Acids Res. **19**, 61 (1991); C. Randak, T. Brabletz, M. Bergenrother, I. Sobotta, E. Serfling, *EMBO J.* **9**, 2529 (1990).
22. J. Kaye *et al.*, *J. Exp. Med.* **158**, 836 (1983).
23. Oligonucleotides and constructs. The IgκB site is described in J. Pierce *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* **85**, 1482 (1988)]. The IL-2κB oligo nucleotide spans −212 to −195 of the IL-2 enhancer. The sequence of these oligonucleotides is in the legend to Fig. 1, except that a 5′-TCGA-3′ overhang exists at the 5′ end. For stable transfectants, minimal c-*fos* promoter-bacterial CAT reporter constructs were prepared as described [M. Lenardo, J. W. Pierce, D. Baltimore, *Science* **236**, 1573 (1987)]. The IgκB construct is "J16" from Pierce *et al.* (*23*). (vide supra). The IL-2κB construct contained six copies of the IL-2κB oligonucleotide in the Sal I site of the Δ56 minimal c-*fos*-CAT vector. Constructs were confirmed by DNA sequencing, and function was checked by transient transfection into EL-4 cells. For stable transfections, A.E7 cells were placed in IL-2 (30 U/ml) (in the form of MLA-144 supernatant) for 3 days and transfected with 25 μg of linearized test plasmid and 2.5 μg of Eco RI–linearized pSV2Neo by electroporation [A. J. Cann *et al.*, *Oncogene* **3**, 123 (1989)] with 300 V, 960 μF in a Bio-Rad gene pulser apparatus. After electroporation, the cells were placed into IL-2–containing medium for 2 days before the addition of G418 (0.8 mg/ml) [Genticin, Bethesda Research Laboratories (BRL)]. The cells were then pooled and grown in G418-containing medium for more than 1 month before use. Two different pools of transfectants were tested for each construct. For stimulations, we used 5 × 10^5 T lymphocytes and 2.5 × 10^5 DCEK cells, with or without 81 to 104 peptide (1 μM) in 2 ml. The cells were incubated for 14 to 16 hours and harvested as described above. The CAT assays were performed with standard procedures [C. Gorman *et al.*, *Mol. Cell. Biol.* **2**, 1044 (1982)] and extracts were normalized by protein concentration. Chloramphenicol conversion was quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Transient transfections into EL-4 cells were performed by the DEAE-dextran procedure as described (*7*).
24. M. B. Urban, R. Schreck, P. A. Baeuerle, *EMBO J.* **10**, 1817 (1991); M. B. Urban and P. A. Baeuerle, *New Biol.* **3**, 279 (1991).
25. R. Sen and D. Baltimore, *Cell* **47**, 921 (1986).
26. P. Baeuerle and D. Baltimore, *Science* **242**, 540 (1988).
27. J. J.-S. Chiu and M. J. Lenardo, unpublished data.
28. We thank R. Schwartz and B. Beverly for advice and assistance in the growth of nontransformed T cell clones; R. Germain for advice on bead separation; D. D. A'Ambrosio and K. Bruhn for laboratory assistance; U. Siebenlist, V. Bours, G. Franzoso, and P. Bressler for the p50 expression constructs, discussions, and sharing unpublished results; H. Shibuya and T. Taniguchi for IL-2 CAT constructs; S. Ghosh and D. Baltimore for the p50 cDNA; and D. Levens, J. Mellentin, U. Siebenlist, and L. Staudt for comments on the manuscript. S.-M. K. was supported by a Howard Hughes Medical Institute–NIH Research Scholarship and M.J.L. is supported by a Cancer Research Institute Investigator Award.

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Nerve Growth Factor Stimulation of the Ras-Guanine Nucleotide Exchange Factor and GAP Activities

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The biological activity of Ras proteins is thought to be controlled by the guanine nucleotide exchange factor and the guanosine triphosphatase activating protein (GAP). Treatment of rat pheochromocytoma PC-12 cells with nerve growth factor (NGF) increased the amount of active Ras guanosine triphosphate complex and stimulated the activities of both the guanine nucleotide exchange factor and GAP. In PC-12 cells that overexpressed the tyrosine kinase encoded by the *trk* proto-oncogene (a component of the high-affinity NGF receptor), the NGF-induced activation of the regulatory proteins was potentiated. These results suggest that the NGF receptor system enhances the activities of both the guanine nucleotide exchange factor and GAP and that the activation of Ras might be controlled by the balance in activity between these two regulatory proteins.

