom panel show that NGF increases TrkA-α-adaptin association from the no-NGF conditions in wild type TrkA but not in the TrkA kinase domain mutant 22.7. The mutants have a higher background of TrkA-α-adaptin association without NGF, but with NGF this association does not increase. The bar graphs express the percent change of TrkA-α-adaptin association from the no-NGF condition to the 2 minute NGF treatment condition.

TrkA and Alpha Adaptin Association Increases With NGF Treatment

Figure 20

anti Trk IP
alpha adaptin probe

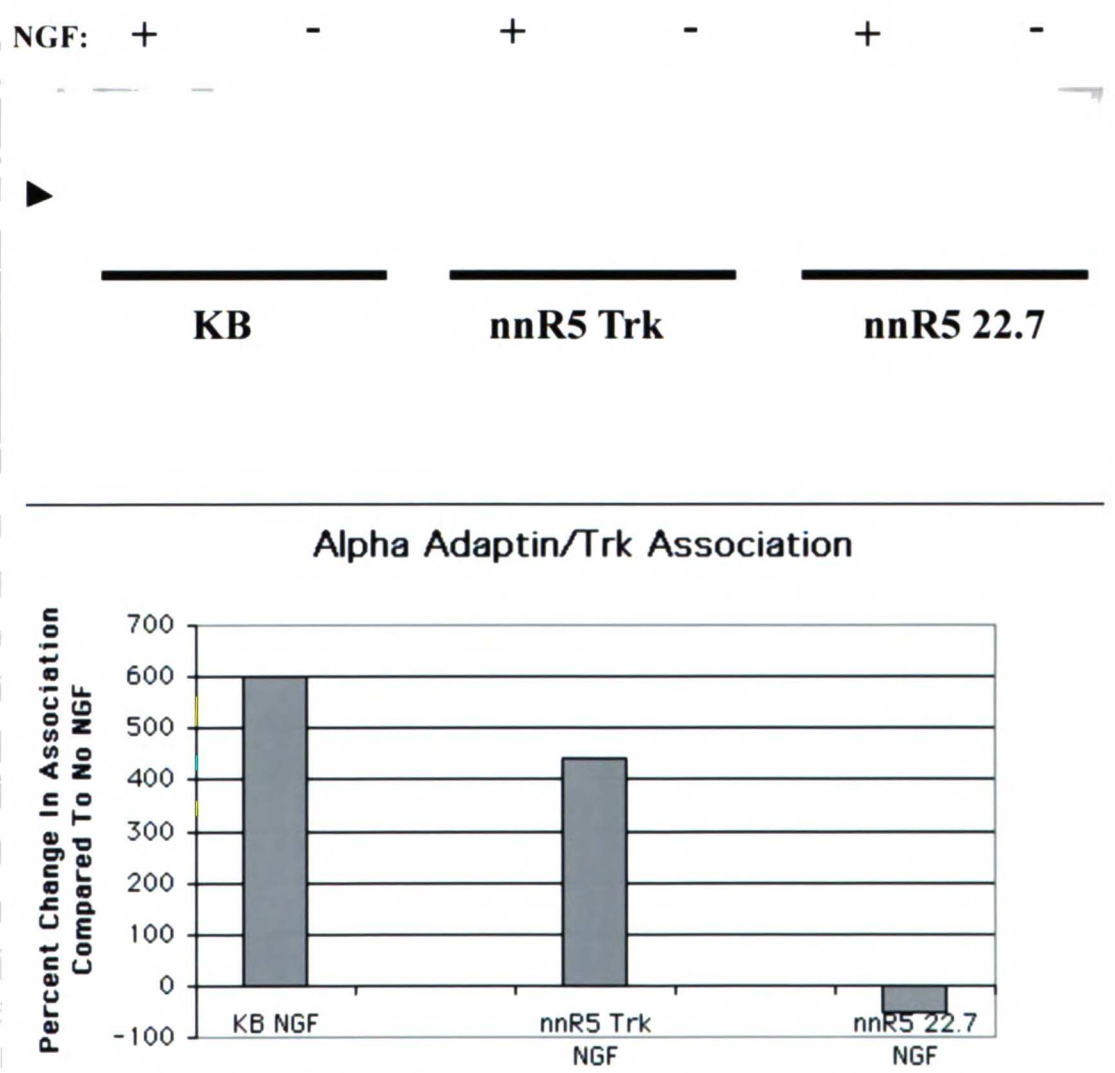


Figure 21. pp60^{src} and TrkA associate in PC12 cells upon NGF application.

