Interaction of Rac with p67^{phox} and Regulation of Phagocytic NADPH Oxidase Activity

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Rho and Rac, two members of the Ras superfamily of guanosine triphosphate (GTP)–binding proteins, regulate a variety of signal transduction pathways in eukaryotic cells. Upon stimulation of phagocytic cells, Rac enhances the activity of the enzyme nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) oxidase, resulting in the production of superoxide radicals. Activation of the NADPH oxidase requires the assembly of a multimolecular complex at the plasma membrane consisting of two integral membrane proteins, gp91^{phox} and p21^{phox}, and two cytosolic proteins, p67^{phox} and p47^{phox}. Rac1 interacted directly with p67^{phox} in a GTP-dependent manner. Modified forms of Rac with mutations in the effector site did not stimulate oxidase activity or bind to p67^{phox}. Thus, p67^{phox} appears to be the Rac effector protein in the NADPH oxidase complex.

The Rho and Rac GTP-binding proteins control signal transduction pathways activated by extracellular factors: Rho regulates the assembly and activity of integrin complexes at cell-substrate and cell-cell contacts, whereas Rac regulates the polymerization of actin at the plasma membrane to form lamellipodia (*1, 2*). The biochemical nature of these signaling pathways is presently unknown, although the cellular responses all appear to involve the formation of multimolecular complexes, associated with polymerized actin, at the plasma membrane (*3*). In phagocytes, Rac regulates the activity of a membrane-bound NADPH oxidase to produce superoxide radicals in response to microbial infection. The active site of this enzyme is located in an integral membrane cytochrome, b_{558}, which consists of two subunits, gp91^{phox} and p21^{phox}, but superoxide production depends on the formation of a complex that includes two cytosolic proteins, p67^{phox} and p47^{phox} (*4*). A fifth component, Rac, is also essential for oxidase activity (*5*).

To identify the functional target protein for Rac in the NADPH oxidase complex, we used an in vitro binding assay. Recombinant human Rac1 was isolated by purification of a glutathione-S-transferase (GST) fusion protein from an *Escherichia coli* expression system and subsequent proteolytic cleavage with thrombin (*2*). The p67^{phox} and p47^{phox} proteins were also purified as GST fusion proteins (GST-p67^{phox} and GST-p47^{phox}) from *E. coli* (Fig. 1A). The p29RhoGAP protein, a catalytic fragment of p50RhoGAP [a guanosine triphosphatase (GTPase)–activating protein that interacts with all members of the Rho subfamily], was used as a positive control in these experiments (*6*). Rac was incubated first with [α-^{32}P]GTP to exchange the bound guanine nucleotide and then with an excess (100 times the concentration of Rac) of GST-p67^{phox}, GST-p47^{phox}, GST-p29RhoGAP, or GST at 4°C for 1 hour (*7*). The GST fusion proteins were collected by addition of glutathione–Sepharose beads and centrifugation. After washing the Sepharose beads, we detected bound Rac by measuring radioactivity. The wild-type Rac protein bound efficiently to p67^{phox} but not to p47^{phox}, RhoGAP, or GST (Fig. 1B).

To look at the interaction in more detail, we used a mutant form of Rac, L61Rac, which has Leu substituted for Gln at codon 61 and which is resistant to GTPase stimulation by RhoGAP. A similar mutation in Rho and in Ras increases their affinities for GAP proteins by 100- and 10-fold, respectively (*8, 9*). We found that L61Rac bound to p67^{phox} with a similar efficiency to that of wild-type Rac, but it also interacted strongly with RhoGAP (Fig. 1B). Neither wild-type Rho nor an activated mutant Rho (L63Rho) interacted with p67^{phox} (Fig. 1B). We cannot determine the strength of interaction between Rac and p67^{phox} with this simple binding assay; however, the affinity of L63Rho for RhoGAP has been determined in competition assays and is estimated to be 10 nM (*8*).

The p67^{phox} protein has two SH3 domains but no other similarity to other known proteins (Fig. 1C) (*10*). To locate the binding site for Rac on p67^{phox}, we expressed as GST fusion proteins two deletion mutants, p67Δ199 and p67Δ246, containing either the NH_2-terminal 199 or 246 amino acids, respectively (Fig. 1A). Both proteins captured [α-^{32}P]GTP-bound Rac as efficiently as full-length p67^{phox}. We conclude that Rac interacts with p67^{phox} somewhere in the NH_2-terminal 199 amino acids of the protein and that neither SH3 domain is required. Attempts to express smaller fragments of p67^{phox} as GST fusion proteins in *E. coli* were unsuccessful (*11*).

