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Translocation of guinea pig p40-phox during activation of NADPH oxidase

Akimasa Someya [a,*], Isao Nagaoka [a], Hiroyuki Nunoi [b], Tatsuhisa Yamashita [a]

[a] *Department of Biochemistry, Juntendo University, School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan*
[b] *Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan*

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Abstract

The superoxide-producing NADPH oxidase consists of membrane-associated cytochrome b_{558} and cytosolic components, p47-phox and p67-phox. Recently, we have found a novel cytosolic component, p40-phox, which is tightly associated with p67-phox. In this study, we examined the translocation of p40-phox during activation of NADPH oxidase in a cell-free system using the membrane and the purified p47-phox/p67-phox/p40-phox complex. p40-phox was translocated to the membrane by arachidonic acid in a dose-dependent manner. The translocation pattern of p40-phox was similar to those of p47-phox and p67-phox. However, immunoprecipitation assay revealed that p40-phox was dissociated from p47-phox and p67-phox during activation. The translocation of three cytosolic components was not affected by the deletion of GTP-γ-s from the reaction mixture. Interestingly, a synthetic peptide corresponding to carboxyl-terminus of p40-phox inhibited the activation of NADPH oxidase and translocation of p40-phox, p47-phox and p67-phox, suggesting that p40-phox might play a role in the activation of NADPH oxidase. These observations suggest that p40-phox is dissociated from p67-phox during activation, and translocates to the membrane by GTP-γ-s-independent mechanism.

Keywords: Superoxide anion; NADPH oxidase; p40-phox; Cytosolic factor; Neutrophil; Cell-free system

1. Introduction

Neutrophils and other phagocytic cells exhibit a stimulus-dependent burst of oxidative metabolism which leads to the formation of superoxide anion and other toxic oxygen derivatives [1–4]. The respiratory burst is induced by the activation of superoxide-producing enzyme, NADPH oxidase, which is a multicomponent electron-transfer complex. The components of the enzyme are distributed in both membrane and cytosol in resting state [5,6]. Cytochrome b_{558} which is composed of two subunits gp91-phox and p22-phox, has been known as the membrane-bound component [7–9]. p47-phox and p67-phox have been identified as the cytosolic components [10–13]. In addition, the cytosolic small GTP-binding protein p21rac has been known as a functional component of the oxidase [14–16]. On cell activation, the cytosolic components translocate to the plasma membrane, where they associate with cytochrome b_{558}, forming the active NADPH oxidase [13,17]. The membrane and cytosolic components are assumed to assemble each other via the protein-protein interaction between

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Phox, phagocyte oxidase factor.

* Corresponding author. Fax: +81 3 3814 9300.

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Src homology 3 (SH3) domains and the polyproline motifs [18–22].

It has been reported that p47-phox and p67-phox are present as a complex in resting neutrophils [23–25]. We have purified the 260 kDa cytosolic complex from guinea pig neutrophils [23]. The complex is essential for the activation of NADPH oxidase, and is composed of p47-phox, p67-phox and a novel 39 kDa protein which is tightly associated with p67-phox. Recently, p40-phox which is associated with p67-phox, has been found in human neutrophils [26,27]. The homologies of amino acid sequences and the tight association with p67-phox indicate that the guinea pig 39 kDa protein and human p40-phox are homologues [3,4]. However, the role of p40-phox in the activation of NADPH oxidase is not clear.

In the present study, to clarify the role of p40-phox, we examined the translocation of p40-phox, and the effect of GTP-γ-s and p40-phox-derived synthetic peptides on NADPH oxidase activity using guinea pig p40-phox (39kDa protein).

2. Materials and methods

2.1. Reagents

Cytochrome *c* (Type VI), arachidonic acid (free acid), diisopropyl fluorophosphate (DFP), phenylmethanesulfonyl fluoride (PMSF), superoxide dismutase (SOD), flavin adenine dinucleotide (FAD:disodium salt), glycogen (TypeII, from oyster), bovine serum albumin (fraction V) and keyhole limpet hemocyanin (KLH) were purchased from Sigma Chemical Co. (USA). Guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-s) was obtained from Boehringer Mannheim (Germany). Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) was obtained from Dojindo Laboratories (Japan). Ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was obtained from Nacalai Tesque Inc. (Japan). β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), was obtained from Oriental Yeast Co., Ltd. (Japan). Trichloroacetic acid solution was obtained from Wako Pure Chemical Industries Ltd. (Japan). Pepstatin A and leupeptin were obtained from Peptide Institute, Inc. (Japan). Goat whole serum was obtained from Immuno-Biological Laboratories (Japan). Other reagents were of analytical grade.

2.2. Preparation of neutrophil membrane and cytosol fractions

Guinea pig neutrophils were obtained from exudates 13–15 h after an intraperitoneal injection of 0.17% glycogen in 0.9% saline [28]. The membrane and the cytosol fractions were prepared as previously described [23]. Briefly, neutrophils (2×10^8 cells/ml) were treated with 5 mM DFP in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) for 30 min at 4°C. After washing with PBS, neutrophils were suspended in 0.34 M sucrose buffer (131 mM NaCl, 8 mM KH_2PO_4, 0.34 M sucrose containing 1 mM EGTA, 0.8 mM PMSF, 10 μg/ml pepstatin A and 50 μg/ml leupeptin, pH 7.0) at 2×10^8 cells/ml, and disrupted by sonication. The sonicates were centrifuged at $8500 \times g$ for 15 min at 4°C to remove granule, nuclei and unbroken cells. The resulting supernatant was further centrifuged at $260\,000 \times g$ for 1 h at 4°C. The obtained supernatant (the cytosol fraction) was used for the purification of the p47-phox/p67-phox/p40-phox complex. The pellet (the membrane fraction) was suspended in 0.34 M sucrose buffer, centrifuged at $260\,000 \times g$ for 1 h, and resuspended in 0.34 M sucrose buffer. The membrane and the cytosol fractions were stored at −80°C until use.

2.3. Purification of the p47-phox/p67-phox/p40-phox complex

The p47-phox/p67-phox/p40-phox complex was purified from the cytosol fraction as described previously [23]. The cytosol fraction (about 500 mg protein) was filtered through a Sephadex G-25 column (Pharmacia LKB Biotechnology, Sweden) equilibrated with buffer A (20 mM sodium phosphate, pH 7.0, containing 0.5 mM EGTA and 0.5 mM PMSF). The void fraction was applied to a S-Sepharose Fast-Flow column (Pharmacia LKB Biotechnology) equilibrated with buffer A, and proteins were eluted with a 0–0.5 M NaCl gradient. Each fraction was monitored for activation of NADPH oxidase in a cell-free assay system using the membrane fraction and arachidonic acid [23]. The active fractions containing

the p47-phox/p67-phox/p40-phox complex identified by Western blot analysis, were dialyzed against buffer B (10 mM sodium phosphate, pH 7.0), applied to a Biofine HAC-5CP hydroxyapatite column (JASCO-Japan Spectroscopic Co., Japan) equilibrated with buffer B