Molecular basis for Rac2 regulation of phagocyte NADPH oxidase

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A Rac GTPase–regulated multiprotein NADPH oxidase is critical for the formation of reactive oxygen species (ROS) in phagocytic leukocytes and other nonphagocytic cells. NADPH oxidase reduces molecular oxygen to form superoxide anion in a two-step process. Electrons are initially transferred from NADPH to cytochrome b–associated FAD, then to cytochrome b heme and finally to molecular oxygen. We show here that Rac is required for both electron-transfer reactions. Mutational and biophysical analysis shows that Rac and p67^{phox} independently regulate cytochrome b to catalyze the transfer of electrons from NADPH to FAD. However, they must interact with each other to induce the subsequent transfer of electrons from FAD to cytochrome b heme and molecular oxygen. This two-step model of regulation by Rac GTPase may provide a means of more effectively controlling the inflammatory responses of phagocytic leukocytes.

NADPH oxidase in phagocytic leukocytes, lymphocytes and nonphagocytic cells generates reactive oxygen species (ROS) that are involved in the regulation of cellular activities ranging from host defense and inflammation to intracellular signaling and transcription[1,2]. Upon stimulation of phagocytes, two cytosolic components of the NADPH oxidase—p47^{phox} (phagocytic oxidase) and p67^{phox}—translocate to the plasma membrane and form a complex with gp91^{phox} and p22^{phox}, the two subunits of a low-potential, membrane-bound flavocytochrome b_{-245} (cyt b)[2]. Homologs of gp91^{phox} have been identified in nonphagocytic cells[3,4,5]. The small GTPase Rac is also required for NADPH oxidase activity both in phagocytic and nonphagocytic cells[1,2,6].

The requirement of Rac2 for formation of ROS in intact phagocytes has been shown with the use of Rac2 antisense oligonucleotides[7]; through selective defects in ROS production by neutrophils of Rac2-null mice[8] and in a patient with an inhibitory mutation in Rac2[9]; and by increased ROS formation and inflammation in the Bcr Rac2 GAP–null mouse[10]. Active Rac GTPase, predominantly Rac2 in human leukocytes, translocates to the plasma membrane simultaneously to, but independently of, the translocation of p47^{phox} and p67^{phox}[11,12,13]. Once formed, the NADPH oxidase complex acts as an electron transporter, conveying electrons from NADPH *via* FAD (step 1) and then to the heme groups of cyt b (step 2), which can directly reduce O_2 due to its low midpoint potential. Reconstitution studies with purified components have shown that Rac is required for activity of the functional (membrane-assembled) NADPH oxidase[14,15].

The molecular mechanisms through which Rac regulates the membrane-assembled NADPH oxidase are not understood. We therefore used a reconstituted cell-free NADPH oxidase system to examine the role of Rac2 in the electron-transfer reactions necessary for the generation of superoxide anion. We show that Rac2 is required for both the step 1 and step 2 electron-transfer reactions catalyzed by the NADPH oxidase. Using site-specific mutants, we also show that Rac2 acts independently of p67^{phox} to regulate the initial transfer of electrons from NADPH to FAD. In contrast, the ability of Rac2 to bind p67^{phox} is necessary to complete electron transfer to molecular oxygen to form superoxide anion. These data suggest that Rac2 directly interacts with cyt b to catalyze the initial electron-transfer reaction. This hypothesis is supported by the inability of a Rac2 insert-domain-deletion mutant, which we show is required for physical interaction of Rac2 with cyt b, to support the first electron-transfer reaction. A direct physical interaction of Rac2 with cyt b is verified with fluorescently labeled Rac2-mant-GppNHp. These data establish a new molecular model for NADPH oxidase regulation by Rac GTPase.

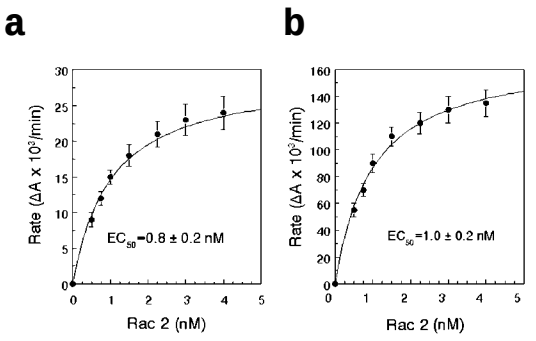

Figure 1. Dependence of NADPH oxidase electron transfer reactions on Rac2 concentration. Electron transfer reactions that measured the rate of (**a**) electron flow from NADPH to FAD *via* INT reduction and (**b**) electron flow from NADPH to O_2 *via* cyt c reduction were determined. The reaction mixtures consisted of cyt b (2 nM), p47^{phox} (141 nM), p67^{phox} (400 nM) and varying concentrations of wild-type (WT) Rac2, as indicated. Data are the mean±s.e.m. values from three independent experiments.

