cells renders the TCRα$^{-/-}$ mouse an invaluable animal model for the study of γδ cell function. In particular, these mice can potentially resolve whether γδ cells provide, as hypothesized, a response to a broad range of epithelial insults (*10*) and whether they respond, as suggested, to specific pathogens such as mycobacteria (*28*), or both.

The development of B cells in the absence of αβ$^+$ T cells is consistent with the independent development of the humoral and cell-mediated immune systems (*29*). However, the de facto influence of T cells on B cell development is poorly understood. Our data indicate that TCRα$^{-/-}$ mice will be useful in resolving this; unlike in nude mice, in which spleens and Peyer's patches are approximately normal in size, the complete elimination of αβ$^+$ T cells has dichotomous effects on B cell development in the spleen and Peyer's patches. Whether this dichotomy is a result of anatomy or whether it is a result of direct effects of αβ$^+$ T cells on B cell development (for example, negative regulation) that are different in the two organs can now be directly tested.

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NF-κB Subunit Regulation in Nontransformed CD4$^+$ T Lymphocytes

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Regulation of interleukin-2 (IL-2) gene expression by the p50 and p65 subunits of the DNA binding protein NF-κB was studied in nontransformed CD4$^+$ T lymphocyte clones. A homodimeric complex of the NF-κB p50 subunit was found in resting T cells. The amount of p50-p50 complex decreased after full antigenic stimulation, whereas the amount of the NF-κB p50-p65 heterodimer was increased. Increased expression of the IL-2 gene and activity of the IL-2 κB DNA binding site correlated with a decrease in the p50-p50 complex. Overexpression of p50 repressed IL-2 promoter expression. The switch from p50-p50 to p50-p65 complexes depended on a protein that caused sequestration of the p50-p50 complex in the nucleus.

Tumor cell lines have proven vital to the study of gene regulation because they are easily grown and manipulated. However, nontransformed T cells exhibit biological characteristics that are not manifested by T cell tumor cell lines, including proliferative responses to antigen, costimulatory requirements, clonal anergy, and propriocidal regulation (*1–5*). Therefore we used major histocompatibility complex (MHC)–restricted T lymphocyte clones that faithfully recapitulate the in vivo cellular response to peptide antigen (*1–5*). These clones are neither transformed nor immortalized and survive in culture by stimulation with antigen and antigen-presenting cells (APCs). T cell clones have not been widely used for gene regulation studies because they appeared to be refractory to DNA transfection and because natural antigen stimulation requires a two-cell interaction with antigen-presenting cells. We devised methods

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to overcome these difficulties and studied the IL-2 gene because of its important function in T cell activation (*2, 4*).

In tumor cell models, a 300–base pair (bp) enhancer of the IL-2 gene responds to T cell activation signals (*6, 7*). The DNA binding protein NF-κB participates in regulation of the IL-2 enhancer (*7–11*). The protein subunits that constitute the NF-κB binding activity are members of the Rel family of proteins, which includes p50 and p65, among others (*12*). The p50-p65 heterodimer (NF-κB) and p65 homodimers stimulate transcription, whereas p50 dimers may activate or suppress transcription (*12, 13*).

The $CD4^+$ T_H1 T cell clone A.E7 responds to a pigeon cytochrome c peptide complexed with the E^k molecule and requires costimulatory signals from APCs to express IL-2 (*5, 15*). We used an antigen stimulation–cell-separation procedure (*15*) to avoid contamination of T cell mRNA and nuclear extracts with material from APCs, allowing us to isolate highly purified (>99%) preparations of antigen-stimulated T lymphocytes. Electrophoretic mobility-shift assays (EMSAs) (*17*) of resting and stimulated A.E7 nuclear extracts revealed induction of NF-κB binding to the IL-2κB site after antigenic stimulation (Fig. 1A). NF-κB bound less tightly to the IL-2κB site than to the immunoglobulin κB (IgκB) site (Fig. 1A). We also detected a faster migrating complex, which we called NF-κC, that was diminished by antigen stimulation and that bound more tightly to the IL-2κB site than to the IgκB site. We found that treatment with APC only, antigen and APC, or either treatment with cyclosporin A (CsA) all induce NF-κB. By contrast, NF-κC was only decreased after antigen stimulation, and this decrease was blocked by CsA. Pharmacologic stimuli also affected NF-κB and NF-κC differently. NF-κB was induced by ionomycin, ionomycin and phorbol 12-myristate 13-acetate (PMA) together, or the protein synthesis inhibitor anisomycin, but not by PMA alone (Fig. 1A). NF-κC was modestly attenuated only by ionomycin and PMA together and, no-