KB PC12 cells were treated with or without NGF for 2 minutes, 37°C as described in methods. Lysates were immunoprecipitated for TrkA, subjected to SDS-PAGE, and the resulting western blot was probed for pp60^{src} (blot in top panel, arrow). The band just below pp60^{src} is the antibody used for immunoprecipitation. The measured band intensities graphed in the bottom panel show that NGF increases TrkA- pp60^{src} dramatically.

Figure 21

c-Src/Trk Association in NGF Treated PC12 Cells

Trk IP
c-Src probe

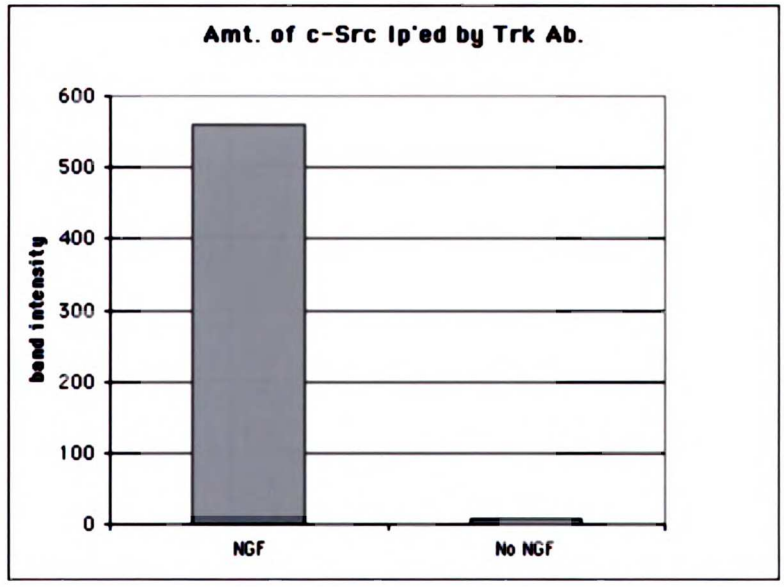
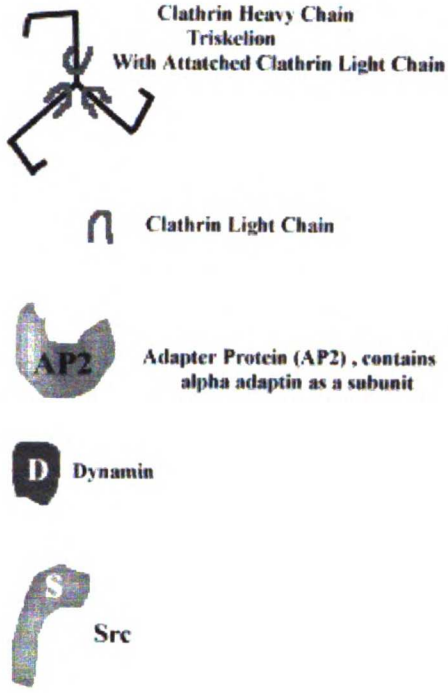


Figure 22. A model for active TrkA's role as a signaling scaffold for key endocytosis proteins.

This model shows some key players in coated pit endocytosis and their approximate locations in the forming pit. Dynamin has been shown to be located at the necks of coated pits. This thesis provides data that places TrkA in such pits with NGF-induced AP2, clathrin, and associations.

