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18 October 1991; accepted 25 November 1991

# Identification of a Zinc Finger Protein That Inhibits IL-2 Gene Expression

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**Transient activation of the *interleukin-2* (*IL-2*) gene after antigen recognition by T lymphocytes is crucial for subsequent T cell proliferation and differentiation. Several *IL-2* gene regulatory elements and binding factors necessary for activation of the IL-2 gene have been defined. However, little is known about negative regulation of IL-2 expression, which is likely to be important in the rapid shut-off of IL-2 transcription. A nucleotide sequence element (NRE-A) that negatively regulates IL-2 expression has been identified within the IL-2 gene. T cell nuclear extracts contained an NRE-A binding activity. A complementary DNA was isolated that encodes a zinc finger–containing protein that suppressed IL-2 gene expression. The observation of negative regulation of the immunoglobulin heavy chain gene enhancer by an element similar to NRE-A suggests that related proteins may regulate multiple immune response genes.**

TRANSCRIPTIONAL ACTIVATION OF the *IL-2* gene is critical for the immune response that follows stimulation of T lymphocytes via the T cell antigen receptor or the CD2, CD28, and interleukin-1 cell surface receptors (*1*). While several DNA sequence elements and binding proteins that participate in transcriptional activation of *IL-2* have been identified, little is known about how this gene is negatively regulated (*2*). Repression of *IL-2* transcription is likely to be important for explaining its transient, inducible, and tissue-specific expression.

Regulation of *IL-2* expression may be studied with the use of the human Jurkat T cell line transfected with hybrid plasmid constructs in which the *IL-2* promoter-enhancer is linked to the bacterial chloramphenicol acetyl transferase (CAT) (*3*) or firefly luciferase (Luc) (*4*) reporter genes. In transient expression assays, IL-2–reporter gene constructs [pIL2(−548)CAT and pIL2(−548) Luc] that contain IL-2 gene sequences extending 548 bp 5′ of the transcription start site display behaviors characteristic of the endogenous IL-2 gene. That is, they are inducible by T cell activation signals only in T lymphoid cells, are silent in unstimulated cells, are inhibitable by cyclosporin A (Cs-A), and employ the proper transcription start site (*3–7*).

The proximal IL-2 promoter extending 130 nucleotides 5′ of the TATA box is essential for induced IL-2 gene expression (*3, 5–7*). This region contains an inducible DNAse I hypersensitive site (*3*) near or at an Oct-1 binding site (*8*) and a binding site for Fos-Jun family heterodimers (*9*). We focused on a sequence (−110 to −101) between the Oct-1 and Fos-Jun binding sites. Using pIL2(−548)CAT and pIL2(−548)-Luc, we generated internal deletion and site-specific mutations within this region. One mutation, pIL2(−106/−101), deleted four nucleotides (residues −105 to −102), replacing them with an 8-bp Xba I linker. A second construct, pIL2(−110/−101), has eight IL-2 nucleotides deleted (residues −109 to −102) and replaced with an 8-bp linker. We transfected these constructs into Jurkat T cells to assess their transient expression in response to T lymphocyte activation signals (Fig. 1A). Mutations of the −110 to −101 region yielded three- to sevenfold increases in Luc and CAT activity in phytohemaglutinin (PHA-P)– and 12-O-tetradecanoylphorbol-13-acetate (TPA)–activated Jurkat cells, suggesting the presence of a negative regulatory element (NRE-A). The enhanced activity seen with alteration of the NRE-A region is not likely

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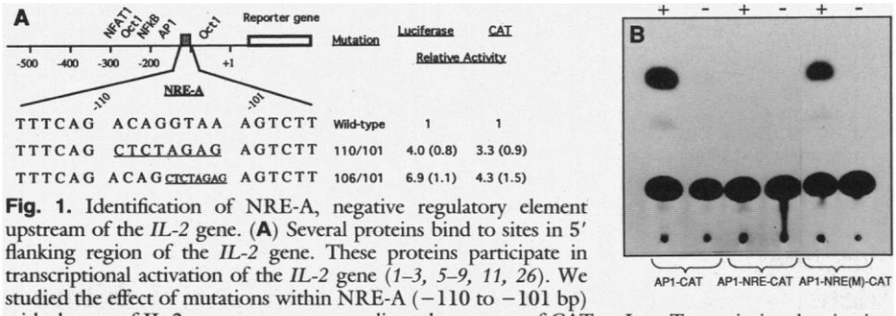


