

Involvement of p40^{phox} in Activation of Phagocyte NADPH Oxidase through Association of Its Carboxyl-terminal, but not Its Amino-terminal, with p67^{phox}

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Summary

Phagocyte NADPH oxidase, dormant in resting cells, is activated upon cell stimulation to produce superoxide anion, a precursor of microbicidal oxidants. Active NADPH oxidase is found on the membrane as an enzyme complex, composed of membrane-integrated cytochrome *b558* (gp91^{phox} and p22^{phox} subunits) and two cytosolic factors (p47^{phox} and p67^{phox}), each of the latter containing two *src* homology 3 (SH3) domains. Recently, we radioactively identified a third cytosolic factor, p40^{phox}, as a molecule that associates with p67^{phox} in human neutrophils. Although it has been found that this p40^{phox} protein is defective in patients with chronic granulomatous disease (CGD) who lack p67^{phox}, evidence to functionally relate it to the NADPH oxidase system has hitherto been lacking. In this study, we raised separate antibodies against both the COOH- and NH₂-terminal polypeptides of p40^{phox} as well as against the COOH-terminal polypeptide of p67^{phox} to examine the mode of interaction between p40^{phox} and p67^{phox} in a complex. The antibody against the COOH terminus of p67^{phox} was able to coimmunoprecipitate p40^{phox} in conjunction with p67^{phox} itself as was expected. Very interestingly, however, the antibody against the COOH terminus of p40^{phox} completely dissociated the p67^{phox} molecule from the p40^{phox}-p67^{phox} complex unit without any detectable coimmunoprecipitation of p67^{phox}, despite their tight association, whereas that against the NH₂ terminus of p40^{phox} had absolutely no dissociation effect. Similar results were found regarding their effects on the O₂⁻-generating ability of cytosol in a cell-free activation system, i.e., inhibition was noted with the COOH terminus antibody but not with that for the NH₂ terminus of p40^{phox}. However, this dissociation did not affect the translocation of the cytosolic components including p47^{phox} to the membrane. Once the NADPH oxidase was activated, the antibody for the COOH terminus did not show any inhibitory effect on catalysis by the activated enzyme. The stimulators of NADPH oxidase, MA and SDS, did not dissociate the p40^{phox}-p67^{phox} complex. These results provide the first demonstration that p40^{phox} is practically involved in the activation of NADPH oxidase through the association of its COOH-terminal, but not its NH₂-terminal, with p67^{phox}.

The production of toxic oxygen species by professional phagocytic cells is important for killing microorganisms (1–3). Recently, it has become evident that related oxidases in B cells and other cells (fibroblasts, carotid bodies, and endothelial cells) that are not involved in host defense also produce small amounts of O₂⁻ anion in well-defined settings (2), suggesting that it plays larger roles in biological systems than just antibactericidal activity. The responsible enzyme, activated NADPH oxidase found in the membrane fraction as an enzyme complex upon cell stimulation, catalyzes the reduction of molecular oxygen to the O₂⁻ anion in conjunction with the oxidation of NADPH.

Ordinarily, this activatable NADPH oxidase is tightly controlled in a dormant state, since microbicidal oxidants are extremely dangerous to their hosts. In resting cells, its membranous components (cytochrome *b558* composed of gp91^{phox} and p22^{phox} subunits), cytosolic factors (p67^{phox} and p47^{phox}), and a cytosolic regulator protein, *rac* p21, are distributed in separate locations. However, once phagocytes encounter microorganisms or soluble stimulants such as PMA (4, 5), myristic acid (MA; reference 6), or SDS (4), these cytosolic components migrate to the plasma membrane where cytochrome *b558* resides to assemble the active enzyme. The significance of this enzyme in the host

defense is well-illustrated by the genetic disorder chronic granulomatous disease (CGD)¹, in which any one of the genes of these four components, excluding *rac* p21, is defective. Patients with CGD suffer from recurrent and life-threatening infections.

Chromatographic analyses have suggested that the cytosolic components exist in a form with a molecular mass of 240–260 kD (7, 8), and recent immunological approaches have provided evidence for an association between p67^{phox} and p47^{phox} (9–11). However, based on the molecular mass, there were indications that other component(s) might be involved. We and others found a third oxidase-related cytosolic component in neutrophils, designated p40^{phox}, which physically and tightly associates with porcine p63^{phox} (6), guinea pig p63^{phox} (12), and human p67^{phox} (13, 14), and demonstrated that it was specifically defective in patients with CGD who lack p67^{phox} (13, 14).

The cytosolic components p67^{phox} and p47^{phox} both contain two *src* homology 3 (SH3) domains and polyproline motifs (15–18). Furthermore, cloning of the cDNA for p40^{phox} revealed one SH3 domain (13). SH3 domains, composed of ~60 amino acid residues, direct cellular localization and signal transduction through specific protein–protein interactions with Pro-rich target sequences (19, 20). Therefore, their presence in these cytosolic oxidase components implies a wide variety of possible protein–protein interactions. In fact, this possibility has been tested by *in vitro* binding assays in which the oxidase components were in the form of glutathione *S*-transferase fusion proteins (21–23). Furthermore, transfection experiments have been conducted with B cell lines from patients with CGD or K562 erythroleukemia cells with a series of cDNA constructs with their SH3 domains or Pro-rich sequences manipulated (23, 24).

O₂⁻-generation in an artificial cell-free system composed entirely of recombinant proteins: p67^{phox}, p47^{phox}, and *rac* p21 with either cytochrome *b*558 (25) or purified cytochrome *b*558 (26, 27), did not require p40^{phox}, in contrast to the absolute dependence upon p67^{phox} and p47^{phox}. Therefore, p40^{phox} has been overlooked. However, its tight linkage to p67^{phox} and its coincident depletion in the cytosol of p67^{phox}-deficient CGD, suggest that p40^{phox} is not merely a functionless protein bound to p67^{phox}, but rather is actively involved in the NADPH oxidase system. Therefore, we raised antibodies against both the COOH- and NH₂-terminal polypeptides of p40^{phox} to examine their effects not only on the NADPH oxidase activity in a cell-free system but also on the mode of interaction between p40^{phox} and p67^{phox}. In the present paper, we document that the COOH-terminal side, but not the NH₂-terminal side, of p40^{phox}, is a key region in its association with p67^{phox} and in subsequent O₂⁻-generation.

