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Forum Review

Regulation of the Phagocyte NADPH Oxidase by Rac GTPase

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ABSTRACT

Phagocytic leukocytes generate reactive oxygen species important for the killing of invading microorganisms. The source of these oxidants is the NADPH oxidase, a tightly controlled multicomponent enzyme made up of a membrane-associated catalytic moiety and cytosolic regulatory components that must assemble to form the active oxidase. The phagocyte NADPH oxidase was the first mammalian system shown to be directly regulated by a Rac GTPase. We review here our understanding of NADPH oxidase regulation by Rac, as well as the regulation of Rac itself, in phagocytic leukocytes. Rather than viewing Rac as a "cog" in the NADPH oxidase machinery, we argue for a view of Rac GTPases as critical "molecular switches" regulating the formation of ROS by phagocytic leukocytes under physiologic and pathologic conditions. *Antioxid. Redox Signal.* 8, 1533–1548.

INTRODUCTION

THE ABILITY OF PHAGOCYTIC LEUKOCYTES to produce reactive oxygen species (ROS) is a vital component of innate immunity. Neutrophils and other leukocytes migrate chemotactically to sites of microbial infection and ingest invading microorganisms by phagocytosis. ROS are formed during this process via a remarkable enzyme, the NADPH oxidase, which acts to kill the ingested microorganisms either directly or through the activation of proteases (1, 2). The NADPH oxidase catalyzes the NADPH-dependent one-electron (e^-) reduction of O_2 to form superoxide anion (O_2^-), from which other ROS, including hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), and hypochlorous acid (HOCl), are derived [reviewed in (1, 2)]. This multiprotein oxidase is dormant in quiescent cells, where its components are segregated into cytosolic and membrane compartments. In response to chemotactic and phagocytic stimuli, the enzyme rapidly assembles at the membrane and becomes activated. Such stimuli include lipid mediators, soluble peptides (*e.g.*, complement component C5a and *N*-formylated peptides, such as fMetLeuPhe, which are produced as by-products of bacterial protein secretion), and opsonized particulate stimuli, all of which bind to specific cell-surface receptors present on neutrophils, macrophages, monocytes, and eosinophils.

The phagocyte NADPH oxidase was the first cellular system shown to be directly regulated by Rac1 or Rac2, members of the Ras superfamily of GTP-binding proteins. The availability of cell-free assays for reconstitution studies and knowledge of the regulatory protein components of the NADPH oxidase derived from genetic studies has led to much insight into the function of Rac GTPase in this system. Genome analysis has demonstrated that the phagocyte NADPH oxidase is one of a group of NADPH oxidases or Nox proteins that exist in a variety of nonphagocytic tissues [reviewed in (3, 4); see also current volume]. The nonphagocyte NADPH oxidases generate much lower levels of ROS than does the phagocyte oxidase and are thought to participate in intra- and intercellular signaling pathways, as well as host defense. Our relatively detailed understanding of the roles of Rac GTPase in regulation of the phagocyte NADPH oxidase currently provides a potential framework for understanding how these novel oxidases may be regulated by Rac GTPases. In this article, we review our understanding of NADPH oxidase regulation by Rac, as well as the regulation of Rac itself, in phagocytic leukocytes. Rather than viewing Rac as a "cog" in the NADPH oxidase machinery, we argue for a view of Rac GTPases as critical "molecular switches" regulating the formation of ROS by phagocytic leukocytes under physiologic and pathologic conditions.

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COMPONENTS OF THE NADPH OXIDASE

The phagocyte NADPH oxidase is a multiprotein enzyme in which cytosolic regulatory components must assemble with membrane-anchored flavocytochrome b_{558} to form O_2^- (Fig. 1). The separation of oxidase components into different subcellular locations in resting cells, along with modulation of reversible protein–protein and protein–lipid interactions, provides for tight regulation of enzymatic activity. Mutations or deletions in the genes for NADPH oxidase components results in an immunodeficiency disorder, chronic granulomatous disease (CGD) (2, 5). Phagocytes of patients with CGD are unable to generate ROS, and these patients have chronic bacterial and/or fungal infections, which may be lethal. We briefly describe here the primary NADPH oxidase regulatory and catalytic components (see also elsewhere in this volume).

Cytochrome b_{558}

The electron transfer reactions of the NADPH oxidase are catalyzed by the integral membrane protein, flavocytochrome b_{558} (cyt *b*). Cyt *b* is a low-potential flavohemeprotein that is composed of two transmembrane subunits, the heavily glycosylated gp91*phox* subunit (or Nox2), and p22*phox* (phagocyte oxidase). The N-terminal 300 amino acids of gp91*phox* are predicted to form six transmembrane α-helices, whereas p22*phox* is predicted to form three transmembrane α-helices. Cyt *b* contains all the redox components for O_2^- production, including two heme groups and binding sites for NADPH and FAD in the C-terminus of gp91*phox* (2). Although cyt *b* provides the catalytic components for the electron-transfer process, catalysis requires interaction with the cytosolic regulatory components. Mutations within gp91*phox* (X-linked) or p22*phox* (autosomal recessive) result in severe forms of CGD.

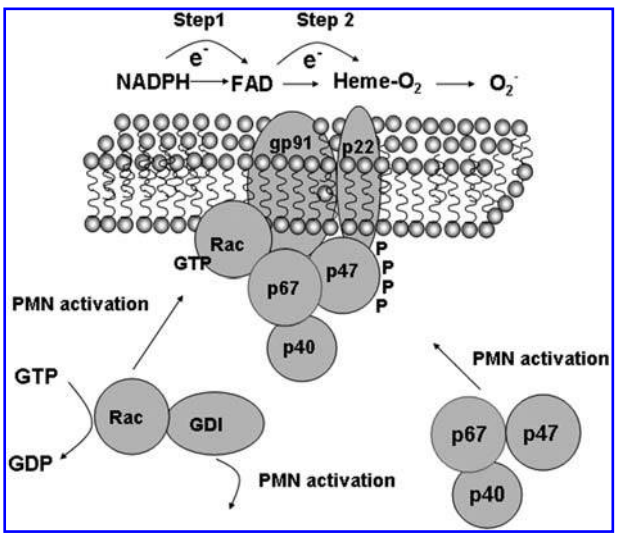

FIG. 1. The phagocyte NADPH oxidase. Components, assembly and activation of the human neutrophil NADPH oxidase are depicted in this figure and described in the text. Transfer of e^- from cytosolic NADPH to molecular oxygen takes place as two distinct reactions: Step 1 involves e^- transfer from NADPH to cyt *b*–bound FAD, whereas Step 2 involves e^- transfer from FAD to cyt *b*-heme and spontaneously on to O_2 to form O_2^-. PPPP represents multiple phosphorylations of p47*phox*, known to be important in the assembly and activation process.

p47phox

The cytosolic NADPH oxidase regulatory component p47*phox* exists both in a free form and in complexes with the other cytosolic oxidase components, p67*phox* and p40*phox* (6, 7). In resting cells, p47*phox* is maintained in an inactive conformation by an intramolecular interaction between internal SH3 domains and a C-terminal polyproline domain that masks the SH3 domains in p47*phox* from binding to proline-rich regions of other NADPH oxidase components [reviewed in (8–10)]. Upon cell activation, p47*phox* is phosphorylated on up to 11 sites through the action of still incompletely defined signaling pathways, inducing a cumulative disruption of the autoinhibited conformation that enables p47*phox* to bind with enhanced affinity to p22*phox*, initiating the translocation of the p47*phox*/p67*phox*/p40*phox* complex to the membrane (11, 12). The phosphorylation of p47*phox* also exposes an internal PX ("phox homology") domain that binds PtdIns(3,4)P_2, and which is required for effective membrane translocation and NADPH oxidase activation in response to cell stimulation (13–15). At the cell membrane, p47*phox* interacts via several binding sites with both subunits of the integral cyt *b* membrane protein to form the active enzyme complex (8–10).

