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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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-

rylation of Rap1A with PKA.

We assessed complex formation between purified recombinant Rap1A (*15*) and purified cytochrome b (*8, 16*) by gel-filtration chromatography (*17*). The incubation of Rap1A bound to GTP-γ-S (Rap1A-γS) with a twofold molar excess of cytochrome b resulted in nearly a complete shift of the Rap1A-γS to a larger molecular size representing a complex with the cytochrome b (Fig. 1). The presence of Rap1A in the fractions containing cytochrome b was verified with antibody to Rap1 (R61), and separate analysis of the Rap1A–cytochrome b complex on an AcA34 gel-filtration matrix gave an apparent hydrodynamic size consistent with the molecular sizes of the two proteins (*14*). Rap1A with bound [α-^{32}P]GTP also formed a complex with cytochrome b (*14*). To determine if there was a specific interaction of Rap1A with cytochrome b, purified H-Ras (*15*) with GTP-γ-S bound (Ras-γS) was incubated with a twofold molar excess of cytochrome b. Essentially no complex was observed (Fig. 1). Rap1A-γS that had been heated for 5 min at 100°C also failed to form complexes with cytochrome b. When a fivefold molar excess of G protein βγ subunits or a threefold molar excess of purified platelet glycoprotein IIb, IIIa was substituted for the cytochrome, there was no interaction observed between these proteins and Rap1A-γS. The latter data indicated that Rap1A-γS was not interacting in a nonspecific manner with hydrophobic or integral membrane proteins. Rap1A-γS prepared with Rap1A purified from human neutrophils (*4, 5*) also formed complexes with the cytochrome. Both neutrophil and recombinant Rap1A are isolated as a post-translationally processed form that has undergone isoprenylation, truncation, and carboxylmethylation (*18*).

The interaction of Rap1A with cytochrome b was stoichiometric (Table 1). Maximal incorporation of Rap1A-γS into the complex (*19*) was obtained after incubation with an equimolar amount or twofold excess of cytochrome b. At a molar ratio of cytochrome to Rap1A-γS of 0.5 to 1, only about one-half of the Rap1A-γS was shifted to the larger size. These data indicate a one-to-one binding of Rap1A-γS to cytochrome b. The interaction of GTP-binding proteins with target molecules is often regulated by the guanine nucleotide bound to the protein, with the GTP-bound form being active (with higher affinity for the target) and the GDP-bound form being inactive (*1*). Rap1A with bound GDP formed a complex with cytochrome b, although it appeared to be somewhat less effective than Rap1A-γS (Table 1). The amount of complex detected was variable, with the extent of complex formation varying between very little (13%) and nearly maximal (56%) under identical incubation conditions (1:2 molar ratio of Rap1A-GDP to cytochrome b).

Rap1A can be phosphorylated by PKA in human neutrophils (*4*), but the effect of phosphorylation on the biological function of Rap1A in these cells is unknown (*5*). The activation of neutrophils by chemoattractants is inhibited by hormones that increase the intracellular concentration of cAMP (*20*). The formation of superoxide by the NADPH oxidase is particularly sensitive to such hormones, being completely abolished by the output of less than 1000 β-adrenergic receptors on a single neutrophil (*21*). Rap1A-γS was phosphorylated by PKA to an extent of 1 mol of phosphate per mole of GTP-γ-S bound (*22*). The phosphorylated protein did not form a complex with the cytochrome b effectively, even when cytochrome b was present in a twofold molar excess (Fig. 2). The lack of complex formation was not due to the presence of PKA or components of the phosphorylation mixture, because (i) Rap1A-γS added to a sample of phosphorylation mixture (that included PKA) was able to effectively complex with cytochrome b, and (ii) removal of ATP from the buffer used for the incubation of phosphorylated Rap1A-γS with cytochrome b had no effect on the results, indicating that a phosphorylation reaction occurring dur-