¹Abbreviations used in this paper: aa, amino acid; CGD, chronic granulomatous disease; GDI, GDP dissociation inhibitor; GTPγS, guanosine 5'-(γ-O-thio)-triphosphate; HRP, horseradish peroxidase; MA, myristic acid; PSL, photostimulative luminescence; SH3, *src* homology 3.

Materials and Methods

Preparation and Fractionation of Neutrophils. Human neutrophils were isolated from the heparinized blood of normal donors or four autosomal recessive CGD patients after informed consent was obtained, as previously described (5, 14). The isolated neutrophils were treated with 2 mM diisopropyl fluorophosphate for 15 min on ice in Ca²⁺/Mg²⁺-free PBS containing 5 mM glucose (PBSG), pH 7.4, washed three times with PBSG, and finally suspended in buffer A: 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, and 10 mM Pipes (pH 7.3) containing 10 μM of leupeptin and 1 mM of PMSF. The cells in ~1 ml of the buffer A were then disrupted on ice by sonication (60–80 W) with 20 1-s pulses, at 1-s intervals, using a micro tip. Cytosol and membrane fractions were obtained from the homogenate by differential centrifugation at 4°C, as previously described (5, 14). The membrane fraction was finally resuspended in buffer A. Proteins were determined with BCA reagents from Pierce Chem. Co. (Rockford, IL) using BSA as a standard.

Preparation of Antibodies Against Cytosolic Components. Polypeptides corresponding to both the NH₂-terminal (aa 1–15) and COOH-terminal (aa 325–339) regions of p40^{phox} (13), and the COOH-terminal regions of p67^{phox} (aa 508–526; reference 17) and p22^{phox} (aa 177–195; reference 28) were synthesized by Fujiya Bioscience Lab. (Tokyo) and confirmed to be specific by mass spectrometry, amino acid composition analysis, and HPLC analysis. Antisera against these synthetic polypeptides were raised in rabbits by repeated injections of their keyhole limpet hemocyanin conjugates. When stated, antisera against p40^{phox} and normal serum were subjected to (HN₄)₂SO₄ fractionation, extensively dialyzed against 20 mM Na-phosphate buffer (pH 8.0) overnight, and applied to DEAE-52 cellulose chromatography in the same buffer to isolate IgG fractions. The IgG fractions were further dialyzed against 20 mM Na-phosphate buffer (pH 7.0) to obtain an optimal pH for *in vitro* activation experiments. The amounts of protein in IgG fractions were determined by the method of Bradford (29) using BSA as a standard.

Immunoprecipitation. Cytosol (0.5–1 × 10⁷ cell equivalents or 300 μg) was incubated with one of the above-mentioned antisera for 90 min on ice in 200 μl of immunoprecipitation buffer: 150 mM NaCl, 10 mM EDTA, 1% (wt/vol) deoxycholate, and 1% (vol/vol) Nonidet P-40 in 10 mM Tris (pH 7.4). After adding 4 mg of protein A–Sepharose CL-4B, the reaction mixture was brought to a final volume of 1 ml with the immunoprecipitation buffer and then rotated for 1 h at 4°C. The protein A–Sepharose beads were washed twice with 1 ml of the immunoprecipitation buffer containing 20% (wt/vol) sucrose at 20,600 g for 5 min. In Figs. 4 A and 5, aliquots of cytosol were first incubated with antisera in 200 μl of activation buffer for NADPH oxidase activation (see below) and then brought to 1 ml with the immunoprecipitation buffer. Furthermore, in Fig. 5, the protein A–Sepharose beads were washed twice at 180 g for 4 min, which was sufficient to spin down the beads, to eliminate nonspecifically pelleted cytosol components due to their complexation with either of MA or SDS.

Immunoblot Analysis. The above immunoprecipitates and cytosol fraction were subjected to SDS-PAGE (10% acrylamide) after heating in the sample buffer for 5 min at 100°C, and proteins were then transferred to a polyvinylidene difluoride microporous membrane (PVDF) as previously described (6). The transferred proteins were probed with the aforementioned primary antisera raised against the synthetic polypeptides (1:300–1,000 dilution), and immunoreactive bands were then localized with secondary horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG

(1:1,000 dilution) followed by development with *o*-dianisidine. For quantitative analysis of the interaction between p40^{phox} and p67^{phox}, they were also revealed with a secondary donkey ¹²⁵I-labeled F(ab')₂ fragment against rabbit Ig (1:1,000 dilution), and then exposed to an imaging plate and developed in a bio-image analyzer (Fuji BAS 2000; Fuji Photofilm Corp., Tokyo). The radioactivities measured with the imaging plate were expressed as photo-stimulative luminescence (PSL) counts.

Cell-free NADPH Oxidase Assay. In vitro O₂⁻-generation was measured as the superoxide dismutase-inhibitable reduction of cytochrome *c* using a dual-wavelength spectrophotometer (model 557; Hitachi, Tokyo), essentially as described previously (6). A mixture of 10 μg of membranes and 300 μg of cytosol was stimulated with 100 μM of MA for 10 min at room temperature in a 200–400-μl volume of activation buffer (65 mM Na, K-phosphate buffer, pH 7.0, containing 5 μM guanosine 5'-(γ-O-thio)-triphosphate [GTPγS], 1.5 mM MgCl₂, 1 mM EGTA, and 2 mM NaN₃). To examine prior effects of antibodies (IgG fractions) on p40^{phox} as well as synthetic peptides corresponding to the COOH and NH₂ termini of p40^{phox}, cytosol was pretreated with one of these for 3 h on ice before the stimulation with MA. To determine posterior effects of antibodies on p40^{phox}, the MA-stimulated cytosol, in which an activated NADPH oxidase resides, was post-treated with either of them for 3 h on ice. Then, the volume of the reaction mixture was brought to 800 μl with the activation buffer supplemented with 30 μM of cytochrome *c* and 225 U/ml of catalase. The reaction was initiated by the addition of 0.2 mM of NADPH, and at maximal velocity, 200 U/ml of superoxide dismutase was added to determine the net cytochrome *c* reduction.

