Antagonistic Cross-talk between Rac and Cdc42 GTPases Regulates Generation of Reactive Oxygen Species*

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Cross-talk between Rho GTPase family members (Rho, Rac, and Cdc42) plays important roles in modulating and coordinating downstream cellular responses resulting from Rho GTPase signaling. The NADPH oxidase of phagocytes and nonphagocytic cells is a Rac GTPase-regulated system that generates reactive oxygen species (ROS) for the purposes of innate immunity and intracellular signaling. We recently demonstrated that NADPH oxidase activation involves sequential interactions between Rac and the flavocytochrome b_{558} and p67^{phox} oxidase components to regulate electron transfer from NADPH to molecular oxygen. Here we identify an antagonistic interaction between Rac and the closely related GTPase Cdc42 at the level of flavocytochrome b_{558} that regulates the formation of ROS. Cdc42 is unable to stimulate ROS formation by NADPH oxidase, but Cdc42, like Rac1 and Rac2, was able to specifically bind to flavocytochrome b_{558} in vitro. Cdc42 acted as a competitive inhibitor of Rac1- and Rac2-mediated ROS formation in a recombinant cell-free oxidase system. Inhibition was dependent on the Cdc42 insert domain but not the Switch I region. Transient expression of Cdc42Q61L inhibited ROS formation induced by constitutively active Rac1 in an NADPH oxidase-expressing Cos7 cell line. Inhibition of Cdc42 activity by transduction of the Cdc42-binding domain of Wiscott-Aldrich syndrome protein into human neutrophils resulted in an enhanced fMetLeuPhe-induced oxidative response, consistent with inhibitory cross-talk between Rac and Cdc42 in activated neutrophils. We propose here a novel antagonism between Rac and Cdc42 GTPases at the level of the Nox proteins that modulates the generation of ROS used for host defense, cell signaling, and transformation.

The process by which cells produce reactive oxygen species (ROS) has gained much interest because of the diverse functions attributed to this class of molecules. In nonphagocytic cells, oxidants affect a variety of cellular processes, including transcription factor activation, proliferation, transformation, and apoptosis. In neutrophils and other phagocytes, oxidants play an important role in cellular innate immune responses. A critical component of the bactericidal activity of phagocytes is the NADPH oxidase, also referred to as “phox” (phagocytic oxidase) (1–3), which generates superoxide anion and, subsequently, a number of other ROS. The phagocyte NADPH oxidase is a multiprotein system whose activity is regulated by the RhoGTPase Rac2 in human cells (4–6). Electrons are transferred from NADPH to molecular oxygen through the action of an integral membrane flavocytochrome b_{558} (cyt b_{558}), composed of subunits gp91^{phox} and p22^{phox}. In addition to Rac2, the activity of the NADPH oxidase is regulated by the cytosolic components p47^{phox}, p67^{phox}, and p40^{crk}, which exist as a heterotrimeric complex in the cytosol of unstimulated neutrophils (7). In a separate cytosolic complex are Rac2 (or Rac1 in certain species) and GDP dissociation inhibitor (8). When neutrophils are activated, a series of interrelated regulatory events take place. p47^{phox} becomes phosphorylated and mediates translocation of the p47^{phox}/p67^{phox}/p40^{crk} complex to the plasma membrane (3). Rac2 and GDP dissociation inhibitor dissociate, followed by the guanine nucleotide exchange factor-mediated exchange of GTP for GDP and membrane localization of Rac2 (9). At the membrane, p67^{phox} and Rac2 interact with cyt b_{558} to form the functional NADPH oxidase complex. We have recently shown that Rac2 regulates NADPH oxidase activity via a two-step mechanism involving an initial functional interaction with cyt b_{558} to catalyze electron transfer to bound FAD, followed by a subsequent interaction with p67^{phox} that results in electron transfer to the cyt b_{558}/bound heme (10).