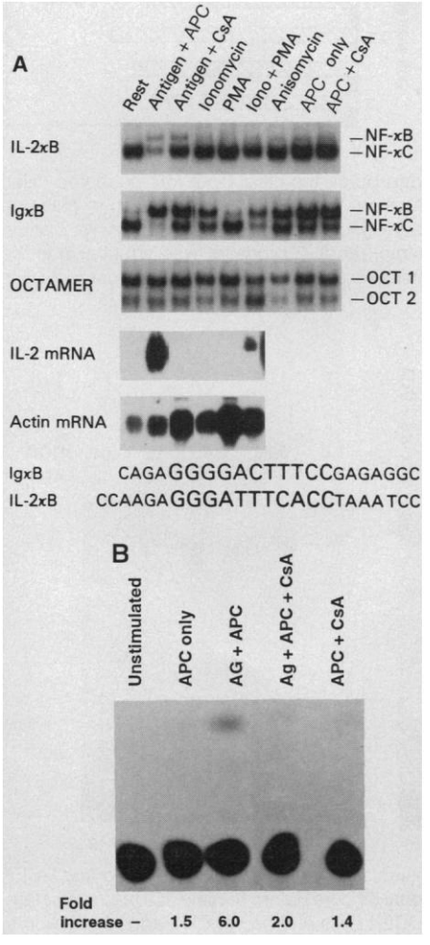

Fig. 1. The κB binding complexes and IL-2 mRNA in A.E7 cells. (**A**) The upper three panels show EMSAs with 2 μg of nuclear extracts from A.E7 cells treated with stimuli as shown above each column of bands and described in (*16, 23*). The oligonucleotides used as probes are shown on the left. The IgκB oligonucleotide was 5′-CAGA**GGGGACTTTCC**GAGAGGC-3′ and the IL-2κB oligonucleotide was 5′-CCAA-GA**GGGATTTCACC**TAAATCC-3′, with the binding sites shown in bold. Only the shifted bands are shown and are identified to the right of each panel; no other specific bands were observed with these probes. Competition analysis revealed that the slight difference in sequence in the sites caused NF-κB to bind approximately fivefold more tightly to the IgκB site than to the IL-2κB site and NF-κC to bind approximately fivefold more tightly to the IL-2κB site than to the IgκB site (*18*). "Rest" indicates T cells that were not stimulated. The lower two panels show a Northern blot, with 10 μg of total RNA prepared from A.E7 cells stimulated as indicated, that was sequentially hybridized with the mouse IL-2 or β-actin gene probes. DCEK cells (APC) induce NF-κB in A.E7 cells by releasing small amounts of tumor necrosis factor α (*19*). Control experiments show that NF-κC reduction was not caused by stimulating A.E7 cells with IL-2. (**B**) Transcriptional activity of IL-2κB–dependent reporter constructs in A.E7 cells (*16, 24*). Stimulation conditions were as follows: unstimulated (T cells alone); magnetic bead-loaded DCEK cells only (APC only); 81 to 104 peptide antigen (1 μM) and magnetic bead-loaded DCEK cells (Ag + APC); 81 to 104 peptide antigen (1 μM), magnetic bead-loaded DCEK cells, and 200 μM CsA; and magnetic bead-loaded DCEK cells and 200 μM CsA. T cells were magnetically separated from APC where required, and samples normalized for protein concentration were analyzed for CAT activity. "Fold increase" indicates the ratio of the percent conversion (chloramphenicol to its acetylated form) of a stimulated sample to the percent conversion of the unstimulated sample. Data are representative of four assays of two independent pools of stable transfectants quantitated by a phosphorimager.