Cell-free Translocation. The translocation experiment was carried out essentially as reported previously (30). An approximately ninefold reaction mixture of in vitro O₂⁻-generation (135 μg of membrane and 2.7 mg of cytosol) was stimulated with 100 μM of SDS in a 1.35-ml volume of activation buffer. When mentioned, cytosol was pretreated with anti-p40^{phox}C IgG (1.8 mg) for 2 h on ice before the stimulation with SDS. After incubation for 5 min at room temperature, the reaction mixture was layered onto a discontinuous gradient composed of 2.5 ml of 15% (wt/vol) sucrose over 0.2 ml of 50% (wt/vol) sucrose, both in activation buffer,

and then centrifuged at 100,000 g for 30 min at 4°C in a swinging bucket rotor (SW 50.1; Beckman, Fullerton, CA). After centrifugation, the supernatant was removed and a 0.2-ml portion was withdrawn from the bottom for use as a membrane fraction. Membranes were located in the 50% sucrose bottom. Aliquot of the membrane fraction (10 μg) was then subjected to an immunoblot analysis, including antibody against p22^{phox} for quantitation of the membrane.

Materials. Catalase, cytochrome *c*, deoxycholate, *o*-dianisidine, EDTA, EGTA, GTPγS, Nonidet P-40, Pipes, protease inhibitors, protein A-Sepharose CL-4B, and superoxide dismutase were purchased from Sigma Chem. Co. (St. Louis, MO). MA and NADPH and SDS were from Nakalai Tesque (Kyoto) and Wako (Tokyo), respectively. DEAE-52 cellulose was from Whatman (Maidstone, England). HRP-conjugated swine anti-rabbit IgG and a donkey ¹²⁵I-labeled F(ab')₂ fragment against rabbit Ig were obtained from Dakopatts (Glostrup, Denmark) and New England Nuclear-DuPont (Boston, MA), respectively. The molecular marker kit for SDS-PAGE and PVDF sheets for Western blotting were obtained from Bio-Rad Lab. (Hercules, CA) and Millipore Corp. (Bedford, MA). All other reagents were of analytical grade.

Results

Specific Recognition of Antibodies Against Neutrophil Cytosolic Components. We previously identified a 40-kD protein by affinity labeling of cytosol with [³²P]GTP dialdehyde as a marker, which consistently behaves with p67^{phox} through immunoprecipitation and column works (14). On the other hand, Wientjes et al. (13) determined an amino acid sequence of a protein with the same molecular mass as ours, which was isolated by SDS-PAGE after immunoabsorption to an anti-p67^{phox} IgG column. Then, we raised antibodies against the sequenced p40^{phox} in rabbits by injecting synthetic polypeptides corresponding to the COOH-terminal and NH₂-terminal amino acids, respectively. Antibody against

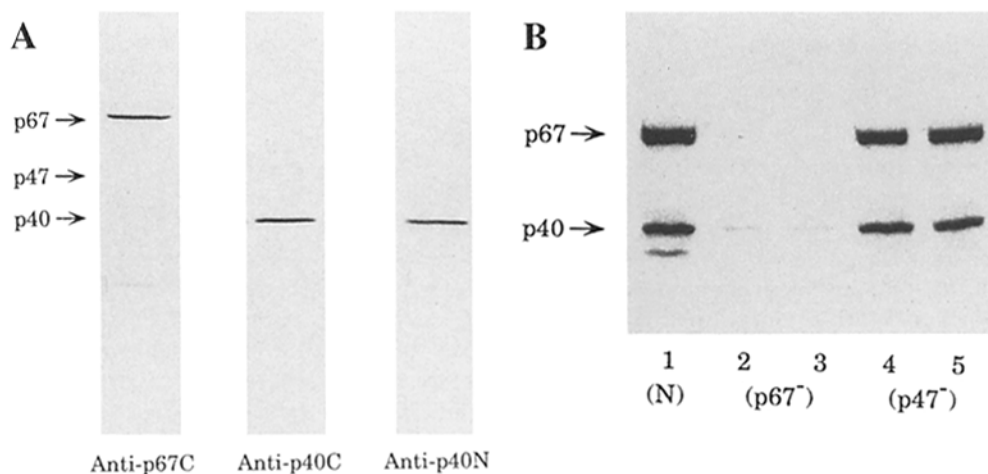


Figure 1. Immunodetection of cytosolic components (A) and coincident reduction of p40^{phox} in patients with CGD lacking p67^{phox} (B). In A, cytosol (1×10^6 cell equivalents) was subjected to SDS-PAGE (10%). Proteins were electroblotted onto a PVDF sheet, which was then cut, blocked, and probed with primary antiserum raised against each synthetic polypeptide. Immunoreactive bands were localized with HRP-conjugated anti-rabbit IgG, followed by development with *o*-dianisidine. C and N denote the COOH-terminal and NH₂-terminal polypeptides of cytosolic components, respectively. In B, cytosol samples (0.75×10^6 cell

equivalents) from normal (N) and p67^{phox}- or p47^{phox}-deficient CGD subjects were immunoblotted with a mixture of antisera against the COOH-terminal peptides of p40^{phox} and p67^{phox}, followed by a ¹²⁵I-labeled F(ab')₂ fragment against rabbit Ig. A PVDF sheet was exposed to an imaging plate, and then analyzed in a bio-image analyzer. The figure shows results for a normal volunteer (No. 1), and p67^{phox}-deficient (Nos. 2 and 3) and p47^{phox}-deficient (Nos. 4 and 5) CGD patients.

