p21ras is believed to be involved in the neuronal differentiation of cells responsive to nerve growth factor (NGF). We show that NGF stimulates the activation of p21ras in embryonic sensory neurons and in PC12 cells. In the initial 5 min of exposure to NGF, the activation is concentration-dependent. In the sensory neurons and PC12 cells, the apparent maximal activation reached at 50 and 10 ng/ml, respectively, with half-maximal activation at ~5 and 2-3 ng/ml, respectively. Kinetic analysis at low concentrations of NGF showed that p21ras activation slowly increases with time in both types of cells, while high concentrations result in rapid activation within 5 min. These results indicate that NGF regulates the activation state of p21ras in these cells and provides evidence suggesting that activation of p21ras is involved in NGF signal transduction. Treatment of PC12 cells with brain-derived neurotrophic factor or neurotrophin-3 (NT-3) failed to activate p21ras, suggesting that binding alone to p75NGFR is insufficient for ras activation. Treatment with the kinase inhibitor, K252a, which inhibits the NGF tyrosine kinase receptor p140trk, abolished ras activation, suggesting that p140ras is the major mediator of p21ras activation by NGF.

During development of the nervous system, nerve growth factor (NGF) functions to regulate the survival and differentiation of certain sensory and sympathetic neurons in the peripheral nervous system, as well as basal forebrain cholinergic neurons in the central nervous system (Thoenen and Barde, 1980; Greene and Shooter, 1981; Barde, 1989). In the adult, NGF serves to maintain the differentiated neuronal phenotype, with sympathetic neurons alone continuing to require NGF for survival. After axonal injury, NGF also plays an important role in the regeneration process. Other homologous members of the neurotrophin family, such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) function to support survival and differentiation of neuronal populations with different and overlapping specificities.

Transduction of the NGF signal begins with binding of NGF to specific cell surface receptors (for review, see Meakin and Shooter, 1992; Chao, 1992). Two types of receptors are known to bind NGF: p75NGFR (Johnson et al., 1986; Radeke et al., 1987) which binds NGF with low affinity (Kd = 10⁻⁸-10⁻⁹), and p140ras, a receptor tyrosine kinase (Kaplan et al., 1991; Klein et al., 1991; Meakin et al., 1992) which either alone (Klein et al., 1991) or in combination with p75NGFR (Hempstead et al., 1991) binds NGF with high affinity (Kd = 10⁻¹⁵-10⁻¹⁷) (Sutter et al., 1990a; Schechter and Bothwell, 1981; Landreth and Shooter, 1980; Bernd and Greene, 1984). As with other types of tyrosine kinase receptors, the binding of NGF to p140ras results in its activation by autophosphorylation of specific tyrosine residues (Kaplan et al., 1991; Klein et al., 1991). It is the high affinity form of the receptor which is believed to mediate the actions of NGF (Greene, 1977; Sonnenfeld and Ishii, 1985; Green et al., 1986). The signal transduction events which follow binding of NGF to its receptors are under active investigation. Based on studies using the NGF-responsive rat pheochromocytoma PC12 cell line (Greene and Tischler, 1976), it has been suggested that the cellular counterpart of the oncogenic protein p21ras plays a key role in mediating the NGF signal in NGF-responsive cells. PC12 cells differentiate into a sympathetic-like neuronal phenotype on treatment with NGF, and introduction of the oncogenic form of p21ras into these cells by microinjection of the protein (Bar-Sagi and Ferrendisco, 1985), transfection with the gene (Guerrero et al., 1986), or infection with virus carrying the gene (Noda et al., 1985) mimicks the effects of NGF on PC12 cells. Similarly, microinjection of the activated p21ras oncoprotein into primary cultures of embryonic NGF-responsive neurons is able to promote their in vitro survival and neurite outgrowth in the absence of exogenous NGF (Borasio et al., 1989). Furthermore, microinjection of anti-ras antibodies into PC12 cells (Hagag et al., 1986), or transfection with a dominant inhibitory mutant (Asn-17 of p21ras) (Széberenyi et al., 1990) results in the inhibition of the phenotypic response of PC12 cells to NGF.

