Tetratricopeptide Repeat (TPR) Motifs of p67phox Participate in Interaction with the Small GTPase Rac and Activation of the Phagocyte NADPH Oxidase*

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The small GTPase Rac functions as a molecular switch in several important cellular events including cytoskeletal reorganization and activation of the phagocyte NADPH oxidase, the latter of which leads to production of superoxide, a precursor of microbialic oxidants. During formation of the active oxidase complex at the membrane, the GTP-bound Rac appears to interact with the N-terminal region of p67phox, another indispensable activator that translocates from the cytosol upon phagocyte stimulation. Here we show that the p67phox N terminus lacks the CRIB motif, a well known Rac target, but contains four tetratricopeptide repeat (TPR) motifs with highly α-helical structure. Disruption of any of the N-terminal three TPRs, but the last one, results in defective interaction with Rac, while all the four are required for the NADPH oxidase activation. We also find that Arg-102 in the third repeat is likely involved in binding to Rac via an ionic interaction, and that replacement of this residue with Glu completely abrogates the capability of activating the oxidase both in vivo and in vitro. Thus the TPR motifs of p67phox are packed to function as a Rac target, thereby playing a crucial role in the active oxidase complex formation.

Rac1 and Rac2, members of the Rho family of small GTPases, play a pivotal role in several important cellular functions including cytoskeletal reorganization, gene expression, and activation of the phagocyte NADPH oxidase following microbial infection (1, 2). Rac serves as a molecular switch cycling between an active GTP-bound and an inactive GDP-bound states. In the active state, Rac interacts with a variety of target (effector) proteins to elicit cellular responses (1, 2). For example, the protein kinase PAK is activated by interacting with Rac in a GTP-dependent manner (3). This interaction is mediated via binding of Rac to a Cdc42/Rac interactive binding (CRIB)1 motif within the N-terminal regulatory region of PAK, a motif that is present in a variety of targets of Rac and Cdc42 (4). Although more than 10 targets of Rac have been discovered (1), molecular natures of the interactions, except the CRIB motif, remain largely unknown. It is thus considered important to study Rac-target interactions especially in functionally well defined systems.

The phagocyte NADPH oxidase, dormant in resting cells, is activated during phagocytosis to produce superoxide, a precursor of microbialic oxidants (5–8). The significance of the enzyme in host defense is indicated by chronic granulomatous disease (CGD) patients suffering from recurrent severe infection caused by defect of the superoxide producing activity (7, 8). Although the NADPH oxidase is originally discovered in phagocytes because of its abundance, it has recently been proposed that the enzyme is expressed in a variety of cells and reactive oxygen species derived from superoxide that play a role in several signal transduction systems (6). The redox core of the oxidase is a membrane-spanning flavocytochrome, cytochrome b558, comprising the two subunits gp91phox and p22phox. Upon cell stimulation three cytosolic proteins, namely p47phox, p67phox, and Rac, translocate to membranes, where they interact with the cytochrome to form an active oxidase complex. All the five polypeptides are required for activation of the NADPH oxidase in vitro, and CGD is caused by defect of any of the genes encoding these proteins except Rac (5–8).

In assembly and activation of the phagocyte NADPH oxidase, protein-protein interactions between the oxidase factors play a crucial role (5, 9, 10). Both p47phox and p67phox harbor two SH3 domains, which mediate specific interactions between the factors: the C-terminal SH3 domain of p67phox interacts with p47phox, while the N-terminal one of p47phox does with p22phox (11–15). At least two events elicited during intracellular signal transduction in stimulated cells appear to function as a switch of the oxidase activation. One of the two is a conformational change of p47phox, the N-terminal SH3 domain of p47phox is normally inaccessible, and, upon cell stimulation, becomes unmasked to interact with p22phox, an induced interaction that is required for the oxidase activation (12, 15, 16).

The other critical event seems to be conversion of Rac to the active state: only the GTP-bound Rac, but not the GDP-bound one, activates the oxidase under cell-free conditions (16–19), and introduction of Rac antisense oligonucleotides or expression of a GTP-γS, guanosine 5′-O-(thio)-triphosphate.

