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 b558 and p67phox 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 b558 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 b558 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 Wischott-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 b558 (cyt b558), composed of subunits gp91phox and p22phox. In addition to Rac2, the activity of the NADPH oxidase is regulated by the cytosolic components p47phox, p67phox, and p40phox, 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. p47phox becomes phosphorylated and mediates translocation of the p47phox/p67phox/p40phox 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, p67phox and Rac2 interact with cyt b558 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 b558 to catalyze electron transfer to bound FAD, followed by a subsequent interaction with p67phox that results in electron transfer to the cyt b558-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 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 p22phox and by p47phox and p67phox 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 O2.
production in Ras-transformed cells (19). Rac1 and, specifically, the insert domain of Rac1 (amino acids 124–135), was necessary for O$_2^-$ production and mitogenesis in fibroblasts (20). Rac1 expression in NIH3T3 cells also led to increased O$_2^-$ 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 GTpase has been proposed (10).

Cross-talk between members of the Rho GTPase family (Rho, Rac, and Cdc42) plays an important role in modulating and coordinating downstream cellular responses resulting from Rho GTPase 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 GTPase signaling occurs through a number of mechanisms. Individual Rho GTPase family members can modulate the activity of guanine nucleotide exchange factors and/or GTPase-activating proteins that control the activity of other Rho GTPases. In addition, the ability of multiple RhoGTPase family members to interact with common effectors also allows for cross-talk. In motile fibroblasts, Rac1 prevents the phosphor-ylation 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 Rho 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 GTPases 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 b$_{558}$. This antagonistic cross-talk between these Rho GTPase family members provides a novel mechanism by which oxidant production may be regulated in neutrophils and in other nonphagocytic cells.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Recombinant p47$^{	ext{phox}}$ and nonprenylated Rho GTPases were expressed and purified as a glutathione S-transferase (GST) fusion protein in *Escherichia coli* (10). p67$^{	ext{phox}}$ and prenylated Rho GTPases were expressed and purified as GST fusion proteins in baculovirus-infected SF9 cells as previously described (10, 26). GST fusion proteins were cleaved with thrombin for use in enzyme assays. Rho GTPases were quantified and preloaded with guanine nucleotides as described previously (27). Rac1 and, specifically, the oxidase-regulatory component and the oxidase-inactive Cdc42, expressed and purified from *E. coli* and quantified by BCA protein assay (Pierce). The proteins were delivered into neutrophils as previously described (31). Briefly, Biopporter reagent (Gene Therapy Systems, La Jolla, CA) was reconstituted with chloroform according to the manufacturer's instructions, and 2–3 $\mu$l was aliquoted into Eppendorf tubes and evaporated overnight. 100 $\mu$l of phosphate-buffered saline containing 50 $\mu$l of WASP-CRB protein was used to rehydrate the Biopporter reagent. Neutrophils (3.0 x 10$^6$) were incubated for 5 min 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 $\mu$l) of each cell was removed after the incubation period, and cytochrome c (100 $\mu$m) and FMLP (10 $^{-7}$ 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, V250A,G251A,D253A) vectors were a kind gift from Dr. Jacques Bertoglio (INSERM, France). The proteins were expressed in *E. coli* and purified using Ni$^{2+}$-agarose chromatography as described (32, 33) under nondenaturing conditions. The proteins were used immediately after removing imidazole by dialysis. Neutrophils (2 x 10$^6$) were incubated with 100 $\mu$g of Tat-WASP-CRIB or Tat-mutWASP-CRIB protein in 1 ml of KRH buffer at 37 °C for 30 min. After 30 min, 0.2 ml of the cells was assayed for superoxide production.

**RESULTS**

Cdc42 and Rac2 Interact with Cyt b$_{558}$ from Human Neutrophils—We previously reported that recombinant Rac2 interacted with cyt b$_{558}$ in vitro as determined using changes in the fluorescence of Rac-bound mant-GppNHp (10). In support of this result, we observed that cyt b$_{558}$ binds to prenylated GST-Rac2 in pull-down assays (Fig. 1A). The interaction of cyt b$_{558}$ 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 b$_{558}$ 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...
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be unable to activate the NADPH oxidase (27, 34, 35). Both
GTPase. This may reflect the need for a strong association of
Cdc42 with the plasma membrane afforded by the
insert domain of prenylated Rac2 and Cdc42, because
mutating only the two Switch I residues enabled 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 b$_{558}$(10). Fig. 3 demonstrates that the insert region (amino acids 124–135) of prenylated Rac2 and Cdc42 was necessary for their interaction with cyt b$_{558}$. We therefore hypothesized that Cdc42 might compete with Rac for binding to cyt b$_{558}$ 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 b$_{558}$ from neutrophil membranes, reconstituted with FAD, was incubated with recombinant p47$_{phox}$, p67$_{phox}$, 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 b$_{558}$. 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 $\Delta$ins) that lacks the insert domain (amino acids 124–135) and used this in place of wild type Cdc42 in our assays. Cdc42 $\Delta$ins in the GTP bound form was also inhibitory but to a slightly lesser degree (curve b), consistent with somewhat decreased binding to cyt b$_{558}$. 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).

Prenylated Cdc42WT was also inhibitory when either prenylated Rac1-GTP$\gamma$S (Fig. 4C) or nonprenylated Rac1-GTP$\gamma$S (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 b$_{558}$.

