The Domain Organization of p67^{phox}, a Protein Required for Activation of the Superoxide-Producing NADPH Oxidase in Phagocytes

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Key Words

NADPH oxidase • Phagocyte • p67^{phox} • Domain organization • Small-angle X-ray scattering • Innate immunity

Abstract

The phagocyte NADPH oxidase, crucial for innate immunity, is dormant in resting cells, but becomes activated during phagocytosis to produce superoxide, a precursor of microbicidal oxidants. In activation of the oxidase, the multi-domain protein p67^{phox} plays a central role: it translocates to the membrane as a ternary complex with p47^{phox} and p40^{phox}, and interacts with the small GTPase Rac to assemble with the membrane-integrated catalytic protein gp91^{phox}, leading to superoxide production. Here we show, using small-angle X-ray scattering (SAXS) analysis, that p67^{phox} adopts an elongated conformation when it exists not only as a monomer but also as the heterotrimer. Although p67^{phox} harbors an N-terminal TPR domain for binding to Rac and a p40^{phox}-interacting PB1 domain, followed by an SH3 domain that associates with p47^{phox}, the present model suggests that no or few apparent associations occur between the domains. The positions of the protein-interaction domains in p67^{phox} contribute to activation of the phagocyte NADPH oxidase: the first

SH3 domain that is located between the TPR and PB1 domains positively regulates oxidase activation only when it is present at the correct position; the PB1 domain placed at this SH3 domain position inhibits the oxidase by interacting with p40^{phox}.

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Introduction

Neutrophils play crucial roles in innate immunity [1, 2]. During phagocytosis of invading microbes, these cells produce superoxide, a precursor of powerful microbicidal oxidants, by the catalysis of the NADPH oxidase [3–9]. The significance of the phagocyte NADPH oxidase in host defense is evident from recurrent and life-threatening infections that occur in patients with chronic granulomatous disease (CGD), whose neutrophils genetically lack the superoxide-producing system [3–9].

The catalytic core of the phagocyte NADPH oxidase is the membrane-integrated cytochrome gp91^{phox}, which

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Fig. 1. Domain architecture of human p67^{phox}. Human p67^{phox} of 526 amino acids harbors an N-terminal domain composed of 4 TPR motifs, an N-terminal SH3 domain [SH3(N)], a PB1 domain (PB1) and a C-terminal SH3 domain [SH3(C)]. Rac in the GTP-bound state binds to the TPR domain, p40^{phox} associates with the PB1 domain and p47^{phox} interacts with the C-terminal SH3 domain.

forms a stable heterodimer with the membrane-spanning protein p22^{phox} [3–9]. The oxidase is dormant in resting cells, and becomes activated during phagocytosis or upon cell stimulation by soluble agents such as chemoattractants and phorbol 12-myristate 13-acetate (PMA). The activation requires membrane translocation of the specialized proteins p67^{phox}, p47^{phox} and p40^{phox}, and the small GTPase Rac, which are present in the cytosol of resting cells. At the membrane of stimulated cells, these factors assemble with the gp91^{phox}-p22^{phox} dimer, activating superoxide production. p67^{phox} forms a ternary complex with p47^{phox} and p40^{phox}; p67^{phox} plays an essential role in oxidase activation [3–9]. p47^{phox} is not crucial for a cell-free activation system in the presence of excess amounts of p67^{phox} and Rac [10, 11], whereas it is required under the conditions where the oxidase is activated in intact cells [3–9]. p40^{phox} is dispensable in both cell-free and whole-cell systems, but enhances oxidase activation by facilitating membrane translocation of the ternary complex in stimulated cells [12–14].

The oxidase activator p67^{phox} comprises 526 amino acids with a molecular mass of 59.8 kDa. It contains an N-terminal domain consisting of four tetratricopeptide (TPR) motifs and two SH3 domains that are separated by a PB1 domain (fig. 1). p67^{phox} constitutively associates with p40^{phox} via the PB1-PB1 interaction [12, 15, 16] and with p47^{phox} via binding of the C-terminal SH3 domain to the p47^{phox} proline-rich region [17–19], thereby forming the ternary complex [12, 20]. Upon cell stimulation, Rac translocates to the membrane independently of the p67^{phox}-p47^{phox}-p40^{phox} complex, and interacts there with the p67^{phox} TPR domain, the interaction of which is essential for activation of the phagocyte NADPH oxidase [21, 22]. Although the target of the N-terminal SH3 domain has not been identified, this module positively regulates the oxidase, which could be mediated via binding to gp91^{phox} [23]. Thus, the functions of individual domains have been intensively studied; however, it is ob-

scure whether domains in p67^{phox} interact with each other and whether the three-dimensional domain organization contributes to oxidase activation.