The formation of ROS in nonphagocytic cells also involves Nox (NADPH oxidase) enzymes (11–13). Nox enzymes are a group of homologues of gp91, the large subunit of the cyt b_{558} in the phagocyte NADPH oxidase (also referred to as Nox2). Recent studies on the Nox proteins indicate that the regulation of ROS production in nonphagocytic cells may parallel in many ways that of the phagocyte NADPH oxidase system, including regulation by p22^{phox} and by p47^{phox} and p67^{phox} or their homologues (14, 15).

Another similarity of ROS production by phagocytic and nonphagocytic cells is regulation by the Rac GTPase. The involvement of Rac2 in the NADPH oxidase of phagocytes has been confirmed by the generation of Rac2 and Bcr (a Rac GTPase-activating protein) null-mice (6, 16, 17) and through the use of Rac antisense oligonucleotides (18). There is also substantial evidence that Rac1 is involved in controlling ROS production in nonphagocytic cells, although a direct link to Nox has not been reported (2). For example, transient expression of constitutively active Rac1 in NIH3T3 fibroblasts increased O$_2^-$ production.
production in Ras-transformed cells (19). Rac1 and, specifically, the insert domain of Rac1 (amino acids 124-135), was necessary for O2 production and mitogenesis in fibroblasts (20). Rac1 expression in NIH3T3 cells also led to increased O2 production in response to various growth factors and hormones (e.g. platelet-derived growth factor and angiotensin II) (21). A direct regulation of Nox function by Rac GTase has been proposed (10).

Cross-talk between members of the Rho GTase family (Rho, Rac, and Cdc42) plays an important role in modulating and coordinating downstream cellular responses resulting from Rho GTase signaling. Many such regulatory interactions between Cdc42, Rac, and Rho have been described in the context of cytoskeletal remodeling during motility, presumably resulting in the coordinated functioning of the cellular cytoskeletal elements to promote smooth, continuous motion (22-24). Cross-talk in Rho GTase signaling occurs through a number of mechanisms. Individual Rho GTase family members can modulate the activity of guanine nucleotide exchange factors and/or GTase-activating proteins that control the activity of other Rho GTases. In addition, the ability of multiple RhoGTase family members to interact with common effectors also allows for cross-talk. In motile fibroblasts, Rac1 prevents the phosphorylation of myosin light chain through its effector p21-activated kinase, which phosphorylates and decreases the activity of myosin light chain kinase, thus decreasing the contractile force exerted by Rac action (25). This process serves to balance the protrusive forces generated by Rac and Cdc42, and the contractile forces initiated by Rho, a critical requirement for directional cell movement. To date, such complex interplay between Rho GTases has not been described for Rac-mediated formation of ROS.

In this paper, we identify cross-talk between Rac2 (and Rac1) and Cdc42 in regulation of ROS production by the phagocyte NADPH oxidase. We show that this inhibitory interaction results from a competition between the active Rac2 (or Rac1) oxidase-regulatory component and the oxidase-inactive Cdc42 for binding to flavocytochrome b558. This antagonistic cross-talk between these Rho GTase family members provides a novel mechanism by which oxidant production may be regulated in neutrophils and in other nonphagocytic cells.

EXPERIMENTAL PROCEDURES

Proteins—Recombinant p47phox and nonprenylated Rho GTases were expressed and purified as a glutathione S-transferase (GST) fusion protein in Escherichia coli (10). p67phox and prenylated Rho GTases were expressed and purified as GST fusion proteins in baculovirus-infected SF2 cells as previously described (10, 26). GST fusion proteins were cleaved with thrombin for use in enzyme assays. Rho GTases were quantified and preloaded with guanine nucleotides as reported (27). Cytochrome b558 was purified from human neutrophil membranes, relipidated, and refolded as reported to generate flavocytochrome b558, abbreviated as cyt b558 throughout (10).

Mutagenesis—The deletion mutant, Cdc42 Δ124−135 P136A, was prepared using standard overlapping PCR and site-directed mutagenesis. Proline 136 was mutated to Ala, as was previously done for Rac2 Δ124−135 (10, 28) to avoid possible structural perturbations. Cdc42 K27A, G30S and Rac2 A27K, G30S were prepared using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA).