Cell-free studies support the concept that p47*phox* serves as a molecular adapter for assembly of p67*phox* into the membrane-active NADPH oxidase, optimally positioning p67*phox* (and perhaps Rac2-GTP) in the active enzyme complex, rather than providing any intrinsic catalytic function (16, 17). Nevertheless, p47*phox* is critical for normal NADPH oxidase activation in response to receptor stimuli and/or phagocytic particles, as evidenced by the occurrence of CGD in its absence (2, 5, 12).

p40phox

p40*phox* is another putative adapter involved in NADPH oxidase regulation [reviewed in (18)]. Originally identified as a component of the 250-kDa cytosolic complex containing p47*phox* and p67*phox* (6, 7, 19), p40*phox* contains an N-terminal PX domain, an SH3 domain, and a PB1 (*P*hagocyte oxidase and *B*em1p) domain. The exact role(s) of p40*phox* in NADPH oxidase regulation remain controversial. Recently, it has been shown that p40*phox* may be required for NADPH oxidase activation by Fc-receptor–dependent particulate stimuli (20). As the PX domain of p40*phox* interacts specifically with PtdIns(3)P (13, 21), and this lipid is generated at a late stage after Fc-receptor–dependent phagocytic engulfment of bacteria (22), p40*phox* could act specifically to recruit cytosolic oxidase components to the phagocytic compartment. It has also been reported that p40*phox* enhances the NADPH oxidase response to G protein–coupled receptor stimuli, including *N*-formylated peptides (23) and a muscarinic receptor–derived activating peptide (24).

p67phox

p67*phox* contains two C-terminal SH3 domains which enable it to interact with p47*phox* and p40*phox* and translocate to the membrane during assembly of the active oxidase (6–9, 11, 12). It also contains a central region (aa. 199–210), identified as an "activation domain," because mutation within this region causes a loss in the ability of p67*phox* to support NADPH oxidase function *in vitro* (25, 26). The activation domain of p67*phox* appears to regulate electron transfer from NADPH to FAD (27). The N-terminus of p67*phox* contains four tetratricopeptide motifs necessary for regulation by Rac GTPase (28), two of which have been shown by x-ray crystallography to interact directly with the Switch 1 region of activated Rac (29).

Unlike p47*phox* (and p40*phox*), p67*phox* is absolutely required for NADPH oxidase activity in reconstituted cell-free systems (16, 17, 30). In the presence of a minimal system containing Rac GTPase and cyt *b*, p67*phox* catalyzes the transfer of electrons from NADPH to cyt *b*–bound FAD (Fig. 1, Step 1). Then, in a second reaction (Fig. 1, Step 2), which may require the ability of p67*phox* to interact both with Rac and cyt *b* (see later), electron transfer from FAD to the cyt *b* heme groups and on to molecular oxygen to generate O_2^- is accomplished. Recent cell-free experiments making use of Rac–p67*phox* chimeric proteins have demonstrated that portions of both proteins are absolutely necessary for NADPH oxidase activation in the absence of activating amphiphiles (31, 32). Analysis of the turnover of p67*phox* (and Rac2) on phagosomal membranes by fluorescence recovery after photobleaching demonstrated that both proteins exhibited continuous translocation and exchange on cyt *b* (33).

Rac GTPase IS REQUIRED FOR ACTIVITY OF THE PHAGOCYTE NADPH OXIDASE

The absolute requirement for active Rac in the phagocyte NADPH oxidase has been confirmed both *in vitro* and *in vivo* [see (34, 35) for reviews]. The initial identification of Rac as an important cytosolic NADPH oxidase regulatory component came from studies using amphiphile-activated cell-free systems to identify a guanine-nucleotide–sensitive cytosolic oxidase regulator, which was shown to be Rac2 in human neutrophils (36) and Rac1 in guinea-pig macrophages (37). We emphasize that Rac and p67*phox* are the minimal cytosolic components necessary and sufficient for O_2^- formation in a semi-recombinant cell-free system (16, 17, 30).

Several lines of evidence support the NADPH oxidase regulatory role of Rac2 GTPase in intact phagocytes. Rac2 translocates to the membrane during NADPH oxidase activation with kinetics and stoichiometries consistent with a role in NADPH oxidase regulation (38). Deletion of the Rac GTPase activating protein Bcr in transgenic mice caused increased Rac2 activation and membrane translocation and enhanced oxidant formation (39). Conversely, use of antisense oligonucleotides to decrease B lymphocyte Rac2 levels inhibited NADPH oxidase activity (40). Ultimately, generation of the Rac2 null mouse confirmed the critical role of Rac2 in phagocyte NADPH oxidase regulation by multiple stimuli (41, 42). Despite high expression levels of the closely related Rac1 isoform in mouse neutrophils, the absence of Rac2 resulted in substantial defects in oxidant production initiated by G protein–coupled chemoattractant receptors (*e.g.,* fMLP receptor), IgG-opsonized particles, and phorbol esters (42, 43). Murine neutrophils with a conditional Rac1 deficiency show no defects in oxidase responses to these same stimuli (44). Similar findings were obtained using murine Rac2$^{-/-}$ macrophages (45). Interestingly, human monocytes [and presumably guinea pig macrophages (37)], appear to be able to use Rac1 to regulate NADPH oxidase, depending on the initiating stimulus (46). A dominant negative Rac2 mutation (D57N) has been identified in humans, resulting in defective NADPH oxidase activity and severe recurrent infections (47, 48).

Rac IS A MEMBER OF THE RHO FAMILY OF SMALL GTPases

The three mammalian *RAC* genes are expressed in different tissues at different levels. Rac2 is the major Rac isoform expressed in human hematopoietic cells, whereas Rac1 appears to be ubiquitous, and Rac3 is widespread, but notably absent in neutrophils (Ulla Knaus, personal communication). Rac GTPases are members of the Rho family of small GTPases. These ~20-kDa monomeric GTPases (including Rac1–3, RhoA-G, and Cdc42) control such disparate biologic activities as growth and cell division, apoptosis, motility, vesicle trafficking, and development (49, 50). This diverse range of activities is paralleled by the interaction of active Rho GTPases with a large and equally diverse array of target or effector molecules.

Rho GTPases can be thought of as "molecular switches," cycling between GTP-bound active forms that stimulate downstream effectors, and GDP-bound inactive states (51). This activation cycle is regulated by three classes of regulatory protein, depicted in Fig. 2. These include GDP-dissociation inhibitors (GDIs), of which two major forms are found in myeloid cells, RhoGDI and D4 GDI (52). GDIs maintain bound Rho GTPases in soluble cytoplasmic complexes by masking the C-terminal prenyl group (see later). The sequestration of the GDP-bound GTPase prevents GDP dissociation, as well as interactions with target and regulatory molecules. GTPase activating proteins (GAPs) stimulate the low intrinsic GTP hydrolytic activity of the GTPases, resulting in the conversion back to the inactive GDP state. GDP/GTP exchange factors (GEFs) catalyze the release of bound GDP, resulting in the formation of the GTP-bound active protein in the normal intracellular milieu. Rho GEFs also direct signaling downstream from the GTPase, probably through a combination of spatially restricted activation within the cell and through formation of specific ternary complexes between GTPase, GEF, and effector molecules [*e.g.* (53, 54)].