To examine whether the interaction of Rac with p67^{phox} is GTP-dependent, we incubated Rac with either [^{3}H]GTP or [^{3}H]guanosine diphosphate (GDP) then assayed for binding to GST-p67^{phox} (*12*). Only the GTP-bound form of Rac interacted with p67^{phox} (Fig. 2A). The interaction of Ras with its target protein c-Raf is also dependent on GTP binding, and c-Raf acts as a weak GTPase-activating protein for Ras (*13, 14*). We detected no RacGAP activity associated with p67^{phox}. The p67^{phox} protein did, however, interfere with the ability of Bcr, a known RacGAP, to stim-

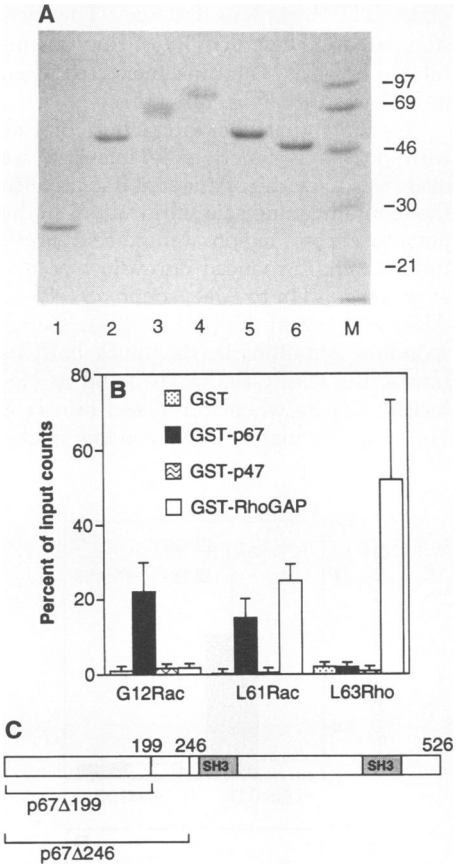

Fig. 1. Interaction of Rac with GST-p67^{phox}. (**A**) Coomassie blue–stained SDS-polyacrylamide gel of purified GST fusion proteins. Lane 1, glutathione-S-transferase (GST); lane 2, GST-p29RhoGAP; lane 3, GST-p47^{phox}; lane 4, GST-p67^{phox}; lane 5, GST-p67Δ246; and lane 6, GST-p67Δ199. Lane M, molecular size standards (in kilodaltons). (**B**) Binding of wild-type Rac (G12Rac), L61Rac, or L63Rho, each complexed with [α-^{32}P]GTP to different GST fusion proteins. The results are the mean of three experiments. (**C**) Structure and position of p67^{phox} deletion mutants and SH3 domains.

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ulate GTP hydrolysis by Rac. The Bcr-stimulated GTPase activity of Rac was inhibited by p67^{phox} but was unaffected when p47^{phox} was used (Fig. 2B) (*15*).

To show that the interaction of Rac with p67^{phox} is functionally relevant, we made use of a series of mutated Rac proteins that contain amino acid substitutions in the putative effector region. Amino acid substitutions were introduced into wild-type Rac at codon 35 (Thr to Ala), codon 38 (Asp to Ala), and codon 40 (Tyr to Lys). Corresponding mutations in Ras block both its interaction with c-Raf in vitro and its biological activity when introduced into cells (*14, 16*). Similarly, corresponding mutations in Rho block its ability to induce focal adhesion formation in fibroblasts (*17*). Each of the three mutated Rac proteins was unable to sustain superoxide production in an in vitro oxidase assay under conditions where recombinant wild-type Rac was fully active (*18*). The three effector mutant Rac proteins did not interact with p67^{phox} in the in vitro binding assay (Fig. 3).