A study using neutron scattering, in combination with analytical gel filtration, suggested that p67^{phox} forms a homodimer, although the protein used had a tendency to aggregate even at low concentrations [24]. On the other hand, analytical centrifugation data indicated that p67^{phox} is an elongated monomer, which explains its apparent high molecular mass on gel filtration analysis [20]. Thus, the oligomerization state of p67^{phox} has been controversial, and the three-dimensional domain organization of this protein remains to be elucidated.

Here we show, using small-angle X-ray scattering (SAXS) analysis, that p67^{phox} exists as a monomer that adopts an elongated conformation with no or few significant associations between the domains. This is in contrast to the structures of p47^{phox} [25] and p40^{phox} [26]; these two proteins show a more compact or globular structure via interdomain interactions. Furthermore, we demonstrate that the positions of the protein-interaction domains in p67^{phox} play important roles in activation of the phagocyte NADPH oxidase.

Materials and Methods

Plasmid Construction

The human cDNAs encoding full-length p67^{phox} (amino acids 1–526), p47^{phox} (amino acids 1–390) and p40^{phox} (amino acids 1–339) were prepared as described previously [12, 19, 21]. PCR-mediated site-directed mutagenesis was performed to obtain the following mutant p67^{phox}: p67^{phox}-mut1, in which the PB1 domain (amino acids 350–432) and the C-terminal SH3 domain (amino acids 458–518) were swapped; p67^{phox}-mut2, in which the N-terminal SH3 domain (amino acids 240–301) and the PB1 domain were swapped; p67^{phox}-mut3, in which the N- and C-terminal SH3 domains were swapped; and p67^{phox}-mut4, which lacked the regions of amino acids 241–457 and 519–526. p67^{phox}-mut1-ΔSH3(N), p67^{phox}-mut2-ΔSH3(N), and p67^{phox}-mut3-ΔSH3(N)

were the proteins with the truncation of SH3(N) in addition to the above-mentioned domain swapping. p40^{phox}-mut carried the D289A substitution. The cDNA for gp91^{phox} were ligated to the mammalian expression vector pcDNA3 (Invitrogen). The cDNAs for p22^{phox}, p67^{phox}, p47^{phox} and p40^{phox} were ligated to the mammalian expression vector pEF-BOS. p67^{phox} was constructed for expression as a Myc-tagged protein; p47^{phox} and p40^{phox} as a Flag-tagged protein. Mutations leading to the indicated amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis. All constructs were sequenced for confirmation of their identities.

Protein Preparation and Analytical Ultracentrifugation

Bacterially expressed p67^{phox}, p47^{phox} and p40^{phox} were purified, and the binary complexes (p67^{phox}-p47^{phox} and p67^{phox}-p40^{phox}) as well as the ternary complex (p67^{phox}-p47^{phox}-p40^{phox}) were prepared, as previously described [27].

To characterize the homogeneity of molecular species, hydrodynamic properties and oligomeric states of the samples, analytical ultracentrifugation experiments were performed using a Beckman Model XL-I analytical ultracentrifuge (Beckman Coulter Inc.) equipped with both UV-visible and Rayleigh interference optical detection system using an An-60 Ti rotor at 20°C. In sedimentation velocity experiments, interference fringe displacement profiles were recorded every 1 min up to 800 experimental curves at the rotor speed of 40,000 rpm. The *c(s)* size distributions were calculated from the experimental curves using the program Sedfit (version 8.9) [28]. The program SEDNTERP (version 1.08) was used to calculate the partial specific volume and solvent density from the amino acid and buffer compositions [29]. All proteins and complexes (p67^{phox}, p47^{phox}, p40^{phox}, p67^{phox}-p47^{phox}, p67^{phox}-p40^{phox} and p67^{phox}-p47^{phox}-p40^{phox}) yielded a single sedimentation boundary, indicating sample homogeneity. Sedimentation equilibrium experiments were performed as previously described [30]. The analyses revealed that p67^{phox}, p47^{phox} and p40^{phox} existed as a monomer, the p67^{phox}-p47^{phox} and p67^{phox}-p40^{phox} complexes as a heterodimer, and the p67^{phox}-p47^{phox}-p40^{phox} complex as a 1:1:1 trimer. The molecular masses of the samples that were estimated from the experiments were in good agreement with those calculated from their amino acid sequences.

