Molecular Cell, Vol. 6, 899–907, October, 2000, Copyright ©2000 by Cell Press

Structure of the TPR Domain of p67^{phox} in Complex with Rac·GTP

Karine Lapouge, Susan J. M. Smith, Philip A. Walker, Steven J. Gamblin, Stephen J. Smerdon, and Katrin Rittinger*
Division of Protein Structure
National Institute for Medical Research
Mill Hill
London, NW7 1AA
United Kingdom

Summary

p67^{phox} is an essential part of the NADPH oxidase, a multiprotein enzyme complex that produces superoxide ions in response to microbial infection. Binding of the small GTPase Rac to p67^{phox} is a key step in the assembly of the active enzyme complex. The structure of Rac·GTP bound to the N-terminal TPR (tetratricopeptide repeat) domain of p67^{phox} reveals a novel mode of Rho family/effector interaction and explains the basis of GTPase specificity. Complex formation is largely mediated by an insertion between two TPR motifs, suggesting an unsuspected versatility of TPR domains in target recognition and in their more general role as scaffolds for the assembly of multiprotein complexes.

Introduction

Phagocytes are important mediators of innate immunity through the generation of reactive oxygen species (ROS) that are capable of killing invading microorganisms. The source of these oxygen species is a superoxide anion (O_2^-) produced by the NADPH oxidase ("the respiratory burst"), a large, membrane-associated enzyme complex (Segal and Abo, 1993; Leusen et al., 1996b; Babior, 1999; Nauseef, 1999). In humans, the NADPH oxidase constitutes the primary defense mechanism against microbial infection, and mutations in any of the enzyme components can result in chronic granulomatous disease (CGD) in which patients suffer from severe bacterial and fungal infections due to defects in superoxide production (Roos, 1994; Thrasher et al., 1994). Since these highly reactive chemical species may also cause severe tissue damage and induce inflammatory responses, the respiratory burst must be tightly regulated. This is achieved by maintaining the oxidase in a dormant, inactive state in which the component proteins are partitioned between the cytosol and the cell membrane.

The NADPH oxidase consists of at least six subunits: four cytosolic proteins, Rac, p40^{phox}, p47^{phox} and p67^{phox}, together with two membrane-bound components, gp91^{phox} and p22^{phox}, which form a heterodimeric flavocytochrome, known as cytochrome b_{558}. In resting cells, p40^{phox}, p47^{phox}, and p67^{phox} exist in a cytoplasmic complex (Wientjes et al., 1993; Park et al., 1994), which, upon activation, translocates to the membrane and associates with cytochrome b_{558} to form the active enzyme. Each of these proteins contains SH3 domains, which bind to proline-rich sequences present in p22^{phox}, p47^{phox}, and p67^{phox}. The pairwise protein–protein interactions in these complexes have been extensively investigated (reviewed in DeLeo and Quinn, 1996; Babior, 1999; Nauseef, 1999), but the intra- and intermolecular rearrangements that take place during activation and translocation to the membrane are still poorly understood.

A crucial step in the assembly and the activation of the NADPH oxidase is the binding of the small GTPase Rac to p67^{phox} (Abo et al., 1991; Knaus et al., 1991; Diekmann et al., 1994). Inactive, GDP-bound Rac exists as a cytosolic complex with RhoGDI (Rho guanine nucleotide dissociation inhibitor) from which it dissociates upon activation to translocate to the plasma membrane, independently of the other cytosolic components (Heyworth et al., 1994; Kleinberg et al., 1994; Dusi et al., 1996). The interaction of Rac with p67^{phox} is strictly GTP dependent, and much effort has been put into defining the regions in Rac and p67^{phox} that are involved in complex formation.

The region in p67^{phox} mediating the interaction with Rac has been mapped to the N-terminal 200 amino acid residues (Diekmann et al., 1994; Ahmed et al., 1998; Han et al., 1998; Koga et al., 1999), which have been predicted to contain four TPR (tetratrico-peptide repeat) motifs (Ponting, 1996). TPR motifs are degenerate 34 amino acid repeats that are present in a variety of organisms, ranging from bacteria to humans (Lamb et al., 1995; Ponting, 1996). TPR motifs often occur in tandem arrays and mediate a range of protein–protein and possibly protein–lipid interactions. In fact, many TPR motif–containing proteins may act as scaffolds for the assembly of multiprotein complexes such as the anaphase promoting complex (APC) or the peroxisomal import receptor complex (Hirano et al., 1990; Sikorski et al., 1990; Ponting and Phillips, 1996).

Rac belongs to the Rho family of small GTPases, which are involved in a large variety of cellular processes. Like all small GTPases, Rac is active in its GTP-bound form and able to interact with a host of downstream effectors to induce specific cellular responses (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). These downstream effectors can be roughly devided into two groups, those specific for Rac and/or Cdc42 and those specific for Rho. A subset of Rac/Cdc42 effectors share a common motif, designated the CRIB motif (Cdc42/Rac interactive binding), which consists of a minimal region of 16 amino acids and is present in PAK (p21-activated kinase), ACK (activated Cdc42-associated kinase), and WASP (Wiskott-Aldrich syndrome protein) (Burbelo et al., 1995). However, many more effectors specific for Rac and/or Cdc42 have been identified that lack the CRIB domain and share no obvious sequence homology in their GTPase binding domain. The recently solved structures of Cdc42 bound to CRIB domain containing peptides derived from ACK, WASP, and PAK revealed how CRIB domain effectors

*To whom correspondence should be addressed (e-mail: katrin.rittinger@nimr.mrc.ac.uk).

recognize their respective GTPase (Abdul-Manan et al., 1999; Mott et al., 1999; Gizachew et al., 2000; Morreale et al., 2000). However, no structural information has been available for complexes with Rac/Cdc42 specific effectors that do not contain a CRIB domain. Here we report the crystal structure of dominantly active Rac·Q61L·GTP bound to the TPR domain of $p67^{phox}$ and biochemical analyses of the interaction. The structure reveals a completely different set of GTPase/effector interactions to those observed in CRIB domain binding and explains the observed biological specificity of $p67^{phox}$ for Rac. Furthermore, the Rac/$p67^{phox}$ complex involves a different mode of TPR domain–mediated protein–protein interaction from those previously described and suggests that TPR motifs may be used in different ways to create binding surfaces for the assembly of multiprotein complexes.

Results and Discussion

Characterization of the Rac/$p67^{phox}$ Complex

Rac2 is the most abundant isoform of Rac in neutrophils and macrophages and shares 92% sequence identity with Rac1 (Figure 1A). The switch I and II regions are completely conserved between the two proteins, and the major differences in sequence are located at the C termini. Both isoforms have been shown to bind to $p67^{phox}$ and activate the NADPH oxidase in a cell-free system (Heyworth et al., 1993; Nisimoto et al., 1997; Ahmed et al., 1998). The binding site for Rac has been localized to the N-terminal 200 amino acids of the $p67^{phox}$ subunit (Diekmann et al., 1994; Han et al., 1998; Koga et al., 1999), which have been predicted to contain four tetratrico-peptide repeat (TPR) motifs (Figure 1B). We expressed the N-terminal TPR motif–containing domain of $p67^{phox}$ (amino acids 1–203, p67TPR) as well as the full-length protein and determined the affinity for activated Rac1 and Rac2 by isothermal titration calorimetry (ITC). Rac1·Q61L·GTP and Rac2·Q61L·GTP bind stoichiometrically to the N-terminal domain of $p67^{phox}$ with affinities of 2.7 and 1.9 μM, respectively (Figure 1C), compared to an affinity of full-length wild-type $p67^{phox}$ for Rac1·Q61L·GTP of 1.7 μM. These data confirm previous reports that the N-terminal region contains all structural features necessary for complex formation with Rac (Diekmann et al., 1994; Han et al., 1998; Koga et al., 1999).