p21ras is a member of the family of GTPases, whose homology resides in the domains responsible for guanine nucleotide binding and hydrolysis (Hall, 1990; Bourne et al., 1991). One important characteristic of p21ras lies in its ability to cycle between inactive and active forms by switching between GDP and GTP-bound forms, respectively. In non-neuronal cells, the conversion of the inactive GDP-bound form of p21ras to the active GTP-bound form is stimulated in response to various growth factors. Platelet-derived growth factor, epidermal growth factor, and insulin are able to stimulate increased levels of active p21ras in fibroblasts (Satoh et al., 1990a, 1990b; Burginger et al., 1991). Stimulation of the antigen receptor of T lymphocytes by an anti-CD3 antibody or phytohemagglutinin causes a rapid activation of p21ras (Downward et al., 1990). The cytokines, interleukins 2 and 3, and granulocyte

The abbreviations used are: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; DME, Dulbecco's modified medium; DRG, dorsal root ganglia.
Poietin (Torti et al., 1992), interleukin 5, steel factor (Duronio et al., 1992), and erythropoietin (Torti et al., 1992) activate p21Warp in hematopoietic cells. NGF, fibroblast growth factor, and interleukin 6, each of which can cause differentiation of PC12 cells, have also been shown to induce increased levels of active p21Warp in PC12 cells (Qiu and Green, 1991; Muroya et al., 1992; Nakafuku et al., 1992).

While it has been shown that NGF activates p21Warp in the PC12 cell line, it is not known whether developing NGF-responsive neurons respond in a similar manner. Because embryonic NGF-responsive neurons need NGF for survival, this requirement has made it difficult to use developing neurons to study signal transduction by NGF. Here, we show that in both embryonic sensory neurons and PC12 cells, NGF regulates the activation state of p21Warp. We also show that the low affinity NGF receptor p75NGFR does not appear to be involved in this activation and suggest that the high affinity NGF receptor involving p140Warp is the major mediator of ras activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Nerve growth factor was obtained from Harlan Bioproducts For Science. Poly-L-lysine, phosphate-free DME, protease inhibitors, mouse anti-r-A-Sepharose, and brome-kinase were obtained from Worthington Biochemical Corp. Mouse laminin was purchased from Upstate Biotechnology, Inc. [32P]Orthophosphate was obtained from ICN Biomedicals, while DME was obtained from Life Technologies, Inc. Supplemented calf serum and horse serum were from HyClone. Rabbit anti-rat IgG was from Vector Laboratories.

**Cell Culture**—Dorsal root ganglia (DRG) were dissected from embryonic day 9 (E9) chick embryos into Dulbecco’s phosphate-buffered saline, 2 mg/ml glucose. Following digestion with 0.08% trypsin for 15 min at 37 °C, the ganglia were diluted 1:5 with 5% heat-inactivated horse serum in DME. The ganglia were pelleted, the supernatant removed, and the ganglia were dissociated by repeated passages through a flame-polished narrow bore Pasteur pipette. The cells were then filtered through a 45-μm nylon mesh (Nitex) and preplated in fresh 5% heat-inactivated horse serum/DME in a 100-mm plastic culture dish for 3.5–4 h at 37 °C under 5% CO2. Non-neuronal cells such as fibroblasts and Schwann cells adhered preferentially to the plastic. The neuron-enriched supernatant was pelleted and resuspended into phosphate-free 5% horse serum/DME, and transferred to 100-mm culture dishes 2 days prior to labeling, after which the media was replaced with phosphate-free 5% calf serum, 2.5% horse serum/DME containing the label.

**Analysis of GuaNucleotides Bound to p21Warp**—Ras activation was assayed according to Satoh et al. (1990a). Dissociated and purified E9 chick DRG were labeled in phosphate-free media containing [32P]orthophosphate (0.5 mCi/ml) in the presence of 0.05 ng/ml NGF and 0.1 mg/ml cytochrome c, for 14–16 h at 37 °C. PC12 cells were labeled in phosphate-free media containing [32P]orthophosphate (0.25 mCi/ml) for 14–16 h at 37 °C. Following labeling, the cells were exposed to fresh media containing either no NGF or with NGF at the appropriate concentration, at 37 °C. When the protein kinase inhibitor K252a was used, 1 μM was added to the media directly and incubated at 37 °C for 30 min prior to treatment with NGF. Following treatment with neurotrophin, the media was removed, and the cells immediately rinsed with ice-cold 50 mM Tris, pH 7.5, 150 mM NaCl. The cells were lysed with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 20 mM MgCl2, 0.5% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM phenylmethylsulfonyl fluoride) and pelleted to remove cellular debris, and the supernatant was cleared with 30 μl of 10% activated charcoal. Cellular p21Warp was immunoprecipitated from the lysates with rotation for 90 min at 4 °C using affinity-purified rat monoclonal anti-v-H-ras antibody Y13-259. Protein A-Sepharose beads pre-ocated (for 3 h) with rabbit anti-r ras IgG was then added, and the immunoprecipitation continued for 1 h. The beads were then washed five times with lysis buffer, and three times with 50 mM Tris, pH 7.5, 150 mM NaCl, 20 mM MgCl2. The guanine nucleotides were eluted from the immunoprecipitates with 20 mM Tris, pH 7.5, 20 mM EDTA, 2% SDS, 0.5 mM GTP, 0.5 mM GDP for 5 min at 55 °C. The guanine nucleotides were separated by thin layer chromatography on polyethyleneimine-cellulose in 0.75 M KH2PO4, pH 3.4, and visualized by autoradiography. The guanine nucleotides GDP and GTP were quantitated by liquid scintillation counting of the cellulose. The percentage of GTP to the total amount of GTP plus GDP was calculated, and ras activation was then represented as percent of control.