This paper is available on line at http://www.jbc.org

CGD, chronic granulomatous disease; TPR, tetratricopeptide repeat; CD, circular dichroism; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate; GTPγS, guanosine 5′-O-(thio)-triphosphate.
sion of a dominant negative form of Rac2 (T17N) inhibits superoxide production in stimulated cells (20, 21). Rac1 in the GTP-bound state can directly interact with the N-terminal region of p67phox, comprising approximately 200 amino acid residues (22). This region lacks a CRIB motif, an established target of Rac (4), but appears to contain tetratricopeptide repeat (TPR) motifs, as suggested solely by sequence alignment with other proteins containing the motif (23, 24). Since Rac proteins with a mutation leading to defective interaction with p67phox are unable to activate the oxidase (22, 25–27), the interaction is considered to be involved in the oxidase activation. The final conclusion that Rac-p67phox interaction is required, however, has awaited studies using mutant proteins of the target p67phox.

Here we demonstrate that the p67phox N-terminal region of about 200 residues is not only sufficient but also required for fully interacting with Rac. Circular dichroism (CD) spectrum of the region reveals that it contains highly α-helical structure, and comparison between human and mouse p67phox supports the idea that the required region contains four TPR motifs, the first three of which are tandemly arranged. TPR motifs, each comprising a pair of antiparallel α-helices (24), are initially identified as a tandemly repeated degenerate 34-amino acid sequence in the nuclear protein Nuc2p (28) and the cell cycle division genes cdc16, cdc13, and cdc27 (29, 30). It is now realized that the motif occurs in a wide variety of proteins present in organisms as diverse as bacteria, archaea, and eukarya (31, 32), and is involved in protein-protein and protein-lipid interactions (33, 34). Little is, however, known about molecular nature of TPR-mediated interactions.

Based on the crystal structure of the TPRs of the protein phosphatase PP5 (24), we have introduced mutations that are expected either to disrupt or to unaffact packing of the TPR helices of p67phox. The present findings show that the N-terminal three TPRs, but the last one, are packed to interact with Rac, and that Arg-102 in the third TPR is likely involved in binding to Rac via anionic interaction. The results here also provide evidence that the interaction between p67phox and Rac is required for the NADPH oxidase activation both in vivo and in vitro. Although the fourth TPR is dispensable for the interaction, it appears to play a essential role in the oxidase activation.

**EXPERIMENTAL PROCEDURES**

**Preparation of cDNAs of Mutant Rac2 and p67phox**—The DNA fragments encoding various forms of human Rac2 were constructed by polymerase chain reaction-mediated mutagenesis, all of which contained the C1895 substitution to avoid being modified by isopenylation (35). Constitutively active and dominant negative forms of Rac2 carried the Q61L and T17N substitutions, respectively. Mutations in the effector loop (D38K and D38R substitutions) were introduced into the active Rac2 to obtain Rac2 (D38K/Q61L) and Rac2 (D38R/Q61L). The DNA fragments encoding mutant forms of p67phox were also constructed by polymerase chain reaction-mediated site-directed mutagenesis. All the constructs were sequenced to confirm their identity.

**Interaction between Rac and p67phox in the Yeast Two-hybrid System**—In the yeast two-hybrid system to investigate interaction between Rac and p67phox, we used yeast strains YEp7c containing two GAL4-inducible reporter genes, HIS3 and lacZ. The multiple cloning sites of pGBT9 (CLONTECH), containing the GAL4 DNA-binding domain, and pGADGH (CLONTECH), containing the GAL4 trans activation domain, were modified so that the inserts from glutathione S-transferase (GST) fusion protein plasmids pGEX-2T (Pharmacia) can be readily transferred in correct orientation and reading frames, to obtain pGBT9g and pGADGHg (14). Yeast cells were co-transformed with pairs of two-hybrid plasmids and selected by growth on medium lacking tryptophan and leucine. Cells containing both plasmids were picked up and plated on a nitrocellolose filter using Hybrid-slot (Life Technologies, Inc.) according to the manufacturer’s protocol. Various p67phox fused to GST (10 μg) were transferred to a nitrocellolose filter using Hybi-slot (Life Technologies, Inc.) according to the manufacturer’s protocol. The filter was incubated with the blocking buffer (3% bovine serum albumin, 0.1% Triton X-100, 0.5 mM MgCl2, 5 mM dithiothreitol) for 2 h, and washed two times with buffer A (10 mM Tris, pH 7.5, 0.1 mM NaCl, 5 mM MgCl2, 0.1 mM dithiothreitol). His-tagged Rac2 (Q61L/C1895S) (0.2 μg) was preloaded for 30 min at 30 °C with 2 μl of [γ-32P]GTP (NEN Life Science Products Inc; 6000 Ci/mmol, 10 μCi/ml, 0.8 μl) in the GTP-loading buffer (50 mM Tris-CH3C pH 7.5, 5 μM EDTA, 0.5 mM mg/ml bovine serum albumin). The freshly prepared probe was incubated for 5 min at room temperature with the GST fusion proteins on the filter in buffer A containing 1 mM GTP and 1 mg/ml bovine serum albumin. The filter was washed 3 times with an ice-cold washing buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20). After the filter was dried, it was exposed to a Fuji Imaging plate (Fuji Photo Co.), and signals were detected with the image scanner STORM (Molecular Dynamics).