The *ras* proto-oncogenes encode membrane-bound proteins that bind guanine nucleotide and have low intrinsic guanosine triphosphatase (GTPase) activity (*1*). Binding of guanosine triphosphate (GTP) activates Ras proteins, and subsequent hydrolysis of bound GTP to guanosine diphosphate (GDP) inactivates signaling by these proteins. In mammalian cells, the former process can be catalyzed by the guanine nucleotide exchange factor or GDP-dissociation stimulator for Ras proteins (*2, 3*), and the latter process can be accelerated by GAP (*4*). Although the biochemical and biological activities of GAP have been extensively studied, little is known about the physiological role of the guanine nucleotide exchange factor in growth factor–stimulated signal transduction.

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Ras participates in NGF-induced differentiation of rat pheochromocytoma (American Type Culture collection CRL 1721) PC-12 cells. In the presence of NGF, PC-12 cells extend neurites and differentiate into cells resembling sympathetic neurons (*5*). The expression of Ha-Ras oncogenic proteins in PC-12 cells mimics some, if not all, of the effects of NGF on the cells (*6*), and the induction of morphological differentiation of PC-12 cells by NGF is blocked by the microinjection of neutralizing antibody to Ras into the cells (*7*). Normal Ras-GTP can also induce neurite outgrowth in PC-12 cells (*8*).

We labeled serum-starved PC-12 cells with [^{32}P]orthophosphate and then incubated them with or without NGF. Analysis of the nucleotides bound to immunoprecipitated Ras showed that NGF rapidly (within 5 min) induced an increase in the ratio of Ras-GTP to Ras-GDP (Fig. 1A). The magnitude of the increase in the amount of Ras-GTP complex was dependent on the concentration of NGF (Fig. 1B), as is the degree of neurite outgrowth induced by NGF (*9*). Similar stimulatory effects of NGF were observed by others (*10*). Dibutyryl adenosine 3′,5′-monophosphate (cAMP) (1 mM) had no effect on the amount of Ras-GTP (*11*), which is consistent with the observation that the induction of morphological differentiation of PC-12 cells by dibutyryl cAMP is not mediated by Ras proteins (*7*). The effect of NGF on the amount of Ras-GTP was transient, and the amount of Ras-GTP in cells treated with NGF for 20 min was similar to that in untreated cells. Because the treatment of cells with NGF for 20 min does not cause neurite outgrowth (*11*), it appears that the increased amount of Ras-GTP is not sufficient to promote differentiation.

We compared the effect of NGF on the amount of Ras-GTP in normal PC-12 cells to its effect in PC-12 cells transfected with the human Trk proto-oncogene (Trk–PC-12 cells). Trk proteins encode a 140-kD membrane protein with tyrosine kinase activity that is a component of the high-affinity NGF receptor. The expression of Trk is required for signal transduction by NGF (*12*). The Trk–PC-12 cells expressed high levels of Trk protein compared to parental PC-12 cells and increased amounts of NGF-induced Trk tyrosine kinase activity (*13*). After a 5-min incubation with NGF, the ratio of Ras-GTP to total bound nucleotides was higher in Trk–PC-12 cells (34.5 ± 3.0%) than in untransfected PC-12 cells (23.0 ± 1.0%) (Fig. 1C). There was no difference in the amount of Ras in the two cell lines as detected by immunoblotting with the antibody to Ras (*11*).

Thus, these results suggest the involvement of Ras in the signaling pathway acti-

vated by Trk. Modulation of binding of GDP or GTP to Ras occurs in response to growth stimulation. An increase in the amount of Ras in the GTP-bound form has been detected in cells stimulated to proliferate by growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (*14*), by cells transformed by oncogenes such as *src*, *erb*-B2, and *abl* (*14, 15*), and by lymphoid cells activated by antibody to CD3 or lymphokines such as interleukin-2 and granulocyte-macrophage colony-stimulating factor (*16*). In contrast, our data indicate that the increased amount of Ras-GTP was correlated with both growth change and differentiation induced by NGF in PC-12 cells (*8*).