Results

NADPH oxidase electron transfer reactions require Rac2

To evaluate the role of Rac2 in NADPH oxidase electron-transfer reactions, we used a cell-free system that consisted of recombinant p47^{phox} and p67^{phox}; cyt b that had been purified from human neutrophils; and

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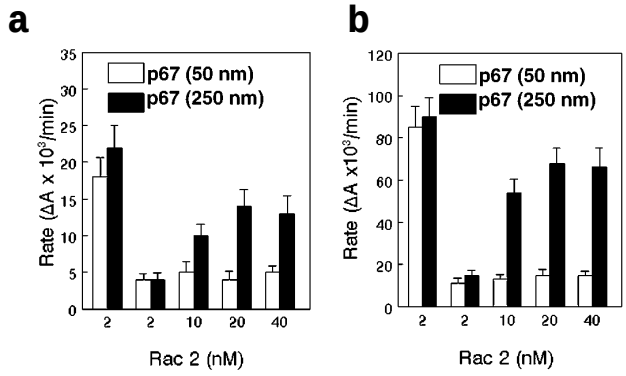

Figure 2. NADPH oxidase activity is p47^{phox} independent. Electron transfer reactions were conducted in the absence of p47^{phox}. (**a**) INT reduction and (**b**) cyt c reduction. (The first sets of bars represent the control data with p47^{phox} included.) The reaction mixtures consisted of cyt b (2 nM) and p67^{phox} (50 nM or 250 nM) + the indicated concentrations of Rac2. Data are the mean± s.e.m. values from three independent experiments.

baculovirus Sf9 cell–expressed, fully processed Rac2[14]. As shown previously[14], effective formation of superoxide anion by this system was completely dependent on the presence of each of the protein components. The initial transfer of electrons from NADPH to the cyt b–bound FAD (step 1) was measured with the artificial two-electron acceptor, iodonitrotetrazolium violet (INT)[16]. This diaphorase activity has an absolute requirement for the components of the NADPH oxidase. In addition, the reduction of INT is not mediated by O_2^- or ROS derived from O_2^-. Rather, it is mediated by the direct transfer of electrons from enzyme-bound FAD to INT, as shown by anaerobic- and oxygen-scavenger studies[16]. This has also been substantiated by an *in vivo* study of neutrophils from a patient who had a point mutation in cyt b that resulted in an inability to catalyze O_2^- production while retaining normal INT reductase activity[17,18].

Transfer of electrons to the cyt b–associated heme and then to molecular oxygen (step 2) was monitored as the superoxide dismutase–sensitive reduction of cytochrome c (cyt c) at 550 nm[19]. In the complete system, electron flux to INT was essentially identical to the flux through the entire system (94±13 *versus* 110±15 mol/s/mol of cyt b), which indicated that we were measuring a full step 1 reaction. Step 1 was completely dependent on the addition of amphiphile, as well as on the presence of Rac2 GTP, with no activity observed when Rac2 was in the GDP-bound form. The EC_{50} of Rac2 for INT reduction (0.8±0.2 nM) was essentially identical to the EC_{50} for cyt c reduction (1.0±0.2 nM) (**Fig. 1**).

p47^{phox} serves an adapter function

It has previously been suggested that p67^{phox} can regulate electron flow from NADPH to FAD in the absence of p47^{phox}, whereas p47^{phox} is required for completion of electron transfer from FAD to cyt b[20]. In contrast, it has also been reported that p47^{phox} is not required for cyt b reduction at high concentrations of p67^{phox} (refs. 21, 22). We observed that both INT reduction and cyt c reduction were markedly decreased in the absence of either p47^{phox} or p67^{phox} (data not shown). However, when the amounts of both Rac2 and p67^{phox} in the reaction were increased by as little as fivefold, we were able to recover more than 70% of the maximal INT- and cyt c–reducing activity, even in the complete absence of p47^{phox} (**Fig. 2**). In contrast, p67^{phox} was required at all concentrations of Rac2 and p47^{phox} (data not shown). These results support the hypothesis that p47^{phox} has no intrinsic catalytic function but serves as an adapter protein to facilitate the interaction of p67^{phox} and/or Rac2 with cyt b.

p67^{phox} and Rac2 independently regulate step 1

It has been shown that Rac binds directly to p67^{phox}[23] and that mutation of Rac (so that it no longer binds p67^{phox}) prevents NADPH oxidase activation[24,25]. However, based upon indirect observations, interaction between Rac and cyt b has also been suggested[25,26]. To investigate the molecular interactions that are necessary for regulation of the active NADPH oxidase complex by Rac2, we generated p67^{phox} and Rac2 mutants that were unable to interact with each other. We then tested the ability of these proteins to support step 1 *versus* step 2 electron-transfer reactions. A p67^{phox} protein that lacked the Rac-binding site (Δ178–184) was able to fully support INT reduction with an affinity that was similar to wild-type p67^{phox} (**Fig. 3a**). This indicated that an interaction with Rac2 was not required for the step 1 reaction. In contrast, there was a dramatic difference in the ability of this construct to support the step 2 reaction because cyt c reduction was markedly reduced (**Fig. 3b**).

