Function of Wild-Type or Mutant Rac2 and Rap1α GTPases in Differentiated HL60 Cell NADPH Oxidase Activation

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Studies of neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation in a cell-free system showed that the low molecular-weight guanosine triphosphatase (GTPase) Rac was required, and that Rap1α may participate in activation of the catalytic complex. Full-length posttranslationally modified Rac2 was active, whereas only the 1-166 truncated form of Rap1α was functional in the cell-free system, and thus, clarification of the function of Rap1α and Rac2 in intact human phagocytes is needed to provide further insight into their roles as signal transducers from plasma membrane receptors. In the present studies, oligonucleotide-directed mutagenesis was used to introduce a series of mutations into human rap1α or rac2 in the mammalian expression vector pSRcYneo. HL60 cells transfected with wild-type or mutated rac2 or rap1α cDNA constructs and control HL60 cells transfected with the pSRcYneo vector containing no inserted cDNA were selected in G418-containing media, then subclones were isolated. Compared with the parent HL60 cells, each of the stable transfected cell lines differentiated similarly into neutrophil-like cells and expressed comparable levels of NADPH oxidase components p47-phox, p67-phox and gp91-phox. The differentiated vector control cell line produced O₂•⁻ in response to receptor stimulation at rates that were not significantly different from parent HL60 cells. O₂•⁻ production by differentiated cell lines expressing mutated N17 Rap1α or N17 Rac2 dominant-negative proteins was inhibited, whereas O₂•⁻ production by the subline overexpressing wild-type Rap1α was increased by fourfold. O₂•⁻ production by the differentiated cell line expressing GTPase-defective V12 Rap1α was also significantly inhibited, a finding that is consistent with a requirement for cycling between guanosine diphosphate- and GTP-bound forms of Rap1α for continuous NADPH oxidase activation in intact neutrophils. A model is proposed in which Rac2 mediates assembly of the p47 and p67 oxidase components on the cytosolic face of the plasma membrane via cytoskeletal reorganization, whereas Rap1α functions downstream as the final activation switch involving direct physical interaction with the transmembrane flavocytochrome component of the NADPH oxidase.

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exotoxin C3 from *Clostridium botulinum*, and in 3T3 fibroblasts, ADP-ribosylated Rho and Rac behave as dominant-negative suppressors, inhibiting adhesion focus formation and submembrane cytoskeletal reorganization, respectively.\(^{18,19}\) Rho and Rac containing N17 mutations also function as dominant-negative suppressors in 3T3 fibroblasts.\(^{18,19}\) A requirement for Rac\(^{19}\) in guinea pig and Rac\(^{21}\) in human neutrophil cell-free NADPH oxidase activation has been established. Cell-free NADPH oxidase activation was stimulated by *smg* GDP dissociation inhibitor (GDI) and inhibited by rhoGDI\(^{22}\) and required posttranslationally processed Rac2 for maximal activity.\(^{23}\) Rac2 was found to be the most abundant neutrophil toxin C(3) substrate in neutrophils; however, botulinum holotoxin D (containing C3)-mediated ADP ribosylation of up to 70% of the available 22-kD substrate in neutrophils was without effect on NADPH oxidase activation.\(^{25}\) Botulinum C3 toxin-mediated ADP ribosylation of Rac2 in neutrophil subcellular fractions also had no effect on cell-free NADPH oxidase activation.\(^{26}\) The absence of NADPH oxidase inhibition after botulinum toxin treatment of intact neutrophils may be caused by the fact that, although Rac2 is the most abundant neutrophil toxin C(3) substrate in neutrophils, 100-fold higher toxin concentration is required to ADP ribosylate Rac than Rho.\(^{19,22}\) The behavior of N17 dominant-negative mutants of Rac2 in intact neutrophils may thus clarify the significance of the lack of botulinum C3 toxin functional effects on the NADPH oxidase system.

