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Rac1 Disrupts p67*phox*/p40*phox* Binding: A Novel Role for Rac in NADPH Oxidase Activation[1]

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Phagocytic cells possess a tightly regulated multicomponent enzyme complex, the NADPH oxidase, which produces superoxide, a reactive oxygen molecule that is an essential component of host defense against infection. Upon stimulation, a functional NADPH oxidase is assembled when the cytosolic proteins, Rac, p67*phox*, p47*phox*, and possibly p40*phox*, associate with the gp91*phox* and p22*phox* transmembrane proteins. Rac is a GTPase that in the GTP-bound state binds p67*phox* to activate NADPH oxidase. The function of p40*phox* is not known; it is believed to have a regulatory function in sequestering p67*phox* and p47*phox* in a cytosolic complex. We investigated binding interactions between p40*phox*, p67*phox*, and Rac and found that Rac1-GTP displaced p67*phox* bound to p40*phox*. In contrast, Cdc42, a GTPase homologous to Rac, did not displace p67*phox* from p40*phox*. A synthetic peptide corresponding to p67*phox* amino acids 170–199, a region identified previously as a Rac binding domain, significantly reduced the ability of Rac1-GTP to disrupt p67*phox*/p40*phox* binding. We hypothesize that Rac-GTP binds the p67*phox* N-terminal domain encompassing amino acids 170–199 that transmits a conformational change which causes p40*phox* to dissociate from its binding site in the p67*phox* C-terminus.

The NADPH oxidase in phagocytic cells, neutrophils, eosinophils, and macrophages is an essential component of the human innate immune response. Activity of this multicomponent enzyme is regulated tightly by a transmembrane signaling pathway that is triggered by binding of soluble and particulate ligands to cell surface receptors. A functional NADPH oxidase is formed upon translocation of cytosolic proteins Rac, p67*phox*, p47*phox*, and possibly p40*phox*, to membrane where they complex with cytochrome b558, a heterodimer composed of gp91*phox* and p22*phox*. Assembly involves a cascade of multiple protein-protein interactions between the oxidase proteins. Once activated, the oxidase transfers electrons from NADPH to molecular oxygen forming superoxide which is converted to other reactive oxygen species. The importance of NADPH oxidase is underscored by chronic granulomatous disease (CGD[1]) in which patients with mutations in genes encoding proteins of the NADPH oxidase are severely impaired in their ability to combat infection (1).

Rac1, Rac2, and Cdc42 are members of the Rho-family of low molecular weight GTPases that cycle between active GTP-bound and inactive GDP-bound states (2) and regulate a number of cellular processes including signaling pathways and cytoskeletal rearrangements (3). Conversion of Rac to its active form through GTP/GDP exchange precedes assembly of the NADPH oxidase (4, 5). Only Rac-GTP is able to activate the NADPH oxidase (6) even though both Rac-GTP and Cdc42-GTP bind p67*phox* (6, 7). Rac-GTP binding to p67*phox* was localized initially to the first 199 amino acids of p67*phox* (8). Subsequent studies showed that Rac1 and Rac2 are able to bind p67*phox* amino acids 170–199 (9).

p67*phox*, p47*phox*, and p40*phox* form a 240 kDa complex in the cytosol of inactive neutrophils (10, 11, 12, 13). Upon neutrophil activation, p67*phox* is translocated to membrane where it facilitates electron transfer by cytochrome b558 (14). p47*phox* phosphorylation coincides with signals that activate NADPH oxidase assembly and appears to induce a conformational change enabling p47*phox* to translocate to membrane and bind p22*phox* (15). p47*phox* is likely a chaperone

Abbreviations used: CGD, chronic granulomatous disease; GST, glutathione *S*-transferase; RB + T, relaxation buffer with 0.2% Tween; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; RBD, Rac-binding domain; PAGE, polyacrylamide gel electrophoresis.

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that facilitates oxidase assembly since p47*phox* is not absolutely required for activation of NADPH oxidase *in vitro* (16, 17).

p40*phox* is the least understood of the oxidase components. Levels of p40*phox* are reduced in CGD neutrophils deficient in p67*phox* (12, 13, 18, 19) suggesting that p40*phox* may stabilize and be stabilized by its interaction with p67*phox*. *In vitro* studies suggest that the entire cytosolic p40*phox* pool is bound in a tight complex with p67*phox* in resting neutrophils (11, 13). CGD patients lacking p40*phox,* however, have not been described. p40*phox* is not required for superoxide production *in vitro,* and p40*phox* overexpression in K562 cells decreases NADPH oxidase activity (20). p40*phox* mediates cytoskeletal attachment of p67*phox* and p47*phox* and may facilitate targeted translocation of p67*phox* and p47*phox* to membrane (21).