SAXS and Molecular Shape Analysis

The samples prepared above (p67^{phox}, p47^{phox}, p40^{phox}, the binary complexes p67^{phox}-p47^{phox} and p67^{phox}-p40^{phox}, and the ternary complex p67^{phox}-p47^{phox}-p40^{phox}) were subjected to SAXS analyses. SAXS data were collected at protein concentrations from 1 mg/ml to 10 mg/ml in the *Q* range 0.015 to 0.443 Å⁻¹ to assess the effects of protein concentration on the structural parameters. All measurements were performed using a SAXS diffractometer in BL-10C beam line installed at the Photon Factory in Tsukuba, Japan, as previously described [31, 32]. The wavelength of the X-ray was 1.488 Å. The sample cell had a volume of 50 µl and a 1-mm path length with quartz windows. The data acquisition time was 600 s for each measurement. Protein scattering was determined by subtracting the solvent scattering as the background trace [30, 32, 33]. The forward scattering *I*(0) and the radius of gyration *R_g* were evaluated from the Guinier approximation [34] and also using the program GNOM [35]. The distance distribution function, *P(r)*, was calculated from the scattering data by the indirect Fourier transformation method using GNOM

[35]. The values of *R_g* were stable from the lowest to the highest concentration. The relative molecular weight of the scattering species was estimated using *I*(0), obtained from the scattering data for bovine carbonic anhydrase and bovine serum albumin (BSA) as standard proteins.

Low-resolution models of p67^{phox} were generated from experimental scattering data by the ab initio shape determination programs DAMMIN [36] and GASBOR [37]. The scattering data in the range of the momentum transfer with a *Q* value of 0.02–0.30 Å⁻¹ for DAMMIN and of 0.02–0.40 Å⁻¹ for GASBOR were used for the fit. Ten independent models were reconstructed, which were superimposed and averaged using the programs SUPCOMB [38] and DAMAVER [39]. The averaged shapes were filtered based on the expected molecular weight of the protein using the program DAMFILT [39].

Activation of the Phagocyte NADPH Oxidase in COS-7 and CHO Cells

Experiments for activation of gp91^{phox} in COS-7 and CHO cells were performed as previously described [23, 40, 41]. COS-7 cells, endogenously expressing p22^{phox} and Rac1, and CHO cells, endogenously expressing Rac1 but not p22^{phox}, were transfected using LipofectAMINE (Invitrogen) with the following plasmids: 0.5 µg of pcDNA3-gp91^{phox}; 0.1 µg of pEF-BOS encoding the wild-type or indicated mutant Myc-p67^{phox}; and 0.5 µg of pEF-BOS-Flag-p47^{phox}. CHO cells, endogenously expressing Rac1 but not p22^{phox}, were transfected using FuGENE6 (Roche Diagnostics) with the following plasmids: 0.5 µg of pcDNA3-gp91^{phox}; 0.1 µg of pEF-BOS-p22^{phox}; 0.025 µg of pEF-BOS encoding the wild-type or indicated mutant Myc-p67^{phox}; and 0.5 µg of pEF-BOS-Flag-p47^{phox}. Total lysates of transfected cells were used for estimation of expression of Myc-p67^{phox} and Flag-p47^{phox}. The lysates were subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and probed with an anti-Myc (Roche Diagnostics) or anti-Flag (Sigma) monoclonal antibody. The blots were developed using ECL-plus (GE Healthcare Biosciences) for visualization of the antibodies, as previously described [23, 40, 41].

Transfected cells were cultured for 24 h, and suspended in Hepes-buffered saline (120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM MgCl₂, 1 mM CaCl₂, and 17 mM Hepes, pH 7.4) at a concentration of 1 × 10⁶ cells/ml. The superoxide-producing activity of transfected cells (1.0 ml) was determined by superoxide dismutase (SOD)-inhibitable chemiluminescence with an enhancer-containing luminol-based detection system (DIOGENES; National Diagnostics), as previously described [23, 40, 41]. After the addition of the enhanced luminol-based substrate DIOGENES, the chemiluminescence was assayed at 37°C in the presence or absence of 200 ng/ml of PMA (Research Biochemicals International) using a luminometer (Auto Lumat LB953; EG&G Berthold).

Activation of the Phagocyte NADPH Oxidase in K562 Cells

Parent K562 cells endogenously expressed both Rac and p22^{phox}. The K562 cells stably expressing gp91^{phox} and p47^{phox}, designated as K562-gp91^{phox}-p47^{phox} cells, were prepared as previously described [12, 19, 21]. K562-gp91^{phox}-p47^{phox} cells were transfected by electroporation with the following plasmids: 10 µg of pEF-BOS encoding the wild-type or indicated mutant Myc-p67^{phox}; 15 µg of pEF-BOS-Flag-p40^{phox} (wt) or the pEF-BOS vector. The cells (5 × 10⁶ cells/ml) were electroporated in

the presence of the plasmids at 170 V, 1,070 μ F using a Gene Pulser (Bio-Rad). After culture for 48 h, cells (1×10^6 cells) were incubated for 5 min at 37 °C, and then stimulated with or without PMA (200 ng/ml). Superoxide production by the cells was determined by SOD-inhibitable chemiluminescence with DIOGENES, as described above. For estimation of Myc-tagged p67^{phox}, Flag-tagged p40^{phox} or p47^{phox}, immunoblot analyses were performed with an anti-Myc (Roche Diagnostics), anti-Flag (Sigma) or anti-p47^{phox} (BD Biosciences) monoclonal antibody, respectively.