Observations made in the cell-free NADPH oxidase system require correlation with intact cell NADPH oxidase activation because certain aspects of the cell-free system may be nonphysiologic. A functional requirement for Rac2 in NADPH oxidase activation in Epstein-Barr virus (EBV)-transformed B lymphocytes has been shown by antisense oligonucleotide suppression of Rac2, which caused 50% to 60% inhibition of *Staphylococcus aureus*-stimulated chemiluminescence.\(^{28}\) Because NADPH oxidase activity in B lymphocytes is several orders of magnitude lower than in phagocytic cells, \(O_2^-\) generation must be estimated by a nonlinearly amplified chemiluminescence assay in B lymphocytes. The HL60 cell line may be differentiated by dimethyl sulfoxide to neutrophil-like cells that generate amounts of \(O_2^-\) after stimulation by physiologic stimuli that are comparable with normal neutrophils.\(^{29}\) For this reason, the HL60 cell line was chosen for the present study to examine the effects of mutated forms of Rac2 and Rap1a in intact cells. The choice of mutations for the present studies was based on previous work that established the dominant-negative behavior of point-17 mutations in Rac2\(^{19}\) and Rap1a\(^{30}\) in vivo, and the rap GTPase activator protein resistant--GTPase-defective V12 mutation of Rap1a\(^{41}\) that potentiates its tumor suppressor activity in vivo.\(^{30}\)

**MATERIALS AND METHODS**

**HL60 cell propagation and differentiation.** The HL60 cell line, a kind gift from D. Skalnik (Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis), was grown in RPMI 1640 (BRL-GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS; BRL-GIBCO), and 1% penicillin/streptomycin under standard cell culture conditions (37°C, humidified 5% CO\(_2\) in air). The cells were passaged twice weekly in fresh media at 1.5 to 2 x 10\(^7\) cells/mL. Differentiation to neutrophil-like cells was induced by resuspension of HL60 cells at 5 x 10\(^7\) cells/mL in fresh media supplemented with 1.5% dimethyl sulfoxide.

**Measurement of stimulated nitroblue tetrazolium (NBT) reduction and quantitate \(O_2^-\) production.** For measurement of stimulated NBT reduction, differentiated HL60 cells were resuspended in RPMI 1640 containing 1.7 mg/mL NBT at 5 x 10\(^7\) cells/mL. Aliquots of the cell suspension were transferred to 96-well microtiter plates and warmed to 37°C. For primed N-formyl-methionyl-leucyl-phenylalanine (FMLP) stimulation, phorbol myristate acetate was added to a final concentration of 10 ng/mL, then FMLP was added to a final concentration of 10\(^{-7}\) mol/L to begin the timed reaction. When phorbol myristate acetate was the only stimulus, a concentration of 100 ng/mL was used. Measurement of \(O_2^-\) production as superoxide dismutase-inhibitable ferricytochrome c reduction was performed as a 10-minute endpoint assay as described\(^{32}\) at a cell density of 5 x 10\(^3\) mL in RPMI 1640 (without phenol red and without serum) using the same stimulus conditions as in the NBT reduction assays.