Structure of the Rac·GTP/$p67^{phox}$ Complex

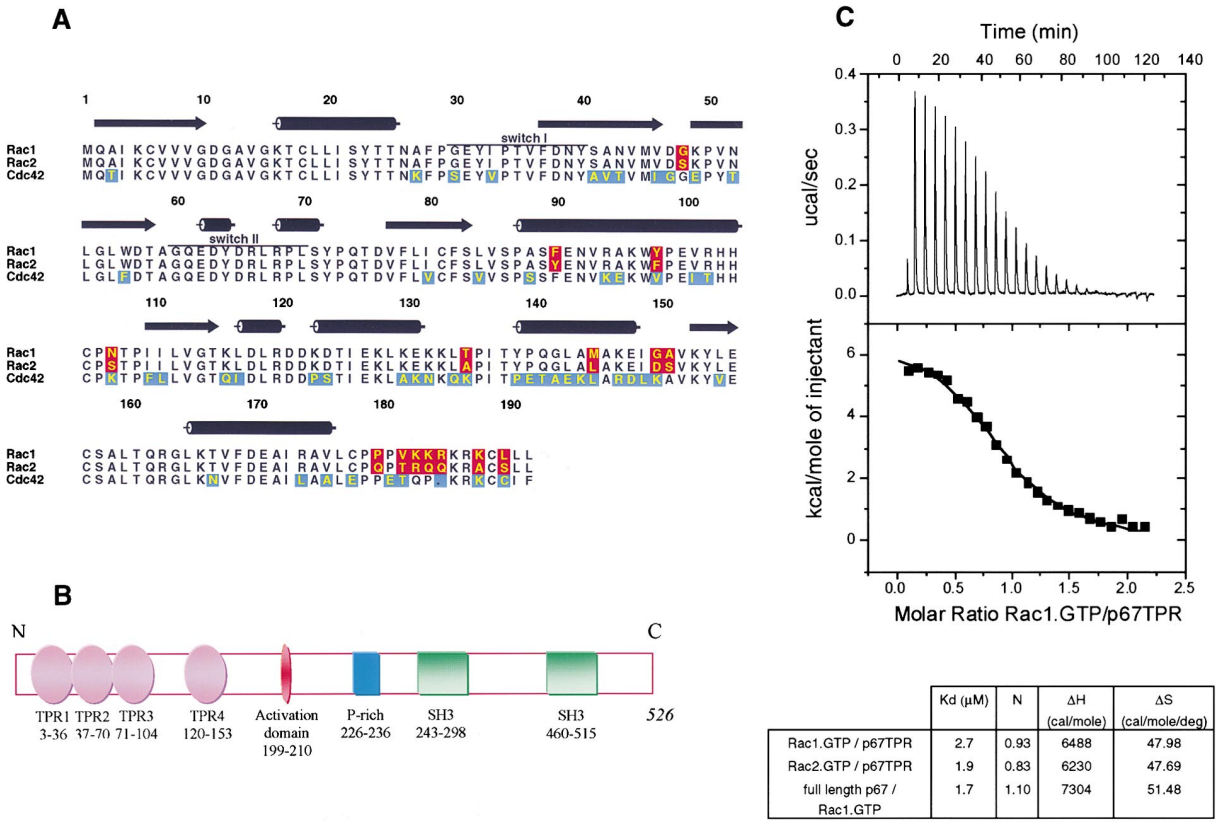
The structure of the Rac1·Q61L·GTP/p67TPR complex was solved by molecular replacement procedures and refined at a resolution of 2.4 Å (Figure 2). The structure of the GTPase-deficient Rac1·Q61L mutant in the complex is very similar to the structure of Rac1 bound to the nonhydrolyzable GTP analog GMPPNP (root-mean-square deviation of 0.65Å for 177 C_α atoms). The most significant differences are associated with the effector or switch I region, which shows strong electron density for residues 32–36 in contrast to the structure of isolated Rac1·GMPPNP (Hirshberg et al., 1997), in which this region is essentially disordered.

The p67TPR fragment contains nine α helices, eight of which form four TPR motifs. Each repeat folds into two antiparallel α helices (termed helix A and B), which pack together in a similar manner to that previously described for the TPR domain structures of protein phosphatase 5 (PP5) and the adaptor protein Hop (Das et al., 1998; Scheufler et al., 2000). The "isolated" C-terminal helix, helix C, packs against the preceding helix of TPR4 in a manner that resembles TPR A–helix interactions with adjacent repeats. Overall, the nine-helical bundle exhibits a right-handed superhelical twist which generates a groove on the A helix face of the domain that binds to the 18 ordered residues C-terminal to helix C, in an extended conformation (Figure 3A). The inside of the groove is rather hydrophobic, and formation of the intramolecular complex with the p67TPR C terminus buries 2742 $Å^2$ of solvent-accessible surface, 75% of which is contributed by nonpolar side chains.

The TPR segment of $p67^{phox}$ contains an insertion of 20 amino acids between the third and fourth repeats that forms two short antiparallel β strands and a 3_{10} helical turn. This β hairpin insertion lies along one edge of the TPR repeat stack and is fixed in position by hydrogen bonds with His-69, Val-72, Glu-96, Leu-98, Ile-99, Glu-122, Val-123, and Leu-124 (Figure 3B). In addition, the β hairpin insertion is further stabilized by nonpolar and polar interactions with the C-terminal extension, in particular by hydrogen bonds of the backbone carbonyls of Gly-113 with Arg-184 and Gln-115 with Lys-181. As described in the following sections, these interactions play crucial roles in the formation of the GTPase binding site.

Contact Regions between Rac and $p67^{phox}$

The binding surface presented by p67TPR to Rac is confined to one face of the TPR domain and is created by the β hairpin insertion and the loops that connect TPR1 with TPR2, and TPR2 with TPR3 (Figures 2 and 4). Formation of the GTPase-p67TPR complex buries 1170 $Å^2$ of solvent-accessible surface and involves direct as well as water-mediated hydrogen bonds (Figure 4A). The effector binding site is localized to the N- and C-terminal regions of Rac. These are located close together in the structure and include residues from helix 1 and the subsequent loop, residues from the N-terminal end of switch I and the loop connecting β5 to α5. No contacts are made with the switch II region or the Rac-inserted helical domain ("insertion helix"). In the following description, the subscripts Rac and p67 will be used to identify amino acids associated with each of the components of the complex. Arg-102_{p67} plays a key role in complex formation with Rac and makes direct hydrogen bonding interactions with main and side chain atoms of no less than four residues from Rac (Ala-159_{Rac}, Leu-160_{Rac}, Asn-26_{Rac}, and Ser-22_{Rac}; Figure 4A). This structural observation rationalizes mutational studies that show that substitution of Arg-102_{p67} by glutamic acid completely abrogates binding to Rac and results in a protein that is not able to support NADPH oxidase activation in vitro or in vivo (Koga et al., 1999). Gln-162_{Rac} forms a hydrogen bond with the side chain of Asn-104_{p67}, which is located within the β hairpin insertion of $p67^{phox}$ and which also forms a second hydrogen bond with the side chain of Asn-26_{Rac}. Additional contacts are made between Asp-108_{p67} in the TPR β hairpin insertion and



	Kd (μM)	N	ΔH (cal/mole)	ΔS (cal/mole/deg)
Rac1.GTP / p67TPR	2.7	0.93	6488	47.98
Rac2.GTP / p67TPR	1.9	0.83	6230	47.69
full length p67 / Rac1.GTP	1.7	1.10	7304	51.48

Figure 1. Complex Formation between Rac and p67phox

(A) Sequence alignment of human Rac1 (P15154), Rac2 (P15153), and Cdc42 (P21181). Residues that are not conserved between Rac1 and Rac2 are highlighted in red, and residues that are not conserved between Rac and Cdc42 are highlighted in blue. Secondary structure elements as well as the positions of switch I and switch II are shown above the sequence alignment. The figure was prepared using Alscript (Barton, 1993).