Formation of the p67^{phox}-p47^{phox}-p40^{phox} Complex

COS-7 cells ectopically expressing the wild-type or indicated mutant Myc-p67^{phox}, HA-p47^{phox}, and Flag-p40^{phox} were lysed with a buffer composed of 1% Nonidet P-40, 142.5 mM NaCl, 2 mM EDTA, 10% glycerol, leupeptin (40 μ g/ml), aprotinin (10 μ g/ml), 2 mM phenylmethylsulfonyl fluoride and 20 mM HEPES, pH 7.4. Proteins in the lysate were precipitated with protein G-Sepharose using the anti-Flag monoclonal antibody. The precipitants were analyzed by immunoblot with anti-Myc (Santa Cruz Biotechnology), anti-HA (Santa Cruz Biotechnology) or anti-Flag (Santa Cruz Biotechnology) antibodies, as previously described [26].

Binding of Rac to the Wild-Type and Mutant p67^{phox} Proteins

Bacterially expressed GST-fusion proteins of p67^{phox} (wt), p67^{phox}-mut1, p67^{phox}-mut2 and p67^{phox}-mut3 were purified using glutathione-Sepharose 4B beads (GE Healthcare) and a Superdex 200 10/300GL column (GE Healthcare). p47^{phox}, p40^{phox} and Rac1 (Q61L) were bacterially expressed and purified using glutathione-Sepharose 4B, followed by cleavage with PreScission protease (GE Healthcare) and further purification using the Superdex 200 column. The wild-type or mutant p67^{phox} (5.0 μ g) were incubated for 10 min at 4 °C with 64 μ g of Rac1 (Q61L) in the presence of p47^{phox} (52 μ g) and p40^{phox} (64 μ g) in 400 μ l of a binding buffer (100 mM KCl and 100 mM potassium phosphate, pH 7.0) containing 0.005% Triton X-100. A slurry of glutathione-Sepharose-4B beads was subsequently added, followed by further incubation for 30 min at 4 °C. After washing 4 times with the binding buffer containing 0.1% Triton X-100, proteins were eluted from the beads with 20 mM glutathione in 200 mM NaCl, 0.1% Triton X-100, and 200 mM Tris, pH 8.0. The eluates were analyzed by immunoblot with an anti-Rac monoclonal antibody (BD Biosciences).

Results and Discussion

SAXS Analysis of p67^{phox}- and p67^{phox}-Containing Complexes

To directly elucidate the conformation of p67^{phox}- and p67^{phox}-containing complexes in solution, we performed SAXS experiments using the p67^{phox} monomer, the p67^{phox}-p47^{phox} and p67^{phox}-p40^{phox} binary complexes, as well as the p67^{phox}-p47^{phox}-p40^{phox} ternary complex. Analysis of the distance distribution function, $P(r)$, revealed that p67^{phox} has a molecular dimension with a D_{max} of 145 Å and an R_g of 48 Å, showing that the

average molecular size of p67^{phox} is much larger than those of globular proteins with a similar molecular mass; for example, BSA with a molecular mass of 65 kDa has an R_g value of about 30 Å. The $P(r)$ shows a curve with 2 broad overlapped peaks located at nearly 30 Å and 50 Å (fig. 2a), indicating that p67^{phox} has a nonglobular structure, since the $P(r)$ of a spherical molecule has a single peak with Gaussian-shape distribution [35]. In addition, the spread of the distribution curve for p67^{phox} extends to 145 Å (fig. 2a). This value is far from the molecular dimension of the globular protein BSA (approx. 85 Å) but similar to that of fully elongated p67^{phox}, which can be estimated at approximately 160 Å on the basis of the dimensions of the four domains of p67^{phox} deposited in the protein data bank: approximately 60 Å for the TPR domain (PDB: 1WM5), approximately 30 Å for the SH3(N) domain (PDB: 2DMO), approximately 40 Å for the PB1 domain (PDB: 1OEY) and approximately 30 Å for the SH3(C) domain (PDB: 1K4U). Hence, p67^{phox} appears to exist as an elongated molecule in solution.

