Remarkable Stabilization of Neutrophil NADPH Oxidase Using RacQ61L and a p67^{phox}−p47^{phox} Fusion Protein†

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ABSTRACT: Activation of the phagocyte NADPH oxidase occurs via assembly of cytosolic p47^{phox}, p67^{phox}, and Rac with the membrane-bound flavocytochrome b_{558}. Recently, we have found that p67^{phox}-(1−210) (p67N) fused with p47^{phox}-(1−286) (p47N) or with Rac efficiently stabilizes the oxidase in a cell-free reconstitution system. In an attempt to further stabilize the oxidase, we herein used a constitutively active Rac, RacQ61L, and examined its effect on the oxidase stability. The half-life ($t_{1/2}$) of the activity reconstituted with wild-type Rac was 12 min at 37 °C, which was extended 6-fold by RacQ61L. Also, the stability of the oxidase without p47^{phox} increased 8-fold using RacQ61L. RacQ61L had a higher affinity for the complex than wild-type Rac and increased the affinity of p67N for the complex. Far-western blotting showed an enhanced binding between RacQ61L and p67N. The oxidase was stabilized by nanomolar FAD, and RacQ61L lowered the FAD concentration required. The combination of RacQ61L and a fusion protein consisting of p67N and p47N produced an extremely stable enzyme ($t_{1/2}$ = 184 min at 37 °C). The effectiveness of RacQ61L and fusion proteins on stabilization was in the following order: p67N−Rac < p67N + RacQ61L ≤ p67N−RacQ61L ≪ p67N−p47N + RacQ61L. These results indicate that a tightly bound ternary complex of p67^{phox}, Rac, and p47^{phox} is very effective in maintaining the oxidase and confirm that the longevity of the activated state requires continuous association of these components. This simple and efficient method of stabilization may provide a useful tool to elucidate the nature of the activated oxidase.

The phagocyte NADPH oxidase is a multicomponent enzyme that produces superoxide anion (O_2^-) in response to exposure of phagocytes to pathogens such as bacteria or fungi (*1*). The enzyme is dormant in resting cells and becomes active upon cell stimulation by pathogens or a receptor-mediated stimulant (*2*, *3*). The activation occurs via assembly of the cytosolic regulatory proteins p47^{phox}, p67^{phox}, and Rac with the membrane-associated flavocytochrome b_{558} (cyt b_{558})[1] (*3*). Cyt b_{558} consists of p22^{phox} and gp91^{phox}, the latter of which is classified as Nox-2 in the Nox/Duox family (*4*). 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.

Interactions among the subunit proteins have been extensively studied (*5*, *6*). SH3 (Src homology 3) mediated interactions among p47^{phox}, p67^{phox}, and p22^{phox} have been demonstrated (*7*−*9*). An interaction between p67^{phox} and Rac has also been revealed (*10*, *11*), and the interaction was shown to occur between the TPR (tetratricopeptide repeat) domain of p67^{phox} (*12*, *13*) and the switch I region of Rac (*13*, *14*). However, actual interactions in the active complex and the whole structure of the complex are still sketchy.

With regard to the role of cytosolic phox proteins in the activation, the following points have been noted: (i) Rac and p67^{phox} are the minimum activating components (*15*, *16*), (ii) p47^{phox} functions as an adapter protein (*15*) and a stabilizer (*17*, *18*), (iii) p67^{phox} is involved in the regulation of electron flow from NADPH to FAD (*19*, *20*), and (iv) an activation domain of p67^{phox} is involved in the regulation (*21*), probably by direct interaction with cyt b_{558} (*20*). Despite these findings, the mechanism for activation of the oxidase has remained unclear.

The activated NADPH oxidase is highly labile, complicating investigations of the subunit structure and preventing isolation of the active enzyme complex (*22*). In earlier studies we found that a chemical cross-linker remarkably improves the stability of the oxidase in crude cell-free systems (*17*). We also applied the methodology to a pure reconstitution system consisting of the recombinant cytosolic components and purified cyt b_{558}, but the effect was not as dramatic as in the crude reconstitution systems.[2]

Recently, we constructed fusion proteins between C-terminal truncated p67^{phox} (p67N) and p47^{phox} (p47N) and found that p67N−p47N, but not p47N−p67N, remarkably improves the stability of the oxidase activity (*23*). Subsequently, fusion between p67N and Rac was also investigated, and it was shown that p67N−Rac, but not Rac-p67N,

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[1] Abbreviations: cyt b_{558}, cytochrome b_{558}; RacQ61L, Rac mutant in which Gln-61 is replaced by Leu; p67N, C-terminal-truncated p67^{phox} (residues 1−210); p67N−RacQ61L, fusion protein between p67N and RacQ61L; p47N, C-terminal-truncated p47^{phox} (residues 1−286); p67N−p47N, a fusion protein between p67N and p47N.

[2] M. Tamura, unpublished results.