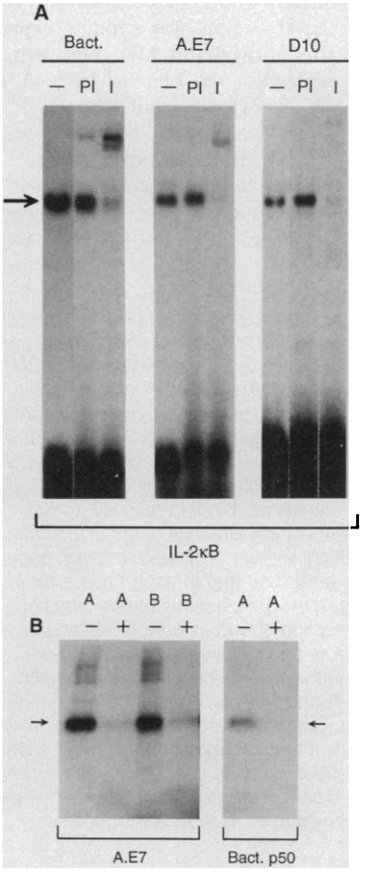

Fig. 2. The NF-κC complex is a p50 homodimer. (**A**) Alteration of the NF-κC DNA complex by anti-p50. The extracts added to each binding assay were as follows: affinity-purified, bacterially expressed p50 (100 ng) (Bact.); resting A.E7 nuclear extract (2 μg) (A.E7); and resting nuclear extract (2 μg) from D10.G4.1, a nontransformed $CD4^+$ T_H2 T lymphocyte clone (D10) (*25*). Nuclear extracts were either untreated (–), mixed with either preimmune rabbit serum (1 μl) (PI), or immune serum (1 μl) (I) for 10 min at ambient temperature, then added to a DNA binding assay that contained the IL-2κB probe. (**B**) Ultraviolet cross-linking of the NF-κC complex from resting A.E7 cells and bacterial p50. Probes were designed such that only half of the IL-2κB sequence was substituted with bromodeoxyuridine (BrdU) and [α-^{32}P]deoxycytosine triphosphate (dCTP) as described (*23, 26*). The A probe contains the 5′ half-site substituted with and the B probe contains the 3′ half-site substituted. Binding reactions contained either resting A.E7 nuclear extracts (A.E7) or affinity-purified, bacterially expressed NF-κB p50 subunit (Bact. p50), and lanes marked + were from binding assays in which an ~50-fold molar excess of unlabeled IL-2κB oligonucleotide was added. The arrow indicates the 50-kD species.

tably, was not increased by any of these treatments. Binding to a probe containing the octamer sequence showed that the extracts were of comparable quality for each experiment.

Northern (RNA) blot analysis revealed that the IL-2 gene was strongly expressed only after antigen and APC treatment, and this expression was blocked by CsA (Fig. 1A) (*5, 7, 21*). A small amount of IL-2 mRNA was seen with PMA and ionomycin, but not with either agent alone. Thus, IL-2 mRNA induction correlated with removal of NF-κC. These results suggested that NF-κC could inhibit IL-2 gene expression through its binding to the IL-2κB site. If NF-κC acted as a repressor, it could block transactivation of the IL-2κB site by NF-κB until removed by antigen stimulation.

We next prepared pools of A.E7 cells stably transfected with multimers of NF-κB binding sites from the IL-2 gene directing the activity of a bacterial chloramphenicol acetyltransferase (CAT) gene (Fig. 1B) (*23*). The IL-2κB binding motif responded well only to antigen and APCs and was relatively insensitive to APCs alone. Activation of the IL-2κB motif was almost completely blocked by CsA. These effects are likely to strongly influence IL-2 gene expression because mutations in the IL-2κB site eliminate ~80% of the activity of the IL-2 promoter (*7, 18*). Stable transfectants of A.E7 cells with constructs containing the IgκB site were activated approximately equally by APC alone, Ag + APC, or either treatment with CsA (*18*). Thus, expression of the IL-2κB motif was specifically antigen-restricted and CsA-sensitive in nontransformed T cells, which correlated well with regulation of the NF-κC complex.

To identify the molecular nature of the NF-κC, we prepared antisera to the bacterially expressed murine p50 subunit of NF-κB (anti-p50) and tested for recognition by EMSA (Fig. 2A). Anti-p50 and not preimmune sera shifted the complex of bacterial p50 with the IL-2κB site. The NF-κC complex in the nontransformed T cells clones A.E7 and D10.G4.1 (*22*) comigrated with that of bacterial p50 and was also efficiently shifted by anti-p50. Ultraviolet (UV) cross-linking studies verified that the NF-κC complex in A.E7 cells consisted of two 50-kD subunits each binding to one half-site of the IL-2κB site (Fig. 2B). These findings, together with the fact that NF-κC had the binding characteristics of purified p50 previously described (*24*), suggest that NF-κC is a homodimer of the NF-κB p50 subunit.