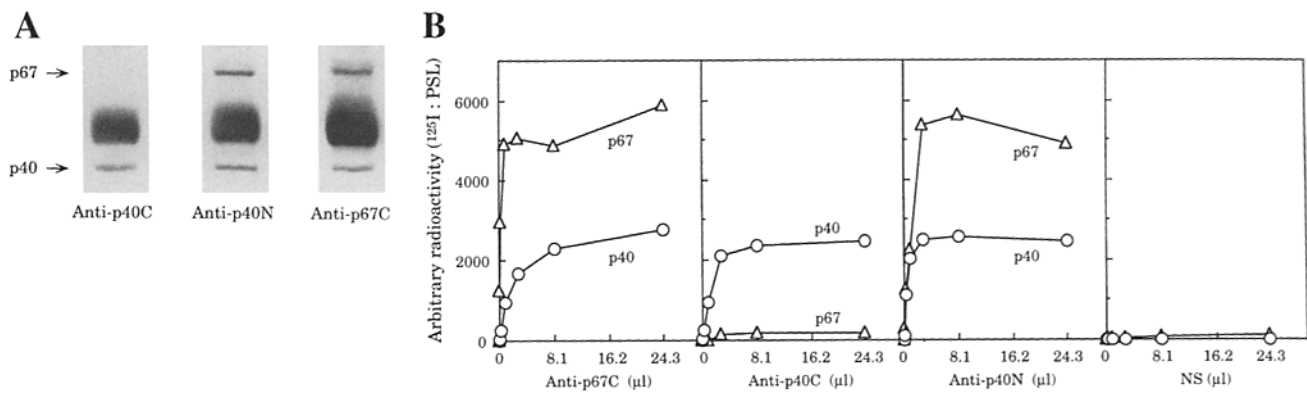


Figure 2. Dissociation of the p40^{phox}-p67^{phox} complex with antiserum against the COOH-terminal polypeptide of p40^{phox}. In *A*, cytosol (1×10^7 cell equivalents) were immunoreacted in 200 μ l of immunoprecipitation buffer with antisera (10 μ l) against the COOH or NH₂ terminus of p40^{phox} and the COOH terminus of p67^{phox} for 90 min on ice, followed by precipitation with protein A-Sepharose CL-4B at 4°C, SDS-PAGE analysis, and transfer to a PVDF sheet. The presence of p40^{phox} and p67^{phox} in the immunoprecipitates was then examined by immunoblot analysis using a mixture of the primary antisera against the COOH termini of p40^{phox} and p67^{phox} plus a secondary HRP-conjugated anti-rabbit IgG. The figure shows representative result of many reproducible experiments. In *B*, aliquots of cytosol (5×10^6 cell equivalents) were immunoreacted with the indicated volumes of each antiserum, and then processed in the same way as in *A*. The amounts of p40^{phox} (○) and p67^{phox} (△) in the immunoprecipitates were finally determined using a secondary ¹²⁵I-labeled F(ab')₂ fragment against rabbit Ig, and expressed as ¹²⁵I-PSL counts. Data are representative of three reproducible experiments.

p67^{phox} was also raised using a synthetic polypeptide corresponding to its COOH-terminal amino acids (17). The immunoblot bands in Fig. 1 *A* demonstrate specific binding to each cytosolic component. Particularly, there was no mutual recognition of p67^{phox} and p40^{phox} with either anti-p40^{phox}N or anti-p67^{phox}C serum (see below). The antiserum against p67^{phox}C alone showed minor bands at molecular masses of 42 and 32 kD, as well as a specific band at 67 kD.

We previously reported the complete absence of p40^{phox} in neutrophils from p67^{phox}-lacking CGD patients using a radioactivity assay (14). However, direct immunoblot analysis with the above antisera against p40^{phox}, probably, which may be a more sensitive method than the radioactivity assay, revealed a slight amount of p40^{phox} in the p67^{phox}-deficient CGD patients (Nos. 2 and 3; Fig. 1 *B*) being consistent with a previous report (13). Cytosol samples from other autosomal recessive patients, i.e., p47^{phox}-deficient CGD (Nos. 4 and 5), showed the same amount of p40^{phox} as seen in cytosol from a normal subject (No. 1).

The p40^{phox}-p67^{phox} Complex Dissociates with Antiserum against the COOH-terminal Site of p40^{phox}. Since p40^{phox} was originally found by immunoprecipitation using antiserum against p67^{phox}, the opposite protocol was adopted to evidence whether this occurs vice versa, i.e., coimmunoprecipitation of p67^{phox} with antiserum against p40^{phox}. First, we raised antiserum against p40^{phox} using its synthetic COOH-terminal polypeptide as usual, with the intention of applying it to coimmunoprecipitate p67^{phox} together with p40^{phox}. Broad bands over a ~50-kD region were present in large quantities due to the heavy chain of IgG used for the immunoprecipitation (Fig. 2 *A*). Nonetheless, SDS-PAGE analysis provided clear immunoreactive bands of p40^{phox} and p67^{phox}. Very surprisingly, however, anti-p40^{phox}C serum completely failed to coimmunoprecipitate p67^{phox} along with a p40^{phox} molecule (Fig. 2 *A*), even though the strong binding between p40^{phox} and p67^{phox} was

not broken by treatment with NaCl, EDTA, or detergents until subjected to SDS-PAGE (14). Therefore, we next raised antiserum using the synthetic NH₂-terminal polypeptide of p40^{phox}. In contrast to the result obtained with anti-p40^{phox}C serum, anti-p40^{phox}N serum coimmunoprecipitated p67^{phox} in addition to p40^{phox} itself (Fig. 2 *A*). Anti-p67^{phox}C serum coimmunoprecipitated p40^{phox} together with p67^{phox} itself.

We next quantitatively analyzed the association and dissociation between p40^{phox} and p67^{phox} molecules using a secondary ¹²⁵I-labeled F(ab')₂ fragment against rabbit Ig. In excess dosages, anti-p67^{phox}C serum was able to coimmunoprecipitate an amount of p40^{phox} almost equal to that directly immunoprecipitated by anti-p40^{phox}C serum itself (Fig. 2 *B*). However, anti-p40^{phox}C serum did not yield any significant PSL count of p67^{phox} in the immunoprecipitates. In light of the fact that p40^{phox} and p67^{phox} are likely to exist in a complex unit at a roughly equimolar ratio in resting cytosol (13, 14), almost all of the p67^{phox} in the complex unit would appear to have been released from p40^{phox} by treatment of cytosol with anti-p40^{phox}C serum. Conversely, anti-p40^{phox}N serum had the capability to coimmunoprecipitate a comparable amount of p67^{phox} as that directly immunoprecipitated by anti-p67^{phox}C serum. Normal sera did not precipitate any detectable amounts of either p40^{phox} or p67^{phox}. The PSL ratio of p67^{phox} to p40^{phox} (~2:1) in excess regions of both anti-p67^{phox}C and anti-p40^{phox}N sera, may be largely due to the difference in their antigenicities, so that it does not directly represent numbers in the immunoprecipitates. Furthermore, there seems to be small amount of free p67^{phox} and p40^{phox} in cytosol as can be speculated by dose responses of anti-p67^{phox}C and anti-p40^{phox}N sera in low dosages. Comparable immunoprecipitation of p40^{phox} with anti-p40^{phox}C and anti-p40^{phox}N sera indicates that their distinct bindings at the NH₂ and COOH termini make little difference in the recognition of p40^{phox} and their