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considerably improves the stability (*18*). From these results we proposed a model for the topology of these phox proteins in the active complex (*18*). Although these fusion proteins reconstitute the oxidase activity that is fairly stable at 25 °C, the activity thus obtained was still not stable enough at higher temperatures (e.g., 37 °C).[3]

In an attempt to further stabilize the oxidase activity and to investigate the nature of the complex, we have used a constitutively active Rac, RacQ61L, and examined its effect on the stability of the oxidase activity in a cell-free reconstitution system. We find that the RacQ61L mutant remarkably stabilizes the oxidase activity and the effect could be ascribed to an enhanced binding of RacQ61L to $p67^{phox}$. We applied a fusion technique to the RacQ61L-containing system and found that when used with a fusion protein, p67N−p47N, RacQ61L produces an extremely stabilized oxidase activity. The mechanism for the excellent stabilization is discussed in relation to the topology model of the complex.

EXPERIMENTAL PROCEDURES

Materials. pGEX-2T, pGEX-6P, *Escherichia coli* BL-21, and glutathione−Sepharose were purchased from Amersham Pharmacia Biotech (Little Chalfont, U.K.). Oligonucleotide primers were synthesized by the same manufacturer. *Eco*RI and *Bam*HI were purchased from Toyobo Inc. (Tokyo, Japan). GTP, GTPγS, GDP, and FAD were purchased from Nacalai Tesque (Kyoto, Japan). NADPH was obtained from Oriental Yeast Co. (Tokyo, Japan).

Preparation of Rac, $p47^{phox}$, and $p67^{phox}$ (1−210). Complementary DNAs encoding human Rac [Rac1(C189S)], $p47^{phox}$, and $p67^{phox}$-(1−210) (p67N) were gifts from Dr. Dave Lambeth (Department of Pathology, Emory University School of Medicine). The protein Rac1(C189S) is referred to as wild-type Rac in this paper. We constructed the RacQ61L mutant on the basis of Rac 1(C189S), which is fully active and more stable than natural Rac1. The pGEX-2T plasmid containing the cDNA for Rac or p67N or the pGEX-6P plasmid containing $p47^{phox}$cDNA was transfected into BL-21 cells. The proteins were expressed and purified as previously described (*23*).

Construction of Recombinant Plasmids for RacQ61L and p67N−RacQ61L Fusion Proteins. The Rac cDNA in pGEX-2T was mutated to convert glutamine-61 (CAA) to leucine (CTA) using a site-directed mutagenesis kit (Stratagene Quik Change). For the fusion protein p67N−RacQ61L, the pGEX-2T containing the p67N−Rac cDNA (*18*) was engineered to convert glutamine-61 (CAA) to leucine (CTA) in the Rac moiety using the Quik Change kit. The sequences of the mutant and fusion proteins were confirmed by dideoxynucleotide-based sequencing.

Expression of Mutant and Fusion Proteins. The pGEX-2T containing RacQ61L, p67N−RacQ61L, or p67N−p47N cDNA was transfected into *E. coli* BL-21, expressed at 37 °C, and lysed using the conditions previously described for Rac (*23*). In the case of p67N−RacQ61L, the cells were frozen and thawed before lysis to improve the protein recovery as described for preparation of p67N−Rac (*18*). The expressed proteins were purified with glutathione−Sepharose beads, concentrated with a Centricon Y-10 (Millipore Corp., Bedford, MA), and stored at −80 °C until use.

Preparation of Cyt b_{558}. Isolation of plasma membranes from porcine neutrophils and purification of cyt b_{558} were performed as previously described (*18*). The cyt b_{558} preparation obtained was relipidated with a mixture of phospholipids and cholesterol (PC:PE:PI:SM:cholesterol = 31:14:7.5:23.2:24.3 wt % of total lipid) and stored at −80 °C until use.

Reconstitution of NADPH Oxidase and Assay for O_2^- Generation. Rac (6 μM) was preincubated with 100 μM GTP at 25 °C for 20 min in buffer A (20 mM potassium phosphate buffer, pH 7.0) and reconstituted with p67N (6 μM) with 1.3 μM $p47^{phox}$ and cyt b_{558} (0.1 μM) in 50 μL of buffer A containing 4 mM $MgCl_2$, 10 μM FAD, and 10 μM GTP. In some experiments, RacQ61L was used instead of Rac without preloading (see Results). The mixture was incubated with 200 μM SDS for 5 min at 25 °C to activate NADPH oxidase. Four 10 μL aliquots of the reaction mixture were then transferred into wells of a 96-well plate and diluted 25-fold with buffer A containing 4 mM $MgCl_2$, 200 μM NADPH, 10 μM FAD, and 80 μM cytochrome *c*. Superoxide generation was measured by monitoring the cytochrome *c* reduction 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 O_2^--dependent cytochrome *c* reduction, and the rate of O_2^- generation was corrected.

