A Fusion Protein between Rac and p67^{phox} (1−210) Reconstitutes NADPH Oxidase with Higher Activity and Stability than the Individual Components†

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ABSTRACT: Activation of the phagocyte NADPH oxidase, a superoxide-generating enzyme, involves assembly of cytosolic p47^{phox}, p67^{phox}, and rac with the membrane-associated cytochrome b_{558}. Following cell-free activation, enzymatic activity is highly labile [Tamura, M., Takeshita, M., Curnutte, J. T., Uhlinger, D. J., and Lambeth, J. D. (1992) *J. Biol. Chem. 267*, 7529−7538]. In an attempt to stabilize the activity and to investigate the nature of the complex, we have produced fusion proteins between rac and a C-terminal truncated form of p67^{phox} (residues 1−210, 67N), which is a minimal active fragment. In a cell-free system, a fusion protein 67N-rac had higher activity and a 3-fold higher affinity than the individual cytosolic proteins, and 67N-Ser3-rac, which has a longer linker, showed a similar activity with the individual proteins. In contrast, rac-67N, a fusion in the opposite orientation, showed considerably lower activity. The enzyme activity reconstituted with 67N-rac showed a 10-fold higher stability and a lower K_m for NADPH than the individual components. In the absence of p47, 67N-rac fusion protein at a high concentration showed nearly full activation, which was higher than that with the individual components. These results indicate that covalent binding between p67N and rac in the correct order produces a more stable complex than the individual components, suggesting that interactions among the subunits significantly influence the duration of the oxidase activation. On the basis of these findings, we propose a model for the topology among rac, 67N, and cytochrome b_{558}.

Neutrophil NADPH oxidase is a multicomponent enzyme complex that produces superoxide (O_2^-) in response to exposure of neutrophils to bacteria or fungi (*1*, *2*). The enzyme is dormant in resting cells and becomes active upon cell activation. The activation is thought to occur via assembly of the cytosolic components p47^{phox} (p47),[1] p67^{phox} (p67), and rac with the membrane-associated flavocytochrome b_{558} (cyt b_{558}), which consists of p22^{phox} (p22) and gp91^{phox} (gp91) (*3*−*5*). Two other factors, p40^{phox} and rap1A, are also assumed to be involved in the enzyme regulation although they are not essential for the activity. The nature of the active complex has extensively been studied for a decade, but the structure of the complex has remained unclear (*4*−*6*).

NADPH oxidase can be activated in cell-free system consisting of the protein components and an anionic amphiphile (*3*). However, the activated enzyme is highly labile (*7*, *8*), complicating investigations of the subunit structure and hampering the isolation of an active enzyme complex. In a previous study (*7*) using a cell-free system consisting of cytosol and plasma membrane, we showed that the stability is dramatically improved by chemical cross-linking and suggested that the deactivation is caused by dissociation of protein components from the complex (*7*). Cross-linking was useful in stabilizing the oxidase, but it was impossible to isolate the active complex because the cross-linked complex resisted solubilization, perhaps due to covalent binding to the cytoskeleton (*9*). We have also attempted cross-linking in a cell-free system comprised of recombinant cytosolic factors and purified cyt b_{558}, but the effect of cross-linking was not as dramatic as in the crude system.[2]

Fusion of interacting proteins has been applied to microsomal electron transport systems in microsomes. Fusion proteins between cytochrome P450s and their reductase, NADPH cytochrome P450 reductase, have been genetically constructed (*10*, *11*). Subsequent studies revealed that fusion facilitates electron transfer from NADPH to several cytochrome P450s (*12*, *13*). This suggested to us that fusion might be useful to stabilize components of the neutrophil NADPH oxidase.

Recently, it was found that the oxidase can be activated in the absence of p47 when rac and p67 are present in excess (*14*, *15*) and that p47 dramatically lowers EC_{50} values both for rac and for p67 (*14*). These facts show that rac and p67

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[1] Abbreviations: p47, p47^{phox}; p67, p67^{phox}; 67N, C-terminal-truncated p67^{phox} (residues 1−210); cyt b_{558}, cytochrome b_{558}; cyt *c*, cytochrome *c*; PM, plasma membrane; PMSF, phenylmethanesulfonyl fluoride; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PC, L-α-phosphatidylcholine; PE, L-α-phosphatidylethanolamine; PI, L-α-phosphatidylinositol; SM, sphingomyelin; SH3, Src-homology region; TPR, tetratricopeptide repeats; PRR, proline-rich region; MESG, 2-amino-6-mercapto-7-methylpurine ribonucleoside.

[2] M. Tamura, unpublished results.

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are the minimum activating components whereas p47 functions as an enhancing factor. Therefore, we have produced fusion proteins between rac and p67 and investigated their abilities to activate the oxidase, as well as the stability of the activated complex.

Another benefit of using fusion proteins is that it provides basic information on the structure of the complex, e.g., topology and interactions among components. On the phagocyte NADPH oxidase, the interaction between rac and p67 was first reported by Diekman et al. (*16*). Subsequently, tetratricopeptide repeats (TPR) of p67 were shown to interact with an effector region of rac (*17*−*19*). Interactive domain structures such as SH3 (Src-homology region) and PRR (proline-rich region) were identified in p67 and p47 (*20*). The latter structure was also found in p22 (*21*), and the interactions between them have been extensively studied (*4*, *22*−*25*). An activation domain is seen in the middle of the p67 molecule (residues 200−210), and it was suggested that the domain acts on cyt b_{558} (*17*). On the other hand, an insert region in rac is also assumed to interact with cyt b_{558} (*27*), whereas the rac C-terminus is thought to interact with plasma membrane (*27*). Despite these lines of information, neither the actual interactions nor the topology of the components in the active complex is completely understood (*5*).

In this study, we have genetically engineered three fusion proteins between a truncated p67 and rac and examined their properties. We find that one of them has good ability to activate the oxidase in a cell-free system in either the presence or absence of $p47^{phox}$. In addition, the fusion produced an oxidase complex with remarkably higher stability than the individual components. On the basis of the results and kinetic studies, we propose a model for the topology of these cytosolic factors in the active complex, in which the activation domain of p67 is fixed by rac and p47.