(B) Schematic representation of the domain structure of p67phox. The positions of the TPR motifs (purple), activation domain (red), as defined by Lambeth and coworkers (Han et al., 1998), proline-rich region (blue), and SH3 domains (green) are indicated. The fragment crystallized in complex with Rac includes amino acids 1–203.

(C) Isothermal titration calorimetry measurements of the binding of Rac to p67phox. Upper part, raw data of the titration of p67TPR (290 μM) into Rac1·Q61L·GTP (25 μM) at 18°C. Lower part, integrated heat changes, corrected for the heat of dilution, and fitted curve, based on a single-site binding model. Under identical experimental conditions, there was no heat change for the titration of p67TPR into Rac1·GDP or Rac2·GDP.

Thr-25$_{Rac}$. Interaction of Rac with the β hairpin insertion accounts for most of the contacts observed in the complex, and the only other direct hydrogen bonds between the two molecules are made by Gly-30$_{Rac}$ at the beginning of switch I with Asp-67$_{p67}$, and by Glu-31$_{Rac}$ with Ser-37$_{p67}$. All of the residues of Rac that are involved in the interface of the two proteins are conserved between Rac1 and Rac2, thus explaining the lack of any significant discrimination between Rac isoforms by p67phox in vitro.

Previously, the interface between Rac and p67phox has been extensively studied biochemically in an effort to define the regions in Rac responsible for binding and NADPH oxidase activation (Diekmann et al., 1994, 1995; Xu et al., 1994; Freeman et al., 1996; Nisimoto et al., 1997; Joneson and Bar-Sagi, 1998; Toporik et al., 1998). The structure presented here allows us to rationalize these data and provides a structural framework for the design of new experiments to further elucidate the role of Rac in NADPH oxidase function. Rac/Rho chimeras have suggested that the N-terminal (residues 22–45) and C-terminal (residues 143–175) regions of Rac are involved in Rac/p67phox complex formation as well as NADPH oxidase activation, in good agreement with the structural data presented here (Figures 4A and 4B). Mutation of a number residues within the switch I region, particularly Ile-33, Asp-38, and Tyr-40, has suggested a role for this region in Rac/p67phox binding and/or in NADPH oxidase activation. However, the Rac/p67TPR structure shows that these residues are not part of the protein–protein interface (Figures 4A and 4B), suggesting that these mutants may either destabilize the native conformation of Rac or induce conformational changes around the effector region that result in disruption of the p67phox binding surface. Mutations in region 103–107$_{Rac}$ as well as the insertion helix of Rac have been shown to cause a decrease in superoxide production, leading to the suggestion that the insertion helix of Rac might be involved in complex formation. Both regions, however, are distant from the Rac/p67TPR interface

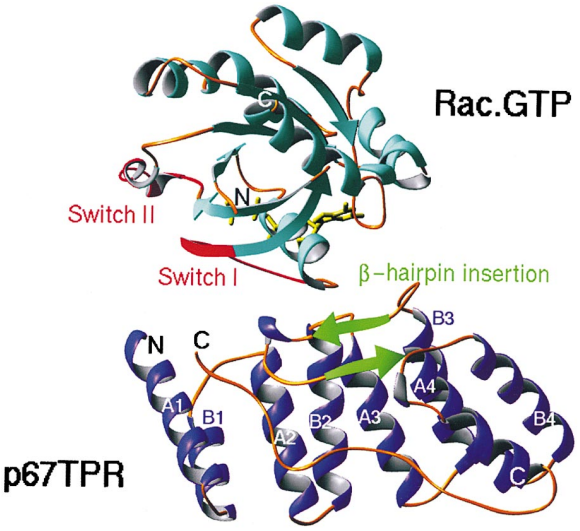


Figure 2. Overall Structure of the Rac1·GTP/p67TPR Complex

Ribbons representation of the complex between Rac1Q61L·GTP (cyan) and p67TPR (purple). The switch I and II regions in Rac are highlighted in red, and the nucleotide and magnesium are shown in yellow in a ball and stick representation. The N and C termini are indicated by the labels N and C. The helices in p67TPR are labeled A1–A4 and B1–B4, in accordance with the terminology introduced for PP5 (Das et al., 1998). The β sheets of the β hairpin insertion are highlighted in green.

(Figure 4B) and do not contribute to complex formation in agreement with binding data from Lambeth and coworkers (Freeman et al., 1996; Nisimoto et al., 1997). Rather, this structure supports the notion that Rac interacts with other components of the oxidase, possibly via the insertion helix, once it has translocated to the membrane (Heyworth et al., 1994; Nisimoto et al., 1997). An "activation domain" in p67^{phox}, spanning amino acids 199–210, has been described to be crucial for NADPH oxidase activation but not required for complex formation with Rac (Han et al., 1998). This is in accordance with the crystallographic study that shows no electron density for residues C-terminal to amino acid 186 in p67^{phox}, making it extremely unlikely that they are involved in complex formation. It instead suggests that the interaction between Rac and p67^{phox} serves to position these two proteins correctly with respect to the catalytic subunit cytochrome b_{558} in the context of the fully assembled, membrane-associated NADPH oxidase complex.

Mutations in p67^{phox} Involved in CGD

Mutations in p67^{phox} associated with chronic granulomatous disease are mainly located in the N-terminal, Rac binding domain and include deletion of amino acids 19–21, deletion of Lys-58, and mutation of Gly-78 to glutamate (Figure 4B) (de Boer et al., 1994; Leusen et al., 1996a; Patino et al., 1999). Intriguingly, none of these mutations map to the interface with Rac but instead are likely to be important for maintaining the structural integrity of the TPR domain. Although TPR sequence motifs are degenerate, a consensus pattern of small and large hydrophobic residues exists whose distribution can be rationalized by their location at the interface of adjoining helices. Deletion of residues 19–21 in the loop connecting helices A1 with B1 as well as Lys-58 in helix B2 would disrupt the regular packing of the TPR helices and thereby either lead to misfolding of p67^{phox} or structural distortions that change the binding surface for Rac. Gly-78 is located in position 8 of helix A3, which is occupied in other TPR motifs by Gly, Ala, or Ser. Mutation of this glycine to glutamate would result in a clash with residues 93 and 94 from helix B3 and thereby disrupt the stacking of the TPR helices.