GTPase Activity. GTPase activities of Rac and its mutants were measured by a filter binding assay using [γ-^{32}P]GTP following the method of Geiszt et al. (*24*) with modifications. Rac or its mutant (6 μM) was loaded with 30 μM [γ-^{32}P]-GTP (7 μCi) at 20 °C for 10 min in 8 μL of buffer B (16 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol) containing 5 mM EDTA. $MgCl_2$ was then added to a final concentration of 20 mM, and the solution was kept on ice for 1 min. Three microliters of [γ-^{32}P]GTP-loaded Rac or RacQ61L was taken, diluted with buffer B (27 μL) containing 1 mM GTP and BSA (1 mg/mL), and incubated at 20 °C for a given time. An aliquot (5 μL) was taken and filtered onto a nitrocellulose membrane (Schleicher and Schell, BA45), followed by washing three times with 2 mL of cold buffer C (50 mM Tris-HCl, pH 7.7, 5 mM $MgCl_2$). The filters were dried, and the radioactivity was counted in a scintillation counter (Beckman LS6000). For the controls, the experiments were sometimes repeated without the proteins.

Far-Western Blotting. Purified p67N (6.6 μg per lane) was electrophoresed in an SDS−polyacrylamide gel (15% gel), and the protein bands were transferred onto nitrocellulose membrane (Schleicher and Schell, BA85). Each band corresponding to p67N (24 kDa) was excised and subjected to far-western blotting. Each piece of membrane was blocked with buffer D (300 mM NaCl and 0.05% Tween 20 in 20 mM Tris-HCl, pH 7.4), and the band was overlaid with Rac or RacQ61L (0.4 μM) at 25 °C for 30 min. For Rac, the protein was preincubated with 100 μM GTPγS at 25 °C for 20 min, and all of the following buffers contained 10 μM GTPγS. After being washed three times (5 min each) with buffer D, each piece of the membrane was incubated with anti-Rac IgG for 12 h at 25 °C. The anti-Rac IgG was a generous gift from Dr. Dave Lambeth (Department of Pathology, Emory University). In a preliminary experiment, we confirmed that there was no difference in anti-Rac IgG

[3] K. Ebisu and M. Tamura, unpublished results.

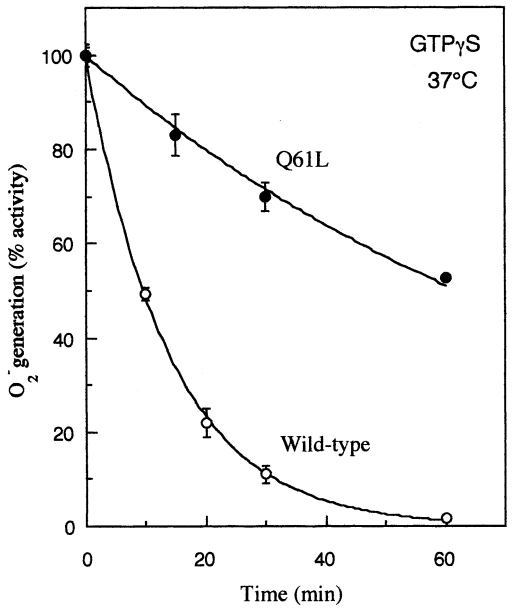

FIGURE 1: Effect of the RacQ61L mutation of Rac on the thermolability of NADPH oxidase. The reconstitution system contained GTPγS-treated Rac or RacQ61L (6 μM), p67N (6 μM), p47^{phox} (1.3 μM), and purified cyt b_{558} (0.1 μM). After activation with SDS for 5 min at 25 °C, the mixture was kept at 37 °C for a given time. NADPH was then added to start the enzyme reaction, and O_2^- generation was measured as described in the Experimental Procedures. Symbols and error bars represent the means ± SD from four determinations. The initial activities with Rac or RacQ61L are shown in Table 1. The graph shows the theoretical curves optimized by curve fitting to a first-order equation.

binding to RacQ61L or wild-type Rac. The membrane was treated with goat anti-rabbit IgG conjugated with peroxidase (Cappel Products) at 25 °C for 2 h and then washed twice with buffer D and once with 20 mM Tris-HCl (pH 7.4) containing 300 mM NaCl. After color development, blots were scanned using a scanner (Epson GT8700) and analyzed with the NIH Image software.

RESULTS

Effect of the Q61L Mutation on the Stability of NADPH Oxidase. The thermostability of NADPH oxidase activity reconstituted with Rac or RacQ61L was examined. Prior to reconstitution, both Rac and RacQ61L were loaded with GTPγS as a conventional procedure. Following activation of the oxidase, the mixture was incubated at 37 °C for 0−60 min and then assayed for O_2^- generation. Figure 1 shows the residual activities of the oxidase incubated. The oxidase with RacQ61L was significantly more stable at 37 °C than that with wild-type Rac. After a 30 min incubation, the residual activities of Rac and RacQ61L were 12% and 70% of the initial activities, respectively. As deactivation was found to be a first-order reaction, the half-lives ($t_{1/2}$) were estimated by curve fitting of the data (Table 1). The half-lives of the oxidase with Rac and RacQ61L were 9.5 and 60.2 min, respectively. This indicates a marked ability of RacQ61L to improve the oxidase stability.

