NADPH oxidase is a plasma membrane enzyme of phagocytes generating superoxide anions which serve as bactericidal agents. Activation of this multicomolecular enzyme minimally requires assembly at the membrane with flavocytochrome b$_{558}$ of cytosolic components p47$_{phox}$, p67$_{phox}$, and Rac proteins. Rac1 and Rac2 are 92% homologous cytosolic small GTPase proteins. Both Rac1 and Rac2 have been implicated with NADPH oxidase activation in vitro; however, Rac2 is largely predominant in human phagocytes. Here, using the yeast two-hybrid system, we provide data demonstrating in vivo interactions between human p47$_{phox}$, p67$_{phox}$, and Rac proteins. Rac proteins interact with p67$_{phox}$ in a GTP-dependent manner, but do not interact with p47$_{phox}$. Moreover, Rac effector site mutants, which are known to be inactive in NADPH oxidase, lose their interaction with p67$_{phox}$; Rac2L61 mutant, which has an increased NADPH oxidase affinity, shows an increased affinity for p67$_{phox}$. Finally, we observe that p67$_{phox}$ interacts 6-fold better with Rac2 than with Rac1. We also show a strong intracellular interaction between p47$_{phox}$ and p67$_{phox}$. These results indicate that activated Rac can regulate NADPH oxidase by interacting with p67$_{phox}$ and that Rac2 is the main p67$_{phox}$-interacting GTPase in human cells.

The Rac Target NADPH Oxidase p67$^{phox}$ Interacts Preferentially with Rac2 Rather Than Rac1*

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NADPH oxidase is a multicomponent plasma membrane-bound enzyme used by phagocytes to produce superoxide anions in response to stimuli from microbial infections (1–3). This massive production of oxygen metabolites, known as the respiratory burst, is an efficient bactericidal mechanism as underscored by severe infections in case of enzyme deficiency in chronic granulomatous disease (4).

Early biochemical and genetic studies (5, 6) in combination with reconstitution of enzyme activity in a cell-free system, characterized the minimal structure of the enzyme (7, 8). This includes the transmembrane flavocytochrome b$_{558}$, consisting of subunits gp91$_{phox}$ and p22$_{phox}$, and three regulatory cytosolic proteins, p47$_{phox}$, p67$_{phox}$ (9, 10), and the more recently isolated small GTPase Rac1 or Rac2 (11, 12). Rac1 and Rac2 are 92% homologous Ras-like proteins, initially isolated from cell cytosol, Rac1 from guinea pig macrophages, and Rac2 from human neutrophils, as the GTP-binding protein enhancing superoxide generation in the cell-free assay (11, 12). The physiological requirement for Rac proteins in NADPH oxidase has then been demonstrated in a whole cell assay, using rac antisense oligonucleotides or a dominant-negative mutant (13, 14).

In addition, the small GTPase Rap1a, found tightly associated with membranous cytochrome b$_{558}$ (15), although dispensable in the cell-free system, is required for enzyme activity in a whole cell environment (14, 16). The recently isolated cytosolic protein p40$_{phox}$ (17, 18) has been reported to interact with p47$_{phox}$ and p67$_{phox}$ components, and may play the role of a chaperone in the activated complex (19).

How is Rac regulating the NADPH oxidase? Clearly the nucleotide state of Rac proteins controls the activity of NADPH oxidase: there is an absolute requirement for GTP in the cell-free system. The recently isolated cytosolic Rap1a, and Rap2, a complex protein whose GTPase activating function acts on Rac in vitro, regulates the activation of the enzyme in vivo (20). An intact Rac effector domain is also required for enzyme activation, as shown by the inefficiency of Rac effector mutants in cell-free assays (21–23). It has been reported that Rac2L61, a GTPase-deficient mutant (therefore mainly in the GTP-bound form) has at least a 3-fold enhanced affinity for the enzyme, as compared to wild type or RacV12 proteins (22). Both Rac1 and Rac2 proteins are able to activate the enzyme and are roughly equivalent for activation of the recombinant cell-free system (24, 25). However, in the presence of human neutrophil cytosol in the assay, Rac2 was a more potent activator than Rac1 (24).