**Fig. 1.** Identification of NRE-A, negative regulatory element upstream of the *IL-2* gene. (**A**) Several proteins bind to sites in 5′ flanking region of the *IL-2* gene. These proteins participate in transcriptional activation of the *IL-2* gene (*1–3, 5–9, 11, 26*). We studied the effect of mutations within NRE-A (−110 to −101 bp) with the use of IL-2–promoter constructs ligated upstream of CAT or Luc. Transcriptional activation of constructs with NRE-A mutations was compared to that of the full-length IL2(−548)Luc and IL2(−548)CAT constructs (assigned as 1) in transfected Jurkat J32 subclone T cells. Averages are from three experiments with standard deviations in parentheses. Plasmids were prepared, cells were stimulated with PHA-P and TPA, and reporter gene assays were performed as described (*4, 7, 27*). Cells were transfected by electroporation (280 V, 960 μF). To control for transfection efficiency variability, activity of IL-2–Luc constructs was normalized with the use of cotransfected pRSV-CAT (*27*). IL-2–CAT construct activity was not similarly normalized; however, Jurkat cells take up similar amounts of pIL2-CAT constructs with a wide range of deletions (*7*). (**B**) Inhibition of AP-1 transcriptional activation by NRE-A. Oligonucleotides that contained two copies of the NRE-A site were ligated into the Bam HI site of AP-1–CAT between the AP-1 binding site and the TK promoter to form AP1-NRE-CAT. A second construct prepared in a similar fashion [AP1-NRE(M)-CAT] was identical to AP1-NRE-CAT except for a T to C point mutation at nucleotide −104 (*28*). AP1-CAT, AP1-NRE-CAT, and AP1-NRE(M)-CAT were transfected into resting (−) or TPA stimulated (+) Jurkat cells and their CAT activity compared after 24 hours. Cells were cotransfected with pRSV-Luc to control for transfection efficiency. A representative of three experiments is shown.

CAT plasmid that contained a binding site for Fos-Jun heterodimers (the TPA-responsive element, TRE) upstream of the thymidine kinase (TK) promoter linked to CAT (*12*). The interposition of the NRE-A domain between the TRE and TK promoter (AP-1–NREA–CAT) virtually abolished TPA-induced activity (Fig. 1B). However, a construct with a point mutation in the NRE-A site retained TPA-induced activity (Fig. 1B).

We next determined whether T cells contained nuclear proteins that bound to the NRE-A domain. Electrophoretic mobility shift assays with an NRE-A oligonucleotide probe indicated that a nuclear activity existed in both resting and PHA + TPA–stimulated Jurkat cells that interacted with the NRE-A region (Fig. 2). While excess unlabeled NRE-A oligonucleotide inhibited binding, an oligonucleotide that contained the Oct-1 binding region could not. The binding activity is not affected by treatment of Jurkat cells with Cs-A and is not present in HeLa cells (*13*). A second complex of slower mobility was also present. Its behavior in competition assays (Fig. 2, lanes 2 and 3) suggested that it is a nonspecific complex.

We used a cDNA expression library prepared from Jurkat cells to identify genes that encoded proteins with NRE-A binding activity. Clones expressing beta-galactosidase fusion proteins were screened for the ability to recognize a multimerized NRE-A domain probe (*14*). A 1207-bp cDNA (lambda-DM8) that encoded a protein that bound the multimerized NRE-A domain but not a control oligonucleotide was isolated (*15*). Rescreening the library by DNA hybridization to a synthetic oligonucleotide from the 5′ end of lambda-DM8 yielded an overlapping 1727-bp cDNA (lambda-8321). Together, lambda-DM and lambda-8321 constitute a 2530-bp cDNA (Nil-2-a, negative regulator of IL-2) that encodes a 735–