Effects of Various Guanine Nucleotides on the Oxidase Activity. Rac and RacQ61L were pretreated with various guanine nucleotides, and the effects on the stability of the oxidase were examined. For wild-type Rac, the stability of the oxidase varied depending on the guanine nucleotide used (Table 1). Rac-GTP produced a slightly higher stability ($t_{1/2}$ = 12.5 min) than that with GTPγS-treated Rac, and untreated Rac produced a slightly lower stability ($t_{1/2}$ = 6.3 min), whereas GDP-treated Rac resulted in very low stability ($t_{1/2}$ = 3.2 min). The stability was in the following rank order: GDP ≪ none < GTPγS ≪ GTP. Table 1 also shows the initial activities with different guanine nucleotides. Unexpectedly, the activity with GTPγS−Rac was slightly lower than that with GTP−Rac, and furthermore GDP−Rac produced a substantial activity (61% of the maximal). The activity was in the following order: none ≪ GDP < GTPγS < GTP.

Table 1: Effect of Guanine Nucleotides on the Stability of NADPH Oxidase Reconstituted with Wild-Type or Q61L Rac[a]

guanine nucleotide	half-life at 37 °C (initial activity)[b]	
	wild type	RacQ61L
GTP	12.5 ± 0.8 (2053 ± 20)	60.1 ± 5.8 (2798 ± 36)
	11.3 ± 0.7 (1920 ± 69)	53.1 ± 2.7 (2571 ± 95)[c]
GTPγS	9.5 ± 0.2 (1540 ± 20)	60.2 ± 3.7 (2558 ± 62)
GDP	3.2 ± 0.1 (1252 ± 13)	57.5 ± 4.1 (2503 ± 42)
none	6.3 ± 1.4 (513 ± 10)	68.0 ± 5.7 (2361 ± 29)

[a] Rac or RacQ61L was incubated for 20 min at 25 °C with or without a guanine nucleotide (100 μM) and used in the cell-free reconstitution system containing the same guanine nucleotide at 10 μM. Other conditions were as described in the Experimental Procedures. [b] The half-lives were determined from the data in Figure 1 and other experiments by curve itting to an equation for a first-order reaction. The activities are expressed as nmol of O_2^- min^{-1} (nmol of cyt b_{558})$^{-1}$. [c] Preincubation with GTP was performed in the presence of 5 mM EDTA. An aliquot of the mixture was added to the activation mixture containing an excess amount of $MgCl_2$ to achieve a final concentration of 4 mM free Mg^{2+}.

In contrast to wild-type Rac, for RacQ61L the stability of the oxidase was not much changed by the nucleotide used. To our surprise, untreated RacQ61L produced a similar, even higher stability ($t_{1/2}$ = 68 min) compared with a guanine nucleotide-treated one. Addition of EDTA during the GTP loading did not change the results. On the basis of these results, in the following experiments, Rac was treated with GTP prior to reconstitution, and RacQ61L was used without GTP loading.

Stability in the Absence of p47^{phox}. The oxidase can be activated without p47^{phox} when p67^{phox} and Rac are used in excess (*15*, *16*). Therefore, we examined how RacQ61L stabilizes the activity under these conditions. As seen in Figure 2, RacQ61L considerably stabilized the oxidase in the absence of p47^{phox}. The stability with Rac ($t_{1/2}$ = 15 min) was improved 8-fold by RacQ61L ($t_{1/2}$ = 117 min) (Table 2). The stability was not much increased even when p47^{phox} was added. This indicates that stabilization by RacQ61L occurs independently of p47^{phox}.

GTPase Activity. To compare the intrinsic GTPase activity of RacQ61L with that of Rac, we performed a filter binding assay using [γ-^{32}P]GTP. The radiolabeled GTP was loaded on Rac or RacQ61L, and GTPase activity was measured at 20 °C. For Rac, GTP was readily hydrolyzed with a rate constant (k_{cat}) of 0.066 min^{-1}. For RacQ61L, only a small population of GTP was exchanged under the conditions, and hydrolysis occurred very slowly with a k_{cat} of 0.003 min^{-1}, which is 20-fold lower than that with Rac. The results indicate that the GTPase activity as well as nucleotide exchange ability is extremely diminished in the RacQ61L mutant. The data, together with Table 1, indicate that