Because the guanine nucleotide exchange factor and GAP can mediate NGF action on Ras, we examined the effect of NGF on the activities of nucleotide exchange factor and GAP in both PC-12 cells and Trk–PC-12 cells. After incubation of the cells in the absence of serum, cells were treated with NGF for 10 min. The cells were lysed, and the soluble fraction (*17*) was added to a solution that contained H-Ras bound to [^{3}H]GDP in the presence of excess unlabeled GTP. Ras was immunoprecipitated, and the quantity of GDP released from the binary complex was determined. The amount of [^{3}H]GDP released in the presence of extracts from NGF-treated PC-12 cells (33.6 ± 3.8%) was twice that released in the presence of extracts from untreated PC-12 cells (17 ± 0.8%) (*18*); we observed further increases in the quantity of [^{3}H]GDP released in the presence of extracts from NGF-treated Trk–PC-12 cells (52 ± 4.0%) compared to that released in the presence of extracts from untreated Trk–PC-12 cells (21 ± 1.8%).

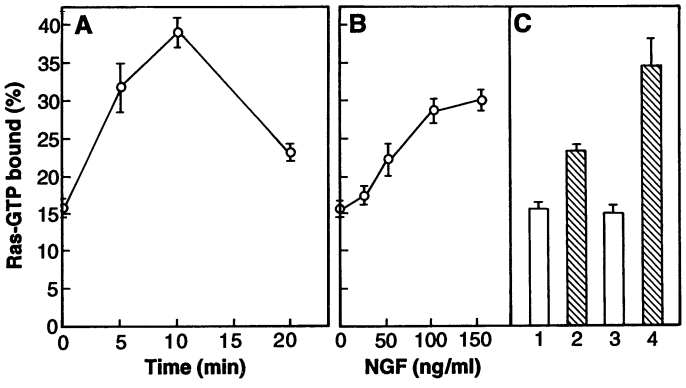


Fig. 1. Effect of NGF on the nucleotides bound to Ras in PC-12 cells. (**A**) Time course of Ras-GTP formation. PC-12 cells (8 × 10^5 per 60-mm dish) were cultured in serum-free Dulbecco's modified Eagle's medium for 18 hours (*28*) and labeled with [^{32}P]orthophosphate (0.2 to 0.5 mCi/ml; Amersham) for 120 min. After the cells were treated with NGF (100 ng/ml; Collaborative Research, Bedford, Massachusetts) for the indicated time intervals, they were washed with phosphate-buffered saline (PBS) and lysed in the buffer [20 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, NP-40 (0.5%), leupeptin (1 μg/ml), and 0.2 mM PMSF]. The lysates were centrifuged at 14,000*g* for 10 min, and the supernatant was incubated with monoclonal antibody to Ras (Y13-259; 40 μg) for 60 min at 4°C. The immune complexes were collected with rabbit antibody to rat immunoglobulin G bound to Protein A–Sepharose (Pharmacia) and washed three times with 1 ml of ice-cold washing buffer [50 mM tris-HCl (pH 7.5) and 20 mM $MgCl_2$]. Nucleotides were eluted from Ras as described (*14*). The extracted samples were analyzed by TLC on polyethyleneimine cellulose in 0.75 M KH_2PO_4 (pH 3.5). Radioactivity was quantitated by scintillation counting. (**B**) Dose response of formation of Ras-GTP. The ^{32}P-labeled PC-12 cells were treated with the indicated amounts of NGF for 5 min. Ras was immunoprecipitated, and nucleotides were separated as in (A). (**C**) Comparison of NGF-induced Ras-GTP formation in PC-12 cells and Trk–PC-12 cells. The ^{32}P-labeled PC-12 (bars 1 and 2) and Trk–PC-12 (bars 3 and 4) cells were treated with (hatched bars) or without (open bars) NGF (50 ng/ml) for 5 min. Ras was immunoprecipitated, and nucleotides were separated as in (A). In (A) through (C), the ratio of GTP to total labeled nucleotides on Ras [(GTP/GTP + GDP) × 100] was calculated. The data in (A) through (C) show the percentage of Ras in the GTP-bound form and represent means ± SD (n = 3).