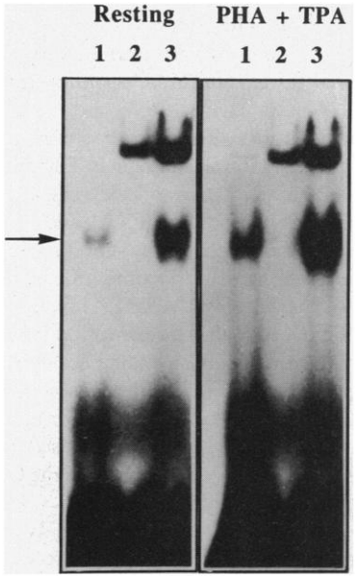


**Fig. 2.** NRE-A domain binding activity from T cell nuclear extracts. In electrophoretic mobility shift assays, a $^{32}$P–end-labeled 24-bp DNA probe that included the −112 to −99 NRE-A domain (Oligo A) (*29*) was incubated with nuclear extracts (*30*) prepared from Jurkat T cells that were either resting or stimulated with PHA + TPA for 5 hours (lane 1 of each panel) (*7*). In oligonucleotide competition experiments, a 100-fold excess of unlabeled Oligo A (lane 2 of each panel) or the 24-bp control Oligo B (*29*) (containing the −81 to −68 region of the IL-2 promoter) (lane 3 of each panel) was added. The arrow denotes the specifically bound complex. DNA binding reactions (*31*) were incubated for 30 min at 23°C and contained labeled probe (1 ng), nuclear extract (2 μg), poly(dI-dC) (3 μg), glycerol (12%), Hepes (12 mM, pH 7.9), and KCl (60 mM). Complexes were separated on polyacrylamide gels in TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0).

to be a result of spacing effects, as pIL2(−110/−101) is a site-specific mutation.

PHA-P and TPA induce IL-2 expression through activation of the T cell antigen receptor and protein kinase C. Mutations in NRE-A also led to increases in IL-2 promoter activity in Jurkat cells stimulated through the CD2 and CD28 pathways (*10*), and in the mouse T cell lymphoma LBRM-331A5 cell line activated with PHA and IL-1–alpha (*11*). Like the full-length construct, pIL2(−110/−101) and pIL2(−106/−101) are inactive in unstimulated Jurkat cells and in Raji B lymphocytes and remain fully inhibitable by Cs-A (*10*).

In order to determine whether the NRE-A domain could suppress the activity of a heterologous construct, we introduced a tandem repeat of the NRE-A sequence into an AP-1–

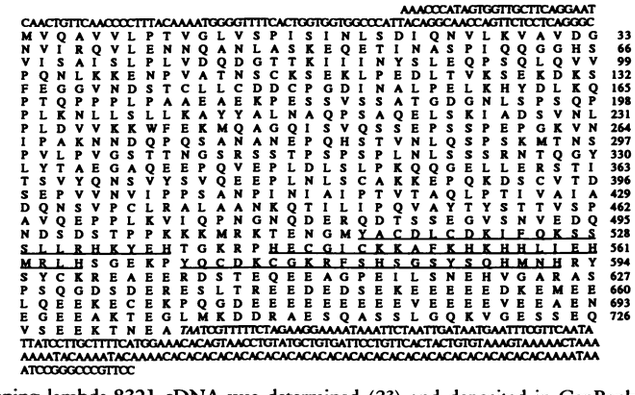


**Fig. 3.** Derived amino acid sequence of Nil-2-a. The zinc fingers are underlined. The lambda gt11 library was constructed with the use of oligo dT and random priming of oligo dT column-purified mRNA isolated from resting Jurkat cells (*32*). We carried out primary filter hybridization screening of 500,000 plaques with an end-labeled catenated NRE-A probe (*29*) and identified one positive colony (lambda-DM8) on duplicate filters. The nucleotide sequence of lambda-DM8 and the overlapping lambda-8321 cDNA was determined (*23*) and deposited in GenBank (accession number M-81699). The nucleotides outside of the open reading frame are single-spaced. The stop codon (*TAA*) is italicized. Numbers at the right indicate the amino acid position. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