EXPERIMENTAL PROCEDURES

Materials. pGEX-2T, pGEX-6P, PreScission protease, *Escherichia coli* BL21 strain, and glutathione−Sepharose were purchased from Amersham Pharmacia Biotech (Little Chalfont, U.K.). Oligonucleotide primers used were synthesized by the same manufacturer. *Eco*RI and *Bam*HI were purchased from Toyobo Inc. (Tokyo, Japan). L-α-Phosphatidylcholine (soybean, PC), L-α-phosphatidylethanolamine (bovine brain, PE), L-α-phosphatidylinositol (bovine brain, PI), sphingomyelin (bovine brain, SM), cholesterol, ω-aminooctylagarose, thrombin (bovine plasma), and cytochrome *c* (horse heart) were from Sigma Aldrich (St. Louis, MO). Phenylmethanesulfonyl fluoride (PMSF), tosyl-L-lysyl chloromethyl ketone (TLCK), GTP, and FAD were purchased from Nacalai Tesque (Kyoto, Japan). 2-Amino-6-mercapto-7-methylpurine ribonucleoside (MESG)/phosphorylase assay kit (EnzChek phosphatase assay kit) was obtained from Molecular Probes, Inc. (Eugene, OR). NADPH was obtained from Oriental Yeast Co. (Tokyo, Japan).

Expression and Purification of Recombinant p47 (Full Length), 67N (1−*210), and Rac.* Human p47 cDNA (in pVL1393) was a generous gift from Dr. Dave Lambeth (Department of Biochemistry, Emory University School of Medicine). The p47 cDNA was amplified by PCR and subcloned into pGEX-6P (*Eco*RI/*Bam*HI fragment) and transfected into the *E. coli* BL21 strain by the TSS method (*28*). Full-length p47 was expressed as glutathione *S*-transferase fusion protein. The cells were incubated with air bubbling at 37 °C for 2.5 h after addition of isopropyl β-D-thiogalactoside. Lysis was performed in the presence of diisopropyl fluorophosphate, and the expressed protein was bound to glutathione−Sepharose beads and released by cleaving the glutathione *S*-transferase moiety by PreScission protease. The preparation was concentrated with a Centricon Y-10 (Millipore Corp., Bedford, MA). By this method full-length p47 was obtained in good yield without any proteolytic fragmentation (Ebisu et al., unpublished results).[3]

Complementary DNAs for human 67N (1−210) and rac [rac1(C189S)] (both in pGEX-2T) were gifts from Drs. Chang-Hoon Han and Dave Lambeth (Department of Biochemistry, Emory University School of Medicine). Rac and 67N were expressed in BL21 cells and purified as above except that thrombin was used to cleave the glutathione *S*-transferase moiety.

Construction of Recombinant Plasmids for Fusion Proteins. For 67N-rac fusion, the 67N cDNA was subcloned into pGEX-2T (*Bam*HI/*Eco*RI fragment) and engineered to remove the stop codon by converting TAA to TCA (Ser) by a site-directed mutagenesis kit Quik Change (Stratagene, La Jolla, CA). Rac cDNA was engineered to change the *Bam*HI site to the *Eco*RI site by converting GGATCC to GAATTC and was ligated to pGEX-2T-p67N (*Eco*RI fragment). The recombinant plasmid, referred to as pGEX-2T-p67N-rac, was transfected into *E. coli* BL-21 as mentioned above. The linker peptide encoded between p67N and rac was Ser-Glu-Phe.

For rac-p67N fusion, the rac cDNA was subcloned into pGEX-6P (*Bam*HI/*Eco*RI fragment) and engineered to eliminate the stop codon by mutating TAA into TCA. The 67N cDNA was engineered to change the *Bam*HI site into the *Eco*RI site and was ligated to pGEX-6P-rac. The recombinant plasmid was referred to as pGEX-6P-rac-67N.

For 67N-Ser3-rac fusion, the pGEX-2T-67N-rac was engineered to insert three serine codons between the 3′-terminus of 67N cDNA and the 5′-terminus of rac cDNA. The primer used in Quik Change was GGTGGATTCA-GAATTCTCGTCGTCGATGCAGGCCATCAAG (three serine codons inserted and the rac initiation codon are underlined). The linker peptide between 67N and rac was Ser-Glu-Phe-Ser-Ser-Ser.

Expression of Fusion Proteins. Fusion proteins between 67N and rac were expressed in *E. coli* at 37 °C for 2.5 h after addition of isopropyl β-D-thiogalactoside. In this procedure the fusion protein formed inclusion body, and the recovery after lysis was extremely low. After several attempts to solve the problem, we found that freezing−thawing before lysis was efficient to improve the recovery. That is, the cells were frozen in the absence of glycerol and thawed immediately before lysis. By this treatment fusion proteins were obtained in a relatively good yield (1−2 mg/L of medium). The purification with glutathione−Sepharose beads was carried out as described above. The preparation was concentrated with Ultrafree Biomax 30k (Millipore Corp., Bedford, MA).

[3] K. Ebisu, unpublished results.

Isolation of Human Neutrophil Plasma Membrane. Human neutrophils were separated from peripheral blood of healthy volunteers with informed consent as described previously (*7*). The fractionation of the plasma membrane (PM) was performed as described (*29*).

Purification of Porcine Neutrophil Cyt b_{558}. Preparation of plasma membrane was essentially performed by the method described by Fujii et al. (*30*) with some modifications (*8*). The purification of cyt b_{558} was essentially followed by Abo et al. (*31*). The suspension was solubilized by 50 μM heptyl thio-β-D-glucoside, and the mixture was immediately centrifuged at 100000*g* for 1 h. The supernatant was subjected to a three-bed column (DEAE-Sepharose, ω-aminooctyl-agarose, and CM-Sepharose) equilibrated with buffer C (50 mM NaCl, 1 mM PMSF, 0.2 mM dithiothreitol, 3 μM TLCK, 17 mM heptyl β-D-thioglucoside, 50 mM PIPES, pH 6.5) containing 16% (v/v) glycerol. The flow-through fractions were applied to a heparin−agarose column equilibrated with buffer C containing 8% glycerol, and the column was eluted with NaCl gradient (0.05−1.5 M) in the same buffer. The fractions containing cyt b_{558} were concentrated with an Ultrafree Biomax 30k (Millipore Corp., Bedford, MA) and immediately mixed with an equal volume of phospholipid suspension (PC:PE:PI:SM:cholesterol = 29:3:27:30:11 wt % of total lipid in buffer C) to give a final lipid concentration of 1 mg/mL of preparation. The specific content of heme in partially purified cyt b_{558} was 2.1−2.5 nmol of heme/mg of protein. The relipidated cytochrome was stored at −80 °C until use.