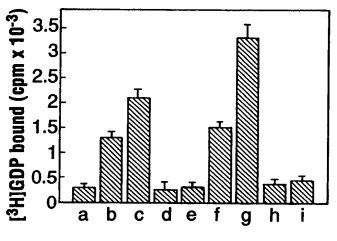


Fig. 2. Acceleration of release and exchange of GDP and GTP-γ-S in the presence of cell extracts from PC-12 and Trk–PC-12 cells. Cell extracts were prepared as described (*18*). Ras (0.25 μg) was incubated with 100 μM unlabeled GDP at 37°C for 30 min and mixed with 18 mM $MgCl_2$. The Ras-GDP complex was incubated for 10 min at 37°C with 10 μCi of [^{3}H]GDP in the presence of the control homogenization buffer (a); with extract (3 μg) from PC-12 cells treated without (b) or with (c) NGF; with GTP-γ-S (1 mM) plus extract (3 μg) from PC-12 cells treated without (d) or with (e) NGF; with extract (3 μg) from Trk–PC-12 cells treated without (f) or with (g) NGF; or with GTP-γ-S (1 mM) plus extract (3 μg) from Trk–PC-12 cells treated without (h) or with (i) NGF. [^{3}H]GDP bound to Ras was immunoprecipitated and eluted (*18*). Data represent means ± SD (n = 3).

We tested whether the promotion of [^{3}H]GDP release by cell extracts reflects catalysis of exchange of GDP for GTP by Ras or nonspecific degradation of bound [^{3}H]GDP. Unlabeled Ras-GDP complex was incubated with [^{3}H]GDP in the presence of extracts from PC-12 or Trk–PC-12 cells or those extracts with 1 mM guanosine-5′-O-(3-thiotriphosphate) (GTP-γ-S) and immunoprecipitated with antibody to Ras (Fig. 2). In comparison to the control buffer, the extracts of PC-12 or Trk–PC-12 cells increased the amount of [^{3}H]GDP bound to Ras approximately fivefold. Treatment of these cells with NGF further stimulated the conversion of GDP to [^{3}H]GDP; nucleotide exchange was stimulated 8- and 13.5-fold in PC-12 and Trk–PC-12 cells, respectively. Moreover, the addition of GTP-γ-S abolished this effect. These results indicate that NGF accelerated the guanine nucleotide exchange reaction of Ras.

To further analyze activity promoting GDP release, we partially purified cell extracts with fast protein liquid chromatography (FPLC) on a Mono Q anion exchange column (Pharmacia). Each fraction was tested for the ability to stimulate the dissociation of bound [^{3}H]GDP from Ras in the presence of excess unlabeled GTP. The active fraction that promoted release of [^{3}H]GDP eluted at ~0.12 M NaCl (*2, 3*). The relative amount of activity increased fourfold in PC-12 cells and tenfold in Trk–PC-12 cells after stimulation by NGF (Fig. 3). Trk from whole-cell extracts was immunoprecipitated with antibody to Trk (*12, 13*) and reacted with Ras-[^{3}H]GDP. Immunoprecipitated Trk or the partially purified baculovirus recombinant Trk had no effect on the GDP-releasing activity, which suggests that the Trk protein itself did not alter the nucleotide exchange activity of Ras (*19*).

To determine whether GAP activity was also altered by the addition of NGF to the cells, we incubated Ras-[α-^{32}P]GTP complexes with cell extracts, and the amount of guanine nucleotide bound to Ras was quantitated by immunoprecipitation and thin-layer chromatography (TLC). The GAP activity in PC-12 cells stimulated with NGF was two times greater than that in unstimulated cells, and the enhancement of GAP activity by NGF was potentiated in Trk–PC-12 cells (Fig. 4, A and B). Similar results were obtained with GAP partially purified from the cell extracts by chromatography on a Mono Q column (Fig. 4, C and D). These data indicate that NGF increased GAP activity. The stimulatory effect of NGF on GAP activity was maximal 10 min after treatment of PC-12 cells with NGF and remained constant after a 20-min incubation. The guanine nucleotide exchange factor activity was maximally stimulated by NGF within 10 min and gradually decreased

thereafter (*11*). We investigated whether the GAP activation was accompanied by its tyrosine phosphorylation; we detected no increase in the tyrosine phosphorylation of GAP in NGF-stimulated PC-12 cells (Fig. 4E), and GAP failed to associate with the activated Trk (*20*). The GAP activity in PC-12 cell extracts appeared as a result of Ras-GAP rather than of neurofibromin (NF1), because we could not detect NF1 by immunoblotting in the extracts we assayed for GAP activity (*21*).