Since p40*phox* binds p67*phox* with high affinity (13, 22), we hypothesized that in one step of NADPH activation p67*phox* must be released from p40*phox* freeing p67*phox* to bind cytochrome b558 and to activate NADPH oxidase. In this report, we show that p67*phox*/p40*phox* binding was disrupted by Rac1-GTP but not Rac1-GDP or Cdc42-GTP. Our results suggest a novel role for Rac in activating NADPH oxidase.

MATERIALS AND METHODS

Construction, purification, and analysis of recombinant p40phox, Rac1, and p67phox. Recombinant GST-p40*phox,* GST-p67*phox* C-terminus (amino acids 301 to 526), GST-Rac1C189S, and GST-Cdc42 were produced in *E. coli* DH5α expressing pGEX-4T-1 into which cDNA encoding these proteins were cloned by the polymerase chain reaction as described (7). *E. coli* expressing GST-p40*phox* was grown at lower temperature (20–26°C) to increase the yield of recombinant protein. Expression was induced with isopropyl β-D-thiogalactopyranoside, recombinant protein was purified, and Rac1C189S and Cdc42 were cleaved from GST with thrombin as described (23). Recombinant p67*phox* was expressed and purified from baculovirus as described by Leto *et al.* (24). Oligonucleotide primer synthesis, DNA sequencing, and peptide synthesis were performed by the Biopolymer Core Facility at the University of Maryland, Baltimore.

Affinity precipitation and immunoblot analysis. 5 μg GST-p40*phox* was bound to 25 μl 50% Glutathione Sepharose 4B (Pharmacia, Piscataway, NJ) for 30 minutes at room temperature. Immobilized GST-p40*phox* was washed with 1× RB + T [relaxation buffer (100 mM KCl, 10 mM PIPES pH 7.4, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP) with 0.2% Tween 20] and resuspended in 50 μl RB + T. Rac1C189S was loaded with either GTPγS or GDPβS by incubating 2.5 μg Rac1C189S in 100 μl 50 mM KCl, 5 mM PIPES pH 7.0, 1.5 mM NaCl, 0.5 mM EDTA and 0.14 mM GTPγS or GDPβS for 4 minutes at 25°C. The reaction was stopped by adding 100 μl 200 mM Tris pH 8.0, 0.8 M NaCl, 240 mM $MgCl_2$, 16 mM dithiothreitol. 1.0 μg p67*phox* and 2.5 μg Rac1-GTPγS or GDPβS were added to the GST-p40*phox* bound resin in 500 μl 1/2× RB + T. After gentle mixing for 2 h at room temperature, the resin was washed with RB + T, extracted with sample buffer (26), and separated through a 8–16% Tris-Glycine gel (Novex, San Diego, CA). Proteins were transferred from the gel onto an Immobilon P membrane (Millipore, Bedford, MA), blocked with 5% nonfat dry milk in 15 mM Tris pH 7.5, 0.5 M NaCl, 0.2% Tween 20, incubated with 1:2500 goat anti-p67*phox* antibody, washed, and incubated with phosphatase conjugated 1:5000 rabbit anti-goat IgG (Kirkegaard and Perry, Gaithersburg, MD). After washing, the membrane was incubated with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium phosphatase substrate (Kirkegaard and Perry) until color development.

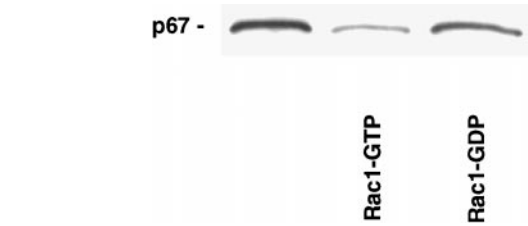

FIG. 1. Rac1-GTP inhibits p67*phox*/p40*phox* binding. Immobilized GST-p40*phox* was incubated with recombinant p67*phox* and Rac1-GTP or Rac1-GDP as described under Materials and Methods. Proteins bound to glutathione Sepharose beads were separated by SDS-PAGE, transferred to Immobilon P membrane, and probed with anti-p67*phox* serum. Immobilized GST-p40*phox* and recombinant p67*phox* were sham incubated with GTPγS (no Rac1) as shown in the left lane. Data presented are representative of 29 independent experiments.