**Cell-free Activation of the Phagocyte NADPH Oxidase**—The membrane fraction of human neutrophils was prepared as described previously (12). The DNA fragment encoding p47phox was subcloned into the His6-tagged fusion protein plasmid pET-28a (+) expression vector (Novagen). The fusion proteins were expressed in *E. coli* strain BL21DE3 (Novagen) and purified by His-bind resin (Novagen), according to the manufacturer’s protocol. The neutrophil membrane (17.5 μg/ml) was mixed with His-tagged Rac2 (7.5 μg/ml) preloaded with 100 μM GTP-S, His-tagged p47phox (3.7 μg/ml), and the indicated concentration of GST-p67phox or its mutants, followed by incubation with an optimal concentration of SDS (100 μM) for 2.5 min at room temperature in potassium phosphate buffer (100 mM, pH 7.0) containing 75 μM cytochrome e, 10 μM FAD, 1.0 mM EGTA, 1.0 mM MgCl2, and 1.0 mM MnCl2. The reaction was initiated by addition of NADPH (250 μM) to the reaction mixture. The production of superoxide was measured at the rate of superoxide dismutase-inhibitable ferricytochrome c reduction at 550–540 nm with a dual-wavelength spectrophotometer (Hitachi S557) (15, 16).

**Transfection of Wild-type and R102E Mutant p67phox in gp91 phox and p47phox-transduced K562 Cells**—We used a retroviral vector system, pSSLCpPause, that utilizes an internal ribosome entry site fragment of the aprt gene (29, 30) to transduce these genes into the leukemia cell line K562 that expresses p22phox (38). A bicistronic retrovirus vector encoding a human multidrug resistance gene (MDR1) and the p47phox gene (pHA-MDR-IRES-p47) (39) were further transduced to the already-transduced gp91phox-expressing K562 cells. The doubly transduced cells were selected with 4 μg/ml vincristine, expanded in a drug-free medium, and used for the following experiments.

Complementary DNA fragments encoding the full-length of wild-type and mutant p67phox carrying the R102E substitution were subcloned into pREP10 (Invitrogen), which were transfected by electroporation to the K562 cells that stably express both gp91phox and p47phox. The K562 cells (2 × 106 cells/ml) were electroporated in the presence of 10 μg of the wild-type or mutant form of p67phox plasmid DNA at 170 V, 960 μF, comprising the two subunits gp91phox and p22phox (38). Cells highly expressing gp91phox were selected using FACS scan with the monoclonal antibody 7D5 to detect functional cytochrome b558, comprising the two subunits gp91phox and p22phox. A bicistronic retrovirus vector encoding a human multidrug resistance gene (MDR1) and the p47phox gene (pHA-MDR-IRES-p47) (39) was further transduced to the already-transduced gp91phox-expressing K562 cells. The doubly transduced cells were selected with 4 μg/ml vincristine, expanded in a drug-free medium, and used for the following experiments.

**Expression of Oxidase Factors in K562 Cells**—For detection of p47phox and p22phox, K562 cells were sonicated and the lysates were applied at 10% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a polyvinylidene difluoride membrane (Millipore), and probed with polyclonal antibodies raised against the C-terminal peptide of p47phox and with an anti-p22phox monoclonal antibody.

For detection of p67phox proteins, proteins were immunoprecipitated from the K562 cell lysates (2 × 107 cells) with rabbit polyclonal antibodies raised against the C-terminal peptide of p67phox and against the N-terminal region of p67phox as well as with an anti-p22phox monoclonal antibody.

mid pProEX-HTB expression vector (Life Technologies, Inc.). The DNA fragments encoding p67phox and its mutants were subcloned into the pGEX-2T expression vector (Amersham Pharmacia Biotech). (His)6-tagged or GST fusion proteins were expressed in the *Escherichia coli* strain BL21DE3 (Novagen) and purified by His-bind resin (Novagen) or glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), respectively, according to the manufacturer’s protocol.
Activation of the NADPH Oxidase in the Whole Cell System—Superoxide production by the K562 cells expressing wild-type or mutant p67phox was determined as superoxide dismutase-inhibitable chemiluminescence detected with an enhancer-containing luminol-based detection system (DIOGENSES; National Diagnostics) as described by de Mendez et al. (37).