As with most small GTPases, Rac GTPases undergo post-translational processing events, including C-terminal isoprenylation [reviewed in (55, 56)]. Modification by the isoprenyl group, usually geranylgeranylation, plays an important role, in combination with the adjacent positively charged

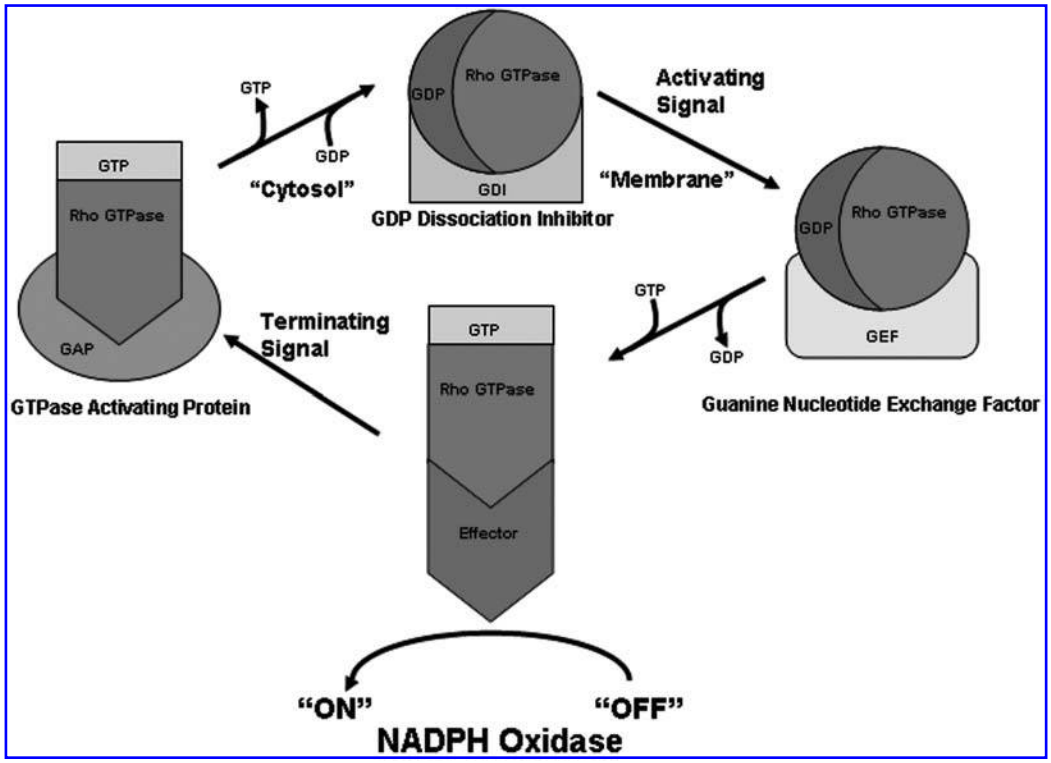

FIG. 2. The Rho GTPase regulatory cycle. The figure emphasizes the critical role of Rac2 as a "molecular switch" for turning on the inactive NADPH oxidase. Rho GTPases in general exist in resting phagocytic cells as cytosolic complexes with GDI proteins. In these complexes, Rho GTPases are sterically hindered from interacting with GEFs, GAPs, or effectors, and cannot interact with membranes because of masking of the prenylated C terminus. When leukocytes are stimulated appropriately, individual Rho GTPases are released from GDIs and are converted to the active GTP form by the action of membrane-associated GEFs. The active GTPases can then bind and activate effector targets. The GTPase response is terminated by intrinsic GTP hydrolysis and/or the stimulation of GTP hydrolysis by GAPs. As indicated, the GTPase activation cycle is linked with a cytosol-to-membrane cycle.

polybasic domain, in mediating membrane association of the free (uncomplexed) GTPase. Prenylation may also affect the ability of Rac to interact with regulatory proteins [*e.g.* (57)] as well as effector targets [*e.g.* (58)].

REGULATION OF Rac DURING NADPH OXIDASE ACTIVATION

The multiple phosphorylations required for $p47^{phox}$ function in NADPH oxidase assembly, and perhaps phosphorylation of other oxidase components, provide a control mechanism to avoid inappropriate ROS formation. However, an additional level of control is accomplished through the regulated release of Rac2 GTPase from inert cytosolic complexes with GDIs. On neutrophil activation, Rac2 must be released from preexisting cytosolic complexes with RhoGDI and D4 GDI (59) by mechanisms that may involve both phosphorylation (52, 60) and the action of lipid mediators (59, 61). GDP is exchanged for GTP on free Rac2 through the action of membrane-localized GEFs (see later section), enabling Rac2 to interact with and activate the assembling NADPH oxidase. Rac2 supports NADPH oxidase activity only in its GTP-bound active form [*e.g.* (58, 62, 63)]. The GTP dependence for oxidase activation by Rac2 indicates that the Rac2 Switch I domain, which changes conformation in a GTP-dependent manner, is involved [the Rac2 Switch II domain undergoes only minimal conformational changes on GTP binding (29, 64)]. Consistent with this, certain point mutations within the Switch I (or effector domain) of Rac2 were unable to support NADPH oxidase activity (65, 66).

The observation that Rac binds directly to $p67^{phox}$ (but not $p47^{phox}$) via the Switch I domain (aa. 27–45) provided important insight into Rac function in the oxidase (67). It was shown that the tetratricopeptide repeat (TPR) in the N-terminus of $p67^{phox}$ was the site of Rac binding (28), and this was confirmed on determination of the crystal structure of the Rac-p67(TPR) complex (29). The structure revealed specific stabilizing interactions between $p67^{phox}$ and Rac, involving amino acids A27 and G30 of Rac and the TPR domain of $p67^{phox}$. Although the Rac insert domain was not involved in $p67^{phox}$ interactions, peptide walking experiments had previously shown that blocking the insert domain of Rac abrogated NADPH oxidase activation (68). Studies using insert domain deletion mutants of Rac have yielded conflicting results, however, concluding that the insert domain was either absolutely required (30, 69) or unnecessary (70, 71) for Rac-

dependent NADPH oxidase activity. More recent studies [see following sections (30)] have indicated that the insert domain is likely to be involved with direct binding of Rac to cyt *b*.

Translocation of Rac2 from the cytosol to the plasma membrane–associated oxidase occurs via a mechanism independent from translocation of the p47*phox*/p67*phox* complex (72, 73). However, Price *et al.* (74) showed that transient expression of constitutively active Rac mutants in Cos-*phox* cells was sufficient to induce the membrane translocation of cytosolic oxidase components and assembly of an active oxidase. These data suggest that Rac GTPase acts to coordinate translocation of the p47*phox*/p67*phox* complex, consistent with the simultaneous translocation kinetics observed for these three cytosolic factors in stimulated human neutrophils by Quinn *et al.* (38). A regulatory action of Rac on p47*phox* might occur through the ability of Rac to modulate the phosphorylation of p47*phox* via the action of Rac-regulated kinases such as p21-activated kinase (PAK). PAKs are abundant in neutrophils, and their activity is stimulated by chemoattractant (75) and Fc receptors (76). Moreover, PAK phosphorylates p47*phox* on several sites of known physiologic significance (75, 77), and inhibition of PAK activity has been shown to impair NADPH oxidase activation (78). Interestingly, Rac-regulated PAK activity also appears to couple the formation of metabolic reducing equivalents involved with maintaining leukocyte NADPH levels with absolute NADPH oxidase activity by inhibiting the glycolytic activity of phosphoglycerate mutase (PGAM)-B (79).