**Construction of plasmid vectors.** The full-length cDNAs for V12, V12 plus E31, and V12 plus R31 rapla (Krev-1) in pSRaneo vector were generous gifts from M. Noda. Full-length rapla in pUC 8 was provided by M. Marshall and full-length rac2 in pUC 219 was provided by R. Weber. General molecular cloning techniques were performed as in standard protocols.\(^{33}\) Mutations in the pUC 219 construct of rac2 and pUC 8 construct of rapla were introduced by oligonucleotide-directed mutagenesis according to the manufacturer's protocol for the Transformer Site Directed Mutagenesis Kit (Clontech Laboratories Inc. Palo Alto, CA). For the N17 Rac2 mutation, codon 17 was mutated from ACC to AAC by using the synthetic oligonucleotide 5'-TGAGAAAGGACTTTCTGGCCACCG-3' corresponding to the antisense strand of the rac2 cDNA. For the N17 Rapla mutation, codon 17 was mutated from TCT to AAT with the oligonucleotide 5'-CTCTTACAGGCTTTCTTCCACCG-3' corresponding to the antisense strand of the rapla cDNA. The sequences of the resultant 17 mutant rac2 and rapla cDNAs were confirmed by the dieoxy chain termination method.\(^{24}\) EcoRI fragments of rac2, N17 rac2 (from pUC 219), and N17 rapla (from pUC 8) were cloned into the EcoRI site of pSRaneo to obtain pSRaneo-rac2, pSRaneo-N17 rac2, pSRaneo-rapla and pSRaneo-N17-rapla. The EcoRI-Sal 1 fragment of rapla was directionally cloned into the EcoRI-Sal 1 site of pSRaneo to obtain pSRaneo-rapla Selection. Production of plasmid clones containing the inserts of interest in the sense direction was determined by partial DNA sequencing of the appropriate pSRaneo cDNA constructs.

**Production of stable G418-resistant pSRaneo-transfected HL60 cell lines.** The pSRaneo vector containing no cDNA insert, and pSRaneo constructs containing rac2; N17rac2; rapla; N17 rapla; V12 rapla; V12, E31 rapla; V12 R31 rapla cDNA inserts in the sense orientation were introduced into HL60 cells by electroporation in a BioRad GenePulser with Capacitance Extender (BioRad Laboratories, Hercules, CA). One milliliter of HL60 cells at 2 x 10\(^7\) x 10\(^7\)/mL in RPMI 1640 plus 30% FBS were mixed with 50 µg plasmid DNA and placed in a BioRad 0.4-cm electrode gap electroporation cuvette. The cells were subjected to a single pulse of 750 volts/cm at a capacitance setting of 960 microfarads. The cuvettes were placed on ice for 30 minutes, then the cells were transferred to 50 mL RPMI 1640 containing 20% FBS and allowed to recover at 37°C for 48 hours. After 48 hours, clones were selected after 2 weeks by plating in semisolid medium containing 1.5 mg/mL G418, and thereafter the sublines were continuously grown in the standard liquid suspension culture containing 1.5 mg/mL G418.

**Southern blotting.** Genomic DNA was isolated according to...
standard methods. The DNA was quantitated and 15 µg was digested with EcoRI overnight. Digested DNA was separated on a 0.7% agarose gel, denatured, neutralized, and transferred onto nitrocellulose as described. The filters were hybridized at 42°C overnight in the following solution: 50% formamide, 0.75 mol/L NaCl, 0.075 mol/L sodium citrate, 1× Denhardt’s solution, 24 mmol/L sodium phosphate [pH 6.5], 4% Herring sperm DNA, 0.625% poly rA, 0.5% poly rC, and 0.5% yeast tRNA with 10% dextran. The final wash was for 20 minutes at 55°C in 0.1× saline sodium citrate.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. SDS-PAGE was performed by the method of Laemmli. Electrophoretic transfer for western blotting and detection of primary antibody by alkaline phosphatase-conjugated rabbit antigoat IgG (Sigma, St Louis, MO) or alkaline phosphatase-conjugated goat-antirabbit (Boehringer Mannheim Biochemicals, Indianapolis, IN), was performed as described. Polyclonal goat antibody to p47-phox and p67-phox provided by T. Leto (Laboratory of Host Defense, NIAID, NIH, Bethesda, MD), and polyclonal rabbit antibody to gp91-phox provided by Mary Dinauer were used at a dilution of 1:1,000 as primary antibodies. For estimates of gp91-phox by western blot, the HL60 cell extract was digested with endoglucosidase F before SDS-PAGE (New England Nuclear, Boston, MA) in the presence of protease inhibitors as described.