Figure 22


Clathrin Heavy Chain
Triskelion
With Attatched Clathrin Light Chain
Clathrin Light Chain
AP2
Adapter Protein (AP2) , contains
alpha adaptin as a subunit
D
Dynamin
S
Src

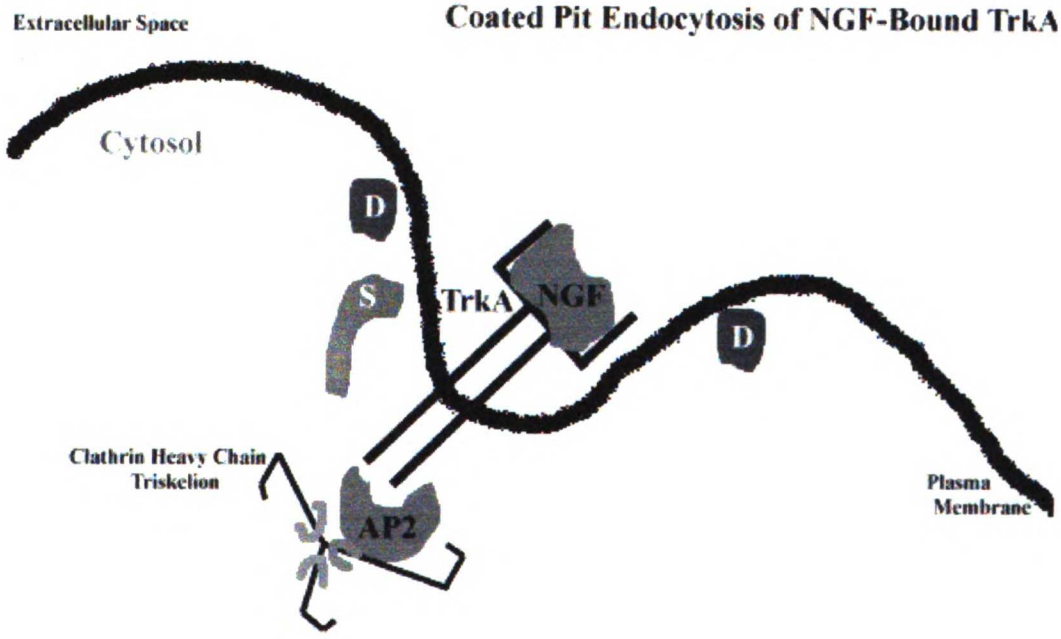
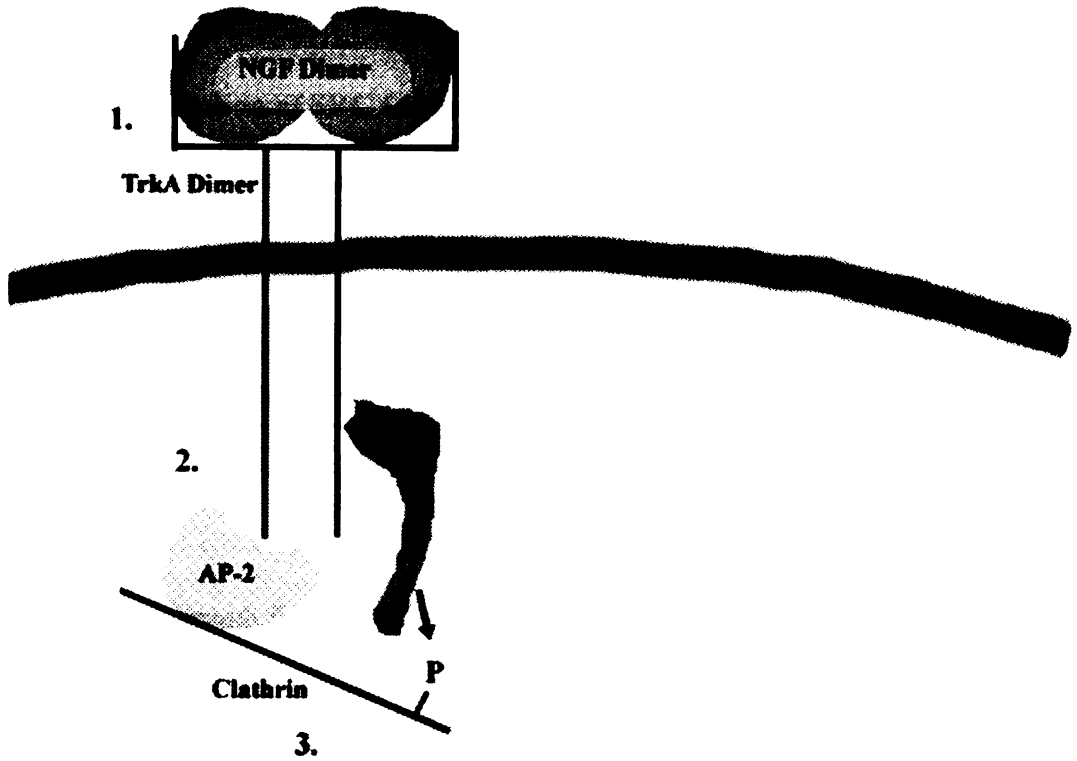