In Vitro Binding Assay Using Purified Proteins—20–50 pmol of GST-Rac1, GST-Rac2, GST-Cdc42, or GST-RhoA (preloaded with either GTPyS or GDP) or an equivalent amount of GST were incubated with 5 pmol of cyt b558 and 30 μl of glutathione-Sepharose beads in 0.5 ml of Relax buffer (10 mM PIPES, pH 7.3, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2) for 18 h with constant inversion at 4 °C. The beads were collected by brief centrifugation and washed with Relax buffer before the addition of SDS-PAGE sample buffer. The precipitated proteins were subjected to SDS-PAGE and Western blot analysis using a monoclonal anti gp91phox antibody that was kindly supplied by Dr. Mark Quinn (Montana State University).

Cell-free Assay for Superoxide Production—Superoxide production was determined as the rate of superoxide dismutase-inhibitable cytochrome c reduction (28), as in Ref. 10. Cell-free assays typically contained cyt b558 (2 nm), p47phox (140 nm), p67phox (50 nm), prenylated Rac1 or Rac2-GTP·S (15 nm), FAD (200 nm), cytochrome c (100 μM), and SDS (60 μM). Nonprenylated Rac1-GTP·S was used at a concentration of 130 nm when substituted for prenylated Rac1-GTP·S. The mixture (145 μl) was incubated for 5 min at 25 °C before the addition of NADPH (0.2 μM). An extinction coefficient at 550 nm of 21.3 μM−1 cm−1 was used to calculate the rate of cytochrome c reduction.

Whole Cell Assay for Superoxide Production—Neutrophils were isolated from human venous blood donated by healthy donors as previously described (27). 4 × 106 neutrophils were incubated with 100 μM cytochrome c in 200 μl of KRGH buffer (25 mM Hepes, pH 7.4, 1.2 mM NaCl, 0.5 mM KCl, 11.8 mM NaHCO3, 1 mM MgCl2, and dextrose) for 5 min at 37 °C. Superoxide production was induced with fMetLeuPhe (10−5 M). For Cos-phox cells (a Cos-7 cell line that stably expresses the phagocytic NADPH oxidase components), 2.5 × 106 cells were incubated with 100 μM cytochrome c in 250 μl of PBS buffer (phosphate-buffered saline supplemented with 0.9 mM CaCl2, 0.5 mM MgCl2, and 7.5 mM glucose).

Cos-phox Cell Culture and Transfection—Cos-7 cells stably transfected with cDNA for p47phox, p67phox, gp91phox, and p22phox were maintained as previously described (30). LipofectAMINE Plus (Invitrogen) was used to transfect 6 μg of total cDNA (pRK5m-RhoGTases)/10-cm plate. Transfection efficiency (50%) was determined by transfecting the manufacturer's instructions, and 2–3 μl was aliquoted into 10-cm Eppendorf tubes and evaporated overnight. 100 μl of phosphate-buffered saline containing 30 μg of WASP-CRIB protein was used to rehydrate the Biporter reagent. Neutrophils (3.0 × 106) in 900 μl of KRGH buffer were added, and the mixture was inverted for 1 h at 25 °C. Triplicate tubes were prepared for each protein, and bovine serum albumin was used in place of WASP CRIB as a reference for 100% activity. An aliquot (200 μl) of cells was removed after the incubation period, and cytochrome c (100 μM) and mLFM (10−5 M) were added to measure the rate of superoxide production as described above.

Transduction of Tat Fusion Proteins into Neutrophils—The pHis-Tat-HA-WASP-CRIB and pHis-Tat-HA-mutant WASP-CRIB (H246A, H249A, and G251A, D253A) vectors were a kind gift from Dr. Jacques Bertoglio (INSERM, France). The proteins were expressed in E. coli and purified from cell lysates using Ni2+–agarose chromatography as described (32, 33) under nonnondenaturing conditions. The proteins were used immediately after removing imidazole by dialysis. Neutrophils (2 × 106) were incubated with 100 μg of Tat-WASP-CRIB or Tat-mutWASP-CRIB protein in 1 ml of KRGH buffer at 37 °C for 30 min. After 30 min, 0.2 ml of the cells were assayed for superoxide production.