**RESULTS**

**p21Warp Activation by NGF in Embryonic Sensory Neurons**—During the period of development when sensory and sympathetic neurons require NGF for survival, their explantation into an in vitro system results in cell death unless NGF is supplemented into the culture medium (Levi-Montalcini and Angeletti, 1963; Cohen et al., 1964). In order to obtain a population of surviving NGF-dependent embryonic neurons which could be responding further to NGF under controlled experimental conditions, neurons were exposed and equilibrated in vitro to low levels of NGF following exposition and dissociation. We cultured dissociated embryonic chick dorsal root ganglion (DRG) cells with a concentration of NGF (0.05 ng/ml) which results in half-maximal survival of NGF-responsive embryonic sensory neurons (Greene, 1977; Sutter et al., 1979b). These neurons were simultaneously radio-labeled and exposed to the subsaturating concentration of NGF overnight to select for a more homogeneous population of neurons which responds only to NGF. These conditions also equilibrated this population of neurons to the in vitro concentration of NGF (0.05 ng/ml) to which it was exposed. In the presence of 0.05 ng/ml NGF, less neurons survived, and exhibited less extensive neuritic networks and fasciculation compared to neurons treated with 50 ng/ml NGF (data not shown). This is in accordance with the NGF concentration-dependent survival of sensory neurons previously demonstrated (Greene, 1977; Sutter et al., 1979b). Following radiolabeling and pretreatment with 0.05 ng/ml NGF, the surviving neurons were exposed to a 1000-fold higher concentration of NGF (50 ng/ml), and the levels of activated p21Warp were assayed. The added NGF stimulated an increase (200–300% of control) in active p21Warp after 5 min (Fig. 1). In the absence of any NGF during radiolabeling, no activation was seen (data not shown). NGF also stimulated p21Warp activation (300–400% of control) in PC12 cells in a similar reaction.
although the cells were not pre-exposed to NGF prior to treatment with the 50 ng/ml concentration (Fig. 1). These results indicated that despite pre-exposure of the embryonic neurons to low levels of NGF, they were able to show further increases in the levels of active p21<sup>rm</sup> in response to higher doses of NGF.

The procedure used to isolate the primary sensory neurons resulted in an enriched population of neurons (80–90%). To confirm that any contaminating non-neuronal cells were not contributing to the stimulation of p21<sup>rm</sup> in response to NGF, the Schwann cells and fibroblasts obtained from preplating the neurons during the neuronal enrichment step were subjected to identical manipulations as that used on the neuronal cultures. They were treated with and without 50 ng/ml NGF for 5 min. No p21<sup>rm</sup> activation was observed (data not shown), indicating that non-neuronal cells did not contribute to the ras activation observed in response to NGF in the neuronal cultures.

**Effect of NGF Concentration on p21<sup>rm</sup> Activation**—Survival and differentiation of embryonic sensory neurons is dependent on NGF concentration (Greene, 1977; Sutter et al., 1979b). In order to determine whether a concentration dependence also existed for activation of p21<sup>rm</sup> by NGF, the cells were assayed for p21<sup>rm</sup> activation in response to varying concentrations of NGF following 5 min of treatment (Fig. 2). In sensory neurons, the level of p21<sup>rm</sup> activation increased with increasing NGF concentration. The apparent maximal activation was reached at 50 ng/ml, with half-maximal activation at approximately 5 ng/ml. In PC12 cells, p21<sup>rm</sup> activation also increased with increasing NGF concentration (Fig. 2). Maximal stimulation was obtained by 10 ng/ml NGF, with half-maximal activation occurring between 2–3 ng/ml. These results indicate that in both sensory neurons and PC12 cells, p21<sup>rm</sup> activation in the initial minutes of NGF exposure is dependent on concentration.