RESULTS

Our laboratory reported recently that Rac binds the p67*phox* C-terminus (7). Since p40*phox* also binds the p67*phox* C-terminus (26, 27), we investigated whether Rac and p40*phox* compete for a common binding site in the p67*phox* C-terminus. GST-p40*phox* was immobilized onto glutathione Sepharose 4B resin and incubated with recombinant p67*phox*. Consistent with previous reports (13), we found that p67*phox* coprecipitated with immobilized GST-p40*phox* (Fig. 1) but not with immobilized GST protein (data not shown). Incubation in buffer containing 30 μM arachidonic acid did not disrupt p67*phox*/p40*phox* binding (data not shown). When Rac1-GTP was added to this coprecipitation, the amount of p67*phox* bound to immobilized GST-p40*phox* was reduced significantly indicating that Rac blocks p67*phox*/p40*phox* binding (Fig. 1). Rac1-GDP minimally reduced the amount of p67*phox* bound to immobilized GST-p40*phox* indicating that the ability of Rac1 to disrupt p67*phox*/p40*phox* binding is GTP-dependent (Fig. 1).

Rac-GTP blocks p67*phox*/p40*phox* binding when all three components are incubated simultaneously (Fig. 1). We investigated whether existing p67*phox*/p40*phox* complexes are disrupted by Rac-GTP binding. p67*phox* was incubated with Rac1-GTP for two hours (Fig. 2, lane 2, "Incubation"). GST-p40*phox* was added and the incubation continued for two additional hours. The amount of p67*phox* that coprecipitated with GST-p40*phox* was reduced significantly with Rac-GTP (Fig. 2, lane 2, "Pulldown") compared to the amount of p67*phox* that coprecipitated with GST-p40*phox* without Rac1-GTP (Fig. 2, lane 1). This result indicates that

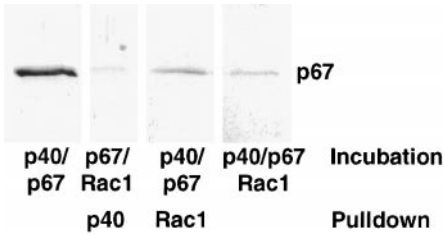

FIG. 2. Rac1-GTP disrupts existing p67*phox*/p40*phox* complexes. Immunoblot of immobilized GST-p40*phox* incubated with recombinant p67*phox* and Rac1-GTP and probed with anti-p67*phox* serum (see text). GST-p40*phox* incubated with p67*phox* (lane 1); p67*phox* incubated with Rac1-GTP for two hours before adding immobilized GST-p40*phox* during pulldown (lane 2); immobilized GST-p40*phox* incubated with p67*phox* for two hours before adding Rac1-GTP during pulldown (lane 3); immobilized GST-p40*phox* incubated simultaneously with p67*phox* and Rac1-GTP (lane 4). Data presented are representative of two independent experiments.

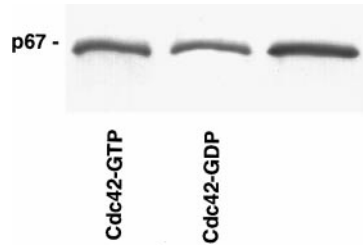

FIG. 3. Cdc42 does not block p67*phox*/p40*phox* binding. Immunoblot analysis of immobilized GST-p40*phox* incubated with recombinant p67*phox* and Cdc42-GTP or Cdc42-GDP and probed with anti-p67*phox* serum. Immobilized GST-p40*phox* and p67*phox* were sham incubated with GTPγS alone in the right lane. Data presented are representative of four independent experiments.

Rac1-GTP blocks p67*phox*/p40*phox* binding. Immobilized GST-p40*phox* was incubated with p67*phox* for two hours to allow p40*phox*/p67*phox* binding (Fig. 2, lanes 1 and 3, "Incubation"). Rac1-GTP was added and the incubation continued for two additional hours. Again, the amount of p67*phox* that coprecipitated with GST-p40*phox* was reduced significantly with Rac-GTP (Fig. 2, lane 3, "Pulldown") compared to the amount of p67*phox* that coprecipitated with GST-p40*phox* without Rac1-GTP (Fig. 2, lane 1). These results indicate that Rac1-GTP binds p67*phox* to disrupt existing p67*phox*/p40*phox* complexes.