Reconstitution of NADPH Oxidase and Assay for O_2^- Generation. For reconstitution with PM, the activation mixture included fused or nonfused 67N and rac (6 μM), p47 (1.33 μM), and plasma membrane (5 μg of protein) in 50 μL of buffer D (8 mM $MgCl_2$, 20 mM potassium phosphate buffer, pH 7.0) containing 10 μM GTP. Rac or fusion proteins were preincubated with 100 μM GTP at 25 °C for 20 min. The mixture was incubated with 120 μM SDS for 5 min at 25 °C to activate NADPH oxidase. Four 10 μL aliquots of the reaction mixture were transferred into wells of a 96-well plate which had been preincubated for 5 min or more at 25 °C. The mixture was supplemented with 240 μL of buffer D containing 200 μM NADPH and 80 μM cytochrome *c* (cyt *c*). Superoxide generation was measured by monitoring the reduction of cyt *c* at 550 nm using a microplate reader (Tecan, Spectra Classic). The assays were sometimes repeated in the presence of superoxide dismutase (80 μg/mL) to verify that O_2^--independent cyt *c* reduction was negligible, and when necessary the rate of O_2^- generation was corrected (*8*).

For reconstitution with purified cyt b_{558}, a one-step dilution method (*29*) was used in the assay. The reconstitution mixture contained 83 nM p47, 375 nM 67N, and 375 nM rac pretreated with 100 μM GTP (or a fusion protein pretreated with GTP) and 5 pmol of cyt b_{558} in 0.8 mL of buffer D containing 0.6 μM GTP, 10 μM FAD, 80 μM cyt *c*, and 1 mM EGTA. In reconstitution without p47, the concentrations of fused or nonfused 67N and rac were increased to 2.35 μM. The mixture was incubated with 80 μM (for the nonfused components and rac-67N) or 120 μM (for 67N-rac and 67N-Ser3-rac) SDS for 5 min at 25 °C. The reaction was started by addition of 200 μM NADPH, and superoxide generation was assayed by following the reduction of cyt *c* at 550 nm using a spectrophotometer (Shimadzu 160A).

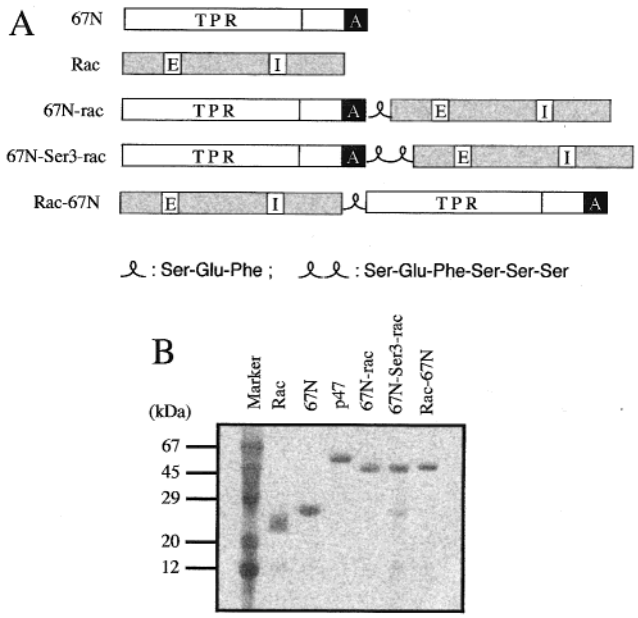

FIGURE 1: Fusion proteins prepared in this work. (A) Rac and 67N (residues 1−210) and their fusion proteins are schematically shown with their domain structures. A tetratricopeptide repeat (TPR) and an activation domain (A) were shown in 67N. An effector region (E) and an insert region (I) are shown in Rac. 67N-rac and rac-67N have a linker Ser-Glu-Phe, and 67N-Ser3-rac has a linker Ser-Glu-Phe-Ser-Ser-Ser. (B) SDS−polyacrylamide gel electrophoresis of the truncations and fusions. The proteins (1 μg each) were loaded on 15% (w/v) gel. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue. Expected molecular masses of the fusions are all around 45 kDa.

Assay for GTPase Activity of Rac and 67N-rac. Intrinsic GTPase activity was determined by the MESG/phosphorylase method described by Webb et al. (*33*) and modified by Cheng et al. (*34*) using an assay kit (Molecular Probes, Inc). Purified rac or fusion protein (67N-rac) was dialyzed against 20 mM Tris-HCl, pH 7.5. An aliquot of rac or 67N-rac was preloaded with 0.1 mM GTP at 25 °C for 20 min and then added to the reaction mixture to afford 5 μM, which included 1 mM $MgCl_2$, 0.2 mM MESG, 0.2 mM GTP, and 1 unit of purine nucleotide phophorylase in 20 mM Tris-HCl, pH 7.5 (1 mL). P_i release was monitored by following the absorbance change at 360 nm at 27 °C. As a blank, the experiment was repeated without a GTPase. Single turnover rate constants of GTP hydrolysis were determined by a first-order plot of the corrected data (sample minus blank) using nonlinear least-squares fits.