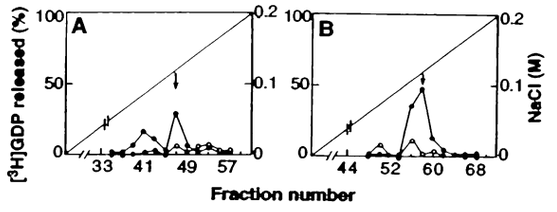
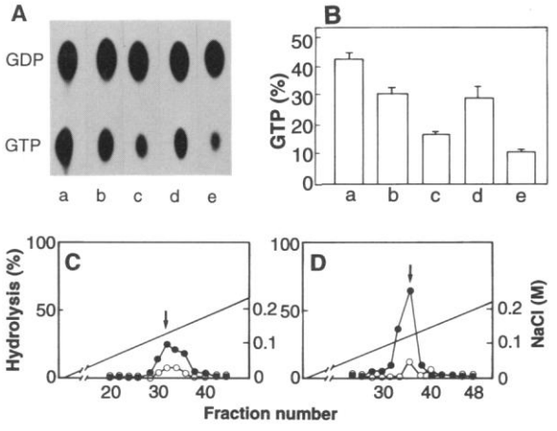


Fig. 3. Anion exchange chromatography of the GDP-releasing activity. (**A**) Activity in PC-12 cells treated with (●) or without (○) NGF. (**B**) Activity in Trk–PC-12 cells treated with (●) or without (○) NGF. Arrows show nucleotide exchange activity eluted at ~0.12 M NaCl. Activity is given as the percentage of bound [^{3}H]GDP released. [^{3}H]GDP (20,000 cpm) was bound to the Ras proteins incubated with the equilibration buffer. After treatment with NGF (50 ng/ml) for 10 min, serum-starved PC-12 or Trk–PC-12 cells (~5 × 10^7 per two ×150-mm-diameter dish) were homogenized in buffer [20 mM sodium phosphate (pH 7.5), 1 mM EGTA, 1 mM DTT, 0.5% NP-40, aprotinin (10 μg/ml), 0.2 mM PMSF, 1 mM sodium orthovanadate, and 10 mM NaF] by sonication, and the homogenates were centrifuged at 100,000*g* for 20 min at 4°C. The supernatants (600 μg) were loaded onto an FPLC Mono Q HR 5/5 column (Pharmacia) equilibrated with buffer [25 mM sodium phosphate (pH 7.5), 1 mM EGTA, 1 mM DTT, 0.2 mM PMSF, and aprotinin (10 μg/ml)]. The proteins were eluted with a linear gradient of NaCl (0 to 0.3 M) in the above buffer, and 0.5 ml of fractions was collected. Each fraction (20 μl) and 0.4 mM GTP were added to 50 μl of the reaction buffer that contained Ras-[^{3}H]GDP (0.25 μg) at 37°C for 10 min. The amount of [^{3}H]GDP associated with Ras was quantitated by filtration on nitrocellulose (*2*).



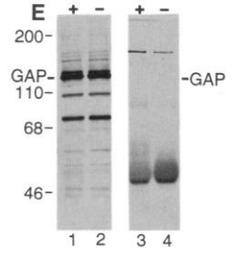


Fig. 4. Effect of NGF-treated cell extracts on Ras GTPase activity and effect of NGF on tyrosine phosphorylation of GAP. Cell extracts in (A) and (B) were prepared from PC-12 and Trk–PC-12 cells treated with NGF (50 ng/ml) for 10 min (Fig. 2). Ras (0.25 μg) was incubated with 0.2 μCi of [α-^{32}P]GTP (3000 Ci/mmol; Amersham) in 50 μl of the reaction buffer for 3 min at 37°C (Fig. 2). Ras-[α-^{32}P]GTP complexes were incubated with the cell extracts (3 μg) or the homogenization buffer at 37°C for 20 min. Ras was immunoprecipitated with the Y13-259 antibody. After being washed three times with washing buffer, the labeled nucleotides were eluted from Ras proteins and analyzed as described (Fig. 1). (**A**) Autoradiogram after TLC of the eluted nucleotides. (**B**) Ratio of GTP to total labeled nucleotides bound to Ras proteins [(GTP/GTP + GDP) × 100]. Ras-[α-^{32}P]GTP was incubated with homogenization buffer (a), or with cell extract from PC-12 cells (b), NGF-treated PC-12 cells (c), Trk–PC-12 cells (d), or NGF-treated Trk–PC-12 cells (e). Data represent means ± SD (n = 3). (**C**) and (**D**) Cell extracts (600 μg) were fractionated by a Mono Q HR 5/5 column with a linear gradient of NaCl (0 to 0.3 M) as described (Fig. 3). Ras (0.25 μg) was incubated with 0.1 μM [γ-^{32}P]GTP (10 Ci/mmol) for 5 min at 37°C, and fractions (20 μl) were added for 10 min at 37°C to 50 μl of the reaction buffer that contained the binary complex. The amount of radioactivity associated with Ras was quantitated as described (Fig. 3); activity in PC-12 cells (C) and Trk–PC-12 cells (D) is shown treated with (●) or without (○) NGF (50 ng/ml). Arrows show the peak of GAP activity. Activities are expressed as the percentage of [γ-^{32}P]GTP bound to Ras that is hydrolyzed relative to the buffer control; 18,000 cpm of GTP remained associated with Ras that was incubated with equilibration buffer. (**E**) PC-12 cells were treated for 5 min with (lanes 1 and 3) or without (lanes 2 and 4) NGF (100 ng/ml) at 37°C. Cell lysates were immunoprecipitated with rabbit antibody to GAP (20 μg) as described (*24*). Immunoprecipitates of GAP on immunoblots were probed with antibody to phosphotyrosine (*24*) (lanes 3 and 4). In lanes 1 and 2, cell lysates were probed by immunoblotting with antibody to GAP. The position of GAP is shown; molecular size markers are in kilodaltons.