The relative molecular masses of the p67^{phox}-p47^{phox} and p67^{phox}-p40^{phox} complexes were calculated by Guinier approximations to be 106 and 99 kDa, which are in good agreement with those calculated from the amino acid sequences (108 kDa and 106 kDa) at a stoichiometry of 1:1 for the binary complexes (table 1). The $P(r)$ of the p67^{phox}-p47^{phox} complex displays a curve with two broad peaks located at nearly 35 and 55 Å, and the curve extends to 180 Å (fig. 2b and c). The finding indicates that this complex adopts an elongated conformation and that p67^{phox} makes a more contribution to the conformation than p47^{phox}; the molecular dimensions of p67^{phox} and p47^{phox} are 145 and 90 Å, respectively (table 1). On the other hand, the $P(r)$ of the p67^{phox}-p40^{phox} complex exhibits a curve extended to 150 Å with a single peak at 45 Å. The value of the molecular dimension is close to that for the p67^{phox} monomer of 145 Å (fig. 2a), implying that p40^{phox} marginally contributes to the maximum dimension of the binary complex.

Fig. 2. SAXS analysis of the p67^{phox} monomer and p67^{phox}-containing complexes. Experimental SAXS profiles for p67^{phox} (a), p67^{phox}-p47^{phox} (b), p67^{phox}-p40^{phox} (c) and p67^{phox}-p47^{phox}-p40^{phox} (d) are shown in upper panels. The distance distribution functions, $P(r)$, for the respective samples were calculated using the program GNOM to fit the experimental scattering data (lower panels). The $P(r)$ values are normalized to unity by their area.

Table 1. Structural parameter obtained from SAXS

Sample	Number of amino acids ¹	Mr (calc.) kDa ²	Mr (Guinier) kDa ²	Rg (Guinier) Å ³	Rg (GNOM) Å ³	Dmax (GNOM) Å ⁴
p67 ^{phox}	540	61.3	58.5 (11.8)	45.2 (1.5)	47.6 (1.9)	145
p47 ^{phox}	407	46.6	38.9 (7.7)	31.1 (0.8)	30.6 (0.7)	90
p40 ^{phox} 5	341	39.2	47.8 (4.8)	28.5 (0.4)	27.7 (0.5)	85
p67 ^{phox} -p47 ^{phox}	947	107.9	105.9 (4.5)	58.0 (1.8)	57.1 (1.8)	180
p67 ^{phox} -p40 ^{phox}	881	100.4	99.4 (6.0)	48.5 (1.0)	48.5 (1.3)	145
p67 ^{phox} -p47 ^{phox} -p40 ^{phox}	1,288	147.1	149.2 (6.3)	57.6 (1.3)	57.6 (1.1)	170

The values in parentheses indicate SD of the structural parameters estimated by SAXS analysis.

¹ Values are calculated including the C-terminal 6-histidine tags.

² Mr (calc.) and Mr (Guinier) are molecular mass calculated from the amino acid sequence and estimated from the scattering data according to Guinier approximation, respectively.

³ Rg (Guinier) and Rg (GNOM) are the radius of gyration, derived from the scattering data using Guinier approximation and the program GNOM, respectively.

⁴ Dmax is a maximum dimension.

⁵ Data from Honbou et al. [26].

We next evaluated the molecular shape of the p67^{phox}-p47^{phox}-p40^{phox} complex. As shown in figure 2d, the $P(r)$ of the ternary complex represents characteristics of an elongated structure with a D_{max} of 175 Å and an R_g of 58.3 Å. The molecular mass was estimated by Guinier analysis to be 149 kDa, which is consistent with the value deduced from the amino acid sequences of the proteins in the 1:1:1 complex (table 1). The dimension of this ternary complex (175 Å) is similar to that of the p67^{phox}-p47^{phox} binary complex, suggesting that p67^{phox} as well as p47^{phox}, but not p40^{phox}, is involved in the elongated structure. Taken together, p67^{phox} likely adopts an elongated conformation when it exists not only as a monomer but also as the trimer with p47^{phox} and p40^{phox}.

Molecular Modeling of p67^{phox}

For visualization of the molecular shape information held in our experimental SAXS data, we reconstructed low-resolution molecular envelope models of p67^{phox} by ab initio molecular shape analyses, using two approaches implemented by the DAMMIN [34] and GASBOR programs [35]. The averaged consensus models are shown in figure 3. Both ab initio calculations of molecular envelopes for p67^{phox} consistently yield an elongated and non-symmetrical conformation, comprising a large globular region and multiple smaller bumps in an extended arrangement of the molecular envelope. These structural elements may be derived from the TPR, SH3(N), PB1 and

SH3(C) domains of p67^{phox}. The model suggests that there exist no or little apparent interactions between the four domains.