RESULTS

Cdc42 and Rac2 Interact with Cyt b558 from Human Neutrophils—We previously reported that recombinant Rac2 interacted with cyt b558 in vitro as determined using changes in the fluorescence of Rac-bound mant-GppNHp (10). In support of this result, we observed that cyt b558 binds to prenylated GST-Rac2 in pull-down assays (Fig. 1A). The interaction of cyt b558 with Rac2 was only slightly enhanced when Rac2 was loaded with GTP versus GDP. Binding did not occur to GST alone or to GST-RhoA. Prenylated GST-Rac1 behaved similarly when substituted for Rac2 (data not shown). Interestingly, however, we observed that cyt b558 bound to prenylated GST-Cdc42 in a manner that was also insensitive to the nucleotide state of Cdc42. This was unexpected, because Cdc42 has been shown to...
be unable to activate the NADPH oxidase (27, 34, 35). Both
GTPase—A and -B were used in the absence of cyt b558.

Cyt b558 could be detected in these assays using GST-Rac2-cyt b558 ratios in the range of 2–10. In the cell-free assays a Rac:cyt b558 ratio of 5 was required for maximal activity using prenylated Rac2 or Rac1 (Fig. 1C).

**Mutation of the Switch 1 Region of Cdc42 Enables Activation of the NADPH Oxidase**—Kwong et al. (35) reported that by mutating Cdc42 at two residues (K27A and S30G) in the Switch I domain, Cdc42 could now mimic Rac1 in activating the NADPH oxidase in a cell-free system. The amino acids at positions 27 and 30 have been shown to be critical for the interaction between Rac and p67phox in the derived crystal structure (36), and these residues are conserved in Rac1 and Rac2 but not Cdc42 or RhoA. We verified that the K27A.S30G Cdc42 mutant was as effective as Rac2 in supporting NADPH oxidase activity in our system (Fig. 2). This result implies that if any other domain(s) of Rac are important for NADPH oxidase function, then those domains must be functionally conserved in Cdc42, because mutating only the two Switch I residues enables Cdc42 to be fully active in the oxidase system. One such oxidase regulatory region is the insert domain of Rac. As noted previously, this region is involved in regulation of NADPH oxidase activity (10, 28, 37), and we have shown that it mediates binding to cyt b558 (10). Fig. 3 demonstrates that the insert region (amino acids 124–135) of prenylated Rac2 and Cdc42 was necessary for their interaction with cyt b558. We therefore hypothesized that Cdc42 might compete with Rac for binding to cyt b558 through this region and thus inhibit superoxide production through the formation of a nonfunctional NADPH oxidase complex.

**Cdc42 Inhibits Activation of the NADPH Oxidase in a Cell-free System**—We tested our hypothesis initially using the well established, semi-recombinant NADPH oxidase cell-free system. Purified cyt b558 from neutrophil membranes, reconstituted with FAD, was incubated with recombinant p47phox, p67phox, Rac2-GTP, and varying concentrations of Cdc42 in the presence of SDS as activator. As shown in Fig. 4A (curve a), using prenylated Rho GTPases, inclusion of Cdc42-GTP in this system inhibited the rate of superoxide production. Cdc42 in the GDP-bound form was also inhibitory but to a slightly lesser degree (curve b), consistent with somewhat decreased binding to cyt b558. When a 5-fold higher concentration of Rac was used in the assay, the degree of inhibition by Cdc42-GTP was decreased (curve c). In contrast to Cdc42, RhoA-GTP over the same concentration range was not inhibitory (curve d).

To test the role of the Cdc42 insert domain in this inhibitory effect, we made a deletion mutant of prenylated Cdc42 (Cdc42 Δins) that lacks the insert domain (amino acids 124–135) and used this in place of wild type Cdc42 in our assays. Cdc42 Δins in the GTP bound form was also inhibitory but to a slightly lesser degree (curve e), consistent with somewhat decreased binding to cyt b558. When a 5-fold higher concentration of Rac was used in the assay, the degree of inhibition by Cdc42-GTP was decreased (curve f). In contrast to Cdc42, RhoA-GTP over the same concentration range was not inhibitory (curve g).