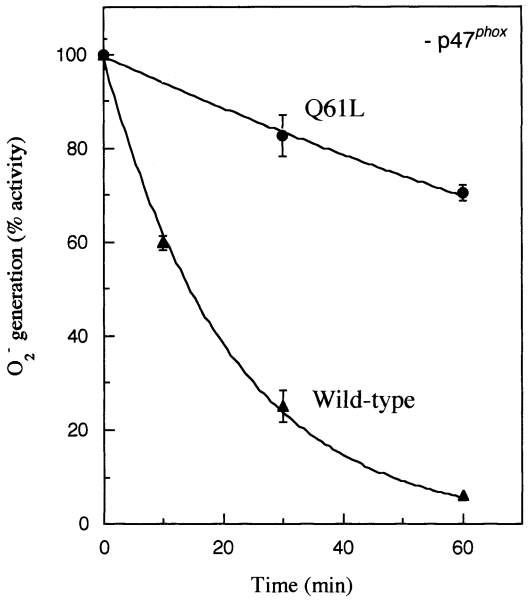

FIGURE 2: Thermolability of NADPH oxidase reconstituted with a high concentration of p67N plus Rac or RacQ61L. The reconstitution system contained 38 μM Rac (triangles) or RacQ61L (circles), p67N, and purified cyt b_{558} (0.1 μM). Other experimental conditions were as described for Figure 1. Data represent the means ± SD from four determinations. The initial activities are shown in Table 3.

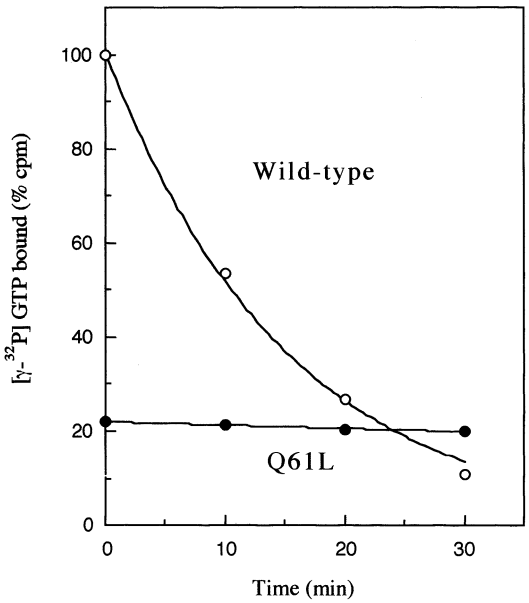

FIGURE 3: Intrinsic GTPase activities of Rac and RacQ61L estimated by a radiolabeled GTP. Rac or RacQ61L (6 μM) was loaded with 30 μM [γ-^{32}P]GTP in the presence of 5 mM EDTA at 20 °C for 20 min. $MgCl_2$ was added to a final concentration of 20 mM to stop the GDP−GTP exchange reaction and start the GTP hydrolysis reaction. The mixture was incubated at 20 °C for a given time in the presence of 2 mM $MgCl_2$, 1 mM GTP, and BSA (1 mg/mL). An aliquot was taken and filtered onto a nitrocellulose membrane. The radioactivity was measured in a scintillation counter. Other experimental conditions were described in the Experimental Procedures.

Table 2: Stability of NADPH Oxidase Reconstituted with High Concentrations of p67N and Rac in the Absence and Presence of p47^{phox} [a]

p47^{phox}	Rac	$t_{1/2}$ at 37 °C (initial activity)[b]
−p47^{phox}	wild type	14.5 ± 0.9 (1491 ± 46)
	RacQ61L	116.8 ± 5.8 (2575 ± 43)
+p47^{phox}	wild type	24.9 ± 5.3 (2909 ± 78)
	RacQ61L	128.1 ± 7.3 (3230 ± 62)

[a] The reconstitution system contained Rac (or RacQ61L) and p67N (38 μM each) in the presence or absence of p47^{phox} (1.3 μM). The half-lives were estimated from the data in Figure 2 by curve fitting to an equation for a first-order reaction. [b] The half-lives ($t_{1/2}$) are expressed as min and the initial activities are expressed as nmol of O_2^- min^{-1} (nmol of cyt b_{558})$^{-1}$.

nucleotide exchange is not necessary for RacQ61L to activate the oxidase. Diminished nucleotide exchange ability was previously reported with Rac2 Q61L by Xu et al. (*25*).

Effect of RacQ61L on Kinetic Parameters. The concentration dependence for RacQ61L in cell-free reconstitution was examined and compared with that for Rac. The EC_{50} values estimated for Rac and RacQ61L were 43 and 16 nM, respectively (Table 3). RacQ61L decreased the EC_{50} value for p67N 4-fold but did not change the EC_{50} value for p47^{phox}, and this is consistent with the concept that Rac does not facilitate the interaction of p47^{phox} with cyt b_{558} (*26*). The above results show that RacQ61L has a higher affinity for the complex and increases the affinity of p67N in the complex.

Table 3 also shows the EC_{50} values for Rac (Q61L) and p67N in the absence of p47^{phox}. With Rac, the EC_{50} values for Rac and p67N were 10.4 and 2.9 μM, respectively. In comparison, with RacQ61L the values for RacQ61L and p67N were 2.3 and 1.6 μM, respectively. The results showed that RacQ61L has a higher affinity than wild-type Rac and increases the affinity of p67N in the complex also in the absence of p47^{phox}. However, the affinities are still far lower than those in the presence of p47^{phox} (Table 3). Thus RacQ61L does not substitute for p47^{phox} as an adapter protein.