Comparison of This Complex with Other Rho Family Protein Structures

The present structure reveals significant differences in the way p67^{phox} interacts with Rac in comparison to other structures of Rho family effector complexes (Figure 5). The CRIB domain effectors ACK, WASP, and PAK bind to and recognize their cognate GTPases in similar ways, although there are differences in the details of target recognition (Abdul-Manan et al., 1999; Mott et al., 1999; Gizachew et al., 2000; Morreale et al., 2000). In general, CRIB domain effectors contact helices α1, α5, and strand β2 as well as the switch I and II regions. Consequently, a large solvent-accessible surface is buried in each of the structurally characterized complexes (WASP 2930 Å^2, ACK 4200 Å^2, and PAK 2500 Å^2). ACK, WASP, and PAK all form an intermolecular antiparallel β sheet with strand β2 of Cdc42, similar to the interactions observed previously in the Rap/Raf and Ras/RalGDS complexes (Nassar et al., 1995; Huang et al., 1998).

Some subfamilies of the GTPase superfamily show significant changes in the conformation of switch II upon cycling between the GDP and GTP-bound states. However, a comparison of the structures of Rac·GTP and Rac·GDP (unpublished results; Scheffzek et al., 2000), as well as that of Cdc42·GTP and Cdc42.GDP (Hoffman et al., 2000), shows that in both nucleotide-bound states, switch II adopts a very similar conformation. This suggests that switch II in Rho subfamily GTPases does not play a significant role in GTP-dependent signaling. Nevertheless, all Rac and Cdc42 specific downstream effectors (apart from p67^{phox}), as well as the regulatory proteins RhoGAP and RhoGDI, make contacts with the switch II region (Rittinger et al., 1997a, 1997b; Hoffman et al., 2000; Scheffzek et al., 2000). Interactions between the switch II region of Rho and RhoGAP play an important role in the stabilization of Gln-63 in Rho that is responsible for the correct positioning of the hydrolytic water molecule for phosphoryl transfer. CRIB domain effectors as well as RhoGDI inhibit GTP hydrolysis and nucleotide dissociation, and it has therefore been suggested that the interaction with switch II might be responsible for this effect (Hoffman et al., 2000; Morreale et al., 2000). Switch II is not in contact with p67TPR in the Rac complex and there is no indication that binding of p67^{phox} to Rac can inhibit the intrinsic GTP hydrolysis rate. It seems likely that the normal turnover of Rac·GTP is essential for tight regulation of the NADPH oxidase. The toxicity of excess superoxide dictates that prolonged signaling by Rac through the inhibition of GTPase hydrolysis is highly undesirable. In this context, comparison of the Rac/p67TPR and the Rho·GDP·AlF_4^-/RhoGAP complexes shows that p67^{phox} and RhoGAP use different binding surfaces of their respective GTPase partners (Figure 5). It is therefore possible that p67^{phox} and a GAP

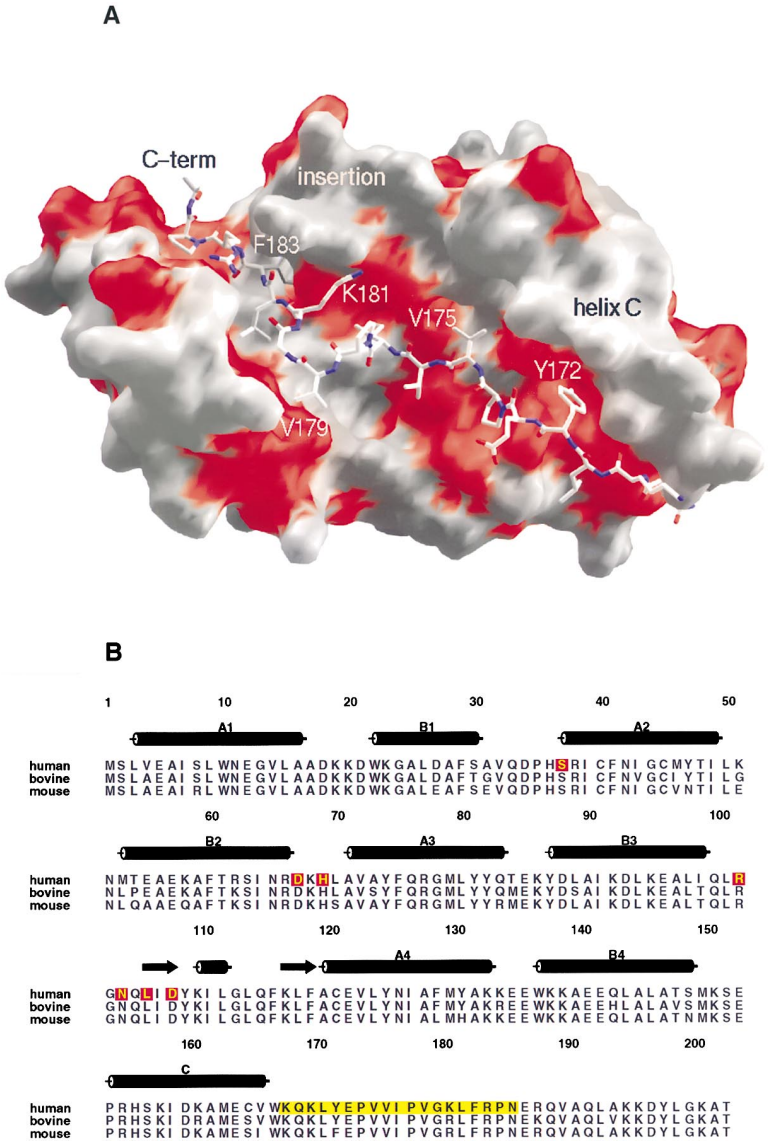


Figure 3. Intramolecular Interactions between the TPR Domain and the C-Terminal Extension

(A) The molecular surface of amino acids 1–167 of p67TPR is shown with hydrophobic residues projected onto the surface in red. Residues 168–186, which line the inside of the TPR groove, are depicted in a stick representation. The figure was prepared using GRASP (Nicholls et al., 1991).

(B) Sequence alignment of the first 203 amino acids of human (1346669), bovine (3687891), and mouse (3061284) p67^{phox}. Secondary structure elements are indicated above the alignment. Amino acids that are involved in the Rac interface are highlighted in red, and the C-terminal extension that lines the inside of the TPR groove is indicated in yellow.

might be able to bind simultaneously to Rac and supply an additional level of regulation of NADPH oxidase activity. Further biochemical studies will be required to test this hypothesis.

Rac Specificity of the p67^{phox} Interaction

The Rac/p67TPR protein–protein interface is formed by residues that are highly conserved between Rac and Cdc42, yet Cdc42 shows no detectable binding. Studies employing point mutations and Rac/Cdc42 chimeras have suggested that Ala-27 and Gly-30 alone account for the specificity of the Rac/p67^{phox} interaction (Kwong et al., 1995). However, this study did not directly address the effects of these mutations on complex formation between Rac and Cdc42 but, instead, tested for their ability to activate the NADPH oxidase in a cell-free assay. In fact, the crystal structure straightforwardly shows that Gly-30 is the only residue not conserved between Rac and Cdc42 that is present at the protein–protein interface (Figures 1A and 4A). Ala-27 is not directly involved in the interface, but the structure suggests that substitution of this residue with Lys, which is present in Cdc42, could lead to a steric clash of its side chain with the β hairpin insertion of p67^{phox}. To directly examine the contribution of Ala-27 and Gly-30 to specificity of the Rac/p67^{phox} complex, we introduced the corresponding mutations into Cdc42 and measured binding to p67TPR by ITC. In combination, these mutations resulted in a protein that bound to p67TPR with an affinity of 6 μM, only about 2-fold lower than wild-type Rac1 (Figure 6). Introduction of the corresponding residues from Cdc42 into Rac results in a protein that is unable to bind to p67TPR (data not shown). These data confirm that specificity of p67^{phox} for Rac is almost exclusively conferred by these two amino acids and that the contribution of other amino acids in the interface is purely to the stability of this complex.