To verify these results, we carried out the reciprocal experiment using a Rac2 mutated within the switch I region to prevent interaction with p67^{phox}. Again, we observed that the Rac2 D38A mutant was able to support INT reduction but was deficient in cyt c reduction (**Fig. 3c,d**). These data indicate that p67^{phox} and Rac2 exert independent regulatory activities on cyt b, which leads to the transfer of electrons from NADPH to FAD. Also, the two proteins must physically and functionally interact to catalyze the subsequent transfer of electrons from FAD to molecular oxygen.

Electron transfer reactions require the Rac insert domain

The insert domain of Rac (aa 124–135) is required for Rac-stimulated ROS generation *in vivo*. When this domain had been deleted, Rac

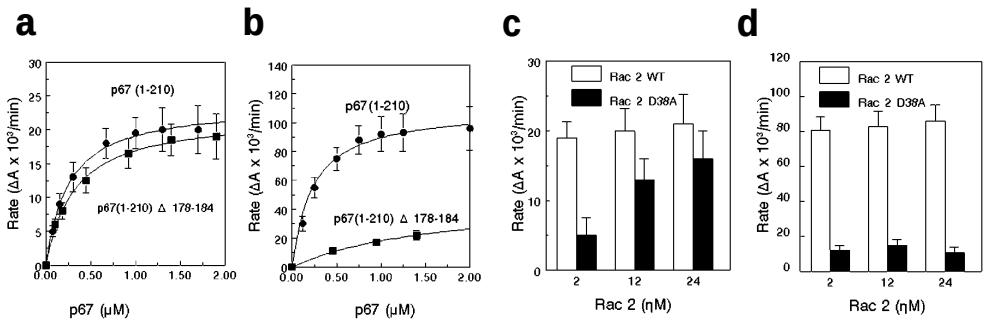

Figure 3. The interaction of Rac2 with p67^{phox} is required for the step 2, but not the Step 1, electron-transfer reaction. (**a,b**) Electron-transfer reactions contained cyt b (2 nM), p47^{phox} (141 nM), Rac2 (2 nM) and the indicated concentrations of either p67^{phox}(1–210) or the Rac-binding–deficient p67^{phox} (1–210)Δ178–184. (**c,d**) Electron-transfer reaction mixtures contained cyt b (2 nM), p47^{phox} (141 nM), p67^{phox} (400 nM) + the indicated concentrations of either WT Rac2 or Rac2 D38A, which is deficient in p67^{phox} binding. (**a,c**) INT reduction and (**b,d**) cyt c reduction. Data are the mean± s.e.m. values from three independent experiments.

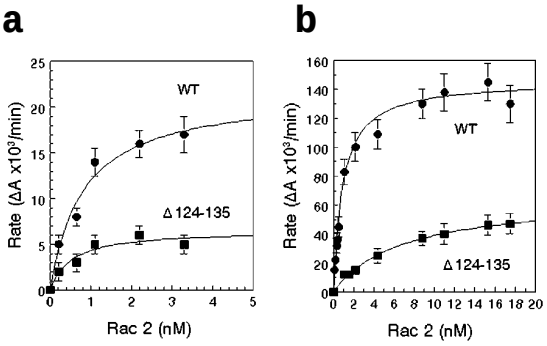

Figure 4. The Rac2 insert domain is required for both step 1 and step 2 electron-transfer reactions. Electron-transfer reactions contained cyt b (2 nM), p47^{phox} (141 nM), WT p67^{phox} (400 nM) + the indicated concentrations of Rac2 WT or the insert domain deletion mutant Rac2Δ124–135. (**a**) INT reduction (**b**) cyt c reduction. Data are the mean± s.e.m. values from three independent experiments.

exhibited a reduced ability to support oxidant production in the cell-free NADPH oxidase[27,28]. It has been hypothesized that this domain interacts directly with the cyt b. We reasoned, therefore, that a Rac2 molecule that lacks a functional insert domain would be deficient in the step 1 reaction, which appears to require direct interaction of Rac2 with cyt b. Insert domain–deficient Rac2 was unable to support the step 1 reaction, as well as the subsequent production of O_2^- (**Fig. 4**). Thus, Rac2 regulates formation of ROS by a two-step regulatory process that involves an initial independent effect on cyt b, followed by a second effect that requires interaction with p67^{phox}.