Both the guanine nucleotide exchange factor and GAP are activated by NGF. The NGF-induced increase in the amount of Ras-GTP may be a result of the dominance of the guanine nucleotide exchange factor activity over GAP activity. The small increase in the amount of Ras-GTP by NGF might reflect the increase in the activities of both the exchange factor and GAP. The accumulated Ras-GTP would be rapidly converted to Ras-GDP by the stimulated GAP activity after the signal transmission to the downstream effector for Ras, which could provide a tight regulation of the Ras-GDP–Ras-GTP cycle in the transient response of the cells to the growth factor. The receptors for PDGF and EGF induce tyrosine phosphorylation of GAP (*22, 23*), and phosphatase inhibitors stabilize the activity of the exchange factor in cell extracts (*3*). In PC-12 cells, NGF induces the tyrosine kinase activity of Trk, which increases the tyrosine phosphorylation of cellular proteins (*24*) and activates several serine-threonine kinases (*25*). Therefore, Trk tyrosine kinase, other nonreceptor kinases, or phosphatases might modulate the exchange factor or the GAP activity. However, NGF may affect GAP activity by inducing phosphorylation of GAP on serine or threonine residues or by altering the interaction of GAP with cellular components, such as p190 and p62 (*22*), because tyrosine phosphorylation of GAP was not detectable in NGF-treated PC-12 cells.

REFERENCES AND NOTES

1. M. Barbacid, *Annu. Rev. Biochem.* **56**, 779 (1986).
2. M. West, H.-f. Kung, T. Kamata, *FEBS Lett.* **259**, 245 (1990); Y. K. Huang, H.-f. Kung, T. Kamata, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8008 (1990).
3. A. Wolfman and I. G. Macara, *Science* **248**, 67 (1990); J. Downward, R. Riehl, L. Wu, R. A. Weinberg, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5998 (1990); T. Yamamoto *et al.*, *J. Biol. Chem.* **265**, 16626 (1990).
4. M. Trahey and F. McCormick, *Science* **238**, 542 (1987); J. B. Gibbs, M. D. Schaber, W. J. Allard, I. S. Sigal, E. M. Scolnick, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5026 (1988).
5. L. A. Greene and A. S. Tischler, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2424 (1976); R. Levi-Montalcini, *Science* **237**, 1154 (1987).
6. M. Noda *et al.*, *Nature* **318**, 73 (1985); D. Bar-Sagi and J. R. Feramisco, *Cell* **42**, 841 (1985).
7. N. Hagag, S. Halegoua, M. Viola, *Nature* **319**, 680 (1986).
8. T. Satoh, S. Nakamura, Y. Kaziro, *Mol. Cell. Biol.* **7**, 4553 (1987).
9. D. Kaplan, unpublished data.
10. K. Muroya, S. Hatponi, S. Nakamua, *Oncogene* **7**, 277 (1992).
11. B.-Q. Li, H.-f. Kung, T. Kamata, unpublished data.
12. B. L. Hempstead, D. Martin-Zanca, D. R. Kaplan, L. F. Parada, M. V. Chao, *Nature* **350**, 678 (1991); R. Klein, S. Jing, V. Nanduri, E. O'Rourke, M. Barbacid, *Cell* **65**, 189 (1991); D. R. Kaplan, B. L. Hempstead, D. Martin-Zanca, M. V. Chao, L. F. Parada, *Science* **252**, 554 (1991); D. M. Loeb *et al.*, *Cell* **66**, 961 (1991).
13. B. L. Hempstead, S. Rabin, L. Kaplan, S. Reid, L. Parada, D. R. Kaplan, in preparation.
14. T. Kamata and J. R. Feramisco, *Nature* **310**, 147