RESULTS

Characterization of the stable transfected HL60 cell lines. Each of the G418-resistant stable HL60 cell sublines contained 1 to 3 genomic copies of the transfected pSRneo plasmid DNA by Southern analysis (Fig 1). In the uninduced state, each of the stable cell lines displayed growth characteristics, doubling times (1.5 to 2 days), and morphologic features that were similar to the parental HL60 cell line. After induced differentiation with 1.5% dimethyl sulphoxide, all of the stable transfected cell lines morphologically differentiated to neutrophil-like cells, showing cessation of proliferation at 1.5 to 2 days, maximal morphologic differentiation at 3 to 4 days, and progressive cell death as assessed by trypan blue uptake at days 4 to 6.

In preliminary studies, assessment of maximal NADPH oxidase expression by the FMLP-stimulated NBT test showed that the parent HL60 cell line and the pSRneo vector control cell line contained the maximal number of strongly NBT-positive cells 3 to 3.5 days after addition of dimethyl sulphoxide. Although the percentage of NBT positive cells, expressed relative to the number of viable cells, was maximal at day 4 after dimethyl sulphoxide addition, the percent nonviable trypan blue-positive cells increased sharply at 3.5 to 4 days (30% to 45%), reaching 40% to 70% at day 5. Thus, to avoid large and variable corrections for the number of nonviable differentiated cells, standard assays of NADPH oxidase activity and estimates of the abundance of NADPH oxidase components were performed after 3 days of exposure to dimethyl sulphoxide.

NADPH oxidase expression. When compared with the vector control HL60 cell subline, FMLP-stimulated NBT reduction by the N17 rapla- and N17 rac2-transfected stable cell lines was markedly decreased (Fig 2). Additionally, the wild-type rapla-transfected cell line consistently showed an increased percent of NBT-positive cells as well as more intense cell-associated NBT deposits (Fig 2). The stable wild-type rac2-transfected cell line also showed a moderate increase in cell associated NBT deposits (Fig 2); however, this was somewhat variable from experiment to experiment. The low levels of FMLP-stimulated NBT reduction by the N17 rapla- and N17 rac2-transfected cell lines shown in Fig 2 did not increase after 4 or 5 days of dimethyl sulphoxide-induced differentiation (not shown), although at these later times the number of viable cells decreased dramatically as noted above. Thus, decreased NADPH oxidase expression in the N17 rapla- and N17 rac2-transfected cell lines was not caused by a delay in dimethyl sulphoxide–induced differentiation and programmed cell death. Similar results were obtained when phorbol myristate acetate alone (100 ng/mL) was used as the stimulus (see below).

These differences in NADPH oxidase expression were confirmed by FMLP-stimulated O2 production as measured by quantitative superoxide dismutase-inhibitable ferriicytochrome c reduction. In this assay, O2 production by the pSRneo vector control cell line was 19 ± 4 nmol/min/107 cells (mean ± SE, n = 6), a value that was not significantly different from the parent HL60 cell line. When expressed relative to the vector control cell line, the wild-type rapla-transfected cell line showed a fourfold increase in O2 production, whereas the dominant-negative N17 rapla-transfected cell line showed a significant (40%; P = 0.03)
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Fig 2. Stimulated NBT reduction by dimethylsulphoxide-differentiated HL60 cell sublines. Production of stable HL60 cell sublines transfected with pSRaneo vector alone or the vector containing cDNA for wild-type Rapla, N17 Rapla, wild-type Rac2 or N17 Rac2 was as described in Materials and Methods. The cells were differentiated with dimethylsulphoxide, primed with substimulatory concentration of phorbol myristate acetate, and stimulated with 10^{-7} mol/L FMLP in the presence of NBT. Photomicrographs were taken 20 minutes after addition of stimulus to the primed differentiated cells.

decrease (Fig 3A). The wild-type rac2-transfected cell line produced O_{2} at rates not different from the vector control line, whereas the dominant-negative N17 rac2-transfected cell line showed a significant (50%; P = .007) decrease in O_{2} production (Fig 3B).