To directly test whether the NF-κB p50 subunit could inhibit IL-2 promoter activity in T cells, we transfected a plasmid expressing the protein-coding sequence of p50 with a 300-bp IL-2 promoter–CAT gene into EL-4 T lymphoma cells (Fig. 3). Stimulation of the transfected cells with PMA and concanavalin A induced IL-2 promoter activity. Increasing amounts of the p50 expression plasmid decreased the response of the IL-2 promoter in a dose-dependent fashion. A plasmid expressing a mutated p50 protein that was incapable of binding to DNA had no effect (Fig. 3).

Using the EMSA, we next tested whether protein synthesis was required for the decrease in p50 binding to the IL-2κB site (Fig. 4). The protein synthesis inhibitor cycloheximide blocked the decrease in p50 after antigen stimulation. By contrast, NF-κB binding was augmented rather than blocked by cycloheximide (*25*). Cycloheximide in the absence of antigen stimulation did not affect p50 binding. Even when antigen was removed after a 3-hour incubation, the p50 complex continued to decrease for at least up to 9 hours. The decrease in the p50 complex depended on continued protein synthesis, because, with addition of cycloheximide after 3 hours, the p50 binding activity after 9 hours was equivalent to the amount of decrease at 3 hours.

The requirement for ongoing protein synthesis suggested that a newly synthesized protein or proteins caused the decrease in p50 binding activity. This protein could

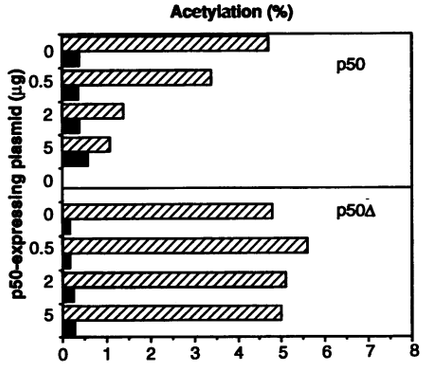

Fig. 3. Repression of IL-2 promoter activity by expression of p50. A 0.3-kb mouse IL-2 promoter-CAT construct (5 μg) was transfected into EL-4 cells with increasing amounts of a construct with the adenovirus major late promoter during expression of either wild-type p50 or a mutant form of p50 that contained a single amino acid deletion in the coding sequence that eliminates binding activity (p50Δ) (*24*). Corresponding amounts of the expression vector DNA without any p50 coding sequence were added so that in each case a total of 10 μg of DNA was used in each transfection. After transfection, the EL-4 cells were incubated for 40 hours and split into two pools. One pool was stimulated overnight with concanavalin A (2 μg/ml) and PMA (20 ng/ml) before harvesting (shaded bars); the other pool was untreated (filled bars). Samples were normalized by protein concentration. We measured conversion of chloramphenicol to its acetylated form using a phosphorimager (Molecular Dynamics, Sunnyvale, California). No effect was seen on an NFAT reporter construct, an IL-2 promoter with a mutation in the IL-2κB site, or the expression plasmid alone (*18*). Similar results were seen in four experiments with <15% variation in replicate samples.