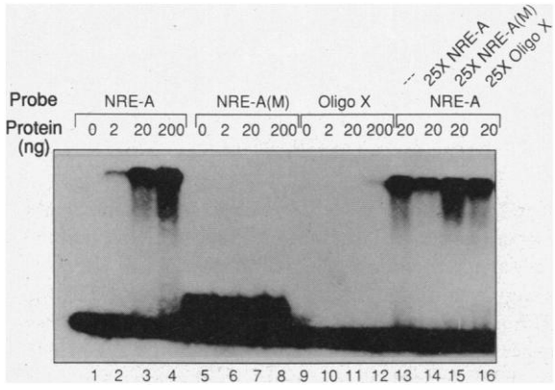


**Fig. 4.** Binding of Nil-2-a peptide to DNA. Electrophoretic mobility shift assays were conducted with a Nil-2-a peptide that contained the zinc finger motifs (*18*). Reactions contained $^{32}$P-labeled oligonucleotides with the NRE-A binding site (1 ng), poly(dI-dC) (1 μg), and the Nil-2 peptide (0, 2, 20, or 200 ng) in a 10-μl reaction buffered with 25 mM Hepes pH 7.9, 50 mM KCl, 10 μM $ZnSO_4$, 10% glycerol, 0.1% NP-40, 0.2 mg/ml bovine serum albumin, and 1 mM DTT. Oligonucleotide probes included the NRE-A domain (Oligo A) (*29*), a point mutation of Oligo A [NRE-A(M), Fig. 1B], or a nonspecific control identical in length to Oligo A [Oligo X, (*15*)]. In competition experiments, a 25-fold excess of the unlabeled Oligo A (lane 14), NRE-A(M) (lane 15), or Oligo X (lane 16) was added to the reactions. Binding reactions and electrophoresis were carried out as described (*33*).

**Table 1.** Repression of IL-2 promoter activity by Nil-2-a. Jurkat T cells were cotransfected with a constant amount (2 μg) of pIL2(−548)Luc or pIL2(−106/−101)-Luc and varying quantities of CMV promoter expression constructs (*24*) encoding either the entire Nil-2-a or a truncated peptide lacking the zinc fingers (Nil-stop) (*25*). Cells were stimulated for 6 hours with PHA-P + TPA. Average light units and relative luciferase activities from three experiments are shown. Standard deviations are in parentheses.

| Nil-2-a | Nil-stop | IL2(−548)Luc | | IL2(−106/−101)Luc | |
|---|---|---|---|---|---|
| DNA (μg) | DNA (μg) | Light units | Relative activity | Light units | Relative activity |
| 0 | 40 | 5,498 | 1 | 19,098 | 1 |
| 10 | 30 | 3,455 | 0.6 (0.1) | 17,734 | 0.9 (0.1) |
| 20 | 20 | 2,017 | 0.4 (0.2) | 21,735 | 1.1 (0.1) |
| 30 | 10 | 1,819 | 0.3 (0.1) | 21,149 | 1.1 (0.2) |
| 40 | 0 | 1,672 | 0.3 (0.1) | 17,402 | 0.9 (0.1) |

amino acid protein with three zinc finger motifs near the COOH-terminal end (Fig. 3). The first two putative fingers are classic $C_2H_2$ motifs, while the third appears to be a variant. Zinc-finger motifs of the $C_2H_2$ class are found in a number of DNA binding transcription factors (for example, see *16*). Nucleotides 103 to 105 of Nil-2-a encode a methionine in a reasonable context for initiation (*17*). Three additional clones obtained by rescreening the library with an Eco RI-Hind III lambda-8321 fragment share identical 5′ ends with lambda-8321. Primer extension analysis demonstrates a major band whose size is consistent with an mRNA start site coincidental with nucleotide 1 of lambda-8321 (*10*).

We next used electrophoretic mobility shift assays to test whether Nil-2-a could specifically recognize NRE-A. *Escherichia coli* were transformed with an expression construct encoding the three Nil-2-a zinc fingers (*18*). The translated Nil-2-a peptide was purified from the bacteria and incubated with oligonucleotide probes. The DNA-protein complexes were separated by electrophoresis (Fig. 4). Nil-2-a bound the NRE-A site in a dose-dependent manner, but failed to recognize an unrelated control oligonucleotide of identical length (*15*) or the NRE-A domain containing a point mutation NRE(M) (see Fig. 1B). An excess of unlabeled NRE-A but not control oligonucleotides inhibited binding of Nil-2-a (Fig. 4).