Despite the similar time course of morphologic differentiation to neutrophil-like cells, it was important to show that the decrease in NADPH oxidase expression by the N17 rapla- and N17 rac2-transfected cell lines was not caused by more subtle or selective defects in differentiation at the molecular level. Thus, the abundance of NADPH oxidase components p47-phox and p67-phox (Fig 4A), and gp91-phox (Fig 4B), were compared by western blot before and after dimethyl sulphoxide-induced differentiation. Compared with the parent HL60 or vector control cell line, the cytosolic p47-phox and p67-phox oxidase components were upregulated similarly in the N17 Rapla- and N17 Rac2-expressing cell lines after induced differentiation. The membrane-associated gp91-phox was present in detectable amounts before differentiation in all of the cell lines, and was not markedly upregulated after differentiation. The abundance of gp91-phox in differentiated N17 Rapla- or N17 Rac2-expressing cell lines was similar to that in the differentiated parent HL60 cell line. Quantitative comparisons of this component were difficult because of the more diffuse mobility of gp91-phox on SDS-PAGE even after enzymatic deglycosylation.

In view of the increased NADPH oxidase activity in the HL60 cell line transfected with wild-type rapla, the effect of an activating V12 mutation in Rapla that results in decreased spontaneous and RapGAP-stimulated GTPase activity was of interest. FMLP-induced NBT reduction by the dimethyl sulfoxide-differentiated V12 rapla-transfected cell line was consistently less than the vector control HL60 cell line as well as the wild-type rapla-transfected cell line (not shown). In quantitative assays, the rate of O_{2} production by V12 rapla-transfected relative to the vector control HL60 cell line was significantly (50%; P = .007) inhibited (Fig 3A). Thus, the interesting situation in which wild-type rapla-transfected cells produce substantially more O_{2}, whereas both dominant-negative N17 rapla- and GTPase-defective V12 rapla-transfected cells produce significantly less O_{2} than control HL60 cells requires an explanation.

Further experiments were designed to determine whether the inhibition of NADPH oxidase by V12 Rapla was dependent on the functionally important domain flanking the amino acid 32-40 effector loop region, which is identical to the corresponding domain of Ras. V12 Rap 1a containing a second mutation adjacent to the effector loop, V12, K31E (substituting the normal Ras residue), or V12, K31R (a more conservative substitution) displayed impaired tumor suppressor activity in vivo compared with V12 Rap 1a. When expressed in stable HL60 cell lines, V12, R31 and V12, E31 Rapla both inhibited NADPH oxidase either FMLP or phorbol myristate acetate stimulation (Fig 5). Quantitative O_{2} production after stimulation with FMLP was inhibited by 65% ± 0.05% SE, n = 4, for V12, K31E, and 60% ± 0.07% SE, n = 4, for V12, K31R. Thus, second mutations at codon 31 in the context of the V12 mutation did not drastically alter the inhibitory action of V12 Rapla on NADPH oxidase activation.

**DISCUSSION**

A model of low molecular-weight GTPase action in neutrophil NADPH oxidase activation is proposed that is capable of explaining most of the experimental findings in this as well as previous studies. In this model, Rac2 and Rap1a participate at distinct steps in NADPH oxidase assembly and activation. In this scheme, Rac2 may facilitate assembly of the cytosolic p47-phox/p67-phox complex with the trans-
membrane gp91-phox/p22-phox flavocytochrome catalytic unit, and the activated GTP-bound form of Rap1a may function via a direct physical interaction with the membrane-associated flavocytochrome. In this role, Rap1a-GTP could promote a conformational change or functional interaction of the p47, p67, and flavocytochrome components that is necessary to allow electron flow through the complex to molecular oxygen.