Extracellular Space
Coated Pit Endocytosis of NGF-Bound TrkA
Cytosol
D
S
TrkA
NGF
D
Clathrin Heavy Chain
Triskelion
AP2
Plasma
Membrane

Figure 23. A model for active TrkA's role as a signaling scaffold for key endocytosis proteins.

The data provided above may be used to construct a model where TrkA activation causes the coalescence of AP2, clathrin, and $pp60^{src}$ around the c-terminus of the receptor. $pp60^{src}$ has previously been localized to endosomes and plasma membrane but our data suggest a more specific localization to activated receptor tails. In this model, activated $pp60^{src}$ would be brought into close proximity to clathrin allowing for the tyrosine phosphorylation of CHC, thus enhancing triskelion aggregation and receptor endocytosis.

Figure 23

A Model For Active TrkA's Role As A Signaling Scaffold For Key Endocytosis Proteins



1. NGF binds two TrkA receptors leading to receptor kinase activation.

2. As a result of TrkA's signaling and C-terminus conformational changes, AP-2 and SRC are recruited to TrkA.

3. SRC phosphorylates the clathrin heavy chain recruited to TrkA.

4. Clathrin heavy chain assembly is enhanced by this phosphorylation, promoting TrkA's endocytosis.

Appendix

EGF activation of SRC family kinases is required for ligand-gated endocytosis and induces phosphorylation and plasma membrane recruitment of clathrin

Andrew Wilde, Eric Beattie, Lawrence Lem, William Mobley,

and Frances M. Brodsky

Summary

Epidermal growth factor (EGF) binding to A431 cells causes rapid phosphorylation of the clathrin heavy chain in the domain controlling clathrin self-assembly, at tyrosine 1477 within a SRC family kinase target sequence. Clathrin phosphorylation parallels its redistribution to the plasma membrane, in response to EGF binding, and is the product of downstream SRC family kinase activity stimulated by activation of the EGF receptor (EGF-R) tyrosine kinase. A specific SRC family kinase inhibitor prevents both clathrin phosphorylation and recruitment to the plasma membrane, indicating that the two processes are linked. These results demonstrate a role for SRC family kinases in activation and recruitment of clathrin during ligand-gated EGF-R endocytosis and thereby defines a novel effector mechanism for regulation of endocytosis by receptor signaling.

References

Bar-Zvi, D., S. T. Mosley, and D. Branton. 1988. In vivo phosphorylation of clathrin-coated vesicle proteins from rat reticulocytes. *Journal of Biological Chemistry*, 263:4408-15.

Beattie, E. C., J. Zhou, M. L. Grimes, N. W. Bunnett, C. L. Howe, and W. C. Mobley. 1996. A signaling endosome hypothesis to explain NGF actions: potential implications for neurodegeneration. *Cold Spring Harbor Symposia on Quantitative Biology*, 61:389-406.

Bernd, P., and L. A. Greene. 1983. Electron microscopic radioautographic localization of iodinated nerve growth factor bound to and internalized by PC12 cells. *Journal of Neuroscience*, 3:631-43.

Bevan, A. P., J. W. Burgess, P. G. Drake, A. Shaver, J. J. M. Bergeron, and B. I. Posner. 1995. Selective Activation of the Rat Hepatic Endosomal Insulin Receptor Kinase - Role For the Endosome in Insulin Signaling. *Journal of Biological Chemistry*, 270:10784-10791.

Bredesen, D. E., and S. Rabizadeh. 1997. p75(NTR) and apoptosis: Trk-dependent and Trk-independent effects. *Trends in Neurosciences*, 20:287-290.