Direct physical interaction of Rac2 with cyt b

To show that Rac2 could interact directly with cyt b, we used a fluorescent analog of nonhydrolyzable GTP, *N*-methylanthraniloyl-GppNHp (mant-GppNHp), which binds tightly to Rac[25]. When the "mant" moiety is subjected to a change in the polarity of its microenvironment, the emission maximum of mant-GppNHp is blue-shifted and there is an increase in its fluorescent intensity[29]. Spectrum A shows the emission spectrum of 65 nM mant-GppNHp alone (**Fig. 5a**). Upon addition of Rac2 to mant-GppNHp, the emission maximum blue-shifted from 440 nm to 433 nm and there was a 1.7-fold increase in fluorescence intensity, which indicated that Rac2 had bound mant-GppNHp (**Fig. 5a**, spectrum B). When cyt b was added to the mant-GppNHp bound to Rac2, the fluorescence intensity increased an additional 10% from 25.5±0.7 to 28.1±0.6 (**Fig. 5a**, spectrum C). This result indicates that Rac can interact directly with cyt b. As controls, we examined the emission spectrum of mant-GppNHp in the presence of p47^{phox} or p67^{phox}. Addition of p47^{phox} to Rac2-bound mant-GppNHp did not increase the fluorescence intensity of the emission maximum any more than that obtained with mant-GppNHp in the presence of Rac2 alone (data not shown). However, the addition of p67^{phox} to the Rac-bound mant-GppNHp did result in an increase in intensity of 10% (**Fig. 5a**, spectrum D), which indicated that Rac2 binds to p67^{phox} as has been reported[25]. The emission spectrum of mant-GppNHp (in the absence of Rac2) + cyt b or p67^{phox} was identical to that of mant-GppNHp alone (data not shown).

To determine whether the insert domain of Rac2 is required for the interaction of Rac2 with cyt b, we substituted a deletion mutant of Rac2 that lacks this domain (Rac2Δ124–135) in the fluorescence study described above (**Fig. 5b**). The emission maximum of mant-GppNHp (**Fig. 5b**, spectrum A) in the presence of Rac2Δ124–135 underwent a blue-shift and increased in intensity 1.3-fold (**Fig. 5b**, spectrum B). This showed that Rac2Δ124–135 is still able to bind mant-GppNHp. The result was as expected because it has been shown that the insert domain is not involved in guanine nucleotide binding. However, upon addition of cyt b, a further increase in fluorescence intensity did not occur (**Fig. 5b**, spectrum C). This indicated that the insert domain of Rac2 is required for the interaction between Rac2 and cyt b. We also found that there was a 24% increase in intensity (21.2±0.8 to 26.4±0.6) when p67^{phox} was added to the mant-GppNHp–Rac2Δ124–135 complex. Thus, the insert domain is not involved in the interaction be-tween p67^{phox} and Rac2.

The dual role of Rac2 was also shown by using the effector-domain mutant of Rac2, Rac2D38A, in the fluorescence assay. Despite a point mutation in its effector domain, this mutant is still able to bind GTP[24], although not p67^{phox}. Addition of Rac2 D38A to mant-GppNHp resulted in a blue-shift and 1.7-fold increase (**Fig. 5c**, spectrum B) in the intensity of the emission maximum of mant-GppNHp (spectrum A). This result showed the ability of this mutant to bind mant-GppNHp. Upon addition of cyt b, there was an additional increase (10 %) in intensity (spectrum C) from 27.1±0.6 to 29.1±0.7. This did not occur when p67^{phox} was added instead of cyt b (spectrum D overlaps B) and supports the concept of a dual functional role for Rac2. In this concept, the insert domain of Rac2 is necessary for an initial and independent interaction with cyt b, although the effector domain is necessary for subsequent binding to p67^{phox} to catalyze the second electron-transfer reaction.