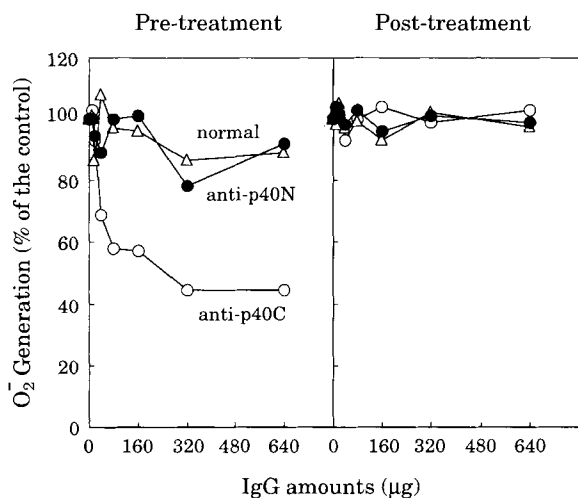


Figure 3. Inhibition of O_2^- -generation with antibody against the COOH-terminal, but not the NH_2 -terminal, polypeptide of $p40^{phox}$. Anti- $p40^{phox}C$ (○) and anti- $p40^{phox}N$ (△) IgGs, as well as normal IgG (●) were extensively dialyzed against 20 mM Na-phosphate buffer (pH 7.0). In the left panel, resting cytosol was pretreated with the indicated amounts of IgG for 3 h on ice. NADPH oxidase was then stimulated in activation buffer by incubating the pretreated cytosol (300 µg) and membranes (10 µg) with 100 µM of MA for 10 min at room temperature. In the right panel, the cytosol was alternatively incubated with the IgGs for 3 h on ice after stimulation with MA in the presence of membranes, and then the activity of the NADPH oxidase was determined. Its activity was stable on 3 h standing on ice. Data are representative of three experiments for pre-treatment and two experiments for post-treatment of cytosol both done in duplicate, and are expressed as the percent of the control O_2^- -generation (399 ± 47 and 421 ± 54 nmol of cytochrome *c* reduced/min/mg protein, respectively: means \pm SD) in the absence of IgG.

ability to immunoprecipitate it, respectively (Fig. 2 B). The results obtained from immunoprecipitation studies suggest that $p40^{phox}$ interacts with $p67^{phox}$ nearby its COOH-terminal but not its NH_2 -terminal site.

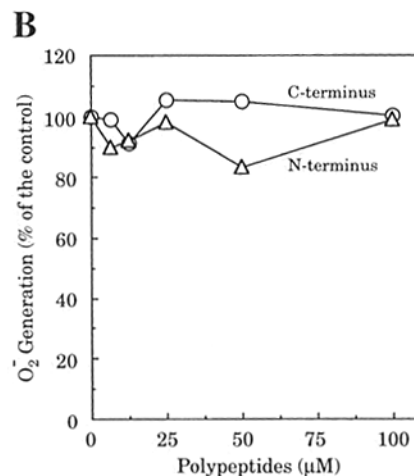
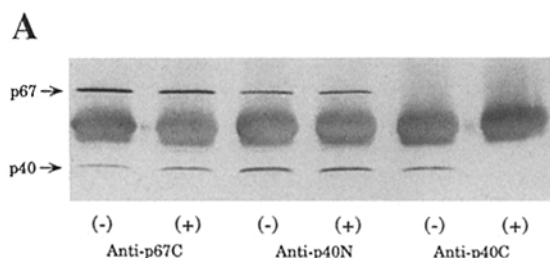


Figure 4. Effect of polypeptide corresponding to the COOH terminus of $p40^{phox}$ on the $p40^{phox}$ - $p67^{phox}$ association (A) and O_2^- -generation (B). In A, cytosol (1×10^7 cell equivalents) was preincubated with (+) or without (-) 100 µM of $p40^{phox}C$ polypeptide in 200 µl of activation buffer for 1 h on ice, and further incubated with anti- $p67^{phox}C$, anti- $p40^{phox}N$, or anti- $p40^{phox}C$ serum for 90 min on ice, followed by precipitation with protein A-Sepharose CL-4B at 4°C. The presence of $p40^{phox}$ and $p67^{phox}$ in the immunoprecipitates was probed with a mixture of anti- $p40^{phox}N$ and anti- $p67^{phox}C$ sera and revealed

by HRP-immunoblot analysis. In B, resting cytosol was preincubated with the indicated amounts of polypeptide corresponding to the either COOH terminus (○) or NH_2 terminus (△) of $p40^{phox}$ for 3 h on ice. Then, its capacity for O_2^- -generation was determined as described in Fig. 3. Data are representative of three experiments done in duplicate, and are expressed as the percent of the control O_2^- -generation (465 ± 46 of cytochrome *c* reduced/min/mg protein, respectively: means \pm SD) in the absence of polypeptide.

An Antibody Against the COOH-terminal Site, but not That Against the NH_2 -terminal Site, of $p40^{phox}$, Prohibits O_2^- -generation in a Cell-free Activation System. The possibility that a component(s) other than $p67^{phox}$, $p47^{phox}$ and *rac* p21 may exist in cytosol, has been deemed unlikely by studies performed with recombinant substitutes using a cell-free activation system (25–27). However, the results of our immunoprecipitation studies led us to test whether or not antibodies against $p40^{phox}$ could affect the O_2^- -generating activity of cytosol. Before testing, the titer of each antibody was assessed by ELISA assay. The amounts of anti- $p40^{phox}C$ and anti- $p40^{phox}N$ IgGs for half-maximal binding to each COOH- and NH_2 -terminal synthetic polypeptide were almost equal at $2^{8.9}$ ng/well and $2^{8.3}$ ng/well, respectively. The effects of these IgG fractions were then examined in the MA-dependent in vitro NADPH oxidase activation system. Cytosol pretreated with either of the IgG fractions was combined with membranes and its O_2^- -generating activity was then stimulated with 100 µM of MA in activation buffer. Anti- $p40^{phox}C$ IgG prevented the in vitro O_2^- -generation in a dose-dependent manner up to $\sim 55\%$ inhibition, whereas anti- $p40^{phox}N$ IgG had little effect, similar to normal IgG (Fig. 3, left). However, addition of anti- $p40^{phox}C$ IgG subsequent to the stimulation with MA did not affect the activity of the stimulated NADPH oxidase (Fig. 3, right). Preincubation of cytosol with 100 µM of polypeptide corresponding to the COOH-terminal site of $p40^{phox}$ was sufficient to prohibit the binding of anti- $p40^{phox}C$ IgG to the $p40^{phox}$ molecule (Fig. 4 A). However, this dosage of polypeptide failed not only to dissociate a $p40^{phox}$ - $p67^{phox}$ complex (Fig. 4 A), but also to inhibit in vitro O_2^- -generation (Fig. 4 B), unlike anti- $p40^{phox}C$ IgG.