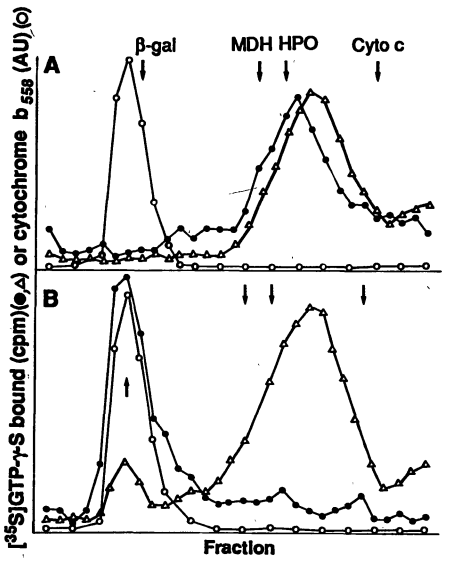

Fig. 1. Gel-filtration analysis of Rap1A–cytochrome b complexes. (**A**) The column profiles represent separate runs of either cytochrome b (○), Rap1A-γS (●), or Ras-γS (△). (**B**) Elution profile after a twofold molar excess of cytochrome b (○) was incubated with either Rap1A-γS (●) or Ras-γS (△) as described (*17*). Cytochrome b was localized by densitometric analysis of autoradiographs obtained from immunoblots probed with antibody to the 22-kD subunit of cytochrome b (*13*) and then with ^{125}I-labeled goat antibody to rabbit IgG. Rap1A-γS and Ras-γS were detected by liquid scintillation counting. Arrows indicate the positions of the marker proteins β-galactosidase (β-gal), malate dehydrogenase (MDH), horseradish peroxidase (HPO), and cytochrome c (Cyto c). AU, absorbance units; cpm, counts per minute.

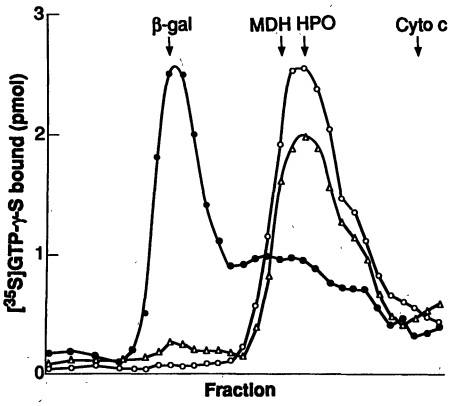

Fig. 2. Failure of phosphorylated Rap1A-γS to form a complex with cytochrome b. Rap1A-γS (●) or phosphorylated Rap1A-γS (○) was incubated with a twofold molar excess of cytochrome b and analyzed (*17*). The elution position of the phosphorylated Rap1A-γS itself (△) was essentially coincidental with that of non-phosphorylated Rap1A-γS. The Rap-γS proteins were localized as described in Fig. 1.

Table 1. Quantitative analysis of complexes containing Rap1A and cytochrome b. Rap1A-γS, Ras-γS, and cytochrome b were prepared and analyzed by AcA44 gel filtration (*17*). The percentage of the total [^{35}S]GTP-γ-S–labeled protein recovered that was shifted to the excluded volume of the column was determined, and is presented as low molecular weight GTP-binding proteins (LMWG) in Complex (%) ±SEM (*19*). Incubations of Rap1A-γS with varying ratios of cytochrome b were performed and analyzed in the same experiment.

Incubation with cytochrome b	Ratio of LMWG to cytochrome b	LMWG in complex (%)	Experiment (no.)
Rap1A-γS	1:2	61.0 ± 1.8	10
Rap1A-γS	1:1	58.7 ± 3.6	7
Rap1A-γS	1:0.5	37.6 ± 0.34	3
Rap1A-GDP	1:2	33.8 ± 9.7	5
Phosphorylated Rap1A-γS	1:2	14.6 ± 8.0	3
Ras-γS	1:2	12.3 ± 5.2	3

ing the incubation with the cytochrome was not inhibiting complex formation. We have confirmed the inhibitory effect of phosphorylation on the Rap-cytochrome interaction by examining the binding of phosphorylated Rap1A-GDP and unmodified Rap1A-GDP to cytochrome immobilized on a heparin Ultragel column (*14*).