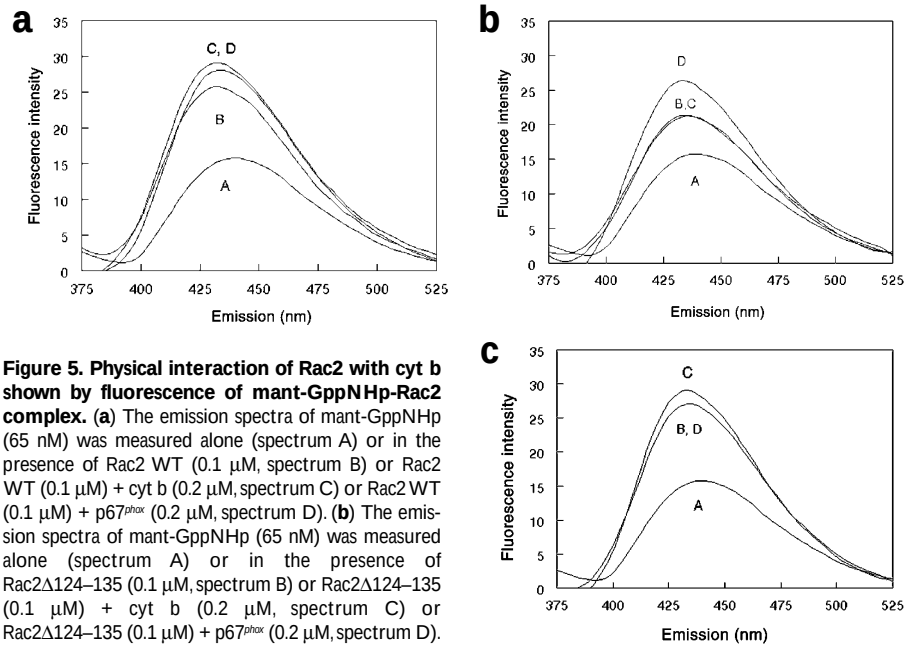

Figure 5. Physical interaction of Rac2 with cyt b shown by fluorescence of mant-GppNHp-Rac2 complex. (**a**) The emission spectra of mant-GppNHp (65 nM) was measured alone (spectrum A) or in the presence of Rac2 WT (0.1 μM, spectrum B) or Rac2 WT (0.1 μM) + cyt b (0.2 μM, spectrum C) or Rac2 WT (0.1 μM) + p67^{phox} (0.2 μM, spectrum D). (**b**) The emission spectra of mant-GppNHp (65 nM) was measured alone (spectrum A) or in the presence of Rac2Δ124–135 (0.1 μM, spectrum B) or Rac2Δ124–135 (0.1 μM) + cyt b (0.2 μM, spectrum C) or Rac2Δ124–135 (0.1 μM) + p67^{phox} (0.2 μM, spectrum D). (**c**) The emission spectra of mant-GppNHp (65 nM) was measured alone (spectrum A) or in the presence of 0.1 μM Rac2 D38A (spectrum B) or Rac2 D38A (0.1 μM) + cyt b (0.2 μM, spectrum C) or Rac2 D38A (0.1 μM) + p67^{phox} (0.2 μM, spectrum D). Data are the mean of spectra from three independent experiments.

Discussion

Phagocytic leukocytes serve as a critical component of the innate immune response, as shown by the prevalence of life-threatening infections in patients who have neutropenia or defects in neutrophil function. However, excessive or inappropriate formation of ROS by phagocytic cells can induce and/or contribute to a variety of inflammatory disorders, including rheumatoid arthritis, reperfusion injury and atherosclerosis. A detailed molecular understanding of the mechanisms regulating oxidant formation by phagocytic leukocytes may thus contribute to the development of more selective and effective anti-inflammatory therapies.

The Rac GTPase has been established as a required component of phagocytic NADPH oxidase that exerts a regulatory effect on the membrane-assembled complex. Earlier studies led to the current dogma that Rac regulates the NADPH oxidase *via* its interaction with $p67^{phox}$, with $p67^{phox}$ serving as the primary activator of electron flux. The identification of an NADPH oxidase "activation domain" on $p67^{phox}$ was consistent with a primary regulatory role of this component[30,31]. Consistent with this model, we have observed that Rac-$p67^{phox}$ interaction is required for the completion of electron transfer to cyt b. However, we have also shown that Rac has an independent function in regulation of the initial NADPH to FAD electron-transfer step of the human neutrophil NADPH oxidase. The data indicate that Rac can exert a direct regulatory effect on cyt b. This effect requires the Rac insert-domain and we show that this domain is required for physical interaction of Rac with cyt b. It has previously been established that the Rac insert domain is not required for interaction with $p67^{phox}$[25].

Regulation of the NADPH oxidase by Rac solely through its interaction with $p67^{phox}$ is inherently problematic because Rac binds to $p67^{phox}$ with a low affinity *in vitro*[23]. One could speculate that the initial interaction of Rac with cyt b may position Rac for more favorable binding to $p67^{phox}$. Alternatively, the interaction of Rac with cyt b is likely to induce a conformational change in that protein. This conformational change, or the Rac–cyt b complex itself, may increase the affinity of Rac for $p67^{phox}$, thereby promoting completion of electron transfer and superoxide formation.

In addition to its implications for the molecular basis for NADPH oxidase regulation in inflammatory leukocytes, this finding is of particular interest in light of the fact that the major production of ROS by nonphagocytic cells might occur by a mechanism similar to that of the phagocyte NADPH oxidase. The recently identified homolog of $gp91^{phox}$, called Mox1 (mitogenic oxidase), has been detected in several tissues, including colon, prostate, uterus and vascular smooth muscle cells[3]. Several other mammalian homologs of $gp91^{phox}$ have been detected in other superoxide-producing tissues such as fibroblasts, endothelial cells, kidney and thyroid. Although excessive ROS production is cytotoxic, moderate to low amounts of ROS can determine the redox state of intracellular proteins and regulate a variety of cell functions. These functions range from cell signaling (in growth and differentiation, apoptotic death and activation of transcription factors) to biosynthetic reactions (iodination of tyrosyl residues during thyroid hormone synthesis). ROS formation in at least some nonphagocytic cells appears to be Rac-dependent but it is not clear whether the other phagocyte NADPH oxidase components ($p22^{phox}$, $p47^{phox}$ and $p67^{phox}$) are present or play any regulatory role. Our data suggest the possibility of direct regulation by Rac of these nonphagocyte cytochromes, independent of other protein cofactors.