RESULTS

Fusion Proteins Prepared in This Study. We designed fused proteins using rac 1[C189S] (designated rac) and the C-terminal-truncated form of p67 (residues 1−210, 67N), which lacks two-thirds of the molecule. The rac mutant and p67 truncation were previously shown to support a full activation in a reconstitution system (*26*, *27*). Figure 1A illustrates domain structures of rac, 67N, and their fusion proteins. The 67N protein contains a tetratricopeptide repeat (TPR) region and the activation domain. The latter has recently been defined by Han et al. (*26*). Rac has an effector region near the N-terminus and an insert region close to the

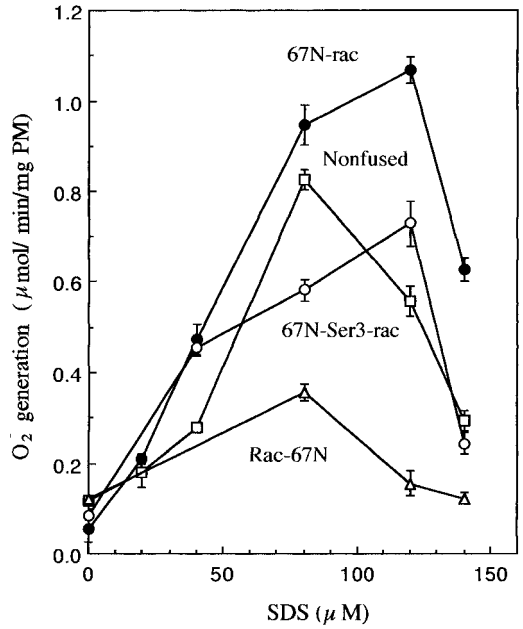

FIGURE 2: NADPH oxidase activity reconstituted with fused or nonfused rac and 67N. The cell-free reconstitution system contained 67N-rac, 67N-Ser3-rac, rac-67N, or nonfused components (6 μM each) with p47 (1.3 μM) and PM (5 μg of protein). The mixture was incubated with various concentrations of SDS. Rac (itself or a fusion with p67N) was incubated with GTP prior to the activation.

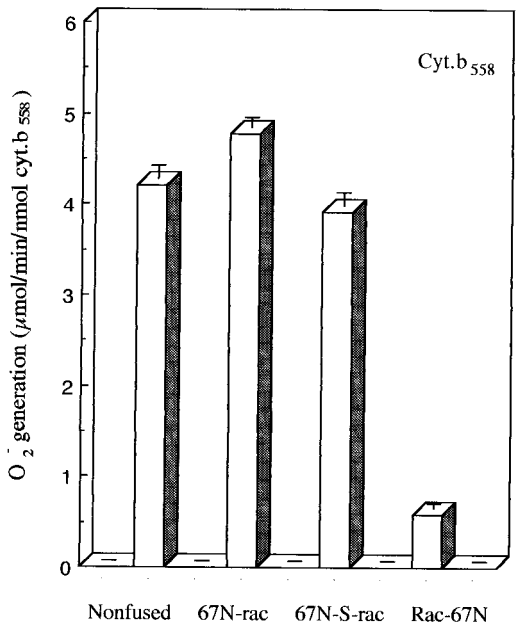

FIGURE 3: NADPH oxidase activity reconstituted with purified cyt b_{558}. The activation mixture contained a fused or nonfused 67N and rac, GTP-loaded rac, and purified cyt b_{558}. Other experimental conditions are as described under Experimental Procedures. The data are expressed as means ± SD from three experiments.

center of the molecule, which is characteristic of rho family GTPase.

We produced three types of fusion proteins designated 67N-rac, 67N-Ser3-rac, and rac-67N, which are different in the length of linker or the order of components. In 67N-rac or 67N-Ser3-rac, the activation domain is located in the middle of the molecule, while in rac-67N it is located at the C-terminus. Figure 1B shows SDS−polyacrylamide gel electrophoresis of the components and fusion proteins expressed in *E. coli* and purified with affinity beads. Fusion proteins showed single major bands at an expected molecular size of ~45 kDa.

Reconstituted Activity of NADPH Oxidase. Figure 2 shows O_2^--generating activity of the oxidase reconstituted with fused or nonfused 67N and rac in the presence of various concentrations of an activating anionic amphiphile SDS. Compared with the nonfused components, 67N-rac produced higher activity at all of the ranges of SDS concentration used, while rac-67N showed much lower activity. Optimal concentration for SDS was 80 μM with the nonfused components, and it shifted to 120 mM when 67N-rac or 67N-Ser3-rac was used but not when rac-67N was used. Compared at each maximum, the activity with 67N-rac was 20% higher than the nonfused components and that with 67N-Ser3-rac was similar, whereas the activity with rac-67N was 43% of that with the nonfused components. The result shows that a fusion in the correct order and distance enhances activation of the oxidase. The higher optimal concentrations for SDS with 67N-rac and 67N-Ser3-rac suggest that these fusions produced a complex, which is more stable under the activating concentrations of SDS.

Concentration Dependence for Cytochrome b_{558}. Figure 3 shows the activity reconstituted with purified cyt b_{558}. In the system, the optimal concentration for SDS was not changed by fusion and was constantly 200 μM. A fusion 67N-rac showed slightly higher activity, and 67N-Ser3-rac showed a similar activity, compared with the nonfused proteins. In contrast, rac-67N showed considerably lower activity. The results showed that, also in the system with cyt b_{558}, 67N-rac produces higher activity and rac-67N does not produce as much activity as in the system with plasma membrane.

EC_{50} Values of Fusion Proteins in a Cell-Free Reconstitution. To examine the effect of fusion on concentration dependence for the components, the oxidase activity was measured with various concentrations of fused or nonfused components (Figure 4), and EC_{50} values were estimated by curve fitting to the Michaelis−Menten equation (Table 1). The EC_{50} values for nonfused, 67N-rac, and rac-67N were 0.43, 0.28, and 1.03 μM, respectively. On the other hand, V_{max} values were in the following rank order: p67N-rac > nonfused components ≫ rac-67N. This indicates that a fusion 67N-rac slightly improves affinity for the complex while rac-67N lowers the affinity.

Concentration Dependence for p47. Figure 5 shows the concentration dependence for p47 in the reconstitution with fused or nonfused 67N and rac. The EC_{50} value for p47 was 0.36 μM with the nonfused components. The value was lowered to 0.15 μM by p67N-rac, and in contrast, it increased to 0.72 μM with rac-67N (Table 2). The result shows that fusion improves the affinity of p47 for other components in the complex.