Prenylated Cdc42WT was also inhibitory when either prenylated Rac1-GTPyS (Fig. 4C) or nonprenylated Rac1-GTPyS (Fig. 4D) was substituted for prenylated Rac2, indicating that Cdc42 also competes with Rac1. The insert domain of prenylated Cdc42 was also required for its ability to inhibit Rac1-dependent superoxide production by the cell-free system (Fig. 4, C and D) Nonprenylated Cdc42 was not inhibitory in any of the cell-free assays. This may reflect the need for the strong association of Cdc42 with the plasma membrane afforded by the additional prenyl group to effectively compete with the higher affinity Rac binding to cyt b558.

**Rac and Cdc42 Antagonize Each Other at the Level of Cyt b558 Binding**—To determine whether the competition between Rac and Cdc42 in the cell-free system occurs because of competition for cyt b558 binding, we included increasing amounts of...
untagged, prenylated Cdc42 in a GST-Rac2 pull-down of cyt b_{558}. As shown in Fig. 5, the amount of cyt b_{558} pulled down by GST-Rac2 decreased as the amount of Cdc42 in the assay increased. A similar result was observed when untagged, prenylated Rac2 was included in the pull-down of cyt b_{558} by GST-Cdc42 and when Rac1 (prenylated or nonprenylated) was substituted for Rac2.

Cdc42 Inhibits Rac-induced Oxidant Production in Cos7-phox Cells—To assess whether Cdc42 was capable of inhibiting NADPH oxidase activity in vivo, we used a genetically engineered Cos7 cell line (Cos-phox cells) (30) that stably express endogenous Rac1 and generates little or no superoxide under non-stimulated conditions (Fig. 6, bar A). However, as reported (30), transfection of constitutively active Rac1Q61L into these cells resulted in superoxide production without any additional stimulation (bar B). Co-transfection of Rac1Q61L plus Cdc42Q61L into these cells resulted in ~50% inhibition of the superoxide formation observed with Rac1Q61L alone (bar C). This level of inhibition is consistent with the efficiency of transfection of both of these Rho GTPases in the Cos-phox cells, which we determined to be ~50%. The hypothesis that the insert domain of Cdc42 is involved in the inhibition of oxidase activity was further supported by the observation that transfection of Cdc42Q61LΔins along with Rac1Q61L resulted in only ~10% inhibition of ROS production (bar D). In contrast to the action of Cdc42, co-transfection of Rac1Q61L with RhoAQ61L resulted in no inhibition (bar E). Transfection of Cdc42Q61L alone (bar F) or of RhoAQ63L alone (bar G) had no effect on ROS production by the Cos-phox cells. The expression of Rac1Q61L was equivalent whether co-transfected with vector alone or in combination with vectors containing cDNA for other Rho GTPases, as determined by Western blot (data not shown).

Inhibition of Endogenous Cdc42 Enhances fMLF-stimulated Neutrophil NADPH Oxidase Activity—In addition to the role of Rac2 in NADPH oxidase activation, both Rac2 and Cdc42 play important roles in neutrophil chemotaxis (31, 38, 39). During
Cdc42 Inhibits Rac-induced Oxidant Production

The effector domains (Switch I) of Rac1/2 and Cdc42 differ by four amino acids, and as shown originally by Kwong et al. (35), mutation of two of these residues, K27A and S30G, enables Cdc42 to function as an activator of the NADPH oxidase. The Rac Switch I domain in its GTP-bound form interacts with the tetratricopeptide repeat of p67phox (36), a direct activator of the NADPH oxidase (43). The crystal structure of the Rac-p67phox complex revealed that Ala27 and Gly30 of Rac are crucial to the stability of this assembly (36). Indeed, the differences in these two key residues in Cdc42 prevent it from binding to the tetratricopeptide repeat of p67phox, thus explaining structurally the inability of Cdc42 to activate the NADPH oxidase. Likewise, the ability of Cdc42 K27A,S30G to fully support NADPH oxidase activity indicates that it is able to interact functionally with p67phox through the mutated Switch I domain, as well as with cyt b558 through the conserved insert domain. This conclusion is supported by the observed loss of cyt b558 binding when the insert domain of Cdc42 is deleted (Fig. 3).