After the selection, K562 cells were resuspended in 1 ml of HBSS buffer (17 mM Hepes, pH 7.4, 120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM MgCl2, and 1 mM CaCl2). After the addition of the enhanced luminol-based substrate (40 μl), the cells were stimulated for 30 min at 37 °C with 200 ng/ml phorbol 12-myristate 13-acetate (PMA). The chemiluminescence was assayed using luminometer (Auto Lumat LB953; EG & G Berthold). The reaction was stopped by the addition of superoxide dismutase (50 μg/ml).

Circular Dichroism (CD) Spectra—GST-p67 (1–203) and GST-p67 (1–203, R102E) were expressed in the E. coli strain BL21DE3 (Novagen) and purified by glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech), as described above. After thrombin digestion to remove the GST tag, the protein fragments, namely p67 (1–203) and p67 (1–203, R102E), were purified on a Q-Sepharose (Amersham Pharmacia Biotech) and RESOURSE S (Amersham Pharmacia Biotech), and their purities were analyzed by 12% SDS-PAGE. The concentrations of the proteins used for these studies were 5 μM in 10 mM sodium phosphate, pH 6.4. CD measurements were performed with a Jasco J-725 spectrometer using rectangular quartz cells of 0.2-cm path length at 20 °C. Far-UV CD spectra were the average of eight accumulations taken at 50 nm/min. Secondary structural components were calculated by the method of Yang et al. (40) using software supplied by Jasco, Inc.

NMR Measurements—The proteins without tags, p67 (1–203) and p67 (1–203, R102E), were dissolved at concentrations of 1 mM in 50 mM sodium phosphate, pH 6.4. CD measurements were performed with a Jasco J-725 spectrometer using rectangular quartz cells of 0.2-cm path length at 20 °C. Chemiluminescence was measured using an enhancer-containing luminol-based detection system (DIOGENSES; National Diagnostics) as described by de Mendez et al. (37).

RESULTS

The N-terminal Region of p67phox Is Both Required and Sufficient for Interaction with Rac—To explore the region of p67phox for binding to Rac2, we prepared a series of deletion mutants of p67phox to use them for the yeast two-hybrid system. A constitutively active form of Rac2, carrying the Q61L substitution, interacted with the full-length p67phox (p67-F) (Fig. 1A), which agrees with the result obtained by the yeast two-hybrid system using a different reporter system (35). Two C-terminal deleted p67phox, p67-N (amino acids 1–242) and p67 (1–203), fully interacted with Rac2 (Fig. 1A). The findings indicate that the N-terminal region of p67phox is sufficient for the interaction with Rac, which is consistent with the results obtained from an in vitro binding assay using purified proteins (22). On the other hand, a dominant negative form of Rac2, namely Rac2 (T17N), was incapable of interacting with p67-F, p67-N, or p67 (1–203) (Fig. 1B and data not shown), confirming that the GTP-bound Rac2, but not the GDP-bound one, binds to p67phox. Further deletion of p67 (1–203) from either its N or C terminus resulted in complete loss of the interaction with Rac2 (Fig. 1A). These results suggest that the p67phox N-terminal domain comprising about 200 residues is both required and sufficient for binding to Rac2.

When Rac1 was used instead of Rac2, the same results were obtained: GTP-bound Rac1 interacted with the N terminus of p67phox (data not shown). These Rac GTPases share 92% amino acid identity with the identical effector loop of amino acid residues 32–40. Some mutations in the loop region result in impaired interaction with p67phox (22, 27) as well as decreased ability to support the NADPH oxidase activation (22, 41). One such mutation is substitution of Asn, a neutral hydrophilic residue, for Asp-38 (27). Replacement of this residue by basic ones (D38K and D38R) also abrogated the interaction with p67phox (Fig. 1B). These observations raise the possibility that Asp-38 may interact with a basic residue in the N terminus of p67phox. To define residues of p67phox involved in Rac binding, we substituted the neutral residue Gln for each of all eight Arg residues that occur in the p67phox N terminus (Fig. 2A). The R102Q substitution resulted in severely impaired interaction with Rac2, while the R38Q or R77Q substitution led to a slight defect of the interaction (Fig. 2B). On the other hand, five other mutant proteins carrying an Arg → Gln substitution at 62, 66, 155, 184, and 188 interacted with Rac2 as strongly as the wild-type one did (Fig. 2B).