NADPH Oxidase-activating Stimulants, MA and SDS, Do Not Disrupt a $p40^{phox}$ - $p67^{phox}$ Complex. A *rac* p21 protein exists as a complex with *rho*GDI (GDP-dissociation inhibitor) under non-activating conditions, and translocates to

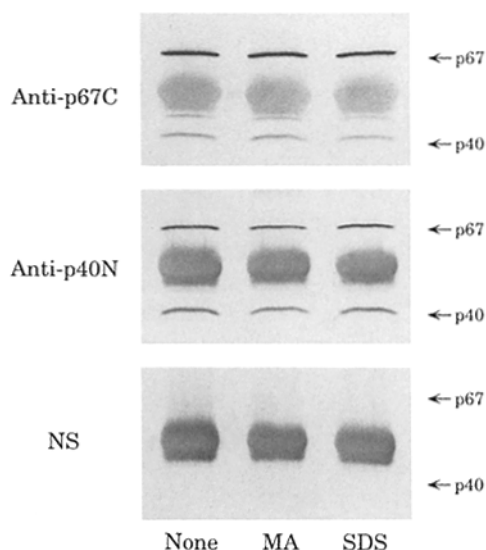


Figure 5. Effect of the NADPH oxidase stimulators, MA and SDS, on the p40^{phox}-p67^{phox} association. Aliquots of cytosol (300 μ g) were first stimulated in 200 μ l of activation buffer with 100 μ M of MA or SDS for 10 min at room temperature. Anti-p67^{phox}C, anti-p40^{phox}N, or normal sera (NS) was then added, followed by incubation for 90 min on ice. After adding protein A-Sepharose, the reaction mixture was brought to a final volume of 1 ml with immunoprecipitation buffer. Here, protein A-Sepharose beads were spun down at 180 g for 4 min to avoid nonspecific pelleting of oxidase components due to complexation with MA or SDS as described in Materials and Methods. The presence of p40^{phox} and p67^{phox} in the immunoprecipitates was also revealed by HRP-immunoblot analysis as described in Fig. 4 A.

the plasma membrane upon cell stimulation (31, 32). This interaction was demonstrated to be dissociable by certain lipids with biological activity, including NADPH oxidase activation (33). Here, cytosol was treated with 100 μ M of either MA or SDS, the optimal concentration for in vitro activation of NADPH oxidase, in an attempt to dissociate a p40^{phox}-p67^{phox} complex in activation buffer and then to separately immunoprecipitate them with either anti-p67^{phox}C or anti-p40^{phox}N serum. However, neither stimulant was effective at disruption of the p40^{phox}-p67^{phox} complex (Fig. 5). Normal serum did not immunoprecipitate any p40^{phox} or p67^{phox} in the presence of MA and SDS, respectively, under the conditions we employed for the immunoprecipitation.

Effect of Anti p40^{phox}C IgG on In Vitro Translocation of Cytosolic Components. The influence of a p40^{phox}-p67^{phox} complex dissociation on in vitro translocation of each cytosolic component was examined here with anti-p40^{phox}C IgG. First, resting cytosol pretreated with anti-p40^{phox}C IgG was combined with membranes, stimulated with 100 μ M of SDS, and then subjected to a discontinuous sucrose gradient to reisolate a membrane fraction. When cytosol and membranes were combined, translocation of cytosolic components occurred (Fig. 6). In the absence of membranes, no translocation was observed with cytosol and SDS alone. Preincubation of cytosol with 200 μ g of anti-p40^{phox}C IgG (1.8 mg in all for a ninefold reaction mixture volume), which corresponds to the \sim 50% inhibition of in vitro

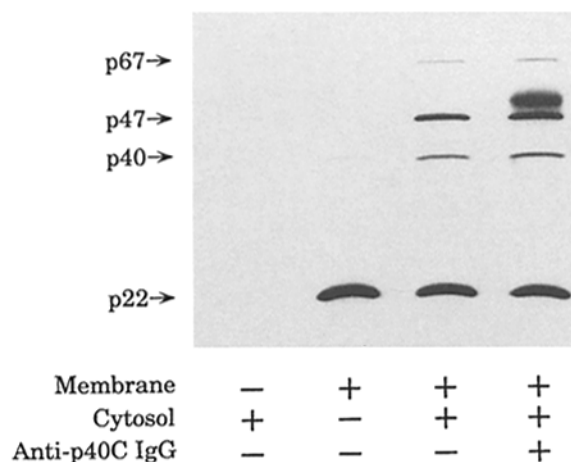


Figure 6. Effect of anti-p40^{phox}C IgG on the cell-free translocation of cytosolic components. After stimulation for 5 min with 100 μ M of SDS, reaction mixtures were layered onto a discontinuous sucrose gradient as described in Materials and Methods. After centrifugation, aliquots of membrane fractions (10 μ g) were subjected to SDS-PAGE, and the presence of p22^{phox}, p40^{phox}, p47^{phox}, and p67^{phox} was revealed by HRP-immunoblot analysis as described in Fig. 4 A. A broad band above p47^{phox} shows the heavy chain of IgG bound to p40^{phox}. + and - denote the presence and absence of each factor. The figure shows representative result of two experiments.

O₂⁻-generation in Fig. 3, did not show any inhibitory effect on the translocation of all cytosolic components. A broad band above p47^{phox} is derived from the heavy chain of IgG bound to the COOH terminus of p40^{phox}. Therefore, in the cell-free system, each cytosolic component seems to translocate independently to the membrane even after dissociation of the p40^{phox}-p67^{phox} complex with anti-p40^{phox}C IgG.