MECHANISM OF NADPH OXIDASE REGULATION BY Rac GTPase

The molecular mechanism(s) through which Rac GTPase regulates NADPH oxidase activity have been studied primarily in cell-free systems derived from phagocytic cells and/or in nonphagocyte cell lines expressing phagocyte NADPH oxidase ("*phox*") components (80). The view that Rac simply mediates the translocation of the other cytosolic regulatory components to the membrane-assembled oxidase has clearly been disproved. In cells genetically deficient in gp91*phox*, p47*phox*, and p67*phox*, Rac2 still translocates to the plasma membrane (72), whereas in Rac2-deficient transgenic mice, p47*phox* and p67*phox* are still recruited to the membrane (41). Similarly, tyrosine kinase inhibitors dissociate Rac2 translocation from translocation of p47*phox* and p67*phox* (73). In adherent neutrophils stimulated with fMLP, p47*phox* and p67*phox* assemble at the membrane in the absence of Rac2 activation and translocation (81). However, as noted earlier, Rac activation can induce NADPH oxidase assembly in a Cos-*phox* model cell system (74), perhaps indirectly through the action of effector kinases such as p21-activated kinase. The latter data suggest that part of the function of Rac is to coordinate NADPH oxidase assembly in response to cell activation.

The fact that Rac (1 or 2) is absolutely required for NADPH oxidase activity in reconstituted cell-free systems under all conditions suggests a more direct role for Rac in regulating the electron-transfer reactions catalyzed by the membrane-assembled oxidase (Fig. 3). This viewpoint is also supported by the lack of NADPH oxidase function of the membrane-assembled cyt *b*/p47*phox*/p67*phox* complex in the absence of active Rac2 in adherent neutrophils (81). Currently, our view of the role of Rac in the system varies depending on whether one considers p67*phox* as the sole mediator of cytochrome *b* regulation [reviewed in (34)].

Rac as an adapter

Several laboratories suggest that p67*phox*, which contains a defined "activation domain" for cyt *b*, is the only protein in-

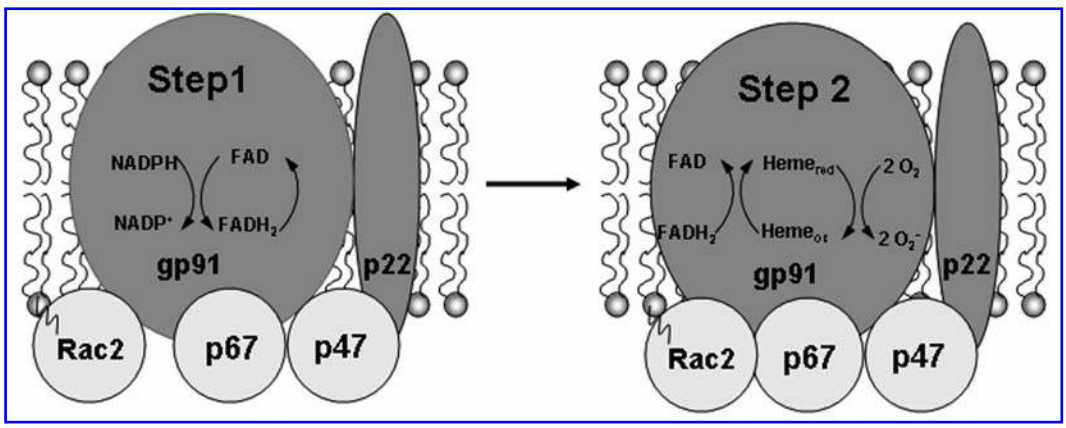

FIG. 3. Two-step regulatory model for regulation of cyt *b* activity by Rac2. Diebold and Bokoch (30) proposed a two-step model for the regulation of NADPH oxidase by Rac2. In Step 1, Rac2 translocates to the membrane and interacts with the phospholipid bilayer *via* its prenylated/polybasic C-terminus. In addition, Rac2, *via* its insert domain, interacts with cyt *b* and contributes to the regulation of electron flow from NADPH to FAD without interacting with p67*phox* (indicated by Rac2 not touching p67*phox* in the left-hand portion of the figure). p67*phox* is still required for electron flow to occur in Step 1 and regulates electron flow *via* its activation domain. The interaction of the insert domain of Rac2 with cyt *b* may induce a conformational change in cyt *b* that modulates the interaction of p67*phox* and cyt *b*. In Step 2, the interaction between the Switch I domain of Rac2 and the Rac-binding domain of p67*phox*, probably inducing a conformational change in p67*phox*, is required for electrons to continue to flow from FAD to the heme groups of cyt *b* (this interaction is indicated in the right-hand portion of the figure by the contact between Rac2 and p67*phox*). p47*phox* (and p40*phox*, not shown) are required as adapters during assembly only.

fluencing the rate-limiting electron transfer step (Step 1) of the NADPH oxidase. These groups then consider Rac primarily as an adapter molecule that binds to the plasma membrane through its polybasic domain and prenylated C terminus, and to p67^{phox} through the Switch I region, aiding in the proper orientation of the p67^{phox} activation domain to cyt *b* [discussed in (9, 31, 82)]. They further suggest that the Rac insert domain may mediate binding to cyt *b* but, as with p47^{phox}, this interaction serves solely to position and facilitate binding of p67^{phox} to cyt *b*. This aspect of the model is based on the report that a nonprenylated Rac1 mutant lacking the insert domain exhibited decreased affinity (EC_{50}) for the oxidase, but had no effect on the maximal rate of superoxide production (69). In addition, the Pick group used prenylated Rac1-p67^{phox} chimeras in the cell-free system and reported that deletion of the insert domain of Rac1 did not affect the ability of this chimera to support superoxide production (31). [Interestingly, this group of investigators originally observed that peptides overlapping the insert domain of Rac inhibited superoxide production in the cell-free system (68)].

Several observations appear to be inconsistent with aspects of this view of Rac solely as an adapter, however. First, as indicated earlier, it is possible to dissociate the membrane translocation of Rac from that of p67^{phox}, which is inconsistent with a required carrier function of Rac for p67^{phox} (72, 73). With regard to a role of the Rac insert domain, a careful examination of the data in Gorzalczany *et al.* (31) shows that a marked decrease (>60%) in the ability of the insert domain deletion mutant to support oxidase activity exists at concentrations of chimera of less than 200 n*M* (*i.e.*, within the normal physiologic range of reactants). Finally, based on effects of exogenous Rac-GTP on the activity of various Rac-p67^{phox} chimeras, the Pick group has recently concluded that Rac must (minimally) play an additional role in modifying the active conformation of p67^{phox} to enable electron flow to take place in cyt *b* (32). They observed that targeting of the p67^{phox} activation domain to the membrane by the construction of a chimera with prenylated Rac1 was insufficient for NADPH oxidase activation. Instead, activation required a productive interaction of Rac with p67^{phox}, mediated by intrachimeric bonds between the Rac Switch I region and the TPR domain of p67^{phox}. Consistent with a possible Rac-mediated conformational change enabling p67^{phox} to interact more effectively with cyt *b*, an ability of Rac1 to dissociate p67^{phox} from p40^{phox} has been reported (83).