The close homology of Rac2 with Rho GTPase would be consistent with a role in cytoskeletal interaction or reorganization that may serve to bring the p47 and p67 components in physical proximity to the cytosolic domains of the gp91/p22 flavocytochrome complex. Activation of NADPH oxidase in the intact neutrophil has been shown to result in an increase in membrane-associated relative to cytosolic Rac2, which may be the result of a shift in dynamic equilibrium or simple mass translocation. Furthermore, the GTP-bound form of Rac2 has recently been shown to physically interact with the p67 oxidase component. This model explains the inhibitory effects of dominant-negative N17 mutants of both Rac2 and Rap1a by inhibition at distinct steps in the activation pathway of NADPH oxidase because the N17 mutation favors binding of GDP over GTP and accumulation of the inactive GDP-bound form of N17 Rac2 or N17 Rap1a inhibits the endogenous wild-type protein possibly by sequestering the guanine nucleotide exchange factor that is essential for activation of the wild-type GTPase.

The inhibitory effect of GTPase-defective V12 Rap1a is associated flavocytochrome. In this role, Rap1a-GTP could promote a conformational change or functional interaction of the p47, p67, and flavocytochrome components that is necessary to allow electron flow through the complex to molecular oxygen.

The inhibitory effect of GTPase-defective V12 Rap1a is explained by the requirement for dynamic cycling between GDP- and GTP-bound forms of Rap1a for each NADPH oxidase activation event in the intact cell. This has clear precedent in the obligatory cyclic function of Rab3 GTPase in synaptic vesicle cycling, in which, after mediating an initial exocytic event, the activated GTP-bound form of Rab3 apparently becomes trapped at the membrane and is unavailable to mediate subsequent events until hydrolysis of the bound GTP to GDP occurs. Thus, the poorly hydrolyzable GTP analogue, GTPyS, was inhibitory to exocytosis and by analogy, the inhibitory effect of V12 Rap1a on HL60 cell NADPH oxidase activation is explained by inability to cycle between the GTP- and GDP-bound form. A cellular deactivation mechanism for NADPH oxidase that necessitates continual activation of NADPH oxidase molecules to maintain O₂ production is operative in intact neutrophils; however, this mechanism is not operative in the cell-free activation system. Thus, the required continuous NADPH oxidase activation in response to FMLP in intact HL60 neutrophil-like cells may be susceptible to inhibition by V12 Rap1a because the GTP-bound form is unable to recycle after mediating a single NADPH oxidase activation event.

The cell-free system, which does not require continuous NADPH oxidase activation, can be fully activated in the presence of poorly hydrolyzable GTPyS. Thus, cycling of the endogenous or exogenously added 1-166 Rap1a is not necessary in the cell-free system.

The inhibitory effect of V12 Rap1a could be explained by postulating a negative regulatory role for Rap1a in NADPH oxidase function, (eg, tonic inhibition of the oxidase by Rap1a-GTP relieved by intrinsic or RapGAP-stimulated hy-

![Graph A](GABIG.png)

**A** Relative O₂⁻ Production (vector control = 1.0)

- **WT**
- **N17**
- **V12**

**P = 0.03**

- **P = 0.03**
- **P = 0.007**

**Fig 3.** Quantitative O₂⁻ production by differentiated HL60 cell sublines. The HL60 cell sublines were prepared and differentiated to neutrophil-like cells as in Fig 2. The differentiated cells were primed with substimulatory concentrations of phorbol myristate acetate, then stimulated with 10⁻⁷ mol/L FMLP and O₂⁻ generation was quantitated as superoxide dismutase-inhibitable cytochrome c reduction in a 10-minute end-point assay as described in Materials and Methods. Cell lines transfected with wild-type, N17- or V12-mutated forms of Rap1a (A) and wild-type or N17-mutated Rac2 (B) were studied. Results are reported relative to the value for the vector control cell line in each experiment (mean ± SE, n = 6). Statistical significance was determined using the paired t-test for each subline compared with the vector control subline. The actual rate of O₂⁻ production by the vector control cell subline was 19 ± 4 nmoi O₂⁻/min/10⁶ cells, mean ± SE, n = 6.