Concentration Dependence for NADPH. The K_m values for NADPH were determined with the oxidase reconstituted with 67N-rac and the nonfused components. The K_m values with the fused and nonfused components were 29 and 36 μM, respectively (Table 2). This showed that fusion between 67N and rac does not much influence the NADPH-binding site, which is thought to be located in gp91^{phox}.

Activity Reconstituted in the Absence of p47. It was reported by two groups that the oxidase can be reconstituted

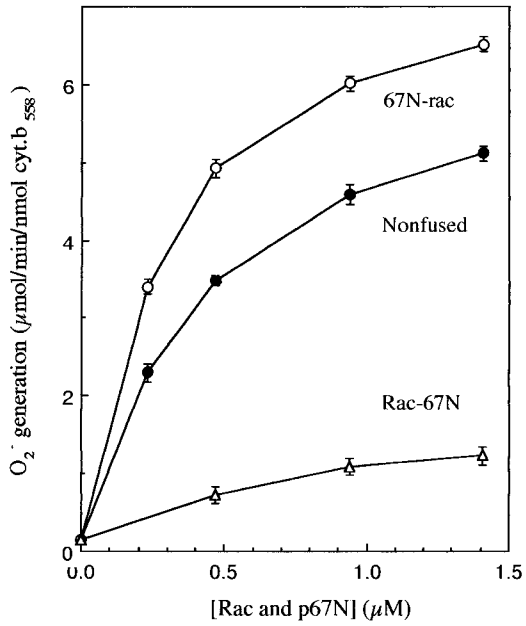

FIGURE 4: Concentration dependence for fusion proteins in the activation of NADPH oxidase. The activation mixture contained different concentrations of fused (67N-rac, rac-67N) or nonfused p67N and rac (nonfused) with p47 and cyt b_{558}. The concentrations of nonfused components were varied, being synchronized. Other experimental conditions are as described for Figure 2. The data are expressed as means ± SD from four experiments.

Table 1: Kinetic Parameters of Fused or Nonfused 67N and Rac in Reconstitution of NADPH Oxidase[a]

p47	rac and 67N	EC_{50} (μM)	V_{max} (μmol/min/nmol of cyt b_{558})
added	nonfused	0.43 ± 0.13[b]	6.74 ± 0.02
	rac-67N	1.03 ± 0.20	1.91 ± 0.19
	67N-rac	0.28 ± 0.03[b]	7.64 ± 0.19
none	nonfused	2.14 ± 0.21	6.71 ± 0.24
	67N-rac	1.68 ± 0.21	7.42 ± 0.33

[a] EC_{50} and V_{max} values were determined by nonlinear least-squares fits from the data in Figure 4 and other experiments (not shown) using the Michaelis−Menten equation. [b] $P < 0.05$ [nonfused, 0.43 ± 0.13 ($n = 4$); 67N-rac, 0.28 ± 0.03 ($n = 4$)].

in the absence of p47 when p67 and rac were present in excess (*14*, *15*). We herein examined the activation in the absence of p47 using 67N-rac or the nonfused components. In the absence of p47, the activity with 67N-rac was higher than that with the nonfused components, but when p47 was added, the activity increased, so the ratios of p47-independent activation to p47-dependent activation were similar in fused and nonfused components (67−68%). The EC_{50} values were determined in the absence of p47 (Table 1). The EC_{50} for the nonfused components was 2.1 μM and was slightly lowered by fusion of 67N-rac. These results showed that, in the absence of p47, 67N-rac produces nearly full activation, having slightly improved affinity for the complex, compared with the nonfused components.

Stability of NADPH Oxidase Reconstituted. Figure 7 shows the stability of oxidase activity reconstituted with fused or nonfused components. The oxidase was activated, incubated at 25 °C for indicated time, and assayed for O_2^- generation. After 30 min incubation, the oxidase with the nonfused components showed 30% residual activity. In contrast, the residual activity with 67N-rac or 67N-Ser3-rac was 80% or

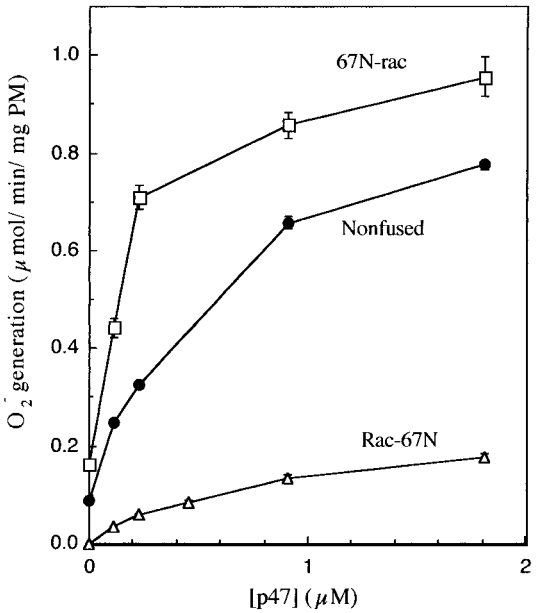

FIGURE 5: Concentration dependence for p47 in the activation with fusion proteins. The reconstitution system contained various concentrations of p47 with PM and fused (67N-rac, rac-67N) or nonfused components (nonfused). Other experimental conditions are as described for Figure 2. The data are expressed as means ± SD from three experiments.

Table 2: EC_{50} Value for p47 and K_m for NADPH of the Activated Oxidase with Fused or Nonfused Components[a]

	p47		NADPH	
rac and p67N	EC_{50} (μM)	V_{max} (μmol/min/mg)	K_m (μM)	V_{max} (μmol/min/mg)
nonfused	0.36 ± 0.12	0.93 ± 0.10	36.0 ± 7.7	1.32 ± 0.13
rac-67N	0.72 ± 0.10	0.24 ± 0.01		
67N-rac	0.15 ± 0.02	1.02 ± 0.11	29.4 ± 3.4	1.49 ± 0.07

[a] EC_{50} and V_{max} values were determined by nonlinear least-squares fits from the data in Figure 5. K_m and V_{max} values for NADPH were determined from the experiment with various concentrations of NADPH (not shown) by the same method.