Brown, M. T., and J. A. Cooper. 1996. Regulation, substrates and functions of src. *Biochim Biophys Acta*, 1287:121-49.

Connolly, J. L., S. A. Green, and L. A. Greene. 1981. Pit formation and rapid changes in surface morphology of sympathetic neurons in response to nerve growth factor. *Journal of Cell Biology*, 90:176-80.

Connolly, J. L., S. A. Green, and L. A. Greene. 1984. Comparison of rapid changes in surface morphology and coated pit formation of PC12 cells in response to nerve growth factor, epidermal growth factor, and dibutyryl cyclic AMP. *Journal of Cell Biology*, 98:457-65.

Corvera, S. 1990. Insulin stimulates the assembly of cytosolic clathrin onto adipocyte plasma membranes. *Journal of Biological Chemistry*, 265:2413-6.

Corvera, S., J. L. Bartels, R. J. Capocasale, K. Cichowski, and J. S. Moore. 1989. Increased assembly of clathrin occurs in response to mitogenic activation of murine lymphocytes. *Journal of Biological Chemistry*, 264:12568-72.

Cunningham, M. E., R. M. Stephens, D. R. Kaplan, and L. A. Greene. 1997. Autophosphorylation of activation loop tyrosines regulates signaling by the TRK nerve growth factor receptor. *Journal of Biological Chemistry*, 272:10957-10967.

Dobrowsky, R. T., M. H. Werner, A. M. Castellino, M. V. Chao, and Y. A. Hannun. 1994. Activation of the Sphingomyelin Cycle Through the Low-Affinity Neurotrophin Receptor. *Science*, 265:1596-1599.

Ehlers, M. D., D. R. Kaplan, D. L. Price, and V. E. Koliatsos. 1995. NGF-stimulated retrograde transport of trkA in the mammalian nervous system. *Journal of Cell Biology*, 130:149-56.

Fallon, R. J., M. Danaher, R. L. Saylors, and A. Saxena. 1994. Defective asialoglycoprotein receptor endocytosis mediated by tyrosine kinase inhibitors. Requirement for a tyrosine in the receptor internalization signal. *Journal of Biological Chemistry*, 269:11011-7.

Ferrari, G., B. L. Anderson, R. M. Stephens, D. R. Kaplan, and L. A. Greene. 1995. Prevention of Apoptotic Neuronal Death By G(M1) Ganglioside - Involvement of Trk Neurotrophin Receptors. *Journal of Biological Chemistry*, 270:3074-3080.

Geisow, M. J., J. Childs, and R. D. Burgoyne. 1985. Cholinergic stimulation of chromaffin cells induces rapid coating of the plasma membrane. *European Journal of Cell Biology*, 38:51-6.

Glickman, J. N., E. Conibear, and B. M. Pearse. 1989. Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. *Embo Journal*, 8:1041-7.

Goodman, O. B., J. G. Krupnick, F. Santini, V. V. Gurevich, R. B. Penn, A. W. Gagnon, J. H. Keen, and J. L. Benovic. 1996. Beta-Arrestin Acts As a Clathrin Adaptor in Endocytosis of the Beta(2)-Adrenergic Receptor. *Nature*, 383:447-450.

Grimes, M. L., E. Beattie, and W. C. Mobley. 1997. A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA. *Proceedings of the National Academy of Sciences of the United States of America*, 94:9909-9914.

Grimes, M. L., J. Zhou, E. C. Beattie, E. C. Yuen, D. E. Hall, J. S. Valletta, K. S. Topp, J. H. LaVail, N. W. Bunnett, and W. C. Mobley. 1996b. Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes. *Journal of Neuroscience*, 16:7950-64.

Grimes, M. L., J. Zhou, E. C. Beattie, E. C. Yuen, D. E. Hall, J. S. Valletta, K. S. Topp, J. H. LaVail, N. W. Bunnett, and W. C. Mobley. 1996a. Endocytosis of activated TrkA: Evidence that nerve growth factor induces formation of signaling endosomes. *Journal of Neuroscience*, 16:7950-7964.

Hamer, I., C. R. Haft, J. P. Paccaud, C. Maeder, S. Taylor, and J. L. Carpentier. 1997. Dual role of a dileucine motif in insulin receptor endocytosis. *Journal of Biological Chemistry*, 272:21685-21691.