TPR Domains Are Versatile Protein–Protein Interaction Modules

TPR motifs occur in a variety of proteins that are, in many cases, part of multiprotein assemblies (Lamb et al., 1995; Blatch and Lassle, 1999). The recently described structures of the isolated TPR domain of protein

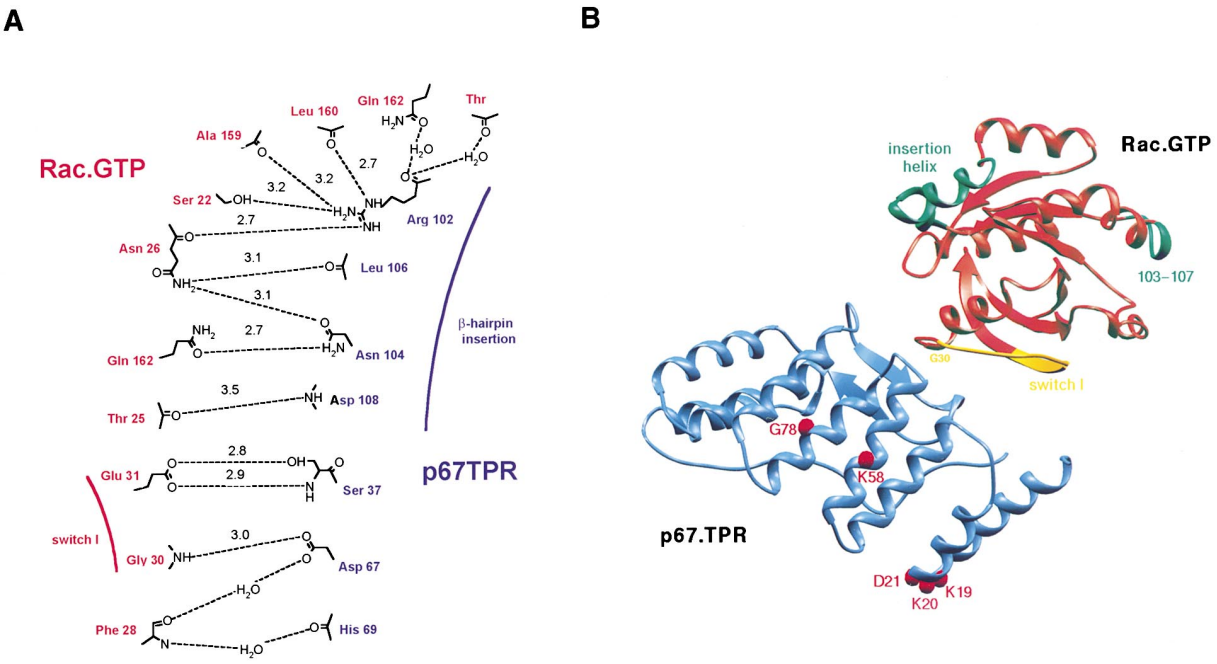


Figure 4. The Interface between Rac and p67TPR

(A) Schematic representation of the hydrogen bond interactions at the interface between Rac and p67TPR. Residues from Rac are labeled in red and residues from p67TPR in blue. Hydrogen bonds are depicted as dotted lines with the bond distances indicated in Å. The positions of switch I and the β hairpin insertion are indicated in red and blue, respectively.

(B) Ribbons representation of the Rac·GTP (red)/p67TPR (blue) complex. The effector loop of Rac is colored in yellow, and amino acids 103–107 and the helical insertion (120–135) are indicated in green. The position of Gly-30 at the N terminus of the effector loop is indicated to show the orientation of switch I. The positions of mutations in $p67^{phox}$ occurring in CGD are shown as red spheres. Figures 2 and 4B were prepared using Ribbons (Carson, 1991).

phosphatase 5 and of complexes between the adaptor protein Hop and peptides derived from Hsp70 and Hsp90 have been importants step toward an understanding of the structural basis of TPR motif–mediated protein–protein interactions (Das et al., 1998; Scheufler et al., 2000). The Hop/Hsp complex structures show that the peptides bind to the TPR groove in an extended conformation. This is reminiscent of the interactions of phosphopeptides with 14-3-3 proteins (Yaffe et al., 1997) supporting the previous suggestion that these two protein families may share a common peptide binding mode (Das et al., 1998; Scheufler et al., 2000). However, in

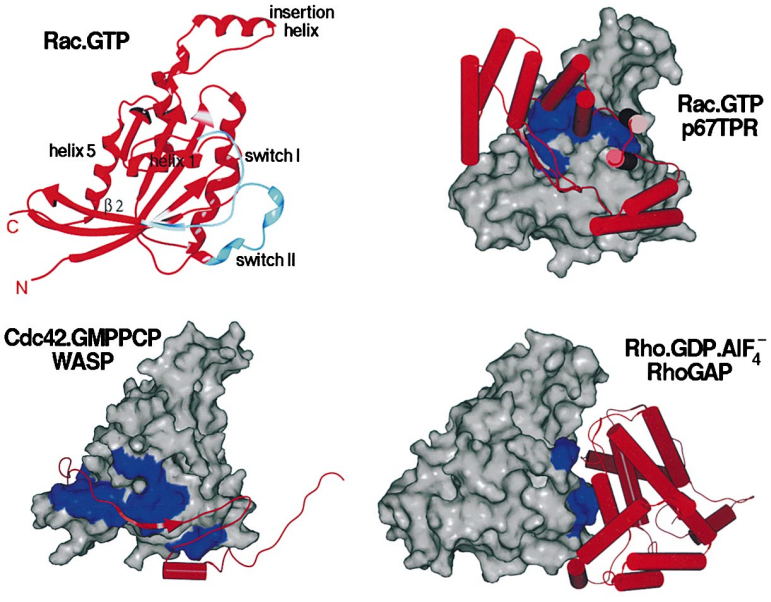


Figure 5. Comparison of the Structure of Rac/$p67^{phox}$ with Rho Family/Protein Complexes

(Upper left) Rac·GTP is shown as a ribbons representation in red with the switch I and II regions highlighted in blue. Surface representation of small GTPases with the residues involved in complex formation with (upper right) p67TPR (Rac), (lower left) WASP (Cdc42), and (lower right) RhoGAP (Rho) projected onto the surface in blue. The structures of the target proteins are shown as arrows (β sheets) and cylinders (helices) in red. The figures were prepared using the program SPOCK.