We conclude from these experiments that p67^{phox} is the functional target for Rac in the phagocytic NADPH oxidase complex. The p67^{phox} protein has no sequence similarity with RhoGAP, BcrGAP, or p65^{PAK}, a protein kinase that binds to Rac and CDC42 in vitro (*19*). Binding of p67^{phox} to Rac could trigger the formation of a p67^{phox}-p47^{phox} complex which might then interact with the membrane components; however, this seems unlikely because such cytosolic complexes can be detected even in unstimulated cells (*20*). The p47^{phox} protein can translocate to the membrane in the absence of p67^{phox} and this, coupled with the experiments described here, would suggest that p47^{phox} translocation is independent of Rac (*21*). We therefore favor a model in which association between the cytochrome b_{558} and a cytosolic p67^{phox}-p47^{phox} complex occurs independently of Rac, perhaps as a consequence of phosphorylation events or through other second messenger signals (*22*). Activation of this complex to produce superoxide would be dependent on the translocation of RacGTP to the membrane and its subsequent interaction with p67^{phox} (*23–25*).

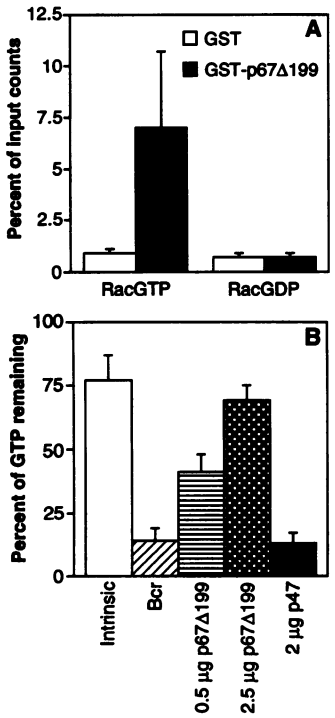

Fig. 2. Binding of p67^{phox} to Rac in a GTP-dependent manner and inhibition of BcrGAP activity. (**A**) Binding of [^{3}H]GTP.Rac or [^{3}H]GDP.Rac to GST-p67Δ199. (**B**) Inhibition of BcrGAP-stimulated GTPase activity of Rac by p67Δ199. The results are the mean of three experiments.

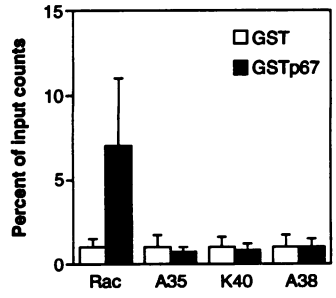

Fig. 3. Ability of Rac effector mutants to bind to GST-p67^{phox}. Binding of wild-type or mutated Rac proteins complexed with [α-^{32}P]GTP to GST-p67Δ199.

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7. The complementary DNAs encoding human p29RhoGAP, p47^{phox}, p67^{phox}, Rac1, and RhoA were cloned into the GST expression vector pGEX-2T (Pharmacia) and the fusion proteins were affinity purified as described (*2, 6*). We constructed deleted forms of p67^{phox} by terminating the reading frame at a unique Dra III site (nucleotide 733) to make p67Δ246 or a unique Sty I site (nucleotide 594) to make p67Δ199 (*10*). As required, fusion proteins were cleaved by overnight treatment with thrombin (5 to 10 U/ml) (Sigma). For the binding assay, thrombin-cleaved Rac and Rho (0.5 μg) were incubated with 50 μCi of [α-^{32}P]GTP (NEN Du Pont; 3000 Ci/mmol) in 250 μl of 50 mM tris (pH 7.5), 5 mM EDTA containing bovine serum albumin (0.5 mg/ml) for 10 min at 30°C, and the exchange reaction was stopped by addition of ice-cold $MgCl_2$ (to 10 mM) followed by addition of dithiothreitol (to 0.1 mM) and GTP (to 10 μM). The amount of radioactivity in a 50-μl sample was measured with the nitrocellulose filter binding assay (*26*). To measure protein interactions, we incubated 50-μl samples containing 100 ng of Rac or Rho with 20 μg of GST–fusion protein on ice for 1 hour. Glutathione–Sepharose beads (15 μl of a 1:1 suspension) (Pharmacia) were added and incubated for 1 hour at 4°C. The beads were centrifuged, washed three times with 1 ml of wash buffer [50 mM tris (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100], resuspended in 300 μl of wash buffer, and collected on nitrocellulose filters for radioactivity counting.
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11. Attempts to express the NH_2-terminal amino acids 1 to 59, 1 to 105, 1 to 135, and 30 to 150 of p67^{phox} as soluble GST–fusion proteins were unsuccessful.
12. Rac (1 μg) was incubated in 150 μl of buffer with 10 μCi of [^{3}H]GTP or [^{3}H]GDP (1 mCi/ml, 7 Ci/mmol; Amersham) (*7*). A sample (50 μl) was analyzed with the nitrocellulose filter binding assay. For the binding assay, labeled Rac complex (50 μl) was incubated with either GST or GST-p67Δ199 (40 μg).
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15. GAP assays were done as described by incubation of Rac complexed with [γ-^{32}P]GTP (2.5 nM) at 17°C for 5 min (*27*). Under these conditions, 20% of the counts bound to Rac are lost as a result of its intrinsic GTPase activity. Sufficient *E. coli*–produced recombinant BcrGAP was added to stimulate the loss of 80% of the counts under the same conditions. For competition assays, 0.5 μg or 2.5 μg of p67Δ199 or 2 μg of p47^{phox} were added to the reaction before addition of BcrGAP. These competing proteins had been cleaved with thrombin and were not GST fusions.
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18. Superoxide production was determined as the rate of cytochrome c reduction that could be inhibited by superoxide dismutase. Solubilized human neutrophil membranes (12 μg) were preincubated with 1 μg each of recombinant p47^{phox}, p67^{phox}, and wild-type or mutant (A35, A38, or K40) Rac complexed with GTP-γ-S as described (*5*). The results (average of two experiments) expressed in nanomoles of O_2^- produced per minute are as follows: No addition, 1.8; wild-type Rac, 14.3; A35Rac, 3.3; A38Rac, 3.6; and K40Rac, 4.6.
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25. Two groups have reported that when complexed to RhoGDI, RacGDP will activate the NADPH oxidase in an in vitro assay (*24, 28*). Because there is as yet no precedent for a GTP-binding