Hanke, J. H., J. P. Gardner, R. L. Dow, P. S. Changelian, W. H. Brissette, E. J. Weringer, B. A. Pollok, and P. A. Connelly. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *Journal of Biological Chemistry*, 271:695-701.

Hendry, I. A. 1992. Retrograde factors in peripheral nerves. *Pharmacology and Therapeutics*, 56:265-85.

Hilborn, M. D., R. R. Vaillancourt, and S. G. Rane. 1998. Growth factor receptor tyrosine kinases acutely regulate neuronal sodium channels through the Src signaling pathway. *Journal of Neuroscience*, 18:590-600.

Hinshaw, J. E., and S. L. Schmid. 1995. Dynamin self-assembles into rings suggesting a mechanism for coated vesicle budding [see comments]. *Nature*, 374:190-2.

Holen, I., P. B. Gordon, P. E. Stromhaug, T. O. Berg, M. Fengsrud, A. Brech, N. Roos, T. Berg, and P. O. Seglen. 1995a. Inhibition of Asialoglycoprotein Endocytosis and Degradation in Rat Hepatocytes By Protein Phosphatase Inhibitors. *Biochemical Journal*, 311:317-326.

Holen, I., P. B. Gordon, P. E. Strømhaug, T. O. Berg, M. Fengsrud, A. Brech, N. Roos, T. Berg, and P. O. Seglen. 1995b. Inhibition of asialoglycoprotein endocytosis and degradation in rat hepatocytes by protein phosphatase inhibitors. *Biochemical Journal*, 311:317-26.

Jackson, A. P. 1993. The isolation of clathrin-coated vesicles and purification of their protein components. *Methods in Molecular Biology*, 19:83-96.

Johnson, E. M., Jr., P. D. Gorin, L. D. Brandeis, and J. Pearson. 1980. Dorsal root ganglion neurons are destroyed by exposure in utero to maternal antibody to nerve growth factor. *Science*, 210:916-8.

Kaplan, D. R., and F. D. Miller. 1997. Signal transduction by the neurotrophin receptors. *Current Opinion in Cell Biology*, 9:213-221.

Khan, M. N., G. Baquiran, C. Brule, J. Burgess, B. Foster, J. J. M. Bergeron, and B. I. Posner. 1989. Internalization and Activation of the Rat Liver Insulin Receptor Kinase Invivo. *Journal of Biological Chemistry*, 264:12931-12940.

Khan, M. N., W. H. Lai, J. W. Burgess, B. I. Posner, and J. J. Bergeron. 1993. Potential role of endosomes in transmembrane signaling. *Sub-Cellular Biochemistry*, 19:223-54.

Kirchhausen, T., J. S. Bonifacino, and H. Riezman. 1997. Linking cargo to vesicle formation: receptor tail interactions with coat proteins. *Current Opinion in Cell Biology*, 9:488-95.

Koliatsos, V. E., M. D. Applegate, B. Knusel, E. O. Junard, L. E. Burton, W. C. Mobley, F. F. Hefti, and D. L. Price. 1991. Recombinant Human Nerve Growth Factor Prevents Retrograde Degeneration of Axotomized Basal Forebrain Cholinergic Neurons in the Rat. *Experimental Neurology*, 112:161-173.

Kremer, N. E., G. D'Arcangelo, S. M. Thomas, M. DeMarco, J. S. Brugge, and S. Halegoua. 1991. Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of src and ras actions. *Journal of Cell Biology*, 115:809-19.

Lamaze, C., and S. L. Schmid. 1995. The emergence of clathrin-independent pinocytic pathways. *Current Opinion in Cell Biology*, 7:573-80.

Martin-Perez, J., D. Bar-Zvi, D. Branton, and R. L. Erikson. 1989. Transformation by Rous sarcoma virus induces clathrin heavy chain phosphorylation. *Journal of Cell Biology*, 109:577-84.

Meakin, S. O., and E. M. Shooter. 1992. The Nerve Growth Factor Family of Receptors. *Trends in Neurosciences*, 15:323-331.

Mobley, W. C., A. Schenker, and E. M. Shooter. 1976. Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry*, 15:5543-52.