Thus, we have identified distinct regulatory interactions controlled by Rac2 GTPase in the regulation of NADPH oxidase activity. In support of our model, it has been observed that neutrophils from a patient with a mis-sense mutation of $gp91^{phox}$ ($Arg^{54} \rightarrow Ser^{54}$ substitution) were able to reduce FAD (as measured by the INT diaphorase assay) to approximately 50% of normal amounts. This occurred even though they were completely deficient in superoxide production[17]. A two-step activation process for electron transfer by the phagocyte NADPH oxidase has also been inferred from kinetic studies[32]. The two-step model of ROS production by Rac2 proposed here may provide a means of more effectively controlling the inflammatory responses of phagocytic leukocytes, as well as for more effectively controlling concentration of ROS generated intracellularly for signaling purposes.

Methods

Measurement of electron transfer steps. Superoxide production was determined as the rate of cyt c reduction that could be inhibited by superoxide dismutase[14,19]. Assays were carried out in a 96-well microtiter plate with a Thermomax kinetic microplate reader (Molecular Devices, Sunnyvale, CA). The typical reaction contained cyt b (2 nM), $p47^{phox}$ (141 nM), $p67^{phox}$ (400 nM), Rac2 (2 nM, loaded with GTPγS), FAD (200 nM) cyt c (100 μM) and SDS (90 μM) in relax buffer (10 mM PIPES at pH 7.3, 100 mM KCl, 3 mM NaCl and 3.5 mM $MgCl_2$). The mixture (145 μl) was incubated for 5 min at 25 °C. NADPH (5 μl) was added to a final concentration of 0.2 mM to initiate the reaction. An extinction coefficient at 550 nm of 21 $mM^{-1}cm^{-1}$ was used to calculate the amount of cyt c that had been reduced. The rate of FAD reduction was determined by substituting INT (100 μM)[16] for cyt c and including superoxide dismutase (30 U) in the reaction. The rate of INT reduction was monitored at 490 nm with an extinction coefficient of 11.0 $mM^{-1}cm^{-1}$.

Proteins. Recombinant $p47^{phox}$, $p67^{phox}$ and mutants of $p67^{phox}$ were expressed in *Escherichia coli* as GST-fusion proteins from pGEX-2T plasmids that were carrying the corresponding cDNAs. The proteins were purified with glutathione-Sepharose followed by thrombin cleavage, as described[33]. Thrombin was removed using p-aminobenzamidine-agarose. Further purification of $p67^{phox}$ was carried out with anion exchange chromatography (Mono Q) and size exclusion chromatography (Sephadex 75, Amershan Pharmaria Biotech, Piscataway, NJ). In Fig. 2 only, to compare these results to similar results that have been published[21], untagged wild-type $p67^{phox}$ was expressed in Sf9 insect cells and purified as described[30]. In Fig. 3a, a truncated form of $p67^{phox}$, p67(1–210), was used instead of full-length $p67^{phox}$ because its purification from *E. coli* yields a much purer protein than that of the wild-type form. This truncated form of $p67^{phox}$ is as active as the wild-type $p67^{phox}$ expressed in *E. coli* in the cell-free NADPH oxidase assay[30]. The $p67^{phox}$Δ178–184 mutant does not bind Rac GTPase[34].

The cDNA encoding Rac2 (or mutant forms of Rac2) was ligated into the baculovirus transfer vector pAcGLT (PharMingen, San Diego, CA). Purified pAcGLT plasmid encoding the GST-Rac2 fusion construct was used for homologous recombination with BaculoGold baculovirus before infection of Sf9 insect cells. The Sf9 culture was amplified twice and a plaque assay carried out to pick virus from individual plaques. The virus was amplified two or tree times and the supernatant from the final amplification was used to infect Sf9 cell cultures at a multiplicity of infection of five to ten. The cells were collected 72 h after infection. The cells were subjected to NH_2 cavitation and cell fractionation, as described[14], to obtain a 0.9% sodium cholate extract of solubilized membrane proteins. The GST-Rac2 was purified from this extract using glutathione-sepharose followed by thrombin cleavage and incubation with p-aminobenzamidine-agarose. The Rac2-containing supernatant was dialyzed for 18 h with Tris (25 mM at pH 7.5), EDTA (1 mM), DTT (1 mM) and $MgCl_2$ (5 mM). Rac2 was concentrated to 5–10 μM using a Centriplus 10 filtering device (Amicon, Danvers, IL). Rac2 was quantified and preloaded with GTPγS, as described[14]. Rac2 D38A has been shown to be deficient in $p67^{phox}$ binding[24,25].