protein being active in the GDP-bound state, we suppose that this complex, purified from cells, is derived from RacGTP:RhoGDI and that after GTP hydrolysis, RacGDP remains in the conformationally active state so long as it is bound to RhoGDI.
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14-3-3 Protein Homologs Required for the DNA Damage Checkpoint in Fission Yeast

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During the cell cycle, DNA is replicated and segregated equally into two daughter cells. The DNA damage checkpoint ensures that DNA damage is repaired before mitosis is attempted. Genetic studies of the fission yeast *Schizosaccharomyces pombe* have identified two genes, *rad24* and *rad25*, that are required for this checkpoint. These genes encode 14-3-3 protein homologs that together provide a function that is essential for cell proliferation. In addition, *S. pombe rad24* null mutants, and to a lesser extent *rad25* null mutants, enter mitosis prematurely, which indicates that 14-3-3 proteins have a role in determining the timing of mitosis.

A sophisticated series of controls has evolved that ensures the integrity of DNA before the initiation of mitosis. These controls include a checkpoint that prevents mitosis after DNA damage induced by radiation (*1*). A key unanswered question is how this radiation checkpoint interacts with the cell cycle machinery. Genetic analysis of *Schizosaccharomyces pombe* (reviewed in *2*) has identified two classes of radiation checkpoint mutants that are distinguishable by phenotype. The first class of mutants are defective in both the DNA damage checkpoint and in replication–mitosis dependency control (*3*, *4*), and they carry mutations in any of six *rad-hus* genes. The second class are defective primarily in the DNA damage checkpoint (*4*, *5*) and carry mutations in either the *chk1* or the *rad24* gene. Cells containing the *rad24.T1* mutation enter mitosis prematurely during normal growth, which indicates that the *rad24.T1* mutant may link the DNA damage checkpoint to the cell cycle machinery (*4*).

To investigate the defect in the *rad24.T1* mutant, we used an *S. pombe* genomic library (*6*) to isolate plasmids that complemented the cells' sensitivity to ultraviolet (UV) radiation. The complementing activity was localized to a 1.8-kb Eco RI–Bam HI fragment, and sequence analysis identified an open reading frame encoding a protein with 58% identity to the highly conserved eukaryotic 14-3-3 proteins (Fig. 1A). This sequence did not contain the *rad24* gene itself (*7*) but carried a multicopy suppressor of *rad24.T1*, which we designated *rad25*. Multicopy *rad25* plasmids completely suppressed the premature mitosis phenotype and the UV sensitivity of *rad24.T1* cells (*8*).