62%, respectively, whereas rac-67N showed a similar activity with the nonfused components. Half-lives ($t_{1/2}$) were estimated from the data in Figure 6 by a first-order rate plot (Table 3). The half-life of the oxidase with nonfused components was 9 min and prolonged to 88 min by using 67N-rac. The half-life was in the following rank order: 67N-rac > 67N-Ser3-rac > nonfused ≥ rac-67N. It should be noted that the activity decay with 67N-rac followed simple first-order plots while that with the nonfused components or rac-67N is biphasic. This suggests that a subpopulation of the complex with a different conformation is not present in the system with 67N-rac.

Time Course of O_2^- Generation by the Activated Oxidase. Figure 8 shows a time course of O_2^- generation by the oxidase reconstituted with 67N-rac or the nonfused components. After addition of NADPH, the absorbance increase at 550 nm (cyt *c* reduction) was followed. There is not much difference between the initial rates of 67N-rac and the nonfused components, but during incubation the oxidase with nonfused components quickly lost activity and actually did not show any activity at 30 min. On the other hand, the oxidase with 67N-rac maintained the activity over 80 min

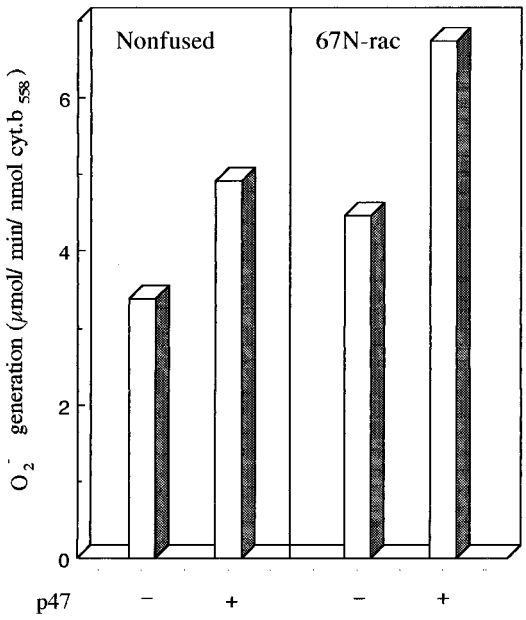

FIGURE 6: NADPH oxidase activity reconstituted in the absence of p47. The activation mixture contained fused or nonfused 67N and rac (2.35 μM) and purified cyt b_{558} (5 pmol) with or without p47 (83 nM). Other experimental conditions are as described for Figure 3. The data are expressed as means ± SD from three experiments.

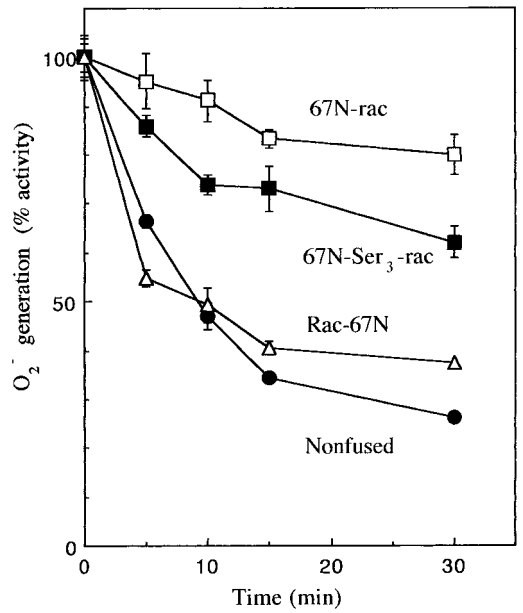

FIGURE 7: Stability of the oxidase reconstituted with fused or nonfused 67N and rac. The reconstitution system contained 67N-rac, 67N-Ser3-rac, rac-67N, or nonfused 67N and rac (6 μM each) with p47 (1.3 μM) and the PM (5 μg). After activation with SDS for 5 min, the mixture was kept at 25 °C for a given time. Then the mixture was supplemented with NADPH, and O_2^- generation was measured as described for Figure 2. Activities were expressed as the percentages of initial activities. The initial activities with p67N-rac and nonfused components were 1.05 ± 0.04 and 0.93 ± 0.03 μmol/min/mg of membrane protein, respectively.

although it gradually decreased. Consequently, the total amount of O_2^- produced with 67N-rac was 3.5-fold higher than that with the nonfused components.

Stability of the Oxidase Reconstituted without p47. We previously reported that p47 stabilizes the oxidase complex (*7*). To know whether fusion-mediated stabilization is related to p47 or not, we examined the enzyme stability reconstituted with fused or nonfused components in the presence or absence of p47. To simplify the analysis, here we used a system containing purified cyt b_{558}, in which the activity decay was found to be monophasic. In the absence of p47, the half-life was 20.5 min and was slightly increased by fusion (Table 4), whereas, in the presence of p47, the half-life was 44 min and was increased to 111.4 min by fusion.

Table 3: Half-Lives of NADPH Oxidase Activity Reconstituted with Fused or Nonfused Components[a]

components	half-life ($t_{1/2}$)	
	first phase	second phase
nonfused	9.4	38.5
rac-67N	6.5	42.7
67N-Ser3-rac	22.9	76.6
67N-rac	87.5	

[a] Half-lives ($t_{1/2}$) were determined by nonlinear least-squares fits from the data in Figure 7.