Effect of Flavin on Thermostability. In the course of the stability experiments, we happened to find that FAD in the incubation mixture influenced the deactivation rate of the

Table 3: Effect of the Q61L Mutation on the EC_{50} Values for Rac, p67N, and p47^{phox} [a]

		Rac		p67N		p47^{phox} [b]	
p47^{phox}	Rac form	EC_{50} (nM)	V_{max}	EC_{50} (nM)	V_{max}	EC_{50} (nM)	V_{max}'
+	wild type	43 ± 6	1805 ± 44	69 ± 17	2002 ± 83	39 ± 16	1821 ± 164
+	Q61L	16 ± 6	2304 ± 124	17 ± 4	2342 ± 105	39 ± 19	2379 ± 197
−[c]	wild type	10400 ± 1900	1255 ± 160	2900 ± 400	475 ± 31		
−[c]	Q61L	2300 ± 100	1657 ± 40	1600 ± 100	1245 ± 35		

[a] The cell-free activation was performed as described in the Experimental Procedures. The V_{max} values are expressed as nmol of O_2^- min^{-1} (nmol of cyt b_{558})$^{-1}$. [b] The activities at [p47^{phox}] = 0 were not negligible, and therefore the data were fitted to a modified Michaelis−Menten equation, $v = V_0 + V_{max}/(1 + K_m/[S])$, where V_0 is the activity at [p47^{phox}] = 0. Furthermore, V_{max}' is defined as follows: $V_{max}' = V_0 + V_{max}$. The V_0 values observed for wild-type and Q61L Rac were 323 and 854 nmol of O_2^- min^{-1} (nmol of cyt b_{558})$^{-1}$, respectively. [c] The system contained 6 μM p67N with various concentrations of Rac or 6 μM Rac with various concentrations of p67N.

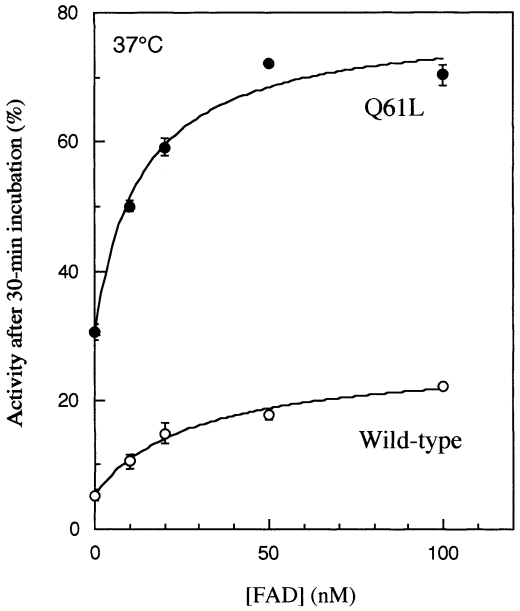

FIGURE 4: Effect of FAD concentration on the thermolability of NADPH oxidase. After cell-free activation, the mixture was incubated for 30 min at 37 °C in the presence of FAD (0−100 nM). After incubation, the mixture was diluted with an assay mixture containing 10 μM FAD and assayed for superoxide generation. Activities were expressed as the percentages of those without 37 °C incubation. The control activities with Rac and RacQ61L were 2023 ± 20 and 2489 ± 54 nmol min^{-1} (nmol of cyt b_{558})$^{-1}$, respectively. Symbols and error bars represent the means ± SD from four determinations.

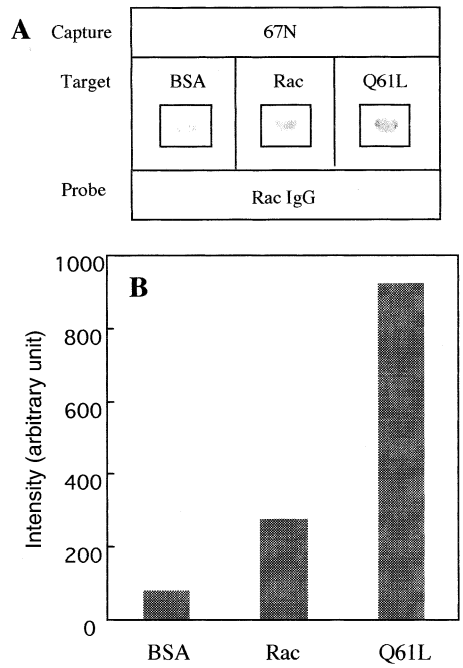

FIGURE 5: Detection of interaction between p67N and Rac or RacQ61L by far-western blotting. Purified p67N was electrophoresed in an SDS−polyacrylamide gel, and the protein bands were blotted onto nitrocellulose membrane. The band corresponding to p67N was excised and subjected to far-western blotting as described in the Experimental Procedures.

oxidase. Therefore, the effect of FAD on the stability of the oxidase complex was investigated in more detail. Following activation, the enzyme was heated at 37 °C for 30 min with various concentrations of FAD and then assayed for O_2^- generation. As shown in Figure 4, the oxidase stability was significantly influenced by the FAD concentration in the incubation. After a 30 min incubation in the absence of FAD, the residual activities with Rac and RacQ61L were 5% and 30%, respectively. When 50 nM FAD was included, they increased 18% and 72%, respectively. The estimated EC_{50} value with Rac was 28 nM, and that with RacQ61L was 13 nM, suggesting that the affinity of flavin for cyt b_{558} is increased by RacQ61L.