Discussion

In earlier studies to evaluate the hypothesis that the NADPH-binding component of the oxidase system is located in the cytosol, we identified 40-kD proteins as radioactive bands in neutrophils, which coimmunoprecipitated with cytosolic component p63^{phox} (porcine; reference 6) and p67^{phox} (human; reference 14). Independently, the presence of a 40-kD protein in guinea pig neutrophils (12) was also indicated by chromatographic works, that in human neutrophils being determined to be a 339-aa protein with one SH3 region and some homology to p47^{phox} (13). Despite identification of the association of p40^{phox} with p67^{phox}, there has hitherto been no evidence to functionally relate it to the NADPH oxidase system. This was mostly due to the apparent reconstitution of O₂⁻-generation in a cell-free system comprised solely of recombinant cytosolic components, p67^{phox}, p47^{phox}, and *rac* p21 with cytochrome b558, but without any addition of p40^{phox} (25-27). However, it is unclear whether this reconstitution system accurately reflects NADPH oxidase activation in intact cells. In fact, as described below, p40^{phox} appears to be directly involved in the process of oxidase assembly and thereby in the resultant O₂⁻-generation.

The p40^{phox} protein level is lowered in patients with CGD who lack the p67^{phox} molecule (Fig. 1 B). Defects in any of the four genes that code for the membranous (gp91^{phox} and p22^{phox}) and cytosolic (p47^{phox} and p67^{phox}) components of the NADPH oxidase system cause CGD. In most cases, a gene defect results in the absence of the respective protein product. The stability of gp91^{phox} depends on the simultaneous presence of p22^{phox}; in p22^{phox}-deficient cells, the gp91^{phox} polypeptide is absent despite the presence of ample mRNA (34). Likewise, the simultaneous reduction of p40^{phox} and p67^{phox} in the cytosol of CGD lacking p67^{phox} suggests that the p40^{phox} molecule is unstable without the presence of the p67^{phox} molecule. In contrast, a deficiency in either p47^{phox} or p67^{phox} does not affect the amount of its counterpart. This fact suggests that their complex formation in cytosol is likely to be indirect through another molecule.

Continuous electron flow leading to formation of toxic oxygen metabolites is highly dangerous to the host. Therefore, we initially considered p40^{phox} to be a locking molecule for p67^{phox} which maintains NADPH oxidase in a dormant state, analogous to the situation with the *rac* p21-*rho*GDI complex (31, 32). All of the *rac* p21 protein exists as a complex with *rho*GDI in resting cells. This interaction is disrupted in the presence of various lipids with biological activity, including arachidonic acid (33), and *rac* p21 translocates to the membrane during the course of NADPH oxidase activation leaving *rho*GDI in the cytosol. Therefore, MA, a potent fatty acid-stimulator of NADPH oxidase, and SDS were used in an attempt to dissociate the p40^{phox}-p67^{phox} complex. However, neither of these stimulators, at their optimal dosages for enzyme activation, exhibited any dissociating influence (Fig. 5). It was previously pointed out that fatty acids and SDS yield nonspecific pelleting of cytosolic components per se (4). However, here, this was overcome by centrifugation of protein A-Sepharose beads at low gravity (180 g). The immunoprecipitation buffer also contributed to decrease nonspecific binding of cytosolic components to the beads after treatment with either MA or SDS. Decreasing the ratio of the immunoprecipitation buffer to the activation buffer tended to increase nonspecific binding to the beads. As shown in Fig. 5, normal serum did not cause any nonspecific pelleting of p40^{phox} and p67^{phox}.

In contrast, anti-p40^{phox}C IgG not only completely cleaved the tight association between p40^{phox} and p67^{phox} (Fig. 2) but also suppressed O₂⁻-generation in the cell-free system (Fig. 3). On the other hand, anti-p40^{phox}N IgG had neither of these effects. Unlike anti-p40^{phox}C IgG, pretreatment of cytosol with p40^{phox}C polypeptide did not affect both the association between p40^{phox} and p67^{phox} and O₂⁻-generation (Fig. 4), despite the fact that this COOH-terminal polypeptide entirely inhibited the immunoprecipitation of p40^{phox} with anti-p40^{phox}C serum. Therefore, the COOH-terminal 15 amino acid residues used for peptide synthesis are unlikely to participate themselves in the association of p40^{phox} with p67^{phox}. Probably, a region near the COOH terminus of p40^{phox} undergoes indirect and physical cleav-

age with anti-p40^{phox}C IgG. Whatever, our results suggest that a region close to the COOH terminus, and not the NH₂ terminus, of p40^{phox}, is functionally involved in the NADPH oxidase system. However, once the NADPH oxidase was activated, its subsequent exposure to anti-p40^{phox}C IgG did not inhibit O₂⁻-generation (Fig. 3).

In a recent report (35), it was proposed that p67^{phox} and p47^{phox} are connected by virtue of p40^{phox} in the resting state, on the basis of findings using the genetic two-hybrid system. In Fig. 7, to facilitate understanding, we have depicted a potential but not definitive model for the existence of a cytosolic complex through p40^{phox}, based on the experimental evidence reported and presented here. The COOH-terminal region of p40^{phox} associates with p67^{phox} by an unknown mechanism in the resting state. The SH3 domain (aa 175–225) of p40^{phox} may contribute to the association with p47^{phox}, at the NH₂-terminal Pro-rich region (aa 70–84) as depicted in Fig. 7. The results obtained by Sumimoto et al. (21), in which the tandem SH3 domains (aa 151–214 and aa 227–284) of p47^{phox} failed to interact with its extensive NH₂-terminal site (aa 1–153) support this notion. In