A GDS interacts preferentially with phosphorylated Rap1B (*6*). We tested whether cytochrome b might exhibit GDS-like properties toward Rap1A. Cytochrome b did not change the kinetics of binding of [^{35}S]GTP-γ-S to Rap1A in either the presence or absence of Mg^{2+} (*23*), indicating that no effect on exchange of endogenously bound GDP occurred. Similarly, in a standard Rap1A-GAP assay (*4, 15*) a twofold molar excess of cytochrome b produced no detectable enhancement in the hydrolysis of [γ-^{32}P]GTP by the Rap1A protein. Thus, the binding of Rap1A-γS to cytochrome b is not associated with stimulation or inhibition of GDP exchange; nor does it stimulate GTP hydrolysis.

A serine residue (Ser^{180}) at the COOH-terminal region of Rap1A has been identified as the site of phosphorylation by PKA (*4*). Because phosphorylation at this site disrupted Rap1A–cytochrome b binding, the COOH-terminal region of Rap1A is a possible site of interaction with cytochrome b. This region is the most variable portion of the many LMWGs. If Rap1A interacts with cytochrome b through this membrane-opposed region, an analogous site may exist in other LMWGs, including Ras, through which they interact with specific membrane-associated effector molecules.

The effect of the Rap1A–cytochrome b interaction on the function of the NADPH oxidase system remains to be determined, particularly in light of the recent identification of Rac2 as a stimulatory regulator of the NADPH oxidase (*24*). Exogenously added Rap1A does not enhance oxidase activity in cell-free systems (*25*), although such activity of a truncated form of Rap1A has been reported (*26*). Cytochrome b is required for $p47_{[phox]}$ translocation to the plasma membrane during activation of the NADPH oxidase (*11*). This macromolecular assembly process may be regulated by the Rap1A–cytochrome b interaction. It is also possible that Rap1A could direct the association of cytochrome b or granules bearing cytochrome b with plasma membrane sites or with the cell cytoskeleton (*27*). The inhibition of Rap1A–cytochrome b interaction by PKA-catalyzed phosphorylation makes Rap1A a potential target for inhibitory regulation by the cAMP pathway in human neutrophils. Such a mechanism could regulate the ability of Rap1A (or Rap1B) to interact with macromolecules in other cells as well.

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17. Rap1A or H-Ras was stoichiometrically labeled with [^{35}S]GTP-γ-S as described (*5*), unbound nucleotide was removed by Sephadex-G25 chromatography, and the labeled protein was concentrated and washed with buffer containing 25 mM tris (pH 8), 1 mM EDTA, 0.5 M dithiothreitol (DTT), 100 mM NaCl, and 5 mM $MgCl_2$ in a Centricon 10 microconcentrator (Amicon). LMWG (30 to 40 pmol) and purified cytochrome b (60 to 80 pmol) were combined in a buffer containing 10 mM Pipes (pH 7.3), 100 mM KCl, 10 mM NaCl, 1 mM adenosine triphosphate (ATP), 1 mM EDTA, 5 mM $MgCl_2$, and octylglucoside (1.0%) and then incubated at 30°C for 5 min and on ice overnight. The 500-μl sample was filtered over an AcA44 column (20 ml) at a flow rate of 0.15 ml/min. Fractions (0.5 ml) were collected and analyzed for [^{35}S] GTP-γ-S–labeled Rap1A or H-Ras by liquid scintillation counting. The position of Rap1A was also confirmed in several experiments with antibodies to Rap1 (*4*). Cytochrome b was detected with antibody to the 22-kD subunit (*13*) and its distribution was quantified by densitometric analysis of the autoradiographs.
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23. The effect of cytochrome b on guanine-nucleotide exchange was assessed by examining the binding of [^{35}S]GTP-γ-S to Rap1A with and without $MgCl_2$ in the presence of a twofold molar excess of cytochrome. Rap1A (3 pmol) was incubated with cytochrome b (6 pmol) for 15 min on ice; then the binding reaction was initiated at 23°C by addition of 25 mM tris (pH 8), 5 mM EDTA, 2 μM [^{35}S]GTP-γ-S (5000 cpm/pmol), and 0.5 mM dimyristoylphosphatidylcholine in the absence or presence of 10 mM $MgCl_2$. Aliquots were removed at 0, 1, 2, 5, 10, 20, 30, and 60 min and bound [^{35}S]GTP-γ-S was determined by filtration analysis.
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