Four TPR Motifs Occur in the Rac-binding Domain of p67phox—The binding experiment using p67phox proteins with substitution of Gln for Arg suggests that approximately 150 residues from the N terminus play a more important role, since the substitution at 155, 184, or 188 did not affect the interaction with Rac2 (Fig. 2). A search of SwissPLOT data base with this region by Blastp algorithm revealed a weak sequence similarity (20–30% identity) to regions of Sanp9, a general transcriptional repressor in Saccharomyces cerevisiae (42), and also, to a lesser extent, those of human CDC27 protein (43) and yeast TOM70, the 70-kDa translocase of outer membrane in mitochondria (44). The regions of these proteins are composed
of TPR motifs. The motif is a degenerate 34-amino acid sequence identified in a wide variety of proteins, present in tandem arrays of 3–16 motifs (24, 28, 29, 31). Although there exists no position characterized by an invariant residue, a consensus sequence pattern of small and large hydrophobic residues has been defined: small hydrophobic residues are commonly observed at positions 8, 20, and 27, while large ones are at 4, 17, and 24 (24, 31). Careful alignment of the N terminus of p67phox suggests that the region comprises four copies of the TPR motif, although the first repeat contains only 31 residues (Fig. 3A), the possibility which is also pointed out by other investigators (23, 24). In all four motifs of p67phox, there exist small hydrophobic residues at positions 8, 20, and 27, and large hydrophobic ones at 4, 17, and 24. In addition, like other TPR sequences, the N-terminal domain of p67phox are quite hydrophilic as estimated from hydrophilicity/hydrophobicity plots (45).

Further support for the identity of the p67phox N-terminal region as a TPR domain came from comparison between human p67phox and its mouse homologue, the sequence of which we have recently determined (46). Since mouse p67phox not only interacts with human Rac2 but also can replace human p67phox in a cell-free activation system of human NADPH oxidase (46), critical residues of p67phox are likely preserved between mouse and human. Alignment of amino acid sequences of human and mouse p67phox revealed that most of substitutions in the TPRs occur at nonconsensus positions; consensus residues are selectively conserved between the two species (Fig. 3B).

To obtain direct information on the structure of the N terminus of p67phox (residues 1–203) as a TPR domain, we isolated the fragment (Fig. 4A) and measured the circular dichroism (CD) spectrum (Fig. 4B). The profile, with the maximum at 190 nm and minima at 208 and 220 nm, is characteristic of an a-helix. The proportions of a-helix, b-sheet, and remaining structures were estimated by the method of Yang et al. (40) to be 76.3, 0, and 23.7%, respectively. This finding supports the idea that the N terminus of p67phox contains TPR motifs, since the motif comprises a pair of antiparallel a-helices (24). Taken together, we concluded that the N-terminal region of p67phox contains four TPR motifs, the first three being tandemly ar-ranged, while 16 extra residues are located between the third and fourth repeats (see Fig. 2A).

Role of p67phox TPR Motifs in Binding to Rac—To clarify roles for each TPR motif of p67phox in binding to Rac, we introduced two types of systematic mutations that are expected to disrupt each TPR architecture, based on the crystal structure of the TPR domain of the protein phosphatase PP5 (24). Each of the three TPR motifs of this domain consists of a pair of antiparallel a-helices of equivalent length, termed helix A and helix B (Fig. 3). Adjacent TPR motifs are packed together...
The first type of the mutations introduced into p67phox (left) carries a substitution of the bulky residue Gln for a conserved small residue at position 8; G13Q, G44Q, G78Q, and A128Q. The second type (right) mutation is deletion of an amino acid residue at position 22 in the TPR motifs; D27Δ, K58Δ, K92Δ, and E142Δ. A, the yeast reporter strain HF7c was co-transfected with recombinant plasmids pGBT9 encoding Rac2 (Q61L) and pGADGH encoding various TPR mutants of strain HF7c was co-transfected with recombinant plasmids pGBT9 encoding Rac2 (Q61L) and pGADGH encoding various TPR mutants of the yeast reporter strain HF7c was co-transfected with recombinant plasmids pGBT9 encoding Rac2 (Q61L) and pGADGH encoding various TPR mutants of strain HF7c was co-transfected with recombinant plasmids pGBT9 encoding Rac2 (Q61L) and pGADGH encoding various TPR mutants of strain HF7c was co-transfected with recombinant plasmids pGBT9 encoding Rac2 (Q61L) and pGADGH encoding various TPR mutants of strain HF7c was co-transfected with recombinant plasmids pGBT9 encoding Rac2 (Q61L) and pGADGH encoding various TPR mutants of.
it is possible that Arg-102 conforms a binding surface and a positive charge of this residue mediates the interaction with Rac. Furthermore, and most importantly, little difference could be observed between $^1$H NMR spectra of the wild-type and R102E protein (Fig. 8), indicating that the mutated TPR domain is correctly folded. Thus the R102E substitution appears to affect the structural integrity of the protein. Taken together with the results obtained by the binding experiments, it is concluded that Arg-102 of p67$^{phox}$ is involved in binding to Rac, probably via an ionic interaction.