**Effect of NGF Concentration on the Kinetics of p21<sup>rm</sup> Activation**—The kinetics of p21<sup>rm</sup> activation were investigated at both low and high NGF concentrations. When the sensory neurons were treated with 0.5 ng/ml NGF for increasing periods of time, the levels of active p21<sup>rm</sup> increased with time (Fig. 3A). At this lower concentration of NGF and by 60 min, the cells approached but had not reached the maximal levels of active p21<sup>rm</sup> observed when they were treated with 50 ng/ml NGF for 5 min. Similarly, when the PC12 cells were exposed to 0.5 ng/ml NGF, p21<sup>rm</sup> activation increased with time (Fig. 3B). As with the neurons, by 60 min the levels of active p21<sup>rm</sup> approached but had not reached the maximal levels observed when treated with 50 ng/ml NGF for 5 min. In both the sensory neurons and PC12 cells, it is likely that given sufficient time, the maximal levels of active p21<sup>rm</sup> would eventually be attained. The kinetics of p21<sup>rm</sup> activation at 50 ng/ml was analyzed to determine how the cells respond to this higher concentration of NGF. In the sensory neurons, maximal levels of active p21<sup>rm</sup> were attained after 5 min (Fig. 4A). By 60 min, the levels of active p21<sup>rm</sup> had slowly declined, but remained elevated at 160% of control. By comparison, when PC12 cells (not previously exposed to NGF) were similarly treated, it resulted in a stimulation (300–400% of control) in the levels of active p21<sup>rm</sup> within 1 min. These high levels remained elevated for up to 60 min (Fig. 4B). Comparison of the p21<sup>rm</sup> activation response between the sensory neurons and the PC12 cells shows an apparent difference in sensitivity to NGF, with the PC12 cells stimulating an apparently greater response. Furthermore, while the levels of active p21<sup>rm</sup> had declined by 60 min in the neurons, those in the PC12 cells remained elevated at their maximal levels. These results indicate that at high NGF concentrations, p21<sup>rm</sup> activation is rapid and maximal activation is achieved within minutes of NGF exposure. However, at these higher concentrations, differences appear between the kinetic profiles of p21<sup>rm</sup> activation in sensory neurons and PC12 cells.

**Involvement of NGF Receptors in p21<sup>rm</sup> Activation**—To investigate the involvement of the NGF receptors p75<sup>NGFR</sup> and p140<sup>NGFR</sup> in p21<sup>rm</sup> activation, assays were performed using the homologous neurotrophins, BDNF and NT-3. Both of these neurotrophins can bind the low affinity receptor p75<sup>NGFR</sup> (Squinto et al., 1991), although with differing association rates (Rodriguez-Tebar et al., 1990, 1992). PC12 cells contain p75<sup>NGFR</sup>, but do not contain the tyrosine kinase receptors TrkB and TrkC for BDNF and NT-3, respectively.

![Fig. 2. Effect of NGF concentration on activation of p21<sup>rm</sup> in embryonic DRG neurons (solid circles) and PC12 cells (open circles). After treatment with varying concentrations of NGF for 5 min at 37°C, the cells were lysed and the p21<sup>rm</sup>-bound guanine nucleotides were analyzed as described under "Experimental Procedures."](image-url)

![Fig. 3. Kinetics of p21<sup>rm</sup> activation with 0.5 ng/ml NGF in embryonic DRG neurons (A) and PC12 cells (B). Cells were treated with 0.5 ng/ml NGF at 37°C, and at the indicated times, were lysed and collected for analysis of p21<sup>rm</sup>-bound guanine nucleotides as described under "Experimental Procedures." Error is presented as mean ± standard deviation (n ≥ 3).](image-url)
protein kinase inhibitor which inhibits the tyrosine kinase activity of p140
(Berg et al., 1992; Tapley et al., 1992), inhibited p21
activation in response to NGF (Fig. 6), suggesting that binding of p140
was indeed necessary for the activation. However, should functional kinase activity of
p140
not be required for p21
activation, inhibition of other protein kinases involved in activation of p21
cannot be ruled out. DISCUSSION
As summarized earlier, evidence has been accumulating to suggest the involvement of p21
in the NGF signal transduction pathway. The studies presented here show that NGF-dependent embryonic sensory neurons stimulate increased levels of active p21
in response to NGF. In both the sensory neurons and PC12 cells, the concentration dependence of p21
activation in the initial minutes of NGF exposure provides evidence suggesting an involvement of p21
in transducing the NGF signal. The results also suggest that this activation is likely to be mediated by the NGF tyrosine kinase receptor p140
and that binding alone to the low affinity NGF receptor p75
NGFR does not appear to be sufficient.