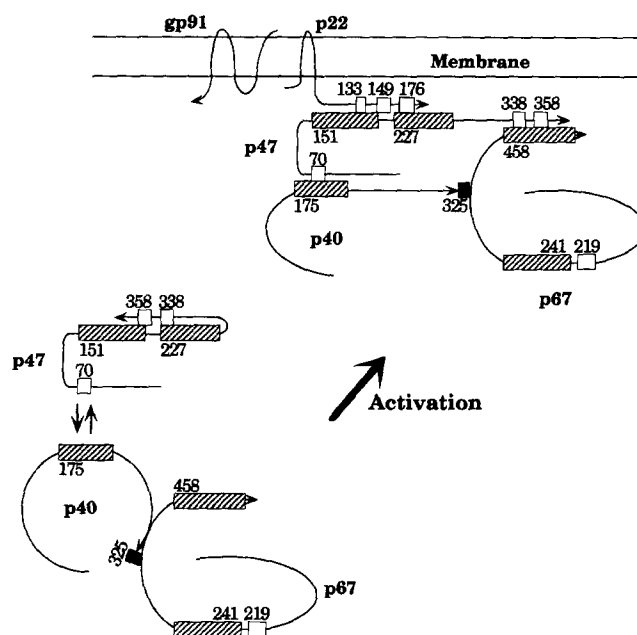


Figure 7. Proposed model for the interaction between p47^{phox} and p67^{phox} with the interposition of a p40^{phox} molecule. SH3 and Pro-rich regions are indicated by hatched and open boxes, respectively (only the numbers of the initial residues are noted). 15 amino acid residues in the COOH-terminal region of p40^{phox} are depicted by a filled box. Arrows indicate COOH-terminal sites. In the resting state, cytosolic components form a complex around p40^{phox}, through interaction between its SH3 domain and either the NH₂-terminal Pro-rich region or at some site in p47^{phox} with some association constant, and between its COOH-terminal region and an undetermined region in p67^{phox}. Disruption of the intramolecular interaction within p47^{phox} during oxidase activation enables new intermolecular binding of SH3 domains in p47^{phox} to the Pro-rich region in p22^{phox}. An unmasked Pro-rich region at the COOH terminus of p47^{phox} binds to the COOH-terminal SH3 domain of p67^{phox}. These interactions lead to the formation of active NADPH oxidase in the membrane. The anti-p40^{phox}C IgG bound to the COOH terminus of p40^{phox} hampers catalysis by this activated enzyme.

contrast, Leto et al. (23) pointed out that these tandem SH3 domains intramolecularly bind both the NH₂ (aa 70–84)- and COOH (aa 338–390)-terminal Pro-rich regions of p47^{phox}. Therefore, two possibilities still remain regarding the NH₂-terminal Pro-rich region of p47^{phox}, i.e., the intermolecular interaction with p40^{phox} and the intramolecular interaction within p47^{phox}. The tandem SH3 domains of p47^{phox}, which bind intramolecularly to its Pro-rich regions, are inaccessible in the resting state (21, 23), but become exposed, on addition of arachidonic acid or SDS, to interact with their target components (21). The two exposed SH3 domains and the COOH-terminal Pro-rich regions of p47^{phox} target Pro-rich regions (aa 133–190) in p22^{phox} (21, 23) and the COOH-terminal SH3 domain (aa 458–526) in p67^{phox}, respectively (22, 23). A second segment (aa 149–162) of the Pro-rich regions in p22^{phox} comprises a critical site in its interaction with the SH3 domains in p47^{phox}, since their interaction is disrupted by a Pro-156 → Gln substitution in p22^{phox} (21, 23, 36), reported in a patient with CGD (37). The minimal Pro-rich sequence in p47^{phox} necessary for interaction with the COOH-terminal SH3 domain of p67^{phox} was demonstrated to be QPAVPPRP (aa 362–369; reference 22). Under physiological conditions, this unmasking of the SH3 domains in p47^{phox} to initiate the translocation of cytosolic components is thought to be achieved by phosphorylation of multiple sites in its COOH-terminal region (38).

p40^{phox} and p67^{phox} exist as a tight complex unit in the resting cytosol. In contrast, our earlier immunoprecipitation (6, 14) studies and another recent report (11), in which the association between p47^{phox} and p67^{phox} was shown to be readily dissociable by increased temperature and high osmolarity, suggest that the interaction between p40^{phox} and p47^{phox} is very fragile. p47^{phox} probably interacts reversibly with p40^{phox} with some association constant, since only a proportion of the p47^{phox} was found to form a complex with p67^{phox} (8). p47^{phox} is entirely free from the cytoskeleton in the resting state (39) and on phosphorylation, it comes to reside in this location (40, 41). In contrast, almost all p67^{phox} is associated with the cytoskeleton in the cytosol (39), so that p40^{phox}, which is tightly associated with p67^{phox},

must also be a cytoskeleton-associated protein. Therefore, we first thought that, when cells are stimulated, freely mobile p47^{phox} in the cytosol will be incorporated into the submembranous cytoskeletal network through p40^{phox} using the above described SH3-dependent translocation mechanism to assemble active NADPH oxidase. However, dissociation of the p40^{phox}-p67^{phox} complex with anti-p40^{phox}C IgG did not show any inhibitory effect on SDS-dependent transfer of all cytosolic components to the membrane (Fig. 6). p47^{phox} and the p40^{phox}-p67^{phox} complex are likely to translocate independently and assemble on cytochrome b558, as suggested by p47^{phox} translocation in neutrophils from CGD patients lacking p67^{phox} (42). The translocation of dissociated p40^{phox} also suggests that the NH₂-terminal Pro-rich region in p47^{phox} might form an intramolecular binding in a resting state as mentioned above and be exposed to form a new intermolecular binding with p40^{phox} on the membrane after stimulation.

The pretreatment of cytosol with anti-p40^{phox}C IgG inhibited O₂⁻-generation only by 55% of the control (Fig. 3). Therefore, the presence of p40^{phox} is unlikely to be essential for catalysis by the activated enzyme in the cell-free system, as demonstrated with recombinant components alone in the absence of p40^{phox} (25–27), but may contribute to dispose cytosolic components in a stereospecifically appropriate state to get optimal catalysis. The antibody bound to the COOH terminus of p40^{phox} might have distorted this disposition. However, this COOH terminus was no longer accessible in the activated oxidase complex (Fig. 3). Since others (13, 43) and we (data not shown) also observed translocation of p40^{phox} to the membrane on intact cell stimulation, p40^{phox} is unlikely to play the role of a chaperone, which should dissociate from the oxidase complex once activation has been accomplished.

In this report, we firstly demonstrated the functional involvement of p40^{phox} in the NADPH oxidase system using anti-p40^{phox}C IgG. However, we speculate the role of p40^{phox} is responsible for a much more critical effect on the stimulation of intact cells with physiological agonists, and further investigations are required.

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