To rule out the possibility that the R102E substitution results in a disrupted structure of the TPR domain, we measured both CD and $^1$H NMR spectra of the protein with this mutation. The CD spectrum of the mutant protein (data not shown) was in complete agreement with that of the wild-type one (Fig. 4B): the estimated proportions of $\alpha$-helix, $\beta$-sheet, and remaining structures in the mutated TPR domain were 78.9, 0, and 23.1%, respectively. We also tested the stability of the proteins by gradually increasing temperature from 20 to 60 °C: the changes in helical content were monitored at 222 nm. The structures in the mutated TPR domain were 76.9, 0, and 23.1%, respectively. We also tested the stability of the proteins by gradually increasing temperature from 20 to 60 °C: the changes in helical content were monitored at 222 nm. The curve for the changes of the R102E mutant protein was the same as that of the wild-type one (data not shown), supporting the idea that the $\alpha$-helices of the p67$^{phox}$ TPR domain are not disrupted by the substitution.

Fig. 7. Effects of substitutions for Arg-102 in p67$^{phox}$ on binding to Rac2. A, Rac2 binding activities of p67$^{phox}$ carrying various substitutions for Arg-102 were tested in the yeast two-hybrid system. The yeast reporter strain Hf7c was co-transfected with recombinant plasmids pGBT9 encoding Rac2 (Q61L) and pGADGH encoding p67$^{phox}$ carrying various substitutions for Arg-102. Its histidine-independent growth was tested as described under "Experimental Procedures." B, SDS-PAGE analysis of wild-type and various mutant forms of p67$^{phox}$ under the cell-free conditions. Each sample (0.4 μg) as GST fusion protein was resolved on a 10% SDS-PAGE and visualized with Coomassie Brilliant Blue. Lane 1, GST-p67; lane 2, GST-p67 (R102Q); and lane 3, GST-p67 (R102E). Position of molecular size standards are indicated to the left in kilodaltons. C, analysis of Rac2 binding activity of mutant p67$^{phox}$ carrying substitutions for Arg-102 by an overlay assay. The wild-type and mutant p67$^{phox}$ GST fusion proteins (10 μg) were put on a nitrocellulose filter, and probed with His-tagged Rac2 preloaded with [$\gamma$-32P]GTP. The filter was exposed to an imaging plate, which was subjected to the image scanner, as described under "Experimental Procedures."
A, respectively (Fig. 9). The order of potency to activate the oxidase (the wild-type R102K, R102Q, R102E) agrees with that to bind to Rac (Fig. 7), providing strong evidence that oxidase activation requires the interaction between p67\textsubscript{phox} and Rac. Thus activation of the NADPH oxidase likely involves an ionic interaction with Rac via Arg-102 in the third TPR of p67\textsubscript{phox}, which is consistent with that this TPR plays a crucial role in the activation (Fig. 6C).

p67\textsubscript{phox} Carrying the R102E Substitution Is Incapable of Supporting the NADPH Oxidase Activation under Cell-free Conditions. Superoxide production was measured as described under “Experimental Procedures” using the indicated concentration of the wild-type or mutant GST-p67-N, His\textsubscript{-}tagged p47\textsubscript{phox} (3.74 μg/ml), His\textsubscript{-}tagged Rac2 (7.3 μg/ml), and human neutrophil membranes (17.5 μg/ml). Open squares, filled circles, filled squares, and open circles indicate superoxide producing activities using p67-N (wild-type), p67-N (R102K), p67-N (R102Q), and p67-N (R102E), respectively.