Rac as a direct regulator of cytochrome b

An alternative paradigm views Rac2 as a key regulator of cytochrome *b* function in its own right (30). It is proposed that differential regulation exists of the two steps involved in electron transfer from NADPH to molecular oxygen (Fig. 3). Rac is required apart from, and in addition to, p67^{phox} for the Step 1 reaction. However, Rac must subsequently then interact with p67^{phox} for the Step 2 reaction to occur. This model is based on the observation (30) that a non–Rac-binding p67^{phox} (a.a. 178–184) deletion mutant was still required for and supported electron transfer from NADPH to FAD (Step 1), but not from FAD to cyt *b* (Step 2). This observation has been subsequently verified with non–Rac-binding p67^{phox} TPR domain mutations (34). Similarly, the reciprocal experiment using the Rac2 D38A mutant that does not bind p67^{phox} also indicated independent roles for Rac and p67^{phox} in Step 1, but not in Step 2 activity. In contrast, the Rac2 insert domain was found to be critical for both the Step 1 and Step 2 electron-transfer reactions. This two-step model is consistent with prior kinetic analysis of oxidase activation (84).

Because the studies described earlier (30) used a reconstituted cell-free system containing cyt *b* and p67^{phox}, but lacking p47^{phox}, this indicated that Rac2 must interact directly with cyt *b* to support the Step 1 reaction. Indeed, such an interaction has been verified: the fluorescence intensity of a GppNHp analogue (mant-GppNHp) bound to Rac2 increased in the presence of cyt *b*, indicating direct interaction between Rac2 and cyt *b*. Use of the insert domain deletion mutant of Rac2 in place of wild-type Rac2 eliminated this interaction, indicating that this domain was important for cyt *b* binding. In support of the fluorescence data, glutathione-S-transferase (GST)-Rac2 can specifically bind cyt *b* purified from neutrophils in pull-down assays (85). These data are consistent with previous observations that membrane translocation of Rac2 was decreased by 75% in neutrophils from cyt *b*–deficient CGD patients (72). Overall, these data strongly indicate that the Rac2 insert domain is important, although perhaps not absolutely required, for both the functional and physical interaction of Rac2 with cyt *b*.

THE Rho FAMILY GTPase Cdc-42 REGULATES ROS PRODUCTION BY COMPETING WITH Rac FOR CYTOCHROME *b* BINDING

Cdc42 is a member of the Rho GTPase family and is closely related to Rac1 and Rac2. However, unlike Rac, Cdc42 cannot activate the cell-free NADPH oxidase system because of differences in two residues within the Switch 1 region, revealed in the crystal structure to form important functional contacts between GTP-Rac and p67^{phox} (29). Interestingly, if these two residues in Cdc42 are mutated to the corresponding residues of Rac, Cdc42 (K27A, S30G) can now support NADPH oxidase activity to the same extent as wild-type Rac (85, 86). Conversely, if these residues in Rac are mutated to those of Cdc42, then Rac (A27K, G30S) completely loses the ability to support superoxide production.

Based on these and other studies demonstrating the lack of activity of Cdc42 in cell-free NADPH oxidase systems [*e.g.* (36)], it was assumed that Cdc42 had no role in the regulation of the NADPH oxidase. It was recently shown, however, that Cdc42 antagonizes the ability of Rac2 to regulate ROS production by the neutrophil NADPH oxidase (85). The ability of both GST-Cdc42 and GST-Rac2 to interact with cyt *b* (but not p67^{phox}) in *in vitro* pull-down assays was demonstrated. This interaction was only partially GTP dependent, but was dependent on the insert domain (aa. 124–135) of Rac2 and Cdc42. In NADPH oxidase cell-free assays, Cdc42 wt, but not Cdc42 Δ124–135, inhibited Rac2 (or Rac1)-induced superoxide production. The inhibitory effect of Cdc42 wt was decreased when the concentration of Rac2 was increased in the assay,

suggesting that Cdc42 competes with Rac2 for binding to cyt *b* via the insert domain (see Fig. 4). This hypothesis was confirmed in a direct competition binding assay in which decreasing amounts of cyt *b* were pulled down by GST-Rac2 in the presence of increasing amounts of Cdc42.

In vivo experiments showed that Cdc42 could inhibit Rac1-induced superoxide production in a Cos *phox* cell line stably expressing a functional NADPH oxidase. Additionally, sequestration of endogenous Cdc42 by introduction of a recombinant Cdc42-GTP–binding domain derived from the Wiscott Aldrich Syndrome protein (WASp) into human neutrophils increased superoxide formation twofold to threefold on stimulation with fMLP, providing further evidence that Cdc42 plays an antagonistic role in regulating Rac-induced oxidant production in intact neutrophils. Normally, Cdc42 is activated nearly simultaneously with Rac2 in chemoattractant-stimulated human neutrophils (87). 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. Based on these findings, Cdc42 may also serve as a tonic regulator to dampen the amount of ROS generated during leukocyte transmigration through tissues. Several autocoids (*e.g.*, bradykinin) reported to inhibit chemoattractant-stimulated ROS production (88) are known to activate Cdc42 (89). Activation of Cdc42 might also provide a mechanism to inhibit full ROS production until phagocytic cup formation and bacterial uptake is completed. This would effectively coordinate oxidant production with the bacterial-uptake process for the most efficient killing.

A recent study on the patterns of activation of RhoGTPases during phagocytosis was conducted using FRET analysis (90). This study showed that Cdc42, Rac1, and Rac2 activation during phagocytosis changed on the subminute timescale. In RAW264.7 macrophages undergoing phagocytosis of opsonized erythrocytes, activation of Cdc42 occurred at the site of particle attachment, immediately after contact with the macrophage. Cdc42 was active only at the advancing edge of the pseudopod and remained active during the pseudopod-extension phase of the phagosome. Before the closure phase, the activity of Cdc42 decreased. During closure, active Rac1 was observed at the base of the phagosome. The activation pattern of Rac2 differed: Rac 2 was only slightly active in the vicinity of the particle and displayed only minor activation during the pseudopod-extension phase. There was, however, a pronounced transient increase in Rac2 activity distributed over the base of the phagosome during closure. The presence of active Cdc42 and the absence of active Rac2 during the early phases of phagocytosis are consistent with the hypothesis that Cdc42 may play an antagonistic role toward NADPH oxidase activation by Rac2 during formation of the phagocytic cup.

Rac2 ACTS AS A MOLECULAR SWITCH FOR ROS PRODUCTION BY ADHERENT NEUTROPHILS

In addition to being a regulatory component of electron flow of the NADPH oxidase, Rac2 serves as a common point of convergence for integrin and chemoattractant receptor cross-talk in neutrophils. Neutrophils adherent to fibronectin- or fibrinogen-coated tissue-culture plates exhibit an initial delay of up to 30–60 min in NADPH oxidase activation induced by chemoattractants or cytokines, followed by a period of enhanced activity (91–95). These effects of adhesion have been shown to be a result of β1 (fibronectin) or β2 (fibrinogen) integrin engagement (93–95). Using the GST-p21–binding domain of PAK1 (GST-PBD) pull-down assay to measure Rac2 activity, it was established that Rac2 activation was also delayed by 30–60 min in adherent neutrophils, but not in suspended cells (81, 96). Analysis of NADPH oxidase assembly showed that adhesion-mediated NADPH oxidase suppression was associated with a lack of Rac2 translocation to the plasma membrane, even though the translocation of p47*phox*