Positional Effect of the N-Terminal SH3 Domain of p67^{phox} on Phagocyte Oxidase Activation

As shown by the present SAXS analyses, p67^{phox} appears to be in an extended form without significant associations between its protein-binding modules. It is thus likely that bindings of p67^{phox} to its target proteins are not regulated via interdomain interactions. However, it is unknown whether the activity of p67^{phox} depends on the positions of the domains or is independent of the domain organization. To address this question, we tested the following mutant p67^{phox} proteins with a distinct domain arrangement: p67^{phox}-mut1, in which the PB1 domain follows the C-terminal SH3 domain; p67^{phox}-mut2, which harbors the PB1 domain preceding the N-terminal SH3 domain; p67^{phox}-mut3, with the N- and C-terminal SH3 domains being interchanged (fig. 4a).

We have previously shown that the p67^{phox} N-terminal SH3 domain, SH3(N), positively regulates activation of the phagocyte NADPH oxidase, which could be mediated via an interaction with gp91^{phox} [23]. When SH3(N) was swapped with the PB1 domain (p67^{phox}-mut2) or with SH3(C) (p67^{phox}-mut3), the ability of p67^{phox} to activate the oxidase was significantly decreased (fig. 4b), suggesting that SH3(N) fails to function at the positions of the

Fig. 3. Ab initio molecular shape analysis for p67^{phox}. The smooth curves correspond to the scattering curves calculated from a dummy atom model derived using the program DAMMIN (a) and an ensemble of dummy residues forming a chain-compatible

model using the program GASBOR (b), which are superposed on the experimental scattering curves (black dots) for p67^{phox}. The orthogonal views of the averaged most probable model of p67^{phox} from the DAMMIN (c) and GASBOR (d) programs are shown.

PB1 and SH3(C). The idea is supported by the findings that oxidase activation by p67^{phox}-mut2 and p67^{phox}-mut3 was not affected by truncation of SH3(N) (fig. 4c); on the other hand, deletion of SH3(N) from p67^{phox} (wt) inhibited superoxide production by 40–50% in the absence of SH3(N) (fig. 4b and c), which is consistent with the previous observation [23]. Hence, the position of SH3(N) appears to be crucial for its role in activation of the phagocyte NADPH oxidase.

Positional Effect of the PB1 Domain of p67^{phox} on Phagocyte Oxidase Activation

The oxidase activator p67^{phox} tightly associates with p40^{phox}, in which the PB1 domain of p67^{phox} directly interacts with that of p40^{phox} [12, 15, 16]. We have previously shown that, via the PB1–PB1 interaction, p40^{phox} enhances membrane translocation of p67^{phox} and facilitates activation of the phagocyte NADPH oxidase [12]. p67^{phox}-mut2, in which the PB1 domain precedes SH3(N),

Fig. 4. Positional effect of p67^{phox}-SH3(N) on NADPH oxidase activation. **a** Structure of mutant p67^{phox} proteins with swapped domains. **b, c** COS-7 cells were transfected with the following plasmids: pcDNA3-gp91^{phox}, pEF-BOS-Flag-p47^{phox}, and pEF-BOS encoding the wild-type or indicated mutant Myc-p67^{phox}. Cells were incubated for 5 min at 37°C, and then stimulated with PMA (200 ng/ml). Chemiluminescence change was continuously monitored with DIOGENES. Each graph represents

the mean + SD of the chemiluminescence values integrated for 10 min, which were obtained from three independent transfections. * $p < 0.01$ versus p67^{phox} (wt); + not significant versus p67^{phox} (wt) (**b**). * $p < 0.01$ versus p67^{phox} (wt); + not significant versus p67^{phox}-mut2 or p67^{phox}-mut3 (**c**). Protein levels of p67^{phox} and p47^{phox} were analyzed by immunoblot, as described in Materials and Methods.

was as active as p67^{phox} (wt) in the absence of p40^{phox} (fig. 5a). Intriguingly, p40^{phox} decreased oxidase activation by p67^{phox}-mut2 to 35% of that without p40^{phox} and 20% of that by the wild-type protein with p40^{phox}. The inhibition was restored when a mutant p40^{phox} carrying the D289A substitution, leading to a defective binding to p67^{phox} [12], was expressed instead of the wild-type one.

On the other hand, p40^{phox} enhanced oxidase activation by p67^{phox}-mut1 or p67^{phox}-mut3 (fig. 5b) as well as by the wild-type protein (fig. 5a). The PB1 domain is thus required to be present C-terminal to the position of SH3(N); otherwise, p40^{phox} binding to p67^{phox} inhibits activation of the phagocyte oxidase.