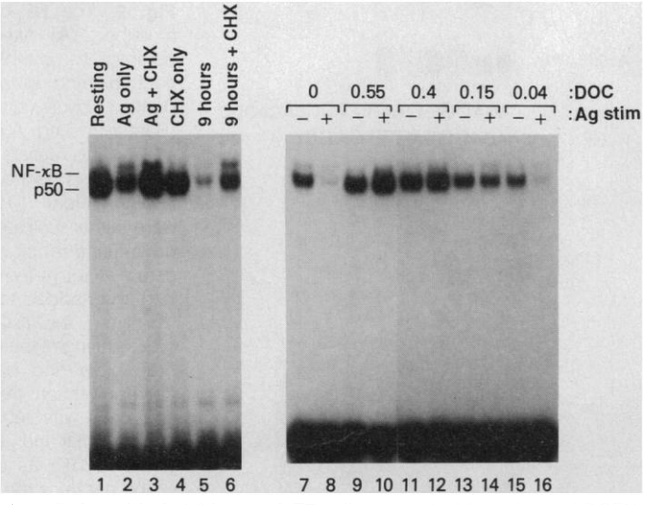

Fig. 4. Nuclear inhibition of p50 binding. The effect of cycloheximide on the antigen-dependent decrease in the NF-κC–DNA complex in A.E7 nuclear extracts is shown in lanes 1 to 6. "Ag" denotes 10 μM 81 to 104 peptide antigen plus DCEK cells, and "CHX" denotes 125 μM cycloheximide (*16, 23*). Lane 1, no stimulation, harvested at 3 hours; lane 2, Ag stimulation, harvested at 3 hours; lane 3, Ag with CHX present at 0 hours, harvested at 3 hours; lane 4, CHX present at 0 hours, harvested at 3 hours; lane 5, Ag added at 0 hours, A.E7 cells separated from Ag and DCEK cells at 3 hours, and allowed to incubate for an additional 6 hours before harvest at 9 hours; and lane 6, Ag added at 0 hours, A.E7 cells separated from DCEK cells at 3 hours, CHX added at 3 hours, then allowed to incubate for an additional 6 hours before harvest at 9 hours. All extracts gave comparable binding to an Ig octamer probe. The effect of DOC on NF-κC binding activity is shown in lanes 7 to 16. Nuclear extracts were from unstimulated (−; lanes 7, 9, 11, 13, 15) and stimulated with 10 μM 81 to 104 peptide antigen plus DCEK cells for 3 hours (+; lanes 8, 10, 12, 14, 16) (*23*). Control experiments showed that the reactivated p50 complex could be supershifted with anti-p50 serum and was competed with the IL-2κB oligonucleotide (*26*).

simply degrade p50 or could bind to p50 and inhibit its ability to bind DNA. The cytoplasmic protein I-κB interacts with NF-κB and inhibits its DNA binding activity. Dissociating agents, such as the detergent deoxycholate (DOC), release NF-κB from IκB in cytosolic extracts and allow NF-κB to bind DNA (*26*). By contrast, treatment of cytosol from either resting or stimulated A.E7 cells with DOC yielded no p50 DNA binding activity (*18*). However, when nuclear extracts from stimulated A.E7 cells were treated with DOC, we detected p50 DNA binding activity that was equivalent to that in resting A.E7 extracts (Fig. 4). The release of p50 required more than 0.04% DOC and was maximal at 0.4%. The recovered binding activity was indistinguishable from p50 on the basis of EMSAs in the presence of competitor oligonucleotides and anti-p50 (*18*). In addition, the inhibitory activity could be separated from p50 by DNA affinity chromatography and was capable of inhibiting p50 binding in an untreated resting A.E7 extract (*27*). Thus, a newly synthesized protein causes p50 to be in a non–DNA binding form in the nucleus of T cells after antigen stimulation.

In T lymphoma cells, expression from the IL-2κB site is activated by inducers of NF-κB, such as lectins or phorbol ester alone, and is resistant to CsA (*7, 21*). In A.E7 cells, we find the IL-2κB site requires full antigen stimulation (antigen + APCs) and is sensitive to CsA. The regulation of the IL-2κB site may begin to explain the observation that IL-2 can be produced after partially inducing signals such as PMA in EL4 cells, or lectins in the case of Jurkat cells, without costimulatory signals (*7, 21*). Such stimuli are never sufficient for IL-2 gene expression or lymphokine production in nontransformed T cells (*5, 16*). Indeed, stimulation of nontransformed T cells by partial signals such as lectins induces a functionally nonresponsive state (anergy) (*5*). Also, complexes in T cell tumor lines such as KBF-1 and TCF resemble the p50 dimers in A.E7 cells, but do not display the same regulatory features, emphasizing the importance of transcriptional studies in nontransformed T lymphocytes (*8, 11, 18*).

Our findings reveal a physiologic function for p50 in the T cell response to antigen in that p50 homodimers appear to inhibit activation of gene expression by NF-κB. The p50-p50 complex is also found in lymph node T cells and thymocytes, which implies a regulatory role in vivo (*18*). One of the paradoxes of NF-κB as a widely used signaling mechanism is that, because it can be induced by so many agents, it lacks the specificity apparently needed to activate genes in response to unique stimuli (*11, 12*). The p50 (NF-κC) control pathway offers one solution to this problem. Many agents increased NF-κB in A.E7 cells, but activities of the IL-2κB site and the IL-2 gene appeared to be blocked unless the p50 complex decreased. Thus, the IL-2κB site may only be active when the appropriate combination of stimuli leads to synthesis of a p50 inhibitory molecule. Inhibition of the p50 complex is a regulatory paradigm that differs from the cytoplasmic regulation of NF-κB by IκB because the non–DNA binding form of the p50 complex appears to be retained entirely in the nucleus.

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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*).
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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-