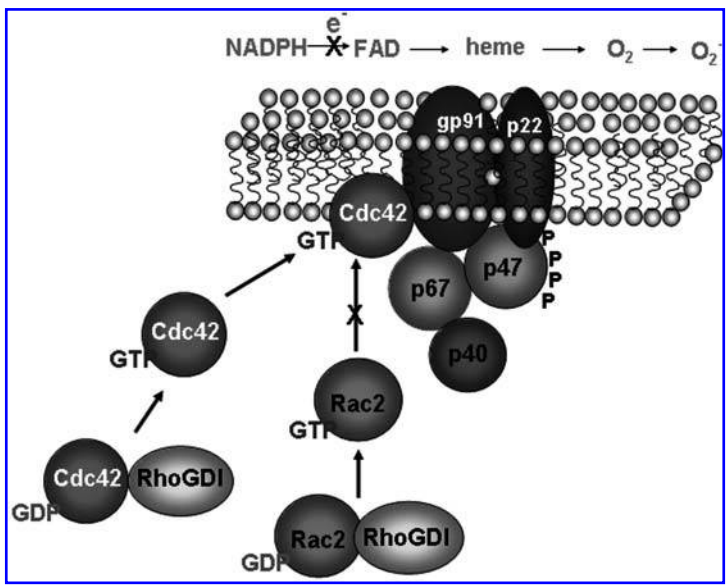

FIG. 4. Cdc42 antagonizes Rac2 by competing for cyt *b* binding. Diagram depicts competition by Cdc42 for Rac2 binding to cyt *b* after cell stimulation, thereby inhibiting e^- transfer from NADPH to form O_2^-.

and p67^{phox} was normal. These data suggested (a) that integrin signaling regulates NADPH oxidase function by specifically controlling the activation and translocation of Rac2, and (b) that Rac2 must be the critical component for activating the membrane-assembled NADPH oxidase. This hypothesis was verified by demonstrating that the suppressive effects of adhesion on the oxidative burst could be reversed by introduction of recombinant constitutively active Rac2G12V into the adherent neutrophils. Rac2 thus served as the critical "molecular switch" for turning on ROS production by the NADPH oxidase (see Fig. 2). It is of interest that, in support of Rac as the primary NADPH oxidase activation signal, it was observed that targeting of prenylated Rac1 to the plasma membrane was sufficient to induce NADPH oxidase activation in a cell-free system consisting of cyt *b* and p67^{phox} (31).

The inability of Rac2 to be activated at early times in adherent neutrophils was localized to loss of a membrane-dependent activity using a cell-free system in which membranes prepared from adherent or suspended neutrophils were reconstituted with cytosol from either adherent or suspended neutrophils in mix–match studies. This observation correlated with inhibition of the activity of the membrane-associated Rac GEF Vav1 in adherent neutrophils. Phosphorylation on the critical Tyr174 site required for Vav1 GEF activity occurred only under conditions in which Rac2 activation and ROS formation was evident. Activation of the tyrosine kinase Syk, which lies upstream of Vav1 and phosphorylates Vav1 on Tyr174, was normal and rapid on adherent neutrophil stimulation with either fMLP or C5a (Fig. 5). Because a delay in Syk activation did not account for the delay in downstream Vav1 activation, the activation of tyrosine phosphatases to dephosphorylate the Tyr174 site during adhesion was postulated. Indeed, the general tyrosine phosphatase inhibitor, sodium orthovanadate, reversed the inhibitory effects of adhesion on Vav1 activation, Rac2-GTP formation, and the kinetics of ROS formation (81).

The prolonged lag period in the oxidative response in neutrophils adherent to surfaces coated with extracellular matrix components is presumed to correspond to the *in situ* situation in which the cells are migrating through the tissue to inflammatory sites. After the cells reach these sites and engulf bacteria, the neutrophils would then respond by generating microbicidal ROS. What might be the signals generated at the inflammatory site that would restore NADPH oxidase responsiveness to the adherent cells? Interestingly, particulate stimuli able to activate various phagocytic receptors were insufficient to overcome the adhesion-mediated inhibition of ROS formation, exhibiting significant delays in oxidative responses in the adherent neutrophils (97). In contrast, exposure of the adherent cells to TNF-α or a number of other cytokines was effective at enabling the rapid onset of ROS production in response to chemoattractant stimulation. The action of TNF-α correlated exactly with restoration of Vav1 activity and Rac2-GTP formation. The effect of these cytokines was associated with their ability to stimulate activation of Pyk2 (proline-rich tyrosine kinase 2). Through the use of a Pyk2-selective inhibitor, tyrphostin A9, and a specific inhibitory fragment of the Pyk2 C terminus (Pyk2 CT), it was shown that the action of TNF-α was indeed dependent on Pyk2 activity. Pyk2-dependent restoration of NADPH oxidase activity by TNF-α correlated with Pyk2-dependent restoration of Vav1 activation and Rac2 activity (Fig. 5). Finally, the activation of Pyk2 led to a decrease in overall tyrosine phosphatase activity in the adherent cells, consistent with the concept that adhesion suppresses NADPH oxidase activation by stimulating dephosphorylation of Tyr174 and deactivation of Vav1 (81).

Interestingly, TNF-α has also been shown to initiate a p38 MAP kinase–dependent inhibitory signal that arrests neutrophil polarization and chemotaxis (98). Combined with the action of TNF-α to overcome the inhibitory effect of adhesion on Rac2 activity leading to ROS formation, this provides additional evidence for TNF-α as a critical mediator of human neutrophil innate immune function at inflammatory sites. Therapies directed at blocking TNF-α have demonstrated therapeutic benefit in the treatment of chronic inflammatory conditions, including rheumatoid arthritis, inflammatory bowel disease, and asthma (99–101).

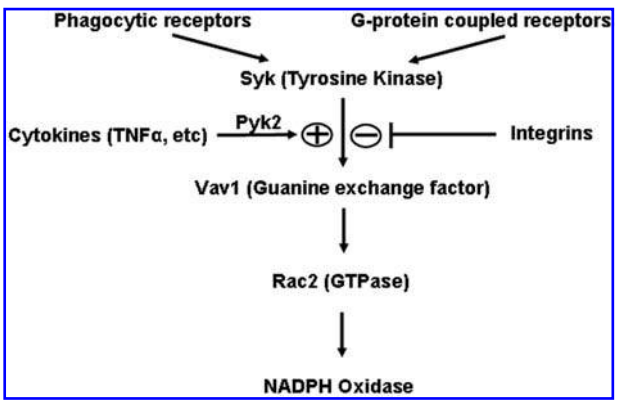

FIG. 5. Signaling pathways in adherent neutrophils modulating NADPH oxidase activity through Rac2. Signaling components whose activities are modulated (positively, +; or negatively, −) in adherent cells to affect Vav1 activation of Rac2 and the NADPH oxidase, as detailed in the text.

ACTIVATION/TERMINATION OF Rac SIGNALING IN THE NADPH OXIDASE

As a critical molecular switch in physiologic (*i.e.*, adherent cell) NADPH oxidase activation, the proteins controlling Rac2 activation and deactivation are likely to play important roles in determining ROS production in response to various inflammatory stimuli (Fig. 2). As noted earlier, Rac2 exists almost entirely in the form of an inactive complex with GDIs (RhoGDI and D4 GDI) in resting neutrophils (59). The regulation of Rac2 dissociation from this cytosolic complex is clearly important in enabling subsequent Rac2 activation and regulation of the membrane-localized NADPH oxidase. The mechanisms through which Rac2-GDI interactions might be controlled are discussed in detail in (52, 102), and are not considered further here. After release from GDIs, Rac2 must be converted to the

active GTP-bound form through the action of membrane-localized guanine nucleotide exchange factors before activation of NADPH oxidase. The formation of Rac1/2-GTP has been shown to be highly regulated both spatially and temporally during neutrophil activation (103). Termination of Rac2 activity and the oxidative response is likely to involve the participation of GTPase activating proteins (104).