Cdc42, a GTPase with extensive homology to Rac, binds the p67*phox* N-terminus in a GTP-dependent manner (6, 7). Despite this homology, Cdc42 is not functionally equivalent to Rac; Cdc42 does not activate NADPH oxidase *in vitro* (28). Incubation with either Cdc42-GTP or Cdc42-GDP did not reduce significantly the amount of p67*phox* that coprecipitated with immobilized GST-p40*phox* (Fig. 3) indicating that Cdc42 does not have the same effect on p67*phox*/p40*phox* binding as Rac1-GTP.

Several laboratories have reported GTP-dependent Rac binding to the p67*phox* N-terminus (7, 8, 9). In addition, we reported a novel GTP-independent Rac binding site in the p67*phox* C-terminus (7). To clarify whether Rac and p40*phox* compete for the same binding site in the p67*phox* C-terminus, we analyzed the binding interactions between immobilized GST-p40*phox*, p67*phox* C-terminus, and Rac-GTP. As expected, p67*phox* C-terminus coprecipitated with immobilized GST-p40*phox* (Fig. 4). Neither Rac1-GTP nor Rac1-GDP, however, was able to disrupt p67*phox* C-terminus/p40*phox* binding (Fig. 4) in contrast to the ability of Rac1-GTP to disrupt binding of full length p67*phox* to p40*phox* (Fig. 1). These results indicate that Rac1 does not compete with p40*phox* for the same p67*phox* C-terminal binding site and suggests that the ability of Rac to block p67*phox*/p40*phox* binding requires an interaction between Rac1-GTP and the p67*phox* N-terminus.

Ahmed *et al.* (9) localized a Rac-binding domain (RBD) to p67*phox* amino acids 170–199. We hypothesized that the ability of Rac to disrupt the p67*phox*/p40*phox* complex involves Rac-GTP binding to the RBD of p67*phox*. Immobilized GST-p40*phox* was incubated with p67*phox*, Rac1-GTP, and a synthetic peptide corresponding to p67*phox* amino acids 170–199. The amount of p67*phox* that coprecipitated with immobilized GST-p40*phox* in the presence of Rac1-GTP was increased significantly with 500 μM p67*phox* (170–199) peptide (Fig. 5, lane 2) compared to the amount of p67*phox* that coprecipitated without peptide (Fig. 5, lane 1). The p67*phox* (170–199) peptide reduced the ability of Rac-GTP to disrupt p67*phox*/p40*phox* binding.

DISCUSSION

p67*phox*, p47*phox*, gp91*phox*, p22*phox*, and Rac are required to assemble an active NADPH oxidase. Several investigators hypothesized that p40*phox* regulates the oxidase (20), prevents spontaneous activation, (22, 26), and functions as a chaperone for p67*phox* (19, 29). p40*phox* was also shown to tether p67*phox* and p47*phox* to the cytoskeleton by its interaction with

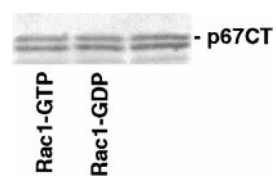

FIG. 4. Rac1-GTP does not inhibit p40*phox* binding to the p67*phox* C-terminus. Immunoblot analysis of immobilized GST-p40*phox* incubated with recombinant p67*phox* C-terminus (p67CT) and Rac1-GTP or Rac1-GDP and probed with anti-p67*phox* serum. The lower molecular weight band represents a p67*phox* C-terminus breakdown product. Immobilized GST-p40*phox* and p67*phox* C-terminus were sham incubated with GTPγS alone in the right lane. Data presented are representative of two independent experiments.

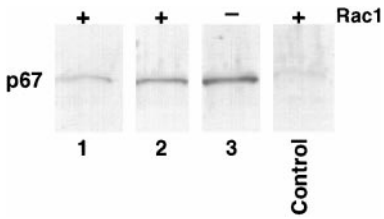

FIG. 5. p67*phox* (170–199) peptide prevents inhibition of p67*phox*/p40*phox* binding by Rac1-GTP. Immunoblot analysis of immobilized GST-p40*phox* incubated with recombinant p67*phox*, Rac1-GTP, and p67*phox* (170–199) peptide and probed with anti-p67*phox* serum. GST-p40*phox* incubated with recombinant p67*phox* and Rac1-GTP (lane 1); GST-p40*phox* incubated with recombinant p67*phox*, Rac1-GTP, and 500 μM p67*phox* (170–199) peptide (lane 2); GST-p40*phox* incubated with recombinant p67*phox* (lane 3); and GST protein incubated with recombinant p67*phox* and Rac1-GTP (Control). Data presented are representative of seven independent experiments.

coronin (21). p67*phox*/p40*phox* binding withstands buffers containing 1% (w/v) deoxycholate and 1% (v/v) Nonidet P-40 (13); 200 μM SDS, and 100 μM arachidonate (22). p40*phox* translocates to membrane upon activation *in vitro* (11, 22) and *in vivo* (12, 13, 19, 21). Even though p40*phox* is not required absolutely for superoxide production by NADPH oxidase *in vitro*, an antibody to the p40*phox* C-terminus disrupted p67*phox*/p40*phox* binding and suppressed NADPH oxidase activation *in vitro* (22) suggesting that p40*phox* regulates oxidase activation. This regulation may be indirect with p40*phox* modulating the activities of p67*phox* and p47*phox*.