Cyt b was purified from human neutrophil plasma membranes, relipidated and reflavinated as described[35] but with modifications[20].

Mutagenesis. The truncated $p67^{phox}$ clone, $p67^{phox}$(1–210), was prepared using full-length $p67^{phox}$ cDNA in pGEX-2T as the template, as described[30]. The deletion mutant, $p67^{phox}$(1–210)Δ178–184 was prepared using standard overlapping PCR with $p67^{phox}$(1–210) cDNA cloned in pGEX-2T as the template. The Rac2 D38A mutant was prepared by site-directed mutagenesis using PCR with Rac2 full-length cDNA cloned in pAcGHLT as the template.

Fluorescence-binding experiments. Fluorescence spectra were recorded as described but with a few modifications[25]. Spectra were taken using a Perkin-Elmer LS50B spectofluorimeter equipped with FLWINLAB software (Norwalk, CT). Mant-GppNHp was from Molecular Probes (Eugene, OR) and diluted in Tris (20 mM at pH 7.5), NaCl (3 mM), KCl (50 mM), EDTA (1 mM) and $MgCl_2$ (100 nM). The concentration of the stock solution was determined with an extinction coefficient of 23,000 M/cm at 255 nm. The emission spectrum of mant-GppNHp was measured with an excitation wavelength of 355 nm and slit widths of 5 nm for both excitation and emission. The spectra were scanned at 50 nm/min. The emission spectrum of mant-GppNHp (65 nM in 0.3 ml) was corrected with the spectrum of buffer alone. Rac2, or one of its mutant forms (100 nM), was added to the mant-

GppNHp solution and incubated for 10 min at 25 °C to allow "loading" of Rac2 with mant-GppNHp. $MgCl_2$ (10 mM) were added before measuring the spectrum again. This spectrum was corrected with the spectrum of Rac2 and buffer in the absence of mant-GppNHp. Cyt b, $p67^{phox}$ or $p47^{phox}$ (200 nM) was added and the solution incubated for an additional 10 min before the spectrum was measured. This spectrum was corrected with the spectrum of the specific protein together with Rac2 in the absence of mant-GppNHp. Volume changes upon the addition of proteins were 7% or less.