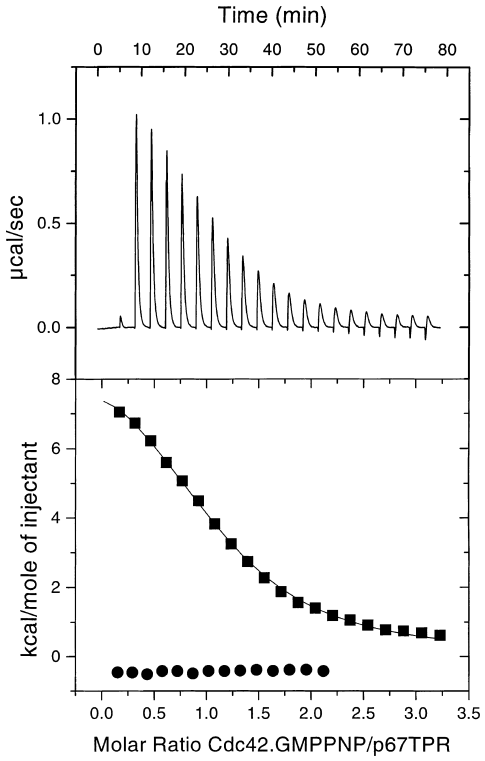


Figure 6. Specificity of the Rac/p67^{phox} Interaction

Isothermal titration calorimetry measurements of the binding of wild-type (closed circles) and mutant (K27A,S30G) (closed squares) Cdc42.GMPPNP to p67TPR. Upper part, raw data of the titration of P67TPR (290 μM) into mutant Cdc42.GMPPNP (25 μM) at 18°C. Lower part, integrated heat changes for the mutant Cdc42/p67TPR titration, corrected for the heat of dilution, and fitted curve, based on a single site binding model. The constants determined are K_d = 6.0 μM, stoichiometry N = 1.1, enthalpy ΔH = 8424 cal/mole.

the present complex, only the loop regions connecting TPR1 through TPR3 are involved in creating the binding site for Rac on the edge of the TPR bundle rather than in the groove itself. The remaining residues of p67^{phox} that form the interface with Rac are supplied by the β hairpin insertion that is not present in the other two TPR domain structures. Thus, the p67^{phox}/Rac structure unexpectedly demonstrates that binding to the TPR groove is not a general structural principle of complex formation between TPR domain–containing proteins and their binding partners. This, in turn, suggests an unsuspected versatility of TPR domains in both target recognition and their more general role as scaffolds for the assembly of multiprotein complexes. Nevertheless, the TPR groove is not empty in this complex but rather occupied by the C-terminal extension of our p67^{phox} construct. In fact, interactions of the C-terminal extension with the β hairpin insertion seem to play an important role in stabilizing a crucial part of the Rac binding surface, suggesting that the reversible formation of this intramolecular interaction might contribute to NADPH oxidase regulation. This hypothesis is supported by the fact that p67^{phox} does not seem to be able to bind to p40^{phox} and Rac simultaneously (Rinckel et al., 1999), implying that significant conformational changes have to occur in the p40^{phox}/p47^{phox}/p67^{phox} complex prior to Rac/p67^{phox} complex formation.

The TPR motif is one of a number of repeating α-helical motifs such as HEAT, armadillo, ankyrin, and leucine-rich families, which all form extended domain structures that are involved in a variety of macromolecular interactions (Groves and Barford, 1999). These architectures all provide the potential for evolution of varied and specific binding surfaces both through variation in the number of repeats and also through the insertion of noncanonical structures between and within the repeat motifs themselves (Sedgwick and Smerdon, 1999). This is exemplified by the structure of p67TPR/Rac, in which binding occurs almost exclusively to the β hairpin insertion element and not to the TPR repeats themselves. Since many proteins appear to contain multiple TPR motifs separated by sequence insertions, it is likely that other structural variations on the TPR domain architecture will be revealed in the future.

Conclusions

The NADPH oxidase in phagocytes plays a key role in host defense against microbial infections. Related NADPH oxidase systems have been found in other tissues, and it is thought that these enzymes might be a source of second messengers in the form of reactive oxygen species. Significant advances have been made recently in our understanding of the mechanism of oxidase activation and assembly. However, there are still many gaps in our knowledge about the molecular details of the protein–protein interactions involved, and the present structure represents an initial step toward an atomic description of this multiprotein complex. This will help us to understand the molecular basis of activation of the NADPH oxidase, which not only plays an important role in the immune defense, but also presents a significant threat if incorrectly regulated.

Experimental Procedures

Protein Preparation

Human p67^{phox} (amino acids 1–203 and the full-length protein), human Rac1, Rac1·Q61L, Rac2, Rac2·Q61L, Cdc42 (amino acids 1–184), and Cdc42·Q61L in pGEX-2T or pGEX-4T-1 (Pharmacia) were expressed in *E. coli* BL21. The proteins were purified by affinity chromatography on Glutathione Sepharose 4B and cleaved on-column with human thrombin (Calbiochem). Further purification was carried out by gel filtration on Superdex 75 (Pharmacia). The proteins were concentrated to 30 mg/ml by ultrafiltration. Mutagenesis was carried out using the QuickChange site-directed mutagenesis kit (Stratagene). Sequences were confirmed by nucleotide sequencing and mass spectrometry of the purified proteins. Expression of full-length Rac and Cdc42 in *E. coli* resulted in proteins that are truncated at the C terminus by eight and seven residues, respectively, as confirmed by electrospray mass spectrometry.

Crystallization, Structure Determination, and Refinement

Crystals of the complex between Rac1·Q61L·GTP and p67TPR were grown at 18°C in hanging drops by vapor diffusion. Equal volumes (2 μl) of the complex at 15 mg/ml in 50 mM Tris (pH 7.5), 50 mM NaCl, 2 mM DTT, 5 mM $MgCl_2$ were mixed with reservoir solution containing 0.1 M Caps (pH 10.5), 0.2 M Li acetate, 0.8 M NaH_2PO_4, 1.2 M K_2HPO_4. Crystals reached their full size (typically 150 × 150 × 200 μm) within 4 weeks. Crystals were transferred into cryosolution by a stepwise transfer into mother liquor containing 5–25% glycerol. Data were collected to 2.4 Å spacing on Station 7.2 (λ = 1.488 Å) at the SRS, Daresbury, UK, on a MARResearch image plate detector

and processed using the HKL suite (Otwinowski and Minor, 1997). The R_{merge} is 8.4% overall for 123,873 observations of 20,975 observations in the 15–2.4 Å resolution range (94% complete with a $<I>/<\sigma I>$ of 16.9) Crystals belong to space group $P3_221/P3_121$ (a = b = 83.2 Å and c = 138.5 Å) with one complex in the asymmetric unit. Molecular replacement calculations (AMoRe, CCP4) revealed a single clear solution using Rac1 as the search model (PDB accession code 1MH1), enabling us to distinguish $P3_221$ as the correct space group enantiomorph. However, searches with several models based on the available coordinates of other TPR repeat proteins (1A17, 1ELW, and 1ELR) were unsuccessful. Although Rac constitutes only 47% of the protein mass of the asymmetric unit, electron density maps phased only from the Rac molecular replacement solution revealed three short regions of α-helical density that were modeled as polyalanine. Rac and the partial p67TPR model were refined against 2.4 Å data using REFMAC and ARP with automated solvent building (CCP4, 1994; Navaza and Saludjian, 1997). The subsequent map was slightly improved and interpretation was aided by the positioning of the three TPR repeats of PP5 by "brute-force" structural alignment with the three helical segments of p67TPR (32 residues in total) using 6D-Lsqman. Using the PP5 model as a guide, further helical segments of p67TPR were discernible, albeit at rather low contour levels (0.3–0.5 σ), enabling a polyalanine model consisting of part or all of five helices of p67TPR to be constructed. This model was further refined as above and the resulting map was of sufficient quality to permit the remainder of the p67TPR fragment to built using the program O (Jones et al., 1991). The current R factor is 24.6% (R_{free} = 28.3% for 10% of data removed from the refinement procedure). The root-mean-squared deviations from ideal bond lengths and bond angles are 0.010 Å and 1.5°, respectively.