Using an NADPH oxidase cell-free system, we found that Cdc42 inhibited superoxide production. This inhibition was only partially GTP-dependent but was dependent upon the insert domain of Cdc42. We have shown that the insert domain of Rac2 mediates a physical interaction with cyt b558 and that the Rac insert domain is necessary for superoxide production (10). The lack of absolute GTP dependence reflects the absence of GTP-induced conformational changes in the insert region (44). Inhibition of cell-free NADPH oxidase activity by Cdc42 was reduced when the concentration of Rac2 in the assay was increased (Fig. 4), suggesting that Cdc42 competes with Rac2 for binding to cyt b558 via the insert domain. This was confirmed in a direct competition binding assay (Fig. 5). Consistent with these in vitro observations, Cdc42 also inhibited Rac-induced ROS formation in intact Cos cells expressing a functional NADPH oxidase (Fig. 6).

We have examined the ability of both prenylated and nonprenylated Cdc42 to inhibit the NADPH oxidase cell-free system. Prenylated Cdc42, but not nonprenylated Cdc42, was inhibitory whether prenylated Rac2, prenylated Rac1, or nonprenylated Rac1 was present in the assay. Prenylation of Rac2 has been shown to be required for Rac2 to translocate and support NADPH oxidase activity in cell-free assays using neutrophil membranes (27) as well as with lipid micelles (46). We observed that nonprenylated Rac2 was not active in the cell-free system using purified, relipidated cyt b558 even at concentrations where nonprenylated Rac1 is active. Nonprenylated Rac1 binds to membranes (or lipid micelles) via its polybasic region (amino acids 183–188), which contains 6 basic residues. In contrast, the polybasic region of Rac2 contains only 3 basic residues, and this is apparently insufficient for membrane targeting. Although nonprenylated Rac1 can support superoxide production in the cell-free system, the concentration of Rac1 required for optimal activity in the cell-free assay was lowered about 10-fold when prenylated Rac1 was used (Fig. 4, C and D).

Nonprenylated Cdc42 was not inhibitory in any of the cell-free assays (Fig. 4, C and D). We speculate that the polybasic region of Cdc42, which contains only four basic residues, is not sufficient to allow binding of Cdc42 to cyt b558-containing lipid micelles, and the subsequent interaction of Cdc42 with cyt b558.

In the GST pull-down assays presented here, we have used the more "physiological" prenylated Rac1, Rac2, and Cdc42 GTPases. We have, however, observed that nonprenylated GST-Rac1 (in the GTP-S- or GDP-bound form) can bind cyt
Cdc42 Inhibits Rac-induced Oxidant Production

b_{558}, but a 10-fold greater ratio of Rac1 to cyt b_{558} is required. As in the cell-free assays, nonprenylated Cdc42 neither competed with nonprenylated Rac1 for binding to cyt b_{558} nor could it pull down cyt b_{558} even at a 10-fold higher concentration than that used for prenylated GST-Cdc42. We have observed that nonprenylated Rac2 and Cdc42 (as well as Rac1) will interact directly with the recombinant, nonprenylated C-terminal tail of GST-Gp91. This suggests that prenylation is not required for the direct interaction of Rac or Cdc42 with cyt b_{558} but only for localization to the membrane or micelles containing cyt b_{558}.

It has been previously described that Cdc42T17N inhibited fMLF-stimulated superoxide production when conditionally expressed in differentiated HL60 cells (47). This study reported that dominant negative Cdc42T17N (but not active Cdc42) expression interfered with GTP-loading of Rac and Ras, the activation of the mitogen-activated protein kinase pathway, and phospholipase C\(\beta\)2 function. Cdc42 T17N did not, however, inhibit superoxide in the cell-free system in that study. These effects appeared to be due to the action of dominant negative Cdc42 on various exchange factors involved in Rac and Ras activation. In contrast, we have demonstrated a direct, insert domain-dependent, competitive effect of Cdc42 on the NADPH oxidase itself, mediated through binding to cyt b_{558}.