Binding Ability to p67N. As the Q61L mutation increased the affinity of Rac and p67N in the oxidase complex, we next tested whether the mutation increased the interaction between Rac and p67N using far-western blotting. When GTPγS-treated Rac was used, a faint band was observed under the conditions (Figure 5). In comparison, with RacQ61L a more prominent band was observed. The intensity of the RacQ61L band was 3.3-fold higher than that with Rac-GTPγS. Thus, RacQ61L binds to p67N more tightly than the GTP-bound form of Rac.

This result is somewhat different from that by Diekman et al. (*10*), who showed that Rac2Q61L has a slightly lower affinity for p67^{phox} than wild-type Rac by using affinity precipitation. The discrepancy might arise from the difference in the forms of p67^{phox} used. They used full-length p67^{phox} fused with glutathione *S*-transferase while we used a C-terminal truncated form of p67^{phox}.

Effect of Fusion on the Stability. In preceding papers, we found that fusion of p67N with p47N or Rac efficiently stabilizes the oxidase. Therefore, here we applied a fusion technique to the system with the RacQ61L mutant. When fused p67N and RacQ61L were used in the system, the stability of the oxidase was slightly higher ($t_{1/2}$ = 86 min) than that with the individual components ($t_{1/2}$ = 64 min) (Table 4). However, the effect of fusion was not as dramatic as that observed with p67N and Rac (*18*). This suggests that RacQ61L and the fusion protein share a common mechanism for stabilization, namely, enhancement of the interaction between p67N and Rac.

Table 4: Effect of RacQ61L and Fusion Proteins on the Stability of NADPH Oxidase[a]

p67^{phox} form	other proteins	$t_{1/2}$ at 37°C (initial activity)
p67N	p47^{phox}, Rac	12.5 ± 0.8 (2053 ± 20)
p67N−Rac	p47^{phox}	35.8 ± 2.6 (2291 ± 44)
p67N	p47^{phox}, RacQ61L	63.6 ± 16.0 (2952 ± 71)[b]
p67N−RacQ61L	p47^{phox}	86.3 ± 21.2 (3049 ± 12)
p67N−p47N	RacQ61L[c]	184.0 ± 33.0 (3501 ± 30)

[a] The reconstitution system contained p67N and RacQ61L or the p67N−RacQ61L fusion protein (6 μM each), p47^{phox} (1.3 μM), and purified cyt b_{558}. Other conditions were as described in the Experimental Procedures. Half-lives ($t_{1/2}$) are expressed as min and initial activities are expressed as nmol of O_2^- min^{-1} (nmol of cyt b_{558})$^{-1}$. [b] When p47N was used, the half-life was 84.0 ± 8.8 min and the activity was 3085 ± 4 nmol of O_2^- min^{-1} (nmol of cyt b_{558})$^{-1}$. [c] p67N−p47N (2 μM) and RacQ61L (6.8 μM) were used in the reconstitution.

In contrast, when RacQ61L was used with the p67N−p47N fusion protein, the oxidase was extremely stabilized. The stability ($t_{1/2}$ = 184 min at 37 °C) was the highest level of all the approaches we tested, including fusion and cross-linking (*17*, *18*, *23*). The effectiveness for stabilization was in the following rank order: p67N, Rac < p67N−Rac < p67N, RacQ61L < p67N−RacQ61L ≪ p67N−p47N, RacQ61L. Of interest is the fact that the stability with

RacQ61L was higher than that with p67N−Rac, a covalently bound form of the protein pair. The remarkable stabilization with p67N−p47N and RacQ61L should be due to the formation of a very stable ternary complex formed among p67N, p47N, and RacQ61L.

DISCUSSION

The Q61L mutation in small GTPases was originally discovered in a Ras variant from human lung carcinoma cells (*27*) and assumed to be the cause of its oncogenic nature. RasQ61L has an impaired GTPase activity as with RasG12V, which might lead to continuous activation of Ras and continuous binding to Raf and, consequently, to nonregulated cell proliferation through intracellular signal transduction.