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1. Finkel, T. Signal transduction by reactive oxygen species in non-phagocytic cells. *J. Leukoc. Biol.* **65**, 337–340 (1999).
2. Babior, B. M. NADPH oxidase: an update. *Blood* **93**, 1464–1476 (1999).
3. Suh, Y. A. *et al.* Cell transformation by the superoxide-generating oxidase Mox1. *Nature* **401**, 79–82 (1999).
4. Banfi, B. *et al.* A mammalian H^+ channel generated through alternative splicing of the NADPH oxidase homolog NOH-1. *Science* **287**, 138–142 (2000).
5. De Deken, X. *et al.* Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *J. Biol. Chem.* **275**, 23227–23233 (2000).
6. Bokoch, G. M. Regulation of the human neutrophil NADPH oxidase by the Rac GTP-binding proteins. *Curr. Opin. Cell Biol.* **6**, 212–218 (1994).
7. Dorseuil, O. *et al.* Inhibition of superoxide production in B lymphocytes by Rac antisense oligonucleotides. *J. Biol. Chem.* **267**, 20540–20542 (1992).
8. Roberts, A. W. *et al.* Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity* **10**, 183–196 (1999).
9. Ambruso, D. *et al.* Human neutrophil immunodeficiency syndrome is associated with an inhibitory Rac2 mutation. *Proc. Natl Acad. Sci. USA* **97**, 4654–4659 (2000).
10. Voncken, J. W. *et al.* Increased neutrophil respiratory burst in Bcr null mutants. *Cell* **80**, 719–728 (1995).
11. Quinn, M. T., Evans, T., Loetterle, L. R., Jesaitis, A. J. & Bokoch, G. M. Translocation of Rac correlates with NADPH oxidase activation: Evidence for equimolar translocation of oxidase components. *J. Biol. Chem.* **268**, 20983–20987 (1993).
12. Philips, M. R., Feoktistov, A., Pillinger, M. H. & Abramson, S. B. Translocation of p21rac2 from cytosol to plasma membrane is neither necessary nor sufficient for neutrophil NADPH oxidase activity. *J. Biol. Chem.* **270**, 11514–11521 (1995).
13. Dorseuil, O., Quinn, M. T. & Bokoch, G. M. Dissociation of Rac translocation from p47phox/p67phox movements in human neutrophils by tyrosine kinase inhibitors. *J. Leukoc. Biol.* **58**, 108–113 (1995).
14. Heyworth, P. G. *et al.* Requirement for post-translational processing of Rac GTP-binding proteins for activation of human neutrophil NADPH oxidase. *Mol. Biol. Cell* **4**, 261–269 (1993).
15. Abo, A., Boyhon, A., West, I., Thrasher, A. J. & Segal, A. W. Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67-*phox*, 47-*phox*, p21 rac1 and cytochrome b_{-245}. *J. Biol. Chem.* **267**, 16767–16770 (1992).
16. Cross, A. R., Yarchover, J. L. & Curnutte, J. T. The superoxide-generating system of human neutrophils possesses a novel diaphorase activity: Evidence for distinct regulation of electron flow within NADPH oxidase by p67phox and p47phox. *J. Biol. Chem.* **269**, 21448–21454 (1994).
17. Cross, A., Heyworth, P., Rae, J. & Curnutte, J. A variant x-linked chronic granulomatous disease patient (X91+) with partially functional cytochrome b. *J. Biol. Chem.* **270**, 8194–8200 (1995).
18. Cross, A., Rae, J. & Curnutte, J. Cytochrome b_{-245} of the neutrophil superoxide-generating sytem contains two non-identical hemes. *J. Biol. Chem.* **270**, 17075–17077 (1995).
19. Bromberg, Y. & Pick, E. Activation of NADPH-dependent superoxide production in a cell-free system by sodium dodecyl sulfate. *J. Biol. Chem.* **260**, 13539–13545 (1985).
20. Cross, A. R. & Curnutte, J. T. The cytosolic factors p47-phox and p67-phox have distinct roles in the regulation of electron flow in NADPH oxidase. *J. Biol. Chem.* **270**, 6543–6548 (1995).
21. Freeman, J. L. & Lambeth, J. D. NADPH oxidase activity is independent of p47-*phox in vitro*. *J. Biol. Chem.* **271**, 22578–22582 (1996).
22. Koshkin, V., Lotan, O. & Pick, E. The cytosolic component p47(phox) is not a sine qua non participant in the activation of NADPH oxidase but is required for optimal superoxide production. *J. Biol. Chem.* **271**, 30326–30329 (1996).
23. Diekmann, D., Abo, A., Johnston, C., Segal, A. W. & Hall, A. Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity. *Science* **265**, 531–533 (1994).
24. Xu, X., Barry, D. C., Settleman, J., Schwartz, M. A. & Bokoch, G. M. Differing structural requirements for GTPase-activating protein responsiveness and NADPH oxidase activation by Rac. *J. Biol. Chem.* **269**, 23569–23574 (1994).
25. Nisimoto, Y., Freeman, J. L. R., Motalebi, S. A., Hirshberg, M. & Lambeth, J. D. Rac binding to p67phox: Structural basis for interactions of the Rac1 effector region and insert region with components of the respiratory burst oxidase. *J. Biol. Chem.* **272**, 18834–18841 (1997).
26. Heyworth, P. G., Bohl, B. P., Bokoch, G. M. & Curnutte, J. T. Rac translocates independently of the neutrophil NADPH oxidase components p47-*phox* and p67-*phox*. Evidence for its interaction with flavocytochrome b_{558}. *J. Biol. Chem.* **269**, 30749–30752 (1994).
27. Freeman, J. L., Abo, A. & Lambeth, J. D. Rac "insert region" is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65. *J. Biol. Chem.* **271**, 19794–19801 (1996).
28. Joneson, T. & Bar-Sagi, D. A Rac1 effector site controlling mitogenesis through superoxide production. *J. Biol. Chem.* **273**, 17991–17994 (1998).
29. Hiratsuka, T. New ribose-modified fluorescent analogs of adenine and guanine nucleotides available as substrates for various enzymes. *Biochim. Biophys. Acta* **742**, 496–508 (1983).
30. Han, C. H., Freeman, J. L., Lee, T., Motalebi, S. A. & Lambeth, J. D. Regulation of the neutrophil respiratory burst oxidase. Identification of an activation domain in p67(phox). *J. Biol. Chem.* **273**, 16663–16668 (1998).
31. Nisimoto, Y., Motalebi, S., Han, C.-H. & Lambeth, J. The p67*phox* activation domain regulates electron flow from NADPH to flavin in flavocytochrome b_{558}. *J. Biol. Chem.* **274**, 22999–23005 (1999).
32. Cross, A., Erickson, R. & Curnutte, J. Simultaneous presence of p47*phox* and flavocytochrome b_{-245} are required for the activation of NADPH oxidase by anionic amphiphiles. *J. Biol. Chem.* **274**, 15519–15525 (1999).
33. Guan, K. & Dixon, J. Eucaryotic protein expression in E. coli: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.* **192**, 262–267 (1991).
34. Ahmed, S. *et al.* Cryptic Rac-binding and $p21^{Cdc42HS/Rac}$-activated kinase phosphorylation sites of NADPH oxidase component $p67^{phox}$. *J. Biol. Chem.* **273**, 15693–15701 (1998).
35. Segal, A. W. *et al.* Cytochrome b_{-245} is a flavocytochrome containing FAD and the NADPH-binding site of the microbicidal oxidase of phagocytes. *Biochem. J.* **284**, 781–788 (1992).