Isothermal Titration Calorimetry

Binding of Rac and Cdc42 to full-length and C-terminally truncated $p67^{phox}$ was measured by isothermal titration calorimetry (Wiseman et al., 1989) using a MicroCal Omega VP-ITC isothermal titration calorimeter (MicroCal Inc., Northampton, USA). All proteins were dialyzed against ITC-buffer (25 mM HEPES [pH 7.0], 50 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT), and experiments were performed at 18°C. Solutions (25 μM) of wild-type and mutant Rac and Cdc42 were titrated by injection of a total of 250 μl of 290 μM p67TPR in 20–30 aliquots. Titrations with full-length $p67^{phox}$ were carried out with 20 μM $p67^{phox}$ in the cell and injection of 200 μM solutions of the GTPase. Heats of dilution were determined by titrating p67TPR, Rac, or Cdc42 into ITC buffer. The heats of dilution were subtracted from the raw titration data before data analysis using the evaluation software, Microcal Origin version 5.0 provided by the manufacturer, assuming a single-site binding model.

Acknowledgments

We thank Pierre Rizkallah at Daresbury for beamtime on station 7.2 at short notice, Emmanuel Skordalakes for help with data collection, Steve Howell for mass spectroscopy analysis, and Michael B. Yaffe and Guy G. Dodson for critical reading of the manuscript. Plasmids for expression of Rac, Cdc42, and $p67^{phox}$ were kind gifts of A. Hall, E. Manser, and M. B.Yaffe.

Received July 5, 2000; revised September 13, 2000.

References

Abdul-Manan, N., Aghazadeh, B., Liu, G.A., Majumdar, A., Ouerfelli, O., Siminovitch, K.A., and Rosen, M.K. (1999). Structure of Cdc42 in complex with the GTPase-binding domain of the 'Wiskott-Aldrich syndrome' protein. Nature *399*, 379–383.

Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C.G., and Segal, A.W. (1991). Activation of the NADPH oxidase involves the small GTP-binding protein p21rac1. Nature *353*, 668–670.

Ahmed, S., Prigmore, E., Govind, S., Veryard, C., Kozma, R., Wientjes, F.B., Segal, A.W., and Lim, L. (1998). Cryptic Rac-binding and p21(Cdc42Hs/Rac)-activated kinase phosphorylation sites of NADPH oxidase component p67(phox). J. Biol. Chem. *273*, 15693–15701.

Babior, B.M. (1999). NADPH oxidase: an update. Blood *93*, 1464–1476.

Barton, G.J. (1993). ALSCRIPT: a tool to format multiple sequence alignments. Protein Eng. *6*, 37–40.

Blatch, G.L., and Lassle, M. (1999). The tetratricopeptide repeat: a structural motif mediating protein-protein interactions. Bioessays *21*, 932–939.

Burbelo, P.D., Drechsel, D., and Hall, A. (1995). A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J. Biol. Chem. *270*, 29071–29074.

Carson, M. (1991). Ribbons 2.0. J. Appl. Crystallogr. *24*, 958–961.

Collaborative Computational Project Number 4 (1994). The CCP4 suite: programs for protein crystallography, Acta Crystallogr. D *50*, 760–763.

Das, A.K., Cohen, P.W., and Barford, D. (1998). The structure of the tetratricopeptide repeats of protein phosphatase 5: implications for TPR-mediated protein-protein interactions. EMBO J. *17*, 1192–1199.

de Boer, M., Hilarius-Stokman, P.M., Hossle, J.P., Verhoeven, A.J., Graf, N., Kenney, R.T., Seger, R., and Roos, D. (1994). Autosomal recessive chronic granulomatous disease with absence of the 67-kD cytosolic NADPH oxidase component: identification of mutation and detection of carriers. Blood *83*, 531–536.

DeLeo, F.R., and Quinn, M.T. (1996). Assembly of the phagocyte NADPH oxidase: molecular interactions of oxidase proteins. J. Leukoc. Biol. *60*, 677–691.

Diekmann, D., Abo, A., Johnston, C., Segal, A.W., and Hall, A. (1994). Interaction of Rac with p67phox and regulation of phagocytic NADPH oxidase activity. Science *265*, 531–533.

Diekmann, D., Nobes, C.D., Burbelo, P.D., Abo, A., and Hall, A. (1995). Rac GTPase interacts with GAPs and target proteins through multiple effector sites. EMBO J. *14*, 5297–5305.

Dusi, S., Donini, M., and Rossi, F. (1996). Mechanisms of NADPH oxidase activation: translocation of p40phox, Rac1 and Rac2 from the cytosol to the membranes in human neutrophils lacking p47phox or p67phox. Biochem. J. *314*, 409–412.

Freeman, J.L., Abo, A., and Lambeth, J.D. (1996). Rac "insert region" is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65. J. Biol. Chem. *271*, 19794–19801.

Gizachew, D., Guo, W., Chohan, K.K., Sutcliffe, M.J., and Oswald, R.E. (2000). Structure of the complex of Cdc42Hs with a peptide derived from P-21 activated kinase. Biochemistry *39*, 3963–3971.

Groves, M.R., and Barford, D. (1999). Topological characteristics of helical repeat proteins. Curr. Opin. Struct. Biol. *9*, 383–389.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509–514.

Han, C.H., Freeman, J.L., Lee, T., Motalebi, S.A., and Lambeth, J.D. (1998). Regulation of the neutrophil respiratory burst oxidase. Identification of an activation domain in p67(phox). J. Biol. Chem. *273*, 16663–16668.

Heyworth, P.G., Knaus, U.G., Xu, X., Uhlinger, D.J., Conroy, L., Bokoch, G.M., and Curnutte, J.T. (1993). Requirement for posttranslational processing of Rac GTP-binding proteins for activation of human neutrophil NADPH oxidase. Mol. Biol. Cell *4*, 261–269.

Heyworth, P.G., Bohl, B.P., Bokoch, G.M., and Curnutte, J.T. (1994). Rac translocates independently of the neutrophil NADPH oxidase components p47phox and p67phox. Evidence for its interaction with flavocytochrome b558. J. Biol. Chem. *269*, 30749–30752.

Hirano, T., Kinoshita, N., Morikawa, K., and Yanagida, M. (1990). Snap helix with knob and hole: essential repeats in S. pombe nuclear protein nuc2+. Cell *60*, 319–328.

Hirshberg, M., Stockley, R.W., Dodson, G., and Webb, M.R. (1997). The crystal structure of human rac1, a member of the rho-family complexed with a GTP analogue. Nat. Struct. Biol. *4*, 147–152.

Hoffman, G.R., Nassar, N., and Cerione, R.A. (2000). Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI. Cell *100*, 345–356.

Huang, L., Hofer, F., Martin, G.S., and Kim, S.H. (1998). Structural

basis for the interaction of Ras with RalGDS. Nat. Struct. Biol. *5*, 422–426.

Jones, T.A., Zou, J.Y., Cowan, S.W. and Kjeldgaard (1991). Improved methods for binding protein models in electron density maps and the location of errors in these models. Acta Crystallogr. A *47*, 110–119.

Joneson, T., and Bar-Sagi, D. (1998). A Rac1 effector site controlling mitogenesis through superoxide production. J. Biol. Chem. *273*, 17991–17994.