(1984); T. Satoh, M. Endo, M. Nakafuku, S. Nakamura, Y. Kaziro, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5993 (1990); T. Satoh *et al.*, *ibid.*, p. 7926.
15. J. B. Gibbs, M. S. Marshall, E. M. Scolnick, R. A. F. Dixon, U. S. Vogel, *J. Biol. Chem.* **265**, 20437 (1990).
16. J. Downward, J. D. Graves, P. H. Warne, S. Rayter, D. A. Cantrell, *Nature* **346**, 719 (1990); T. Satoh, M. Nakafuku, A. Miyajima, Y. Kaziro, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3314 (1991).
17. Consistent with other reports (*3*), nucleotide exchange activity was recovered more in the cytosol fraction than in the membrane fraction when phosphatase inhibitors of serine-threonine phosphatases or tyrosine phosphatases were added. The activities in both fractions were indistinguishable from each other in the elution profile on either the anion-exchange or gel filtration column. These activities may represent an identical exchange factor (M. J. Subuleski, H.-f. Kung, T. Kamata, unpublished data).
18. PC-12 or Trk–PC-12 cells were incubated without serum for 48 hours and treated with or without NGF (50 ng/ml) for 10 min. The cells were sonicated in 0.5 ml of Buffer A [20 mM 3-(*N*-morpholino)propanesulfonic acid) (pH 7.5), 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 200 mM sucrose, 10 μM phenylmethylsulfonyl fluoride (PMSF), leupeptin (10 μg/ml), and pepstatin (1.7 μg/ml)] that contained phosphatase inhibitors as described (*3*). The homogenates were centrifuged at 100,000*g* for 20 min at 4°C, and the supernatants were used. Normal H-Ras expressed in *Escherichia coli* was purified (*26*), and Ras (0.25 μg) was incubated with 2 μM [^{3}H]GDP (10 to 15 Ci/mmol; Amersham) in 50 μl of the reaction buffer [25 mM tris-HCl (pH 7.5), 5 mM EDTA, 1 mM DTT, and bovine serum albumin (100 μg/ml)] for 30 min at 37°C. Cell extracts (3 μg) were added to the Ras-[^{3}H]GDP complex in the presence of 2 mM unlabeled GTP and 18 mM $MgCl_2$ at 37°C for 10 min. Ras was immunoprecipitated with the Y13-259 antibody, and the immune complexes were washed three times with washing buffer. Nucleotides were eluted, and the radioactivity was quantitated as described (Fig. 1). Activity is given as a percentage of bound [^{3}H]GDP dissociated. [^{3}H]GDP (15,000 cpm) was bound to Ras incubated with the control homogenization buffer. Data represent means ± SD (n = 3).
19. T. Kamata, B.-Q. Li, H.-f. Kung, D. Kaplan, unpublished data.
20. D. Kaplan and S. Rabin, unpublished data.
21. We fractionated PC-12 cell homogenates by spinning them at 100,000*g*. By immunoblotting using the antibody to a peptide derived from NF1 (NF1 GRD, serum 31) (provided by D. R. Lowy), we detected NF1 protein in the particulate (P100) fraction but not in the cytoplasmic (S100) fraction, as reported (*27*). Because we used the cytoplasmic fraction (S100) for the GAP assay (Fig. 4), this indicates that our cytoplasmic extract preparations do not contain NF1. The extracts contained Ras-GAP as detected by immunoblotting. In another set of experiments, the particulate fractions (P100) from PC-12 cells were solubilized in lysis buffer and immunoprecipitated by the NF1 antibody. Although the immunoprecipitated NF1 exhibited the activity to stimulate Ras GTPase as reported (*27*), treatment of PC-12 cells by NGF did not enhance the GTPase-stimulating activity of NF1.
22. C. Ellis, M. Moran, F. McCormick, T. Pawson, *Nature* **343**, 377 (1990).
23. D. R. Kaplan, D. K. Morrison, G. Wong, F. McCormick, L. T. Williams, *Cell* **61**, 125 (1990); C. J. Molloy *et al.*, *Nature* **342**, 711 (1989).
24. D. R. Kaplan, D. Martin-Zanca, L. F. Parada, *Nature* **350**, 158 (1991).
25. T. G. Boulton *et al.*, *Cell* **65**, 663 (1991).
26. T. Kamata, S. Kathuria, Y. Fujita-Yamaguchi, *Biochem. Biophys. Res. Commun.* **144**, 19 (1987).
27. J. E. DeClue, B. D. Cohen, D. R. Lowy, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9914 (1991).
28. We starved the cells of serum in medium that contained 0.2% horse serum and 0.1% fetal bovine serum to minimize potential effects of the various serum components on NGF action, which may have complicated the interpretation of the results.
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Cytochrome b_{558}: The Flavin-Binding Component of the Phagocyte NADPH Oxidase