The order of potency to activate the oxidase (the wild-type > R102K > R102Q > R102E) agrees with that to bind to Rac (Fig. 7), providing strong evidence that oxidase activation requires the interaction between p67\textsubscript{phox} and Rac. Thus activation of the NADPH oxidase likely involves an ionic interaction with Rac via Arg-102 in the third TPR of p67\textsubscript{phox}, which is consistent with that this TPR plays a crucial role in the activation (Fig. 6C).

The proteins. The transduced cells expressed functional cytochrome b\textsubscript{558} comprising the two subunits gp91\textsubscript{phox} and p22\textsubscript{phox} (data not shown; see “Experimental Procedures”) and p47\textsubscript{phox} (Fig. 10).

The doubly transduced K562 cells were subsequently transfected with the episomal vector pREP10 that contained cDNA encoding the full-length wild-type p67\textsubscript{phox} (p67-F) or full-length p67\textsubscript{phox} with the R102E substitution, namely p67-F (R102E). The wild-type p67\textsubscript{phox}-expressing cells fully produced superoxide when stimulated with PMA (Fig. 10). On the other hand,
the cells transfected with the p67-F (R102E) cDNA were unable to support superoxide production in response to PMA, although the protein was expressed at a similar level as the wild-type p67phox in the control cells (Fig. 10). Thus the mutant p67phox with the R102E substitution is incapable of activating the phagocyte NADPH oxidase under both cell-free and whole cell conditions.

**DISCUSSION**

Here we present that TPR motifs of p67phox are involved in the interaction with the small GTPase Rac, both structurally and functionally. The binding to Rac requires an overall structure of the p67phox N-terminal domain comprising about 200 amino acid residues in the proper conformation. The domain contains four TPR motifs, the N-terminal three being tandemly arranged, while 16 extra residues are located between the third and fourth TPRs. The present results show that the first three TPRs, but not the last one, play an essential role in the binding to Rac, via directly interacting with the GTpase and/or via being folded for the correct packing of the TPR domain. In particular, the third TPR appears to be directly involved in the interaction with Rac: Arg-102 in the third TPR, a residue that is likely irresponsible for the packing, participates in the interaction, probably via an ionic bond.

The structure of the TPR domain of the protein phosphatase PP5 reveals that each TPR motif of this domain consists of a pair of antiparallel α-helices of equivalent length, helix A and helix B (24). Adjacent TPR motifs are packed together in a parallel arrangement such that a tandem TPR motif structure is composed of a regular series of antiparallel α-helix: each α-helix shares two immediate α-helix neighbors and the protein fold may be defined as an overlapping array of three-helix bundles (24). Since a small residue at position 8 is located at the position of closest contact between the A and B α-helices of a TPR (24), substitution of the residue for the bulky residue Gln may lead to incorrect packing of the helix. This prediction is supported by a mutation of the p67phox gene in a patient with CGD: the mutant protein with substitution of position 8 in the third TPR (Gly-78) for Gln appears unstable in phagocytes (47), probably due to misfolding of the TPR. In addition, mutations at this position within TPRs 5 and 7 of cdc23 result in defect of protein function (49). Position 20 on helix B also resides between both helices A and B, while position 27 is located at the interface of three helices (A, B, and A′) within a three-helix bundle (24). This bundle may be incorrectly packed by one amino acid deletion in the region of residues 21–26 within helix B. Both types of mutations (substitution of Gln for a residue at position 8 and deletion of a residue at position 22) in the first to third TPRs of p67phox result in defective interaction with Rac (Fig. 5). Thus the three TPRs are folded such that the TPR domain interacts with Rac. The conclusion can explain how CGD is caused by three reported mutations within the first to third TPRs of p67phox: deletion of three amino acid residues (Lys-19, Lys-20, and Asp-21) in the first TPR (50), deletion of Lys-58 in the second TPR (48) and substitution for Gly-78 in the third TPR (47), the latter two of which are reported to result in decreased amounts of the proteins in neutrophils (47, 48).

Arg-102, on the other hand, resides at position 32 of the third TPR. Since the position is located at the C terminus of helix B (24), Arg-102 is not likely involved in the packing of the TPR helices. This is supported by the finding that the protein carrying the R102E substitution appears to be as stable as the wild-type p67phox in vivo (Fig. 10), and confirmed by the observations that substitution resulted in little change in both CD (data not shown) and 1H NMR spectra (Fig. 8). This mutation thus does not affect the structural integrity of p67phox. The basic residue is rather considered to constitute a binding interface for Rac. Substitution of the basic residue Lys for Arg-102 slightly reduces the capability of binding to Rac, while replacement by a neutral or acidic residue leads to little or no interaction with Rac, respectively. Thus Arg-102 plays a crucial role in binding to Rac, probably via an ionic interaction. This may explain that replacement of Asp-38 in the effector loop of Rac by a neutral or basic residue abrogates binding to p67phox (Fig. 1B; and Refs. 22 and 27). Taken together with the present experiments using mutant proteins, the binding to Rac requires a specific block of the TPRs of p67phox, the first three motifs, containing Arg-102 as an interacting residue.