The transcriptional activity of Nil-2-a was assessed by co-transfecting Nil-2-a expression constructs and IL2(−548)Luc or IL2(−106/−101)Luc into PHA + TPA stimulated Jurkat cells (Table 1). The effect of cotransfection of varying amounts of a construct including the entire Nil-2-a and a construct lacking the zinc fingers (Nil-2-stop) was compared. Overexpression of Nil-2-a inhibited (IL2(−548)Luc activity by up to 70%. This effect required an intact NRE-A binding site since the activity of the deletion mutation IL2(106/101)Luc was not significantly inhibited by Nil-2-a overexpression. These results suggest that binding of Nil-2-a to the NRE-A site inhibits IL-2 gene expression in activated T cells. Whether this effect simply involves displacement of another NRE-A-bound factor by Nil-2-a or requires both the zinc fingers and separate domains with repressing activity is not known. Nil-2-a does contain proline-glutamine and aspartic-glutamic acid rich regions which function as transcriptional control domains in other proteins. The proximity of NRE-A to IL-2 promoter positive control elements which bind Oct-1 and AP-1 suggests that Nil-2-a's effects might be mediated through interference with the activity of these transcription factors.

Comparison of Nil-2-a with a DNA and protein database (*19*) showed that Nil-2-a was not identical to any previously sequenced genes or proteins. The Nil-2-a zinc fingers share 75% amino acid identity with three tandemly linked $C_2$-$H_2$ fingers of the zfh-1 gene, which encodes both nine zinc finger motifs and a homeodomain and may play a role in Drosophila central nervous system development (*20*). Alignment of the two cDNA sequences revealed no similarity between the zfh-1 homeodomain motif and any region of Nil-2-a.

IL-2 mRNA concentrations peak and then decline rapidly after IL-2 gene activation (*1*). This decline is mediated by transcriptional and post-transcriptional mechanisms (*1, 21*). Nil-2-a is likely to be one factor responsible for transcriptional repression of the *IL-2* gene. A negative regulatory element in the immunoglobulin heavy chain enhancer (mu-E5) that is similar to NRE-A has been defined, suggesting that Nil-2-a–related proteins may negatively regulate multiple immune response genes (*22*).

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Hind III site (5′-GGCATAAGCTTGGACGTTCT-TCCGCTTCTCTCTTAC-3′) and target Nil-2-a cDNA (1 μg). The 320-bp PCR product was digested with Bam HI and Hind III and ligated to similarly digested expression vector pDS56-6×-His. The sequence of the resulting construct [pDS56-6×His-Nil-2 (502-604)] was confirmed (*23*). The Nil-2-a peptide was purified as described [E. Hochuli, H. Doebeli, J. Schacher, *J. Chromatogr.* **411**, 177 (1987); R. Gentz, C. H. Chen, C. A. Rosen, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 821 (1989)] from *Escherichia coli* transformed with pDS56-6×His-Nil-2 (502-604), and the purified preparation contained a major species of the predicted molecular size (15 kD) as deduced by polyacrylamide gel electrophoresis.


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34. We acknowledge the generous support of L. Jarett and R. E. Anders, and the helpful comments of F. Barr, the work of Y. Wu and G. Montoya. We thank L. Kricka for the use of the luminometer, M. Karin for the AP-1–CAT construct, and C. Burks for sequence analysis. Supported by NIH grants AI23879 (J.A.K.); CA54428 (T.M.W.); CA23413 and CA52009 (F.J.R.); NIH core grant CA10817; the McCabe Fund (T.M.W.); the W. W. Smith Charitable Trust (F.J.R.); and the Hansen Memorial Trust (F.J.R.). F.J.R. is a Pew Scholar in the Biomedical Sciences. T.M.W. is supported by an American Cancer Society Junior Faculty Research Award.