Overly, C. C., H. I. Rieff, and P. J. Hollenbeck. 1996. Organelle Motility and Metabolism in Axons Vs Dendrites of Cultured Hippocampal Neurons. *Journal of Cell Science*, 109:971-980.

Page, L. J., and M. S. Robinson. 1995. Targeting Signals and Subunit Interactions in Coated Vesicle Adaptor Complexes. *Journal of Cell Biology*, 131:619-630.

Pearse, B. M. 1988. Receptors compete for adaptors found in plasma membrane coated pits. *Embo Journal*, 7:3331-6.

Pearse, B. M., and M. S. Robinson. 1990. Clathrin, adaptors, and sorting. *Annual Review of Cell Biology*, 6:151-71.

Pley, U. M., B. L. Hill, C. Alibert, F. M. Brodsky, and P. Parham. 1995. The interaction of calmodulin with clathrin-coated vesicles, triskelions, and light chains. Localization of a binding site. *Journal of Biological Chemistry*, 270:2395-402.

Riccio, A., B. A. Pierchala, C. L. Ciarallo, and D. D. Ginty. 1997a. An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. *Science*, 277:1097-1100.

Riccio, A., B. A. Pierchala, C. L. Ciarallo, and D. D. Ginty. 1997b. An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons [see comments]. *Science*, 277:1097-100.

Ross, A. H., M. C. Daou, C. A. McKinnon, P. J. Condon, M. B. Lachyankar, R. M. Stephens, D. R. Kaplan, and D. E. Wolf. 1996. The Neurotrophin Receptor, Gp75, Forms a Complex With the Receptor Tyrosine Kinase Trka. *Journal of Cell Biology*, 132:945-953.

Santini, F., and J. H. Keen. 1996. Endocytosis of activated receptors and clathrin-coated pit formation: deciphering the chicken or egg relationship. *Journal of Cell Biology*, 132:1025-36.

Sato, K., A. Sato, M. Aoto, and Y. Fukami. 1995a. c-Src phosphorylates epidermal growth factor receptor on tyrosine 845. *Biochemical and Biophysical Research Communications*, 215:1078-87.

Sato, K., A. Sato, M. Aoto, and Y. Fukami. 1995b. Site-specific association of c-Src with epidermal growth factor receptor in A431 cells. *Biochemical and Biophysical Research Communications*, 210:844-51.

Schmid, S. L. 1997. Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annual Review of Biochemistry*, 66:511-48.

Schmid, S. L., and H. Damke. 1995. Coated vesicles: a diversity of form and function. *Faseb Journal*, 9:1445-53.

Stephens, R. M., D. M. Loeb, T. D. Copeland, T. Pawson, L. A. Greene, and D. R. Kaplan. 1994. Trk Receptors Use Redundant Signal Transduction Pathways Involving Shc and Plc-Gamma-1 to Mediate Ngf Responses. *Neuron*, 12:691-705.

Wilde, A., and F. M. Brodsky. 1996. In vivo phosphorylation of adaptors regulates their interaction with clathrin. *Journal of Cell Biology*, 135:635-45.

Wolf, D. E., C. A. McKinnon, M. C. Daou, R. M. Stephens, D. R. Kaplan, and A. H. Ross. 1995. Interaction With Trka Immobilizes Gp75 in the High Affinity Nerve Growth Factor Receptor Complex. *Journal of Biological Chemistry*, 270:2133-2138.

Wolf, D. E., C. McKinnonThompson, M. C. Daou, R. M. Stephens, D. R. Kaplan, and A. H. Ross. 1998. Mobility of TrkA is regulated by phosphorylation and interactions with the low-affinity NGF receptor. *Biochemistry*, 37:3178-3186.

Yuen, E. C., C. L. Howe, Y. W. Li, D. M. Holtzman, and W. C. Mobley. 1996. Nerve Growth Factor and the Neurotrophic Factor Hypothesis. *Brain & Development*, 18:362-368.

Zhou, J., J. S. Valletta, M. L. Grimes, and W. C. Mobley. 1995. Multiple Levels For Regulation of Trka in Pc12 Cells By Nerve Growth Factor. *Journal of Neurochemistry*, 65:1146-1156.

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