The observation that Cdc42 can antagonize NADPH oxidase activation by Rac is interesting in light of the fact that Cdc42 is activated essentially simultaneously with Rac2 in chemottractant-stimulated human neutrophils (40). Activation of Cdc42 is most likely required for the cell polarization necessary for leukocyte chemotaxis, as well as for assembly of the motile actin machinery via the WASP-Arp2/3 complex (48, 49). However, the increased level of active Cdc42 appears to act to suppress ROS formation, as evidenced by a 2–3-fold increase in ROS formation when active Cdc42 is sequestered by introduction of the WASP-CRIB domain into the neutrophil (Fig. 7). We speculate that Cdc42 may thereby serve as a tonic regulator to “dampen” the amount of ROS generated during leukocyte migration through tissues. Cdc42 might also inhibit full oxidant production until actin reorganization has taken place to form the phagocytic cup and bacterial uptake is completed. This would coordinate oxidant production with the bacterial uptake process for the most efficient killing. It was reported that WASP is required for efficient phagocytosis of apoptotic cells by macrophages and dendritic cells (50). Perhaps another role for endogenous WASP is to relieve the inhibition of superoxide production by Cdc42 during the phagocytic microbial killing process. Additionally, we propose that the release and activation of Cdc42 could serve as a means for acute regulation of ROS formation. We hypothesize that stimuli that can directly activate Cdc42 but not Rac, for example bradykinin (51), might exert an inhibitory effect on NADPH oxidase output. Indeed, bradykinin has been reported to inhibit NADPH oxidase activity of leukocytes (52). Finally, certain bacteria are known to secrete virulence factors that influence the activation state of Rac and Cdc42 to evade killing by innate immune responses (53, 54). The ability of such bacteria to modulate Cdc42 activity may play a role in suppressing the formation of ROS by the host cell, thereby blocking the bactericidal activity of the infected cell.

Regulation of oxidant production through a competitive mechanism involving the acute regulation of Cdc42 may be even more relevant to controlling ROS formation in nonphagocytic cells. The existence of cyt b_{558} homologues, termed Nox proteins, in nonphagocytic cells has been demonstrated (11–13). The formation of ROS in nonphagocytes is thought to be involved in intracellular signaling processes involved in regulating transcription, proliferation, and cell death, as well as possibly in innate immunity. These systems appear to be regulated in a manner analogous to the phagocyte NADPH oxidase, including activation by Rac1 GTPase (19, 55), through direct interactions of Rac1 with the Nox homologues themselves. If so, then we hypothesize that a competitive inhibition by Cdc42 is likely to occur that would effectively suppress the generation of the low levels of ROS formed by these oxidases, consistent with the competitive inhibition of Rac1-mediated ROS production observed here (Fig. 4, C and D). Rac1 has been shown to lead to a decrease in GTP-bound Rho in fibroblasts through the generation of ROS, which act to inhibit activity of a low molecular weight protein-tyrosine phosphatase (45). This results in the prolonged phosphorylation and activity of p190 Rho GTPase-activating protein, thereby inhibiting Rho by promoting GTP hydrolysis. Our current data suggest the possibility that Cdc42 activity might also play a role in the complex interplay among Rho GTPases in such systems, providing additional means to regulate such GTPase-regulated signaling events.

In summary, we have demonstrated an antagonistic effect of Cdc42 on ROS formation by the prototypal phagocyte NADPH oxidase. This effect occurs because of a previously unrecognized interaction of Cdc42 with the cyt b_{558} component of the NADPH oxidase. This mechanism provides for a direct means of modulating the formation of ROS in phagocytic leukocytes and, potentially, in nonphagocytic cells in which ROS are generated through Nox homologues.

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