The TPRs of p67phox by themselves, however, do not seem sufficient for the interaction, since the protein fragment comprising the first three or all TPRs (p67 (1–122) or p67 (1–167), respectively) was incapable of binding to Rac2 (Fig. 1). A region outside of the TPRs may be required for the structural integrity of the TPR domain and/or for stable interaction between p67phox and Rac. A recent report has shown that p67phox amino acid residues 170–199 can bind to Rac, but to a much lesser extent (51). It can be excluded that the TPR motifs do not physically interact with Rac but provide the structural framework to present residues 170–199 effectively to Rac, because Arg-102 in the third TPR appears to directly bind to Rac: p67phoxArg-102Q, containing both residues 170–199 and TPRs with a mutation unaffecting the structural integrity, is incapable of binding to Rac (Fig. 7). There may be two (or more) sites of p67phox that directly interact with Rac, both of which are required for stable interaction and activation of the NADPH oxidase. The protein that contains residues 170–199 but lacks the first three or all TPR motifs (p67 (126–242) or p67 (170–242), respectively) is not capable of activating the oxidase at all, as shown in this study (Fig. 6B).

Interactions of Rac with p67phox has been considered to be required for activation of the phagocyte NADPH oxidase, based on the observations that mutant forms of Rac, defective in the interaction, are incapable of activating the enzyme in vitro (22, 25–27). The requirement, however, has not been evidenced by experiments using mutant forms of the target protein p67phox, except a report showing that a protein containing deletion of Lys-58, being unstable, neither binds to Rac nor activates the oxidase (48). The present study demonstrates that a series of TPR mutants of p67phox defective in Rac binding, were all devoid of activity in the cell-free activation system of the oxidase (Fig. 6C). Among mutant proteins of p67phox carrying substitution for Arg-102, the Rac binding activity correlates well with the capability of activating the oxidase in vitro (the wild-type > R102K > R102Q > R102E) (Fig. 9). Furthermore, the protein with the R102E substitution, leading to a complete loss of interaction with Rac, is also inactive in the whole cell activation system of the oxidase (Fig. 10). These observations provide strong evidence that the binding of Rac to p67phox plays an essential role in activation of the NADPH oxidase both in vitro and in vivo.

On the other hand, the interaction between Rac and p67phox is not sufficient for activating the NADPH oxidase. The correctly packed fourth TPR of p67phox, in contrast to the other TPRs, does not seem involved in the interaction (Fig. 5), but is required for activation of the NADPH oxidase (Fig. 6C). The fourth TPR may be packed independently of the N-terminal three TPRs; it is rather conformed together with other regions, presumably forming an interface to interact with other oxidase factors, p47phox or a cytochrome b558 subunit (gp91phox (p55phox). In this context, it should be noted that about 10 residues C-terminal to the Rac-binding domain of p67phox (residues 203–212) are also required for the oxidase activation (52, 53). It has been shown that, in some proteins harboring mul-
multiple copies of TPR motifs, specific blocks of TPR motifs mediate interactions with particular target proteins and are assigned to specific biological functions. The N-terminal three TPR motifs of Ssn6p associate with the co-repressor Tup1p, whereas other combinations of TPR motifs mediate interactions with different transcription factors, which accounts for the diverse gene expression patterns regulated by Ssn6p (42, 54). TPR motifs 5–7 of p58, an inhibitor of the RNA-dependent protein kinase PKR, are responsible for interactions with PKR, while the N-terminal TPR motifs direct homotypic interactions (55).

Activation of the phagocyte NADPH oxidase is under strict control, since active oxygen species derived from superoxide are toxic to not only invading pathogens but also host cells, and thus unregulated production of superoxide results in damage of surrounding tissues accordingly. Since three indispensable proteins for the oxidase activation, p47phox, p67phox, and p58, an inhibitor of the RNA-dependent protein kinase PKR, are responsible for interactions with particular target proteins and are assigned to multiple copies of TPR motifs median TPR motifs direct homotypic interactions (55).

Although the TPR domain of p67phox as a Target for Rac GTPase
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