Rac2 activation by GEFs

As indicated earlier, adherence appears to suppress neutrophil NADPH oxidase activity by inhibiting the activation of the Rac GEF, Vav1 (81). The importance of Vav1 in regulating NADPH oxidase activity has been confirmed in the Vav1 knockout mouse (105). ROS production elicited by fMLP was reduced by threefold in bone marrow–derived neutrophils from $Vav1^{-/-}$ mice. The response to PMA stimulation was also decreased, but only by 25–35% with this nonreceptor stimulus. Vav1 thus appears to be required for at least fMLP-induced NADPH oxidase activity. The basis for this requirement for Vav1 remains to be established, however, as the authors of this study were unable to detect any significant decreases in fMLP-stimulated Rac1-GTP or Rac2-GTP formation in the Vav1 null cells. It was suggested that there may be decreases in the activity of a small fraction of Rac and/or a compensatory increase in the activity of other Rac GEFs that obscured changes in the relevant pool of Rac-GTP. The observation that the Vav1 null mice were also deficient in F-actin assembly in response to fMLP despite apparently normal levels of Rac-GTP supports the latter suggestion. It is also consistent with the concept that GEFs are not functionally redundant but may act in the context of specific downstream signaling pathways.

More recently, evidence was obtained that another Rac GEF, P-Rex1, regulates NADPH oxidase–associated Rac2 activation downstream of trimeric G protein–coupled receptors (GPCRs) in neutrophils. P-Rex1 was originally isolated from neutrophil cytosol as a PtdIns[3,4,5]P_3- and G protein βγ-regulated Rac GEF (106). Both signaling via the pertussis toxin-sensitive Gi protein and the subsequent generation of PtdIns[3.4,5]P_3 via PI 3-kinase are known components of chemoattractant receptor-stimulated pathways that are required for Rac2 (87) and NADPH oxidase activation [reviewed in (107)]. P-Rex1 has been shown to be a preferential GEF for Rac2 both *in vitro* and *in vivo* (108). Thus, it binds Rac2 with a higher affinity than Rac1, and Rac2 (but less so Rac1) activation induced by fMLP was significantly diminished in neutrophils derived from the P-$Rex1^{-/-}$ mouse (108, 109). Neutrophils from these animals are compromised in their ability to activate NADPH oxidase by chemoattractants such as fMLP, but not by PMA or LPS. This confirms the initial findings obtained by antisense knockdown of P-Rex1, where ROS formation induced by C5a was partially inhibited (106). These results indicate an important and apparently direct role of P-Rex1–regulated Rac2 activation in NADPH oxidase activity.

Rac2 deactivation by GAPs

Because the formation of Rac2-GTP catalyzed by GEFs in response to upstream signals serves as a molecular switch for physiologic NADPH oxidase activation, it is likely that termination of oxidase activity can result from turn-off of the Rac2-GTP signal (Fig. 2). In support of this, NADPH oxidase activity is dependent on Rac2-GTP *in vitro* (58, 62), and replacing GTP by GDP in the active, electron-transporting enzyme complex results in termination of O_2^- production (110). The conversion of active Rac-GTP to inactive Rac-GDP is catalyzed by the action of GTPase–activating proteins or GAPs.

A number of Rac GAPs have been identified in neutrophils. These include the cytosolic GAPs p190RhoGAP and Bcr, as well as the membrane-associated p50RhoGAP (104, 110, 111). A role for Bcr in downregulating NADPH oxidase associated Rac2 activity was demonstrated by the enhanced O_2^- production observed in response to endotoxin challenge in the Bcr null mouse (39). Addition of Bcr or the other cytosolic GAP, p190RhoGAP, to a cell-free NADPH oxidase system reduced O_2^- production if added during the activation/assembly phase, but had no effect on the assembled active enzyme complex (111, 112). The latter was taken as evidence that these GAPs do not have access to Rac2-GTP in the assembled complex. The ability of fluoride, which inhibits GAP activity, to promote NADPH oxidase output even in the assembled enzyme may suggest that the membrane-associated GAP(s) do have access to oxidase-associated Rac2 (110). How, or whether, the activity and/or localization of these GAPs is regulated during NADPH oxidase activation remains largely unknown, although p190RhoGAP has been shown to be induced to translocate from the cytosol to the plasma membrane on stimulation of neutrophils with fMLP (113).

THE BASIS FOR DISTINCT NADPH OXIDASE REGULATION BY Rac1 VERSUS Rac2

It is clear that Rac1 and Rac2 have distinct abilities to support NADPH oxidase activation in intact neutrophils. This is somewhat surprising, given that these proteins are 92% identical, and have an identical effector (Switch I) domain (aa. 26–45), a critical site of interaction with both GEFs and downstream protein targets. Most of the sequence differences between Rac1 and Rac2 (15 of 192 amino acids overall) are concentrated in the C-terminal tail (aa. 183–188), adjacent to the prenylated cysteine residue that mediates membrane insertion. Whereas Rac1 has six sequential basic residues (KKRKRK) in this polybasic domain, Rac2 has only three basic residues interspersed with neutral amino acids (RQQKRA), thus disrupting its basic nature. Additionally, the ability of Rac1 versus Rac2 to interact productively with many biologic effectors in *in vitro* and *in vivo* assays is similar, although their relative affinities may differ. For example, Rac2 has been shown to have a greater affinity for binding to $p67^{phox}$ than does Rac1 (114). However, Rac1 more effectively binds and activates PAK1 than does Rac2 through their distinct polybasic domains (115), and similar results have been obtained for interaction with phosphoinositide 5-kinase and the adapter, Crk (116).

As noted previously, human monocytes can use Rac1 for NADPH oxidase regulation instead of Rac2 (46), and both Rac2 and Rac1 support cell-free NADPH oxidase function [*e.g.* (36, 37)]. This suggests that the intrinsic ability of Rac1 to regulate downstream NADPH oxidase target proteins is similar to that of Rac2 [however, we note that the actual molecular mechanism(s) of NADPH oxidase regulation by Rac1 versus Rac2 have not been shown to be identical]. What then might account for the differences noted between regulation of NADPH oxidase, as well as other leukocyte functions, by these two Rac isoforms in intact neutrophils?

Various studies have indicated that Rac1 is present at one fortieth (38, 72) to one fourth (43) the level of Rac2 in human neutrophils. However, the two isoforms are present at roughly equal levels in murine neutrophils (43), and more than 90% of the Rac in human monocytes is Rac1 (46). In general, the differential biologic activities exhibited by Rac1 versus Rac2 in studies of genetically targeted mice do not appear to be simply a result of an overall reduction in Rac levels [Filippi *et al.* (117) and Yamauchi *et al* (118)]. There thus seem to be true differences in the regulation and/or function of Rac1 versus Rac2. One possibility is that these isoforms may be differentially localized in leukocytes. As noted earlier, the C terminus of Rac1 contains a highly positively charged polybasic domain that is largely absent in Rac2. Various studies have indicated the importance of the polybasic domain in GTPase membrane targeting in nonleukocytic cells, and differences in the subcellular membrane localization of Rac1 versus Rac2 were demonstrated [*e.g.* (119)]. Both Rac1 and Rac2 are maintained as cytosolic complexes with GDI in resting neutrophils, and both have been reported to translocate to the plasma membrane on cell activation. However, differences in the subcellular distribution of the Rac isoforms have been observed in murine neutrophils by Filippi *et al.* (117). They reported that Rac1 translocated from a predominantly cytosolic localization to a region in the cell periphery overlapping with F-actin. In contrast, Rac2 was observed to redistribute from a cytosolic and perinuclear pool to the cell periphery, where it localized interior to the F-actin, with little overlap. Both Filippi *et al*. (117) and Yamauchi *et al*. (118) convincingly showed that the polybasic region is required for specific regulation of NADPH oxidase activity by Rac2.