Rac and Cdc42 Antagonize Each Other at the Level of Cyt b$_{558}$ Binding—To determine whether the competition between Rac and Cdc42 in the cell-free system occurs because of competition for cyt b$_{558}$ 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 expresses the required components of the NADPH oxidase (gp91
phox, p22
phox, p47
phox, and p67
phox). This cell line expresses 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 Cdc42Q61L alone 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...
As an alternative method of inhibiting endogenous Cdc42, we transduced neutrophils with a Tat-CRIB fusion protein (42) that was shown to bind endogenous Cdc42 in a T-cell line and inhibit stromal cell-derived factor 1-induced chemotaxis (32). 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 b<sub>558</sub> 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 b<sub>558</sub>-containing lipid micelles, and the subsequent interaction of Cdc42 with cyt b<sub>558</sub>. 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 GTPyS- or GDP-bound form) can bind cyt generated during neutrophil activation might act to "dampen" NADPH oxidase activity, thereby limiting the amount of superoxide produced upon neutrophil stimulation.

**DISCUSSION**

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 p67<sub>fphox</sub> (36), a direct activator of the NADPH oxidase (43). The crystal structure of the Rac-p67<sub>fphox</sub> complex revealed that Ala<sup>57</sup> and Glu<sup>30</sup> 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 p67<sub>fphox</sub>, 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 p67<sub>fphox</sub> through the mutated Switch I domain, as well as with cyt b<sub>558</sub> through the conserved insert domain. This conclusion is supported by the observed loss of cyt b<sub>558</sub> 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 b<sub>558</sub> 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 b<sub>558</sub> 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).

Cdc42 inhibits Rac-induced Oxidant Production

![Fig. 7. WASP-PBD inhibits endogenous Cdc42 and increases superoxide production by neutrophils.](image-url)

A, human neutrophils (3 × 10<sup>6</sup>/ml) in KRHG buffer was incubated with a mixture of Bioporter reagent and 30 µg/ml bovine serum albumin (bar A), 10 µg/ml His-WASP-PBD (bar B), or 30 µg/ml His-WASP-PBD (bar C) or with 10 µg/ml Tat-protein (bar D) or 30 µg/ml His-WASP-PBD (bar E) mutared His-WASP-PBD (F271C,H246D,H249D) at 25 °C for 1 h. After 1 h the cells were tested for superoxide production as described under “Experimental Procedures.” B, human neutrophils (2 × 10<sup>6</sup>/ml) were incubated with Tat-protein (bar A), mutant His-Tat-HA-WASP-PBD (H246A, H249A,Y250A,G251A,D253A) protein (100 µg/ml) (bar B), or His-Tat-HA-WASP-PBD protein (100 µg/ml) (bar C) for 30 min at 37 °C. The neutrophils were analyzed for FML-induced superoxide production as described under “Experimental Procedures.” The rate of superoxide production is the average of three experiments using different blood donors.

stimulation of human neutrophils with the chemoattractant fMLF, we previously demonstrated that both Rac2 and Cdc42 are activated very early in the response with similar time courses (40). This suggested the hypothesis that Cdc42 might act as an endogenous inhibitor of superoxide production that limits the amount of ROS generated upon leukocyte stimulation. To specifically inhibit Cdc42 during neutrophil activation, the CRIB domain of WASP, which binds Cdc42 but not Rac (or Rho) (41), was introduced into human neutrophils using Bioporter. We have previously shown that this method successfully delivers fluorescently labeled p21-activated kinase CRIB domain into human neutrophils (31). As shown in Fig. 7A, neutrophils that had WASP-CRIB protein introduced into them generated 2–3-fold more superoxide (bars B and C) compared with neutrophils that were treated with Bioporter plus bovine serum albumin (bar A). As a negative control, a triple mutant of the WASP CRIB domain (F271C,H246D,H249D), which cannot bind Cdc42 (32) was introduced into neutrophils. These cells showed no enhancement of superoxide production (bars D and E).

As an alternative method of inhibiting endogenous Cdc42, we transduced neutrophils with a Tat-CRIB fusion protein (42) that was shown to bind endogenous Cdc42 in a T-cell line and inhibit stromal cell-derived factor 1-induced chemotaxis (32). An extensive study on the uptake of Tat fusion proteins by neutrophils showed that 90% of neutrophils could be transduced within 30 min with Tat-protein (33). Incubation of neutrophils with 100 µg/ml Tat-CRIB resulted in a 3-fold increase in fMLF-induced superoxide production (Fig. 7B, bar C). In contrast, a mutant fusion protein, Tat-mutCRIB (H246A, H249A,Y250A,G251A,D253A) that is unable to bind activated Cdc42 (32) did not significantly increase fMLF-induced superoxide production (bar B). These results indicate that Cdc42...
b\textsubscript{558}, but a 10-fold greater ratio of Rac1 to cyt b\textsubscript{558} is required. As in the cell-free assays, nonprenylated Cdc42 neither competed with nonprenylated Rac1 for binding to cyt b\textsubscript{558} nor could it push down cyt b\textsubscript{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.\textsuperscript{2} This suggests that prenylation is not required for the direct interaction of Rac or Cdc42 with cyt b\textsubscript{558} but only for localization to the membrane or micelles containing cyt b\textsubscript{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 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\textsubscript{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 chemotactant-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\textsubscript{558} homologues, termed Nos 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), perhaps through direct interactions of Rac1 with the Nos 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\textsubscript{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 Nos homologues.

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