Kleinberg, M.E., Malech, H.L., Mital, D.A., and Leto, T.L. (1994). p21rac does not participate in the early interaction between p47-phox and cytochrome b558 that leads to phagocyte NADPH oxidase activation in vitro. Biochemistry *33*, 2490–2495.

Knaus, U.G., Heyworth, P.G., Evans, T., Curnutte, J.T., and Bokoch, G.M. (1991). Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac *2*. Science *254*, 1512–1515.

Koga, H., Terasawa, H., Nunoi, H., Takeshige, K., Inagaki, F., and Sumimoto, H. (1999). Tetratricopeptide repeat (TPR) motifs of p67(phox) participate in interaction with the small GTPase Rac and activation of the phagocyte NADPH oxidase. J. Biol. Chem. *274*, 25051–25060.

Kwong, C.H., Adams, A.G., and Leto, T.L. (1995). Characterization of the effector-specifying domain of Rac involved in NADPH oxidase activation. J. Biol. Chem. *270*, 19868–19872.

Lamb, J.R., Tugendreich, S., and Hieter, P. (1995). Tetratrico peptide repeat interactions: to TPR or not to TPR? Trends Biochem. Sci. *20*, 257–259.

Leusen, J.H., de Klein, A., Hilarius, P.M., Ahlin, A., Palmblad, J., Smith, C.I., Diekmann, D., Hall, A., Verhoeven, A.J., and Roos, D. (1996a). Disturbed interaction of p21-rac with mutated p67-phox causes chronic granulomatous disease. J. Exp. Med. *184*, 1243–1249.

Leusen, J.H., Verhoeven, A.J., and Roos, D. (1996b). Interactions between the components of the human NADPH oxidase: intrigues in the phox family. J. Lab. Clin. Med. *128*, 461–476.

Morreale, A., Venkatesan, M., Mott, H.R., Owen, D., Nietlispach, D., Lowe, P.N., and Laue, E.D. (2000). Structure of Cdc42 bound to the GTPase binding domain of PAK. Nat. Struct. Biol. *7*, 384–388.

Mott, H.R., Owen, D., Nietlispach, D., Lowe, P.N., Manser, E., Lim, L., and Laue, E.D. (1999). Structure of the small G protein Cdc42 bound to the GTPase-binding domain of ACK. Nature *399*, 384–388.

Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995). The 2.2 Å crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with Rap1A and a GTP analogue. Nature *375*, 554–560.

Nauseef, W.M. (1999). The NADPH-dependent oxidase of phagocytes. Proc. Assoc. Am. Physicians *111*, 373–382.

Navaza, J., and Saludjian, P. (1997). AMoRe: an automated molecular replacement program package. In Methods Enzymol. *276*, 581–594.

Nicholls, A., Sharp, K.A., and Honig, B. (1991). Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. Proteins *11*, 281–296.

Nisimoto, Y., Freeman, J.L.R., Motalebi, S.A., Hirshberg, M., and Lambeth, J.D. (1997). Rac binding to p67(phox). Structural basis for interactions of the Rac1 effector region and insert region with components of the respiratory burst oxidase. J. Biol. Chem. *272*, 18834–18841.

Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. In Methods Enzymol., pp. 307–326.

Park, J.W., Benna, J.E., Scott, K.E., Christensen, B.L., Chanock, S.J., and Babior, B.M. (1994). Isolation of a complex of respiratory burst oxidase components from resting neutrophil cytosol. Biochemistry *33*, 2907–2911.

Patino, P.J., Rae, J., Noack, D., Erickson, R., Ding, J., de Olarte, D.G., and Curnutte, J.T. (1999). Molecular characterization of autosomal recessive chronic granulomatous disease caused by a defect of the nicotinamide adenine dinucleotide phosphate (reduced form) oxidase component p67-phox. Blood *94*, 2505–2514.

Ponting, C.P. (1996). Novel domains in NADPH oxidase subunits, sorting nexins, and PtdIns 3-kinases: binding partners of SH3 domains? Protein Sci. *5*, 2353–2357.

Ponting, C.C., and Phillips, C. (1996). Rapsyn's knobs and holes: eight tetratrico peptide repeats. Biochem. J. *314*, 1053–1054.

Rinckel, L.A., Faris, S.L., Hitt, N.D., and Kleinberg, M.E. (1999). Rac1 disrupts p67phox/p40phox binding: a novel role for Rac in NADPH oxidase activation. Biochem. Biophys. Res. Commun. *263*, 118–122.

Rittinger, K., Walker, P.A., Eccleston, J.F., Nurmahomed, K., Owen, D., Laue, E., Gamblin, S.J., and Smerdon, S.J. (1997a). Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP. Nature *388*, 693–697.

Rittinger, K., Walker, P.A., Eccleston, J.F., Smerdon, S.J., and Gamblin, S.J. (1997b). Structure at 1.65 Å of RhoA and its GTPase-activating protein in complex with a transition-state analogue. Nature *389*, 758–762.

Roos, D. (1994). The genetic basis of chronic granulomatous disease. Immunol. Rev. *138*, 121–157.

Scheffzek, K., Stephan, I., Jensen, O.N., Illenberger, D., and Gierschik, P. (2000). The Rac-RhoGDI complex and the structural basis for the regulation of Rho proteins by RhoGDI. Nat. Struct. Biol. *7*, 122–126.

Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F.U., and Moarefi, I. (2000). Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. Cell *101*, 199–210.

Sedgwick, S.G., and Smerdon, S.J. (1999). The ankyrin repeat: a diversity of interactions on a common structural framework. Trends Biochem. Sci. *24*, 311–316.

Segal, A.W., and Abo, A. (1993). The biochemical basis of the NADPH oxidase of phagocytes. Trends Biochem. Sci. *18*, 43–47.

Sikorski, R.S., Boguski, M.S., Goebl, M., and Hieter, P. (1990). A repeating amino acid motif in CDC23 defines a family of proteins and a new relationship among genes required for mitosis and RNA synthesis. Cell *60*, 307–317.

Thrasher, A.J., Keep, N.H., Wientjes, F., and Segal, A.W. (1994). Chronic granulomatous disease. Biochim. Biophys. Acta *1227*, 1–24.

Toporik, A., Gorzalczany, Y., Hirshberg, M., Pick, E., and Lotan, O. (1998). Mutational analysis of novel effector domains in Rac1 involved in the activation of nicotinamide adenine dinucleotide phosphate (reduced) oxidase. Biochemistry *37*, 7147–7156.

Van Aelst, L., and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. Genes Dev. *11*, 2295–2322.

Wientjes, F.B., Hsuan, J.J., Totty, N.F., and Segal, A.W. (1993). p40phox, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. Biochem. J. *296*, 557–561.

Wiseman, T., Williston, S., Brandts, J.F., and Lin, L.N. (1989). Rapid measurement of binding constants and heats of binding using a new titration calorimeter. Anal. Biochem. *179*, 131–137.

Xu, X., Barry, D.C., Settleman, J., Schwartz, M.A., and Bokoch, G.M. (1994). Differing structural requirements for GTPase-activating protein responsiveness and NADPH oxidase activation by Rac. J. Biol. Chem. *269*, 23569–23574.

Yaffe, M.B., Rittinger, K., Volinia, S., Caron, P.R., Aitken, A., Leffers, H., Gamblin, S.J., Smerdon, S.J., and Cantley, L.C. (1997). The structural basis for 14-3-3:phosphopeptide binding specificity. Cell *91*, 961–971.

Protein Data Bank ID Codes

The coordinates have been deposited in the Protein Data Bank with ID code 1e96.