Another reason for the differential biologic activities of Rac1 versus Rac2 may relate to their differential regulation by GEFs and/or GAPs. Preferential activation of Rac2 (vs. Rac1) by P-Rex1 has been demonstrated (108, 109), and Rac2 was more efficiently activated *in vivo* by Vav1 (74). Although Rac1 and Rac2 are identical in the Switch I region, long-range conformational effects of divergent residues may result in differential binding of GEFs or downstream effectors (66). Biophysical analyses indicate that Rac2 exhibits less flexibility in the Switch I and insert domain regions than does Rac1, potentially accounting for its differential regulatory properties (120). It has been proposed that GEFs regulate spatial localization and timing of Rac activation, as well as the downstream targets regulated by these GTPases. Thus, differences in availability or susceptibility to distinct Rac GEFs (or GAPs) may play important roles in Rac1 versus Rac2 signaling specificity.

REGULATION OF NONPHAGOCYTE NADPH OXIDASES BY Rac GTPase

As described elsewhere in this volume, nonphagocyte homologues of gp91^{phox} and the regulatory components p47^{phox} and p67^{phox} have been identified [reviewed in (3, 4, 121, 122)]. These nonphagocyte NADPH oxidases (Noxs) have been implicated in intra- and intercellular signaling and in physiologic processes ranging from host defense to regulation of blood pressure. It has been known for some time that the formation of ROS in nonphagocytic cells requires and can be regulated by Rac1, the predominant Rac isoform in nonmyeloid cells. For example, growth factor (PDGF, EGF) and cytokine (TNF-α, IL-1β)-stimulated ROS production in NIH3T3 cells was stimulated or inhibited by expression of dominant active/negative Rac1 mutants (123), and the adenoviral vector-mediated expression of dominant negative Rac1 in vascular smooth muscle prevented ROS production and the resulting reperfusion injury in a mouse hepatic ischemia/reperfusion injury model (124). However, the source of the Rac-dependent ROS production in the majority of such studies was not definitively established as Nox enzymes. In the cases in which Rac-dependent ROS formation was convincingly shown to involve Nox activity, no evidence was found that the regulation of Nox function by Rac was direct, rather than through the action of Rac on upstream signaling pathways. Several studies have reported that blockade of molecules acting upstream of Rac1 activation [PI 3-kinase (125), βPix (126)] decrease ROS production in Nox1-expressing cells. Direct regulatory interactions of Rac with components of nonphagocyte Nox proteins remain to be established, however. The binding of GTP-Rac1 to the tetratricopeptide (TPR) motif of Noxa1 has been demonstrated (127), fueling the expectation that Rac participates in the regulation of Nox activation. However, the activity of these nonphagocyte NADPH oxidases appears to be regulated in a significantly different manner from the phagocyte NADPH oxidase. Indeed, anecdotal evidence suggests that even though Rac2 is required for activity of the phagocyte NADPH oxidase (Nox2), Rac1 may not be absolutely necessary for the activity of the nonphagocytic Noxs.

CONCLUSION/UNRESOLVED QUESTIONS

We have made substantial progress in understanding how Rac GTPase is regulated in leukocytes, as well as how Rac regulates the activity of the phagocyte NADPH oxidase (Fig. 6). General agreement now exists that Rac is more than a simple adapter that acts to position the activation domain of p67^{phox} for NADPH oxidase regulation, and that Rac is required for effective electron transfer by cyt *b*. Nevertheless, our knowledge remains incomplete. The exact role(s) of Rac in regulating the electron-transfer activity of cytochrome *b* (*i.e.*, does direct regulation occur vs. only indirect regulation through p67^{phox}) remain to be resolved. How Rac release from cytosolic complexes with GDI is regulated, and which GEFs and GAPs control Rac activity in response to specific up-

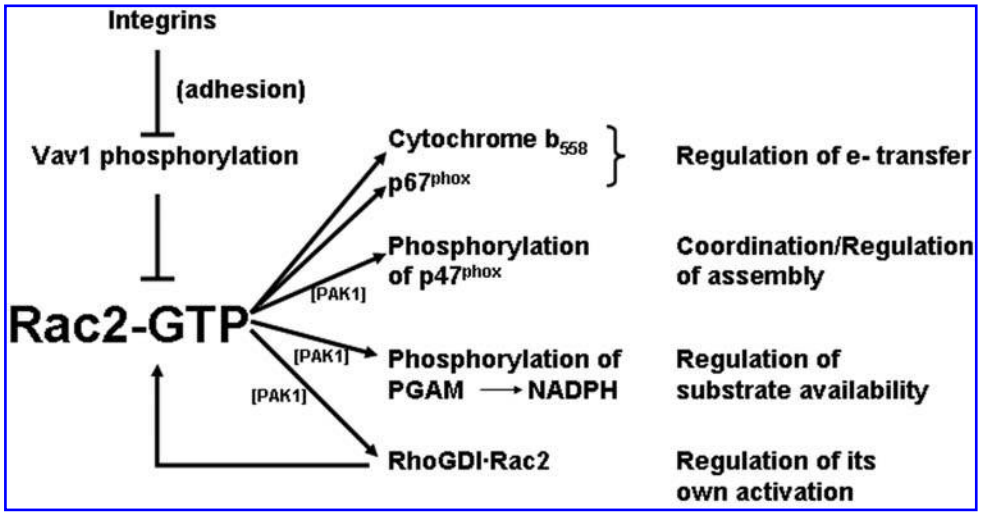

FIG. 6. Multiple modes of NADPH oxidase regulation by Rac GTPase. Figure depicts the multiple means by which Rac2 GTPase may regulate ROS formation by the phagocyte NADPH oxidase.

stream mediators remains to be clarified. The molecular basis for differences in the regulatory activities of Rac1 versus Rac2 in leukocytes will require a more detailed understanding of how these regulatory GTPases act biologically. The answers to these questions are likely to come from functional analysis of transgenic knockout animals, structural studies of NADPH oxidase components and their assembly, and biochemical/biophysical determination of the dynamics of Rac GTPase regulation, as well as of conformational changes in protein components of the NADPH oxidase induced during the assembly, activation, and deactivation process.

The production of ROS through the multiprotein enzyme NADPH oxidase is crucial for our innate immune responses against invading microorganisms but can also produce tissue damage and promote inflammatory diseases. The identification of Rac as a critical molecular switch in NADPH oxidase activation and the regulation of this switch by such factors as adhesion and cytokine signaling suggest Rac to be a critical point of control that may contribute to the development of disease and/or serve as a target for antiinflammatory disease therapy. An example of this is the repression of Rac2 mRNA expression and thus NADPH oxidase–mediated ROS production by the organism, *Anaplasma phagocytophila,* the causative agent of human granulocytic ehrlichiosis (128). The exact contributions of Rac-regulated ROS formation to various inflammatory diseases is currently under investigation using Rac1/2-deficient mouse models. A deeper understanding of Rac signaling should lead to even greater opportunity for novel disease therapies.

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