The Q61L mutant of Rac has often been used as a constitutively active form of Rac, i.e., as a persistently GTP-bound form. In this study, we used RacQ61L in a pure reconstitution system of NADPH oxidase and found that it remarkably improved the stability of the oxidase. The data showed the following properties of RacQ61L and the RacQ61L-containing system: (i) the affinity of Rac and p67N in the complex is increased by the mutation, (ii) the stabilization occurs in either the presence or absence of $p47^{phox.}$, (iii) RacQ61L has a diminished guanine nucleotide exchange ability, and (iv) in combination with p67N−p47N, RacQ61L produced an extremely stable activity of the oxidase.

As expected, RacQ61L had an impaired GTPase activity, but this nature does not seem to be a major reason for the improved stability, because GTPγS-loaded Rac did not stabilize the oxidase as effectively as RacQ61L. Rather, a diminished nucleotide exchange, i.e., a suppressed GTP release (*25*), might contribute to the stability.

Another possibility is a conformational change in the Rac structure by the RacQ61L mutation. The crystal structure of wild-type Rac revealed that Gln at position 61 is located close to switch I (*28*), which interacts with p67N (*13*). Leu substituted for Gln-61 may have contact with switch I (particularly Pro-34) and move the loop or induce a conformational change in switch I, which is favorable to p67N binding. Xu et al. reported that Rac2Q61L loses the ability to bind GDS (*25*) and reverses the inhibitory effect of mutations in the effector domain such as D38A (*29*). These facts suggest that a drastic conformational change occurs in RacQ61L and that RacQ61L has a partially different conformation from that of RacG12V, which shares the properties of low GTPase activity and low responsiveness to GAP (*29*). Thus the stabilization of the oxidase activity by RacQ61L may be due to a conformational change in RacQ61L induced by the mutation, which results in an enhanced binding between RacQ61L and p67N and leads to the complex stabilization.

In the present study, we have established a remarkably stabilized NADPH oxidase by combination of RacQ61L and a fusion protein p67N−p47N. The stability is at the highest level of all of the approaches we tested (*17*, *18*, *23*). Our recent studies showed that the p67N−Rac or p67N−p47N fusion protein stabilizes the oxidase and suggested that the orientation of the activation domain of $p67^{phox}$ at the correct position is centrally important for activation (*23*). The data predicted that if the N-termini of p47N and Rac were both linked to the C-terminus of p67N, stabilization would be more synergistically improved. However, the construction of a triple fusion protein of such an arrangement is impossible using genetic engineering. Fortunately, in the present study we found a tight binding between RacQ61L and p67N. This provided us with the opportunity to overcome the experimental limitations and produce a mostly stable ternary complex among p47N, p67N, and Rac.

It is widely accepted that interaction between cytosolic phox proteins and cyt b_{558} is a major process in the oxidase activation. In addition, the stoichiometry of the protein components in the complex formation has been postulated (*5*, *6*, *30*). However, Cross et al. reported that the activation process is primarily catalytic and not through the formation of a stoichiometric complex (*31*). Furthermore, Quinn et al. showed that complex formation is required but not enough for activation (*32*). Thus, the contribution of the stoichiometric complex to the duration of the activation has not been established. Our present results show that complex formation is centrally important in the duration of the oxidase activity.

One of the most interesting findings in this paper is that the oxidase stability is greatly influenced by FAD. FAD increased the stability 4-fold at a nanomolar order of concentration. The finding suggests that FAD, a chromophore of NADPH oxidase, has a role in maintaining the oxidase complex. The presence of FAD on $gp91^{phox}$ contributes to the conformational stability of the molecule. We also found that the flavin concentration required for stabilization is lowered by the RacQ61L mutant. It is tempting to speculate that p67N, which is tightly bound to $gp91^{phox}$ through RacQ61L, may prevent FAD dissociation either directly or indirectly.

The present experiments showed that GDP-bound Rac can produce substantial activity although the stability is low. Canonical small GTPases bind to their effector proteins as a GTP-bound form (*33*) although there are atypical small GTPases that bind to their effector proteins in GDP form (e.g., ran and rab) (*34*, *35*). With regard to the phagocyte NADPH oxidase, the Rac form that activates the oxidase is controversial. Many groups have reported that the GTP form is necessary for the activation (*36*−*39*), but some groups have demonstrated that the GDP form is also capable of activating the oxidase (*40*−*42*). In addition, it has recently been demonstrated that the interaction between $p67^{phox}$ and cyt b_{558} is enhanced by either the GTP or GDP form of Rac (*43*). Further studies will be required to clarify this point.

In summary, using a mutant of Rac and a fusion protein, we have accomplished the production of an extremely stabilized NADPH oxidase in a pure reconstitution system. This indicates that a tightly bound ternary complex among $p67^{phox}$, Rac, and $p47^{phox}$ is very effective in maintaining the oxidase activity and confirms the concept that the longevity of the activated enzyme requires continuous association of these cytosolic components as suggested in our earlier study (*17*).

Finally, we emphasize that this simple and efficient method to stabilize the oxidase may provide a useful tool for elucidating the nature of the activated complex, which is transient in both cells and cell-free systems.

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