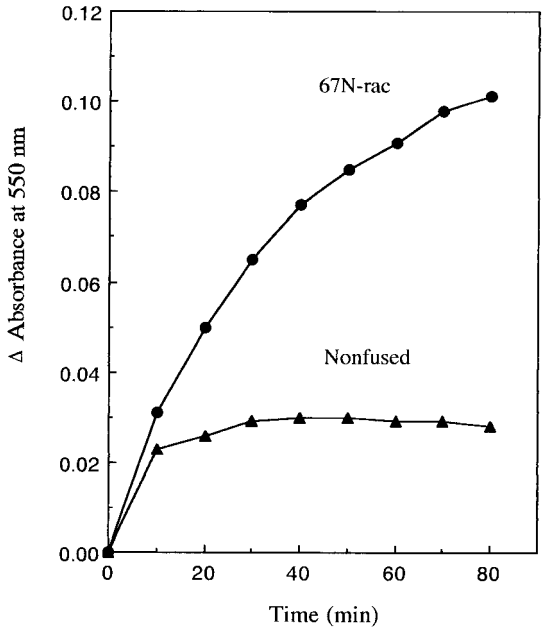

FIGURE 8: Time course of O_2^- generation by reconstituted NADPH oxidase. The reconstitution mixture contained p67N-rac or nonfused components (6 μM) with PM (5 μg) and p47 (1.3 μM), and the oxidase was activated with SDS. After addition of NADPH, the mixture was kept at 25 °C, and O_2^- generation was followed by measuring the absorbance at 550 nm at every 10 min up to 80 min and expressed as ΔA ($A_t - A_{t=0}$).

Table 4: Stability of NADPH Oxidase Activity Reconstituted in the Absence and Presence of p47[a]

p47	67N and rac	$t_{1/2}$ (min)
−p47	nonfused	20.4
	67N-rac	30.7
+p47	nonfused	44.0
	67N-rac	111.4

[a] The activation mixture contained GTP-loaded rac and 67N or GTP-loaded 67N-rac fusion and purified cyt b_{558} (5 pmol) with or without p47 (83 nM). The concentrations of rac and 67N (fused or nonfused) used with or without p47 were 0.375 and 2.35 μM, respectively. The initial rates with nonfused (fused) components were 1.96 (3.84) and 5.36 (5.79) μmol/min/nmol of cyt b_{558} in the absence and presence of p47, respectively. Other conditions are as described under Experimental Procedures.

Table 5: Rate Constants of Intrinsic GTPase Activities of Rac and 67N-rac[a]

sample	k_{cat} (min^{-1})
rac	0.149 ± 0.012 (n = 3)
67N-rac	0.140 ± 0.015 (n = 3)

[a] GTP-loaded rac or 67N-rac (final concentration 5 μM) was added to the reaction mixture containing 1 mM $MgCl_2$, 0.2 mM MESG, 0.2 mM GTP, and 1 unit of purine nucleotide phosphorylase in 1 mL of 20 mM Tris-HCl, pH 7.5, and the time courses of the absorbance change were recorded for 20 min at 27 °C. The experiment was repeated without a GTPase as a blank. Single turnover rate constants (k_{cat}) of GTP hydrolysis were determined as described under Experimental Procedures.

The result showed that stabilization by fusion is more effective in the presence of p47, suggesting that the stabilization partially involves p47 molecules. Enhanced binding of p47 might contribute to the stabilization of the oxidase.

Effect of Fusion on Intrinsic GTPase Activity of Rac. Conversion of activated rac to an inactive form by its intrinsic GTPase activity has been suggested to regulate the duration of activation of the oxidase complex (*35*). Therefore, it is plausible that fusion-mediated stabilization might be due to an impaired intrinsic GTPase activity by fusion. To clarify this point, we measured GTPase activities of rac and 67N-fused rac by the MESG/phosphorylase method (*34*). As shown in Table 5, the single turnover rate constant of rac GTPase was estimated to be 0.149 min^{-1}, and the rate was not altered by using 67N-rac (0.140 min^{-1}). Thus the improved stability of the complex by fusion is not due to inhibition of GTP hydrolysis.

Role of the C-Terminus of p47. In the present study, full-length p47 was used in the reconstitution system. However, the oxidase activity, either with 67N-rac or with the nonfused components, was not changed when the C-terminal truncated form of p47 (1−286) (47N) was used (data not shown). This is consistent with the concept that the C-terminus of p47 is not required for activation itself as suggested recently (*8*, *36*).

DISCUSSION

In this study, three novel fusion proteins between rac and the C-terminal truncated form of p67 were made to investigate how they influence the stability of the oxidase and to investigate the arrangement of cytosolic components in the complex. We found that one of the fusions, 67N-rac, produces higher activity with markedly higher stability than the nonfused cytosolic components. In contrast, rac-67N, in which the components are fused in the opposite orientation, produced considerably lower activity and stability.

The linker between two protein components is a tripeptide Ser-Glu-Phe in 67N-rac and rac-67N. The amino acid sequence was deduced from a nucleotide sequence mutated to change a stop codon and include a restriction site for *Eco*RI with a minimal substitution. When the linker was extended to a hexapeptide, Phe-Glu-Ser-Ser-Ser-Ser, both activity and stability decreased slightly. This shows that a shorter linker is more suitable than a longer one in this case, which may provide more flexibility to the topology between two components. The result indicates that tight binding of two components is favorable for activation, suggesting that the C-terminus of the activation domain is very close to the N-terminus of rac in the native complex.

To understand the mechanism for stabilization of the oxidase activity by 67N-rac, we performed kinetic studies with fusion proteins including 67N-rac. The data revealed that 67N-rac has the following properties, superior to the nonfused components or rac-67N: (i) improved affinity to the complex, (ii) improved affinity for p47, and (iii) improved stability under activating conditions including SDS. We have previously shown that some of activated enzyme molecules start to deactivate during the activation of other enzyme molecules (*7*).

Direct interaction of p67 with p47 or rac has been demonstrated, but direct interaction between rac and p47 has not. Therefore, one of the interesting findings here is that rac-fused p67N increases its affinity for p47. Covalent binding of rac may induce a conformational change in p67N, causing an increased affinity for p47. In relation to this, our recent study on fusions between C-terminal truncated forms of p47 (47N) and p67 (67N) showed that 47N-fused 67N considerably increases its affinity for rac (*8*). Taken together, the results indicate that the binding of p67 with one partner (p47 or rac) strengthens its interaction with the other partner.

As for the sites of interactions among p67, rac, and cyt b_{558}, the TPR region of p67 and the effector region of rac have been demonstrated (*17*−*19*). The rac insert region and p67 activation domain were suggested to interact with the cyt b_{558} (*17*, *26*). More recently, evidence for direct interaction between p67 and cyt b_{558} has been demonstrated (*37*).