5 September 1991; accepted 18 November 1991


# Inhibition of Rap1A Binding to Cytochrome $b_{558}$ of NADPH Oxidase by Phosphorylation of Rap1A


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**Rap1A is a low molecular weight guanosine triphosphate (GTP)–binding protein in human neutrophil membranes whose cellular function is unknown. Rap1A was found to form stoichiometric complexes with the cytochrome $b_{558}$ component of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system. The (guanosine-5′-O-(3-thiotriphosphate) (GTP-γ-S)–bound form of Rap1A bound more tightly to cytochrome $b_{558}$ than did the guanosine diphosphate–bound form. No complex formation was observed between cytochrome $b_{558}$ and H-Ras–GTP-γ-S or Rap1A–GTP-γ-S that had been heat-inactivated, nor between Rap1A–GTP-γ-S and hydrophobic proteins serving as controls. Complex formation between Rap1A–GTP-γ-S and cytochrome $b_{558}$ was inhibited by phosphorylation of Rap1A with cyclic adenosine monophosphate (cAMP)–dependent protein kinase. These observations suggest that Rap1A may participate in the structure or regulation of the NADPH oxidase system and that this function of the Rap1A protein may be altered by phosphorylation.**


A SUPERFAMILY OF LOW MOLECULAR weight GTP-binding proteins (LMWG) structurally related to Ras has been identified (*1*). These proteins undergo a cycle of GTP binding and hydrolysis analogous to that observed with the heterotrimeric G proteins. This analogy suggests that the LMWG may function in regulatory capacities within the cell. Proteins that affect the guanine-nucleotide binding and hydrolysis activity of various LMWG have also been identified, including guanosine triphosphatase activating proteins (GAPs), proteins that stimulate guanine nucleotide exchange, and proteins that inhibit guanosine diphosphate (GDP) dissociation (*2*). The Rap1A protein is identical to Ras in the putative effector domain between amino acid residues 32 to 42 and can form an inactive complex with Ras-GAP that may account for the ability of Rap1A to suppress the transformation of cells by oncogenic K-Ras (*3*). In nontransformed cells the biological function of Rap1A is unknown, but Rap1A is an abundant substrate for cAMP-dependent protein kinase (PKA) in human neutrophils (*4*). Phosphorylation of Rap1A has no effect on its guanine-nucleotide binding kinetics, intrinsic rate of GTP hydrolysis, or ability to be stimulated by a cytosolic Rap-GAP (*5*). Phosphorylation of Rap1B affects its subcellular localization and enhances its ability to bind to a Rap guanine-nucleotide dissociation stimulator (GDS) (*6*).


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The microbicidal activities of human neutrophils include formation of superoxide anion via a plasma membrane–localized superoxide generating system, from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), which transfers electrons to a terminal carrier believed to be cytochrome $b_{558}$ (cytochrome b) and, ultimately, to molecular oxygen (7). Cytochrome b has been purified and cloned and was shown to be a heterodimer of 22-kD α and 91-kD β subunits (*8*). The absence of cytochrome b in some forms of chronic granulomatous disease results in neutrophils unable to generate superoxide, underscoring the essential role of the cytochrome b in this enzyme system (*9*). Activation of the NADPH oxidase requires the association of cytochrome b with a number of other protein components, including $p47_{[phox]}$, $p67_{[phox]}$, an NADPH-binding protein, and a GTP-binding protein (*10*). Translocation of $p47_{[phox]}$ to the plasma membrane requires the presence of cytochrome b (*11*) and possibly a GTP-binding component (*12*). A 22-kD LMWG co-purifies with cytochrome b and remains associated with the cytochrome even after immunoaffinity purification on antibody matrices composed of antibodies to cytochrome b (*13*). Analysis of this LMWG by protein immunoblotting and immunoprecipitation with a polyclonal antibody to Rap1A or Rap1B (R61) (*4*) identified the endogenous protein as Rap1 (*14*). The protein is likely to be Rap1A, because that is the predominant form of Rap1 in human neutrophils (*4*). We now demonstrate that purified Rap1A can form specific complexes with purified cytochrome b and that this interaction can be regulated by phospho-