Fig. 5. Positional effect of the p67^{phox} PB1 domain on NADPH oxidase activation. K562-gp91^{phox}-p47^{phox} cells were transfected with pEF-BOS encoding the wild-type or indicated mutant Myc-p67^{phox} and the wild-type or mutant Flag-p40^{phox}. Cells were incubated for 5 min at 37°C, and then stimulated with PMA (200 ng/ml). Chemiluminescence change was continuously monitored with DIOGENES. Each graph represents the mean + SD of the chemiluminescence values integrated for 10 min, which were ob-

tained from three independent transfections. * $p < 0.01$ versus p67^{phox}-mut2 without p40^{phox}; + not significant versus p67^{phox}-mut2 without p40^{phox} (a). * $p < 0.05$ versus p67^{phox} (wt); ** $p < 0.01$ versus p67^{phox} (wt); ° $p < 0.01$ versus p67^{phox}-mut1 without p40^{phox}; °° $p < 0.02$ versus p67^{phox}-mut3 without p40^{phox} (b). Protein levels of p67^{phox}, p47^{phox} and p40^{phox} were analyzed by immunoblot, as described in Materials and Methods.

Positional Effect of the p67^{phox} Domains on p67^{phox}-p47^{phox}-p40^{phox} Complex Formation

The finding that oxidase activation by p67^{phox}-mut2, in which the SH3(N) and PB1 domains are swapped, is inhibited by binding to p40^{phox} (fig. 5a) raised the possibility that the mutant protein is incapable of forming the p67^{phox}-p47^{phox}-p40^{phox} complex. To address this question, we tested the ability to form the p67^{phox}-p47^{phox}-p40^{phox} complex using co-immunoprecipitation analysis. As shown in figure 6a, p67^{phox}-mut2 as well as p67^{phox}-mut1 and p67^{phox}-mut3 was fully complexed with both p47^{phox} and p40^{phox}. Thus, inhibition of oxidase activation by p40^{phox} binding to p67^{phox}-mut2 appears to occur at a step after formation of the ternary complex. Although the precise mechanism underlying the inhibition is presently unknown, it is unlikely that p40^{phox} binding to p67^{phox}-mut2 prevents the association of p67^{phox} with Rac, a process required for oxidase activation [21]. This is

because p67^{phox}-mut2 as well as p67^{phox}-mut1 and p67^{phox}-mut3 interacted with Rac to the same extent as the wild-type protein in the presence of p40^{phox} and p47^{phox} (fig. 6b).

Positional Effect of the C-terminal SH3 Domain of p67^{phox} on Phagocyte Oxidase Activation

As shown in figure 4a, p67^{phox}-mut1 expressed in COS-7 cells was almost as active as the wild-type protein in activation of the phagocyte NADPH oxidase, although this mutant protein was slightly but significantly less active in K562 cells (fig. 5a). It is thus likely that p67^{phox}-SH3(C) is able to function at the position of the PB1 domain. On the other hand, p67^{phox}-mut3, in which SH3(C) is interchanged with SH3(N), was about 3 times less effective in oxidase activation than p67^{phox} (wt) (fig. 4c), raising the possibility that SH3(C) does not play a role sufficiently at the position of SH3(N). In contrast, however,

Fig. 6. a Effect of the domain rearrangement of p67^{phox} on formation of the p67^{phox}-p47^{phox}-p40^{phox} complex. Proteins of lysates prepared from COS-7 cells transiently expressing the wild-type or indicated mutant Myc-p67^{phox}, HA-p47^{phox}, and Flag-p40^{phox} were immunoprecipitated (IP) with the anti-Flag IgG or control IgG, and then analyzed by immunoblot with the anti-Myc, anti-HA, or anti-Flag antibodies. The data are representative of results from three independent experiments. **b** Effect of p67^{phox} domain rearrangement on binding to Rac. The wild-type and indicated mutant GST-p67^{phox} were purified and subjected to SDS-PAGE, followed by staining with Coomassie Brilliant Blue (upper panel). GST-p67^{phox} (wt), GST-p67^{phox}-mut1, GST-p67^{phox}-mut2 or GST-p67^{phox}-mut3 was incubated with Rac1 (Q61L) in the presence of p47^{phox} and p40^{phox}, and precipitated with glutathione-Sepharose beads; the precipitants were analyzed by immunoblot with the anti-Rac monoclonal antibody (lower panel). The data are representative of results from four independent experiments.

p67^{phox}-mut4 [a mutant protein that contains SH3(C) at the position of SH3(N) but lacks the SH3(N) and PB1 domains; fig. 7a] activated the phagocyte oxidase to the same or even higher extent than p67^{phox} (wt) (fig. 7b and c). The activation was abrogated by the W494R substitution in SH3(C) (fig. 7a), a mutation leading to a loss of the interaction with p47^{phox} [19], confirming that p67^{phox}-mut4 serves in cooperation with p47^{phox} (fig. 7b and c). These findings indicate that SH3(C) is capable of fully functioning at the position of SH3(N), although the reason for the discrepancy between p67^{phox}-mut3 and p67^{phox}-mut4 is presently unknown. Taken together, the C-terminal SH3 domain of p67^{phox} properly contributes to oxidase activation in a manner independent of its position.