Upon cell stimulation, cytosolic regulatory proteins translocate to the plasma membrane and/or membrane-associated cytoskeleton to form the active enzyme complex (26–28). Components, p47$_{phox}$ and p67$_{phox}$, tightly associated as a cytosolic complex (18, 29), translocate to the membrane where p47$_{phox}$ interacts with cytochrome b$_{558}$ subunit p22$_{phox}$, probably through SH3 and proline-rich domain interactions (30, 31). Although contradictory data have been published about Rac recruitment at the membrane (32), overall it appears that Rac-GTP activates NADPH oxidase at the membrane level (33) and that in whole cells, Rac translocation seems to correlate with enzyme activation (27, 28, 34, 35).

Interactions between p47$_{phox}$ and p67$_{phox}$ have been widely documented recently (30, 31, 36–39), but direct interactions of Rac proteins with NADPH oxidase components are so far poorly described. After we began this study, Diekmann et al. (21) reported, using glutathione S-transferase fusion proteins, that Rac1-GTP was able to interact with p67$_{phox}$ through its effector domain, suggesting that p67$_{phox}$ was a molecular target...
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for Rac proteins. However, several points can be raised about this result: a 100 times molar excess of p67phox over Rac recombinant proteins was necessary to observe a rather weak interaction; only Rac1 has been studied even though Rac2, mainly expressed in NADPH oxidase expressing cells, is highly predominant over Rac1 in human phagocytes (34, 40); and in a more biological environment, Sf9 cells overexpressing p47phox, p67phox, and p21Rac1, Rac1-p67phox complex was undetectable (33). Another study (41) also very recently reported a weak interaction in vitro between Rac1-GTP and p67phox recombinants proteins.

Here, we have used a two-hybrid system in yeast (42-44) to study in an intracellular environment, interactions between the three regulatory cytosolic factors, p47phox, p67phox, and p21Rac. Our results provide clear evidence showing that p47phox and p67phox interact in a stable complex, that p67phox is an in vivo target for Rac-GTP, and that Rac2 in a whole cell system, interacts much better than Rac1 with p67phox.

EXPERIMENTAL PROCEDURES

Two-hybrid System: Yeast, Vectors, and Methods—In order to investigate protein-protein interactions in the two-hybrid system, we used the yeast strain HF7c (45) containing two GAL4-inducible reporter genes, HIS3 and LacZ, or the yeast strain L40 (kindly provided by Dr. A. Vojtek) where the same reporter genes are under the control of multicyclopases of LexA operon (46).

Proteins fused to the GAL4 DNA binding domain were expressed from plasmid pGBT10 (45). Proteins fused to the GAL4 activation domain (GAL4 AD)1 were expressed from plasmid pGAD-GH (47). Proteins fused to the LexA protein were expressed from either plasmid pBTM116 (46) or from pVJ L10, a derivative of pBTM116 with modified polylinker.2 HF7c or L40 cells were cotransformed with pairs of two-hybrid plasmids and selected by growth on medium lacking tryptophan and leucine. Cotransformed colonies were patched on the same selective medium and subsequently replicated to test for protein interaction, using β-galactosidase activity assay on filter with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside or using growth in the absence of histidine in the medium, see indications in figure legends (44, 48).

Cloning of rac, p47phox, and p67phox in Two-Hybrid Plasmids—Alleles of rac1, rac1-1wt, rac1-V12, rac1-V12N17, rac1L21A35 and rac1V12A38 have a Ser-189 mutation and are fused to a Myc epitope (49). They were amplified by polymerase chain reaction when necessary and cloned in-frame with LexA protein. p47phox and p67phox cDNAs, a gift of Dr. T. Leto, were cloned in-frame with GAL4 DNA binding domain in plasmid pGBT10 and, in-frame with GAL4 AD in plasmid pGAD-GH. Complementary DNA of rac-V12, rac2-N17, and rac2-L61, amplified by polymerase chain reaction when necessary, were inserted in-frame with LexA protein. In order to insure correct comparisons between rac1 and rac2 alleles, rac1 alleles used for such comparisons (Fig. 4) were reamplified by polymerase chain reaction and cocloned, without myc-tag and Ser-189 mutation, in-frame with LexA protein. All alleles derived from polymerase chain reaction products were entirely sequenced.