![Graph B](GABIG.png)

**B** Relative O₂⁻ Production (vector control = 1.0)

- **WT**
- **N17**

**P = 0.007**

**NS**
Fig 4. Western blot analysis of NADPH oxidase components. Undifferentiated and dimethyl-sulphoxide-differentiated HL60 cells and stable sublines transfected with pSRneo vector, pSRneo-N17 rapla, or pSRneo-N17 rac2 mutants were used to compare total cellular levels of p67-phox and p47-phox (A) or gp91-phox (B). For each cell type, indicated in labels at bottom, the undifferentiated cells are in lanes indicated by (-), and the differentiated cells are in lanes indicated by (+). Each lane was loaded with the cellular protein from $5 \times 10^6$ cells.

hydrolysis of bound GTP to GDP or Rap1a-GTP-driven reversal of the Rac 2 effect). This would fail to explain the stimulatory effect of recombinant 1-166 Rap1a, the inhibitory effect of antibodies to the 32-40 effector loop of Rap1a on cell-free NADPH oxidase activation, and the stimulatory effect of overexpressed wild-type Rap1a in the present study. Likewise, the finding that highly purified cytochrome b558 copurified with Rap1a as a complex that supported NADPH oxidase activation, whereas depletion of Rap1a from the cytochrome b complex in the final purification step was accompanied by loss of the ability to support NADPH oxidase activity strongly argues against a negative regulatory role for Rap1a.

Additional independent lines of evidence support both a positive regulatory role for Rap1a in NADPH oxidase activation and also the existence of trafficking between membrane-associated and cytosolic forms of Rap1a: (1) The GTP-bound form of Rap1a associates with purified cytochrome b 558 in a biochemical assay, whereas the GDP-bound form does not. (2) Protein kinase A phosphorylates neutrophil Rap1a at S180 and this phosphorylated form of Rap1a-GTP fails to associate with purified cytochrome b 558. (3) In platelets, adenylate cyclase-protein kinase A activation results in the phosphorylation of membrane-associated Rap 1b and its partial translocation to the cytosolic fraction. (4) Adenylate cyclase-coupled receptors (e.g., prostaglandin E2) that activate neutrophil protein kinase A also down-regulate NADPH oxidase activation in intact neutrophils. Thus, the composite results suggest that phosphorylation of Rap1a by protein kinase A may mediate the inhibition of NADPH oxidase activation in response to adenylate cyclase-coupled agonists by promoting dissociation of Rap1a from the membrane and preventing reassociation of Rap1a with cytochrome b 558.

The experimental findings thus support the function of both Rac2 and Rap1a as required positive regulators of neutrophil NADPH oxidase activation. It is possible that Rac2 and Rap1a could interchangeably mediate a single step in NADPH oxidase activation; however, the divergent amino acid homology between Rac and Rap, especially in the amino acid 28-45 effector loop and flanking regions, make this hypothesis less attractive. The inhibitory effects of the dominant-negative N17 mutants of Rac2 and Rap1a could be mediated by competition of the GDP-bound form of either N17 mutant for a common nucleotide exchange factor.
(smg GDS) required for the function of the wild-type GTPases. This cross-talk mechanism could be operative whether Rac2 and Rap1a function at the same or distinct points in the signal transduction pathway. However, the effects of both wild-type and V12 Rap1a in the present study as well as the previously reported effects of antisense suppression of Rac2 in B lymphocyte NADPH oxidase function serve as independent lines of evidence that both Rac2 and Rap1a are required for NADPH oxidase activation in intact cells. Cell-free and intact neutrophil NADPH oxidase activation are appropriate experimental systems to further test the effect of mutations within or flanking the effector loop domains of Rac2 and Rap1a to further clarify the structural basis of low molecular-weight GTPase function in this signal transduction pathway.

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