Concluding Remarks

In the present study, using SAXS analysis, we show that p67^{phox} exists as a monomer that adopts an elongated conformation (fig. 2, 3). The conclusion is consistent with the previous finding obtained from analytical ultracentrifugation analyses [20].

In addition, the present model deduced from SAXS data suggests that no or little apparent interdomain associations occur in p67^{phox} (fig. 3). This explains well the previous finding that Rac binding to the TPR domain of p67^{phox} is not affected by the presence of the remainder of the protein [20, 42]. Similarly, neither p40^{phox} association with the PB1 domain nor p47^{phox} interaction with SH3(C) is known to be inhibited by the other regions of p67^{phox} [19, 43]. Thus, the protein-interaction domains in p67^{phox} appear to be normally in a state accessible to their target proteins. In addition, the formation of the ternary complex and the interaction of p67^{phox} with Rac are both independent of the positions of the PB1 and SH3 domains (fig. 6). Such an elongated, ‘beads-on-a-string’ structure is also found in the tyrosine kinase Btk [44]. Like Src and Abl kinases, Btk contains a conserved cassette formed by SH3, SH2 and protein kinase domains, but differs from them by the presence of an N-terminal PH and Tec-homology domains; Btk displays an extended conformation where domains are arranged in a linear manner with little or no interactions between them [44]. This is in contrast with the structures

Fig. 7. Positional effect of p67^{phox}-SH3(C) on NADPH oxidase activation. **a** Structure of mutant p67^{phox} proteins lacking the N-terminal SH3 and PB1 domains. **b** CHO cells were transfected with the following plasmids: pcDNA3-gp91^{phox}, pEF-BOS-p22^{phox}, pEF-BOS-Flag-p47^{phox}, and pEF-BOS encoding the wild-type or indicated Myc-p67^{phox}. **c** K562-gp91^{phox}-p47^{phox} cells were transfected with pEF-BOS encoding the wild-type or indicated mutant

p67^{phox} protein fused to Myc. Cells were incubated for 5 min at 37°C, and then stimulated with PMA (200 ng/ml). Chemiluminescence change was continuously monitored with DIOGENES. Each graph represents the mean + SD of the chemiluminescence values integrated for 10 min, which were obtained from three independent transfections. Protein levels of p67^{phox} and p47^{phox} were analyzed by immunoblot, as described in Materials and Methods.

of Src- and Abl-family kinases, in which SH3 and SH2 domains turn inward and lock the tyrosine kinase domain in the auto-inhibited state via intramolecular interactions [45].

The present study also demonstrates that the order of the domains in p67^{phox} is important at a step after ternary complex formation in activation of the phagocyte oxidase. The N-terminal SH3 domain positively regu-

lates oxidase activation only when it is present at the correct position (fig. 4). This SH3 domain is not required for oxidase activation but enhances superoxide production probably by increasing the affinity of the p67^{phox}-containing complex for gp91^{phox} [23]. The mechanism whereby SH3(N) functions may explain the reason why it must be positioned close to the p67^{phox} N-terminal domain that is required for oxidase activation (fig. 1). Rac binding to

the TPR domain in the N-terminus is considered to induce a conformational change of p67^{phox}, which likely allows a region between the TPR and SH3(N) domains to interact with gp91^{phox}, leading to superoxide production [3–9]. The present study also shows that the PB1 domain placed at the position of SH3(N) does not affect formation of the ternary complex or Rac binding to p67^{phox} (fig. 6), but inhibits oxidase activation by interacting with p40^{phox} (fig. 5), whereas SH3(C) is capable of functioning even at the SH3(N) or PB1 positions (fig. 4, 7). Binding of p40^{phox} to the position of SH3(N), but not binding of p47^{phox} to the same position, may interfere with the interaction of the p67^{phox} N-terminal domain with gp91^{phox}. The significance of the domain organization in human

p67^{phox}, as shown in the present study, is consistent with the fact that the order of the domains in the oxidase activator is completely conserved during evolution [9].

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