In the kinetics of p21
activation, the high NGF concentration elicited a much more rapid activation than did the low concentration. At the high concentration, aside from the similarity of rapid kinetics, the response was quite different between the sensory neurons and PC12 cells. The PC12 cells appeared to be more sensitive, with an apparently greater activation in response to NGF. The difference in sensitivity may reflect the greater numbers of NGF receptors on PC12 cells compared to those on the neurons. Whereas DRG sensory neurons possess approximately 3 \times 10^5 high affinity receptor binding sites and 4.5 \times 10^4 low affinity sites (Sutter et al., 1979), PC12 cells contain approximately 1.5 \times 10^4 high affinity sites and numbers of low affinity sites similar to those on the sensory neurons (Schechter and Bothwell, 1981). Another possibility is that the prior exposure of the sensory neurons to the low NGF concentration may have resulted in the presence of a NGF-induced basal level of p21
activation in effect before initiation of the assay. The slight decline in p21
activation by 60 min may be a consequence of the overnight NGF pre-exposure in these cells. However, when PC12 cells were similarly pre-exposed to 0.35 ng/ml of NGF during radiolabeling, the activation profile remained the same as without pre-exposure (data not shown), showing no decline in either magnitude of p21
activation nor any decline in levels of active p21
by 60 min. Alternatively, these differ-

Furthermore, PC12 cells do not respond to BDNF or NT-3 (Squinto et al., 1991). When the PC12 cells were treated with BDNF and NT-3 at 50 ng/ml, no p21
activation was observed (Fig. 5). Sensory neurons possess p75
NGFR and also respond to BDNF and NT-3 (Lindsay et al., 1985; Maisonpierre et al., 1990). Treatment of these neurons with BDNF and NT-3 resulted in ras activation (Fig. 5). These observations indicated that binding to low affinity NGF receptors alone was not sufficient to activate p21
, and suggested rather, that binding to the appropriate neurotrophin tyrosine kinase receptor may be required for p21
activation. Treatment of both sensory neurons and PC12 cells with K252a, a
NGF-induced Ras Activation in Neurons and PC12 Cells

References could be due to the differing states of differentiation between the sensory neurones and the PC12 cells at the time of the assay. The neurones were already differentiated at the time of extraction while the PC12 cells were not.

The role of the low affinity NGF receptor p75NGFR in NGF signal transduction has not been completely resolved. Previous studies have suggested that expression of p75NGFR is necessary for generation of both low and high affinity NGF receptors in NR18 cells, mutant PC12 cells which lack these receptors (Hempstead et al., 1989), and that the cytoplasmic domain of p75NGFR can mediate neuronal differentiation by interacting with other signaling molecules present in PC12 cells (Yan et al., 1991). Further support for this comes from studies suggesting that p75NGFR together with p140Ras may be the major mediator of p21ras activation by NGF.

The present study has characterized the activation of p21ras induced by NGF in both embryonic sensory neurones and PC12 cells, and shown that NGF regulates the activation state of p21ras in these cells. A recent study has shown that the phenotypic changes induced by neuronal differentiation factors NGF and fibroblast growth factor in PC12 cells is associated with prolonged ras activation (up to 16 days), while ras activation induced by other growth factors such as epidermal growth factor is transient (Qiu and Green, 1992). Given the observation that increased NGF concentrations can further stimulate ras activation in the sensory neurones, together with the dose-response data and the kinetic analyses at high and low NGF concentrations, it is tempting to speculate that each concentration of NGF may determine a different plateau level of activated p21ras which is sustained for the duration of exposure to the particular NGF concentration. The magnitude of the level of active p21ras may then be involved in determining the observed concentration dependence of survival and differentiation in neurones exposed to NGF. It is increasingly clear that neuronal differentiation by NGF involves the activation of multiple effector pathways (Sugimoto et al., 1988; Szebeneyi et al., 1990; Kremer et al., 1991; Wood et al., 1992; Qiu and Green, 1992). The exact role of p21ras in these pathways is presently being elucidated. Characterization of how p21ras responds to NGF in both sensory neurones and PC12 cells will ultimately contribute to our understanding of its particular involvement in transducing the NGF signal.

Acknowledgments—We thank Dr. Laureli Bolin for advice and help on preparation of the neuronal cultures and Dr. S. Squinto for the BDNF and NT-3.