As shown by two groups (*14*, *15*), when used at high concentrations, p67 and rac produces full activation in the absence of p47. The fusion protein 67N-rac also showed full activation in the absence of p47 when used at a high concentration. This confirms the concept that p67N and rac are minimal factors for activation and p47 functions as a modulator. EC_{50} values for the components p67N and rac were slightly lowered by fusion but still much higher than that in the presence of p47, suggesting that fusion between 67N and rac cannot substitute for the function of p47. Fusion-induced stabilization of the complex is not effective in the absence of p47, indicating that the stabilization involves the p47 molecule.

Recently, Lapouge et al. (*38*) have analyzed the crystal structure of the complex between p67-(1−203) and rac-(1−184) by X-ray analysis and determined the coordination of p67 (residues 2−186) and rac (residues 1−178). Grizot et al. have analyzed a longer fragment of p67 alone and determined the coordination (residues 1−193) (*39*). According to the crystal structure of the complex, the effector region (switch I) of rac interacts with the β-hairpin insertion of the p67 TPR region (*38*). In the structure, the N-terminus of rac is relatively close to the C-terminus of p67 TPR (residue 186), while the C-terminus of rac is in the opposite side of the N-terminus of p67N. The topology is well consistent with our results obtained here. In the 67N-rac fusion protein, covalent binding between the rac N-terminus and the 67N C-terminus (residue 210) may not impair a natural topology of the components, providing that the activation domain, the coordination of which is not yet determined, is inserted between them (cf. Figure 9). Whereas, in the rac-67N fusion protein, binding between the rac C-terminus and the 67N N-terminus may cause a drastic change in the topology. Thus

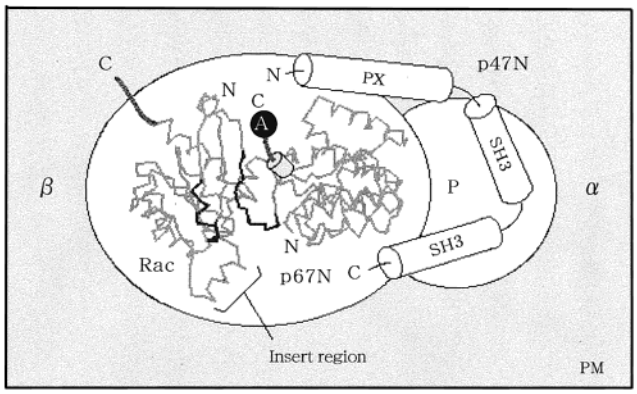

FIGURE 9: Possible model for topology among p67N, rac, and cyt b_{558} in the active complex. The figure is a view from the cytoplasmic side of the complex. Gray background represents the cytoplasmic surface of the plasma membrane (PM). Coordination of the rac−p67TPR complex [rac-(1−178) and p67-(2−186)] (*38*) (PDB: 1E96) is superimposed on gp91^{phox} (β). The rac C-terminus (179−181) and 67N C-terminus (187−210) are drawn in bold line by free hand considering the crystal structures of rac-(1−181) (*41*) and p67N-(1−193) (*39*). An α-helix at the 67N C-terminus identified by Grizot et al. (*39*) is shown by a gray cylinder. Switch I (residues 30−40, effector) and switch II (residues 60−70) regions are drawn in darkened lines. The N-terminus and C-terminus of cytosolic proteins are shown by N and C, respectively. A represents an activation domain, and P shows a proline-rich region in p22^{phox} (α). The PX domain and SH3 domains in p47 are shown by PX and SH3, respectively. The C-terminal region of p47 is omitted because the region was found to be not essential for activation (*8*).

orientation of the activation domain with 67N-rac should be just correct while that with rac-67N should not. When the linker between 67N and rac is extended by a tripeptide, the orientation may become loose, resulting in a modest stabilization.

As mentioned above, we have recently produced fusion proteins consisting of 67N and p47N (*8*). One of the fusion proteins 67N-47N was found to have excellent features in activating and stabilizing the oxidase. On the basis of the kinetic studies, we proposed a model for the topology of the components in the complex. In the model, the C-terminus of 67N (activation domain) is close to the N-terminus of p47 (PX domain), and two SH3 domains of p47 simultaneously interact with the proline-rich region of p22^{phox} (*25*, *40*).

On the basis of recent findings of subunit interactions and the crystal structures of the p67 fragment (*39*), rac (*41*), and their complex (*38*), we propose a new model for the topology of rac, p67N, and p47N on cyt b_{558} (Figure 9). We superimposed a model of the 67N-rac complex onto cyt b_{558} considering the following: (i) the rac insert region interacts with cyt b_{558} (*42*), (ii) the rac C-terminal region interacts with the plasma membrane (*27*), (iii) the activation domain (199−210) is close to the p47 N-terminus (*8*), and (iv) the activation domain is also close to the rac N-terminus (this work). Although the structure of p67-(194−210) including the activation domain has not been determined (*38*, *39*), we orient the domain toward the N-terminus of p47 on the basis of our results with fusion proteins between p47 and p67 fragments (*8*). Consequently, the activation domain (199−210) is located between both the N-termini of p47 and rac in the model. We speculate that the orientation of the activation domain at the correct position is centrally important for activation. Of interest in this context, it has recently been reported that the activation domain of p67^{phox} regulates electron transfer from NADPH to flavin, and valine-204 plays a crucial role in the step (*43*). More recently, Diebold et al. proposed that both rac and p67 are involved in flavin reduction and subsequent electron transfer to heme and molecular oxygen (*42*).

Rac is known to be prenylated in cells. Here we used nonprenylated rac as a component of fusion proteins, which has a higher EC_{50} value in a cell-free reconstitution than prenylated rac (*27*). For the next step, it will be an interesting approach to use prenylated rac as a component of fusion proteins with p67N.

Finally, we emphasize that protein fusion may be useful as a general technique to stabilize multicomponent enzymes or functional assemblies of proteins. The approach may not always be valid owing to the steric hindrance or topological limitations of the complexes, but even in the case, data will be informative to know the arrangement of the components in the complex.

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