The 240 kDa cytosolic complex of p67*phox*, p47*phox*, and p40*phox* produced low levels of oxidase activity that were increased significantly by adding recombinant p67*phox* in a dose dependent manner (30). The authors concluded that p40*phox* represses NADPH oxidase activity and suggested that an essential component of the 240 kDa complex is inactivated by p40*phox* (30). Lysates containing p40*phox* from baculovirus-infected cells inhibited *in vitro* oxidase activity in a dose-dependent manner, and p40*phox* over-expressed in K562 cells with p67*phox* and p47*phox* inhibited *in vivo* oxidase activity (20). The authors of this latter study concluded that the inhibitory effects of p40*phox* involve a p47*phox*/p40*phox* interaction not a p67*phox*/p40*phox* interaction since deletion of the p40*phox* C-terminal domain that binds p67*phox* did not decrease the inhibitory effects of p40*phox* (20).

Our present data clarify how p67*phox*/p40*phox* binding is regulated by Rac-GTP during activation of NADPH oxidase. Rac1-GTP not only blocks high affinity association of p67*phox* with p40*phox* (Fig. 1), it disrupts binding between existing p67*phox*/p40*phox* complexes (Fig. 2). This latter situation mimics more closely that found *in vivo* where p67*phox*/p40*phox* complexes are present in the cytosol of resting phagocytes (10, 11, 12, 13). Release of p67*phox*/p40*phox* binding appears to be specific to Rac. The Rac homologue, Cdc42, which also binds the p67*phox* N-terminus in a GTP-dependent manner (6, 7), did not disrupt p67*phox*/p40*phox* binding (Fig. 3).

Our laboratory reported a novel GTP-independent p67*phox* C-terminal binding site for Rac and Cdc42 (7). In this report, we show that Rac1 did not disrupt binding of p40*phox* to the p67*phox* C-terminus (Fig. 4) suggesting that Rac does not compete with p40*phox* for a common binding site in the p67*phox* C-terminus. We conclude that Rac-GTP interacts with a site in the p67*phox* N-terminus to disrupt p67*phox* C-terminus/p40*phox* binding. The fact that the p67*phox* (170–199) peptide corresponding to the RBD inhibits disruption of p67*phox*/p40*phox* binding by Rac1-GTP (Fig. 5) is additional evidence that Rac-GTP binds to the p67*phox* N-terminus. p67*phox* has been reported to change its conformation after activation *in vitro* (31) which possibly affects p40*phox* binding to the p67*phox* C-terminus. We hypothesize that Rac1-GTP binding to the p67*phox* RBD at amino acids 170–199 induces a conformational change in the p67*phox* C-terminus that reduces affinity of p67*phox* for p40*phox*. Cdc42-GTP binding to the p67*phox* N-terminus is not equivalent to Rac1-GTP binding since Cdc42-GTP does not activate NADPH oxidase (28). Cdc42-GTP binds the p67*phox* N-terminus but does not induce the conformational change needed to disrupt p67*phox*/p40*phox* binding.

We propose that the NADPH oxidase exists in an inactive conformation where p40*phox* sequesters p67*phox* and p47*phox* in an inactive 240 kDa complex. p40*phox* maintains p67*phox* and p47*phox* in an inactive state by blocking access to p67*phox* and p47*phox* binding domains required for oxidase assembly. Thus, the p67*phox*/p40*phox* interaction is an important control point in NADPH oxidase activation. During activation, Rac-GDP is converted to Rac-GTP and translocates to membrane (32). We propose that membrane-bound Rac-GTP binds the p67*phox* RBD, inducing a conformational change in the p67*phox* C-terminus that disrupts the p67*phox*/p40*phox* complex leading to release of p40*phox*. Our data present a novel role for Rac in oxidase activation in addition to its role in mediating the interaction and activation of cytochrome b558 by p67*phox*.

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