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The phagocyte respiratory burst oxidase is a flavin-adenine dinucleotide (FAD)–dependent dehydrogenase and an electron transferase that reduces molecular oxygen to superoxide anion, a precursor of microbicidal oxidants. Several proteins required for assembly of the oxidase have been characterized, but the identity of its flavin-binding component has been unclear. Oxidase activity was reconstituted in vitro with only the purified oxidase proteins $p47^{phox}$, $p67^{phox}$, Rac-related guanine nucleotide (GTP)–binding proteins, and membrane-bound cytochrome b_{558}. The reconstituted oxidase required added FAD, and FAD binding was localized to cytochrome b_{558}. Alignment of the amino acid sequence of the β subunit of cytochrome b_{558} ($gp91^{phox}$) with other flavoproteins revealed similarities to the nicotinamide adenine dinucleotide phosphate (reduced) (NADPH)–binding domains. Thus flavocytochrome b_{558} is the only obligate electron transporting component of the NADPH oxidase.

Phagocytic white blood cells contain a multicomponent electron transfer chain that is responsible for production of microbicidal oxidants. To identify its proximal flavin and NADPH-binding component we explored the superoxide generating capacity of a cell-free system comprised solely of purified proteins. In this cell-free system, complete reconstitution of the oxidase required the presence of the cytosolic factors $p47^{phox}$, $p67^{phox}$, and Rac-related GTP-binding proteins (*1–3*), and the membrane component cytochrome b_{558} (*4–7*) (Fig. 1). In the optimally reconstituted purified protein system (*8*) the concentration of NADPH required for half maximal activity (30 to 40 μM, n = 2) was identical to that observed with the enzyme reconstituted from crude cytosol and membranes (30 to 40 μM, n = 3). This purified protein system produced superoxide only in the presence of added FAD (Fig. 2), the concentration of FAD required for half-maximal superoxide production was from 1 to 20 nM (n = 4). Neither $p47^{phox}$ nor $p67^{phox}$ bind flavin (*2*) and, on the basis of structural data from the Ras superfamily of low molecular weight GTP-binding proteins, binding of flavin to members of the Rac/G25K family also seemed unlikely. Accordingly, we focused our attention on cytochrome b_{558}.

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Relipidated cytochrome b_{558} was incubated with FAD and subjected to gel filtration to separate bound from free flavin (Fig. 3A). A small peak of flavin fluorescence coeluted with the purified cytochrome b_{558}, which was well resolved from the major peak of free flavin. The early eluting peak of flavin was not detected when mixtures of the purified cytosolic components plus FAD or phospholipid vesicles, alone, plus FAD were subjected to gel filtration.

A functional comparison of flavin-reconstituted or unflavinated cytochrome b_{558} was made in the cell-free assay with the purified cytosolic components in the pres-

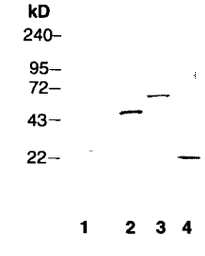

Fig. 1. Protein components of the NADPH oxidase [SDS–polyacrylamide gradient gel (8 to 16%) stained with Coomassie Blue R-250]. Lane 1, Superdex-75 peak fraction (4 μl) showing the 26-kD and 21-kD bands identified by microsequencing as rhoGDI and Rac-related GTP-binding proteins, respectively (*3*); lane 2, recombinant $p47^{phox}$ (1.5 μg); lane 3, recombinant $p67^{phox}$ (1.5 μg); and lane 4, cytochrome b_{558} (2.7 μg; 74 pmol). The specific content of peak cytochrome b_{558}–containing fractions (18 to 33 nmol of heme per milligram of protein) and the molar ratio of FAD to heme (<1:100) in three preparations of purified cytochrome b_{558} were comparable to reported values (*4–6*).