The 14-3-3 proteins have been identified in most eukaryotic cells (*9*) and diverse biochemical properties have been ascribed to them. Mammalian cells contain a minimum of seven 14-3-3 isoforms, and the single known *Saccharomyces cerevisiae* 14-3-3 homolog (BMH1) is nonessential (*10*). These observations suggested that functionally redundant 14-3-3 homologs might exist in *S. pombe*. To search for such homologs, we used degenerate primers corresponding to highly conserved sequences in the 14-3-3 proteins (Fig. 2) in polymerase chain reactions (PCRs). This analysis revealed two 14-3-3 genes, one of which was *rad25* (*11*). The predicted product of the second 14-3-3 gene (Fig. 1B) was 71% identical to that of *rad25*, and a null mutant had essentially the same phenotype as the *rad24.T1* mutant (Fig. 3). This gene mapped (*12*) to the same locus as the *rad24.T1* mutation and thus corresponds to the *rad24* gene. The *rad24-rad25* double null mutant was inviable (*13*), which indicates an essential role for the 14-3-3 homologs in *S. pombe*.

The *rad24* null cells were sensitive to UV and to ionizing radiation (Fig. 3, A and B), and quantitative analysis demonstrated that the duration of the delay to mitosis that was caused by DNA damage was reduced by one-half (Fig. 3C). This is an unusual phenotype for a null mutant of an *S. pombe* checkpoint gene (*3*, *4*). We previously reported (*4*) that *rad24.T1* mutants show a mitotic catastrophe phenotype and die rapidly when DNA ligase function is disrupted by the temperature-sensitive *cdc17.K42* mutation, and that loss of *wee1* function in *rad24.T1* cells is lethal. Equivalent observations have been made with the *rad24* null mutant. In addition to these phenotypes, which have been linked to the loss of the radiation checkpoint pathway (*1*, *5*), *rad24* null cells had a cytokinesis defect similar to that of *S. pombe* protein kinase C mutants (*14*); showed an unusual cone-shaped cell morphology (Fig. 4); and

A

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MSNSRENSVYLAKLAEQAERYEEMVENMKKVACSNDKLSVEERNLLSVAY 50
KNIIGARRASWRIISSIEQKEESRGNTRQAALIKEYRKKIEDELSDICHD 100
VLSVLEKHLIPAATTGESKVFYYKMKGDYYRYLAEFTVGEVCKEAADSSL 150
EAYKAASDIAVAELPPTDPMRLGLALNFSVFYYEILDSPESACHLAKQVF 200
DEAISELDSLSEESYKDSTLIMQLLRDNLTLWTSDAEYNQSAKEEAPAAA 250
AASENEHPEPKESTTDTVKA
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B

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MSTTSREDAVYLAKLAEQAERYEGMVENMKSVASTDQELTVEERNLLSVA 50
YKNVIGARRASWRIVSSIEQKEESKGNTAQVELIKEYRQKIEQELDTICQ 100
DILTVLEKHLIPNAASAESKVFYYKMKGDYYRYLAEFAVGEKRQHSADQS 150
LEGYKAASEIATAELAPTHPIRLGLALNFSVFYYEILNSPDRACYLAKQA 200
FDEAISELDSLSEESYKDSTLIMQLLRDNLTLWTSDAEYSAAAAGGNTEG 250
AQENAPSNAPEGEREPKATHR
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Fig. 1. Predicted sequences of *rad24* and *rad25* gene products (*21*). (**A**) The *rad25* gene was cloned as a multicopy suppressor of the *rad24.T1* mutant. A null allele was constructed by replacement of a 480–base pair Sph I fragment with a *ura4*$^+$ marker gene (*6*). The *rad25::ura4*$^+$ null mutant divided at a slightly reduced size, was mildly radiation-sensitive, and had a slight defect in the radiation checkpoint. (**B**) Fragments of the *rad24* gene were cloned by degenerate PCR (*11*) with different combinations of primers FTT1 (YTNYTNWSNGTNGCNTAYAARAA), FTT2 (TAYYTNAARATGAARGGNGAYTA), FTT3 (YTTCATYTTNTRRTARAANACYTT), and FTT4 (YTCRTARTARAANACNSWRAARTT) (Fig. 2). Thirteen FTT1-FTT2, five FTT3-FTT4, and six FTT1-FTT4 fragments were cloned and sequenced. Of these, seven were derived from *rad25* and seventeen defined a second gene, *rad24*. A null allele of *rad24* was constructed by introduction of a *ura4*$^+$ marker at the Bgl II site in the coding region (*6*). The *rad24::ura4*$^+$ null mutation maps to the same locus as, and produces a similar phenotype to, the *rad24.T1* mutation. The European Molecular Biology Laboratory database accession numbers for the DNA sequences are X79206 (*rad24*) and X79207 (*rad25*).

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