Analysis of LexA Fusion Proteins Expression—Total yeast extracts were prepared in SDS sample buffer according to standard procedures (48), resolved by SDS-polyacrylamide gel electrophoresis (10%) gel, and electrophoried onto nitrocellulose. Blots were processed as described (13) with rabbit anti-LexA antibodies kindly provided by Dr. P. Moreau (50), and developed using the ECL detection system (Amersham).

Quantitative Assay for β-Galactosidase Activity—In addition to the non-quantitative β-galactosidase test on filter, we used the protocol when enzyme activity needed to be quantified. Cotransformed yeast colonies, grown in selective liquid medium lacking leucine and tryptophan, were harvested while in the exponential growth phase. Similar numbers of cells were processed for the different cotransformed yeasts. Cells were disrupted by vortexing with glass beads, at 4°C in 0.1 M Tris, pH 8. Protein extracts were quantified and used to determine β-galactosidase activity in: 100 mM phosphate buffer, pH 7, 10 mM MgCl2, 1 mM MgSO4, and 50 mM β-mercaptoethanol, with O-nitrophenyl-β-D-galactopyranoside as substrate (0.7 mg/ml final). Reactions were carried out at 30°C, stopped by addition of 250 mM sodium carbonate, and enzyme activity determined by measuring absorbance at 420 nm (51). Linearity of β-galactosidase activity versus quantity of yeast cell extract was checked.

RESULTS AND DISCUSSION

Since previous evidence suggested an interaction between p67phox and p47phox (18, 29), we first tested the ability of these two proteins to interact in the two-hybrid system. A plasmid expressing p47phox fused to the GAL4 activation domain and a plasmid expressing p67phox fused to the GAL4 DNA binding domain were introduced into yeast reporter strain HF7c (45). Transformants were tested for their ability to grow in the absence of histidine. The interaction between a hybrid of SNF1 and a hybrid of SNF4, two yeast proteins known to interact with each other, provided a positive control (42). All transformants grew at similar rates in the presence of histidine, but only cells containing GAL4 AD-p47phox and GAL4 DNA binding domain-p67phox hybrids maintained their growth in the absence of histidine (Fig. 1). The reciprocal combination GAL4 AD-p67phox/GAL4 DNA binding domain-p47phox had the same effect. Growth capacity on medium lacking histidine was not induced when p47phox or p67phox were replaced by SNF1 or SNF4 proteins (Fig. 1). Therefore, we conclude that expression of the reporter gene HIS3 results from the interaction between p47phox and p67phox. This interaction was initially suspected from observations showing that a large protein complex found in phagocyte cytosol contained a fraction of both proteins (18, 29), and that p67phox translocation to the membrane depended on the presence of p47phox (52). A direct interaction between both proteins in cell cytosol, involving SH3 and proline-rich domains of each protein has been observed (30, 33, 36, 37). Consistent with these data, the results of our two-hybrid study provide evidence for the formation of a complex between p47phox and p67phox proteins in the intact cell. They further indicate that this complex is independent of Rac. p47phox, p67phox interactions in the two-hybrid system provide a means for investigating in an intracellular environment how this interaction could be modulated by biological signals. It should be feasible, for example, to stimulate or inhibit in yeast, pathways known to activate NADPH oxidase in phagocytes (protein kinase C activation or arachidonate synthesis) and to analyze their effect on the p47phox/p67phox complex.

Interactions between p21Rac and p47phox or p67phox were first investigated using the same two-hybrid system based on GAL4

1 The abbreviations used are: GAL4 AD, GAL4 activation domain; ECL, enhanced chemiluminescence.
2 V. Jung and J. Camonis, unpublished data.
reconstitution in reporter yeast HF7c. Different alleles of Rac1 were used: Rac1 wild type; Rac1V12, an activated mutant of Rac1 blocked in GTP-bound form; and Rac1V12N17, a dominant-negative mutant of Rac1 blocked in the GDP-bound form (49). Rac proteins undergo an isoprenylation of their C-terminal part, at cysteine 189, which probably allows this cytosolic protein to bind membrane (53). To avoid decreasing detection sensitivity due to partial membrane localization of proteins in yeast, as already reported in two-hybrid study of Ras-Raf interactions (54), we tested Rac1 alleles with a serine 189 mutation, thus preventing C-terminal isoprenylation and membrane localization. In the first set of experiments (data not shown) cotransformed colonies expressing the different Rac alleles with p47phox or p67phox proteins were checked for their ability to grow in the absence of histidine. Only transformants expressing Rac1V12 in combination with p67phox showed a significant histidine-independent growth. Although the signal was rather weak, it was significant and suggested that an activated form of Rac1 was required for Rac1-p67phox interaction. To increase signal intensity, we decided to use another yeast two-hybrid system, which in our hands appeared more sensitive.

This system, based on LexA DNA binding protein and yeast strain L40 (46), which carries HIS3 and LacZ as reporter genes, allowed us to make a more detailed analysis of Rac-p67phox interactions. The same Rac1-serine 189 alleles were used as fusion protein with LexA, to look at interactions with p67phox or p47phox fused to the GAL4 activation domain (Fig. 2). The interaction between RasV12 hybrid and c-Raf1 hybrid provided a positive control in L40 cells (Fig. 2B). The various cotransformed cells were able to grow at similar rates in the presence of histidine, indicating no particular toxicity of expressed hybrid proteins. The ability to grow in the absence of histidine was clearly induced when Rac1V12 and p67phox hybrid proteins were coexpressed (Fig. 2A). The “deactivation” of Rac1V12 by the Asn-17 mutation resulted in a dramatic decrease of histidine-independent growth of Rac1V12N17-p67phox transformants (Fig. 2A). By contrast, Rac1V12-p47phox or Rac1V12N17-p47phox combinations were completely inefficient in promoting histidine-independent growth (Fig. 2A). These results therefore indicate that only the activated GTP-bound form of Rac1 is capable of interacting with cytosolic regulator of NADPH oxidase, p67phox, but not with p47phox. We also conclude that isoprenylation of the C-terminal part of Rac proteins is not essential for Rac-p67phox interactions, consistent with previous studies of Rac activity in a cell-free system (24).

To check for specificity of the interaction, various activated small GTPase proteins were also tested for interaction with p67phox in L40 cells. RasV12, an activated form of Ras, did not interact with p67phox in the assay. Similarly, RhoGV12CAAX, an activated form of RhoG lacking the C-terminal CAAX box, and an activated RhoB mutant, RhoBV14, showed no interaction with p67phox able to stimulate growth in the absence of histidine (Fig. 2B). Activated forms of Rap2 (RapV212) and RalA (RalAV23 with 18 C-terminal amino acids deleted) were also negative in a two-hybrid assay with p67phox (not shown). The various GTPases used were able to interact with their own partners (unpublished two-hybrid assays). We conclude that p67phox interacts specifically with the NADPH oxidase activator Rac.

To further investigate whether p67phox is a functional target for Rac, we used Rac proteins mutated in the putative effector region (amino acids 32 to 40). Substitutions of conservative residues at codon 35 (Thr→Ala) or codon 38 (Asp→Ala) have demonstrated in Ras studies that the effector domain is crucial for biological effects of Ras and for direct interaction with Raf.
ment with in vitro results obtained with recombinant proteins and recently published by Diekmann et al. (21) and Prigmore et al. (41). Since both sets of results strongly support the view that p67\textsuperscript{phox} is a direct target for Rac in phagocyte NADPH oxidase.

To complement our study of Rac-p67\textsuperscript{phox} interaction, we wondered if activated RacL61 mutant, known to have a higher affinity for NADPH oxidase than RacV12 or Rac wild type (22), also had a higher affinity for the p67\textsuperscript{phox} component. L40 cells were cotransformed with p67\textsuperscript{phox} encoding plasmid and plasmids encoding Rac1L61 or Rac1V12 fused to LexA. The Rac1L61 mutant appeared highly toxic in yeast cells. Therefore, although Rac1L61-induced signals appeared stronger than those from Rac1V12 (data not shown), accurate quantification of the difference could not be obtained.

Because Rac2 is the predominant Rac protein in human phagocytes (34, 40) and because the functional "L61" data were obtained using Rac2 (22), we decided to use Rac2 alleles to further investigate effects of L61 mutation. Activated Rac2V12 (GTP-bound), deactivated Rac2N17 (GDP-bound), or activated Rac2L61 (GTP-bound) mutants, fused to LexA, were coexpressed in L40 cells with p67\textsuperscript{phox}. We observed that the Rac2V12-p67\textsuperscript{phox} combination induced a clear histidine-independent growth (Fig. 3A). Rac2N17 allele expressed concomitantly with p67\textsuperscript{phox} allowed no growth in the absence of histidine as compared to control cells expressing Rac2N17 alone. On the contrary, Rac2L61 mutant induced a highly efficient growth which is clearly stronger than the growth induced by the Rac2V12 mutant. Identical observations were made using the second reporter gene, LacZ, as shown by a stronger β-galactosidase activity in a liquid assay from the exact same number of cells.

We observed that Rac2-p67\textsuperscript{phox} interactions were strong and comparable to positive controls but that Rac1-p67\textsuperscript{phox} interactions appeared relatively weak. A more precise comparison of Rac1 and Rac2 affinity for p67\textsuperscript{phox}, using Rac V12 or L61 activated mutants fused to LexA proteins, was made directly on the same experiment. Cells coexpressing Rac1V12 or Rac2V12 and p67\textsuperscript{phox} grew at normal rates in the presence of histidine and were tested for their capacity to grow in the absence of histidine. In several independent experiments, Rac2V12 allowed a much better rate of growth than Rac1V12 (Fig. 4A). Similar observations were made using the other reporter gene, LacZ, as shown by a stronger β-galactosidase activity (Fig. 4B). Quantification of Rac1- and Rac2-p67\textsuperscript{phox} interactions was obtained by measurement of β-galactosidase activity in a liquid assay from the exact same number of cells. We observed that Rac2V12 induced around 6-fold more enzyme activity than Rac1V12 (Fig. 4C). It shows that LacZ reporter gene, as well as HIS3 reporter gene, are both stimulated to higher levels by the Rac2V12-p67\textsuperscript{phox} complex than by the Rac1V12-p67\textsuperscript{phox} complex. Expression of both Rac mutant proteins was checked by Western blot and were found to be similar. We tried further to compare Rac1 and Rac2 affinity for p67\textsuperscript{phox} using the Rac L61 mutants. Again Rac2L61-induced signals appeared clearly stronger; however, due to the high toxicity of Rac1L61 mutant we cannot provide a reliable estimation of this difference. In all, these data indicate that p67\textsuperscript{phox}, in an intracellular environment, has a much better affinity for Rac2 protein than for Rac1 protein.

NADPH oxidase activation by small GTPase led to isolation of Rac1 from guinea pig macrophages (11) and to Rac2 from human phagocytes (12). Whether this difference reflects a species specificity remains to be determined. However, the predominant interaction of p67\textsuperscript{phox} with Rac2 that we observed provide new information relevant to this question. Our data, in combination with the fact that human phagocytes contain predominantly Rac2 rather than Rac1 (34, 40), show that the Rac-p67\textsuperscript{phox} interaction in a whole cell environment is almost specific for Rac2 protein.

This raises the question of the molecular basis for the difference between Rac2 and Rac1. We showed that the Rac-p67\textsuperscript{phox} interaction involved the Rac GTP-binding site and required the Rac effector domain. Rac2 and Rac1 are almost identical in these areas; however, the C-terminal portions of the two pro-
Interactions between $p67^{\text{phox}}$, $p47^{\text{phox}}$, and $p21^{\text{Rac}}$.

**Fig. 4.** Comparison between Rac1 and Rac2 proteins for interaction with $p67^{\text{phox}}$. In yeast strain L40, Rac1V12 and Rac2V12 fused to LexA, were tested for interaction with $p67^{\text{phox}}$ fused to the GAL4 activation domain. Protein interactions are demonstrated by histidine-10. Leto, T. L., Lomax, K. J., Volpp, B. D., Nunoi, H., Sechler, J. M., Nauseef, W. M., Clark, R. A., Gallin, J. I., and Malech, H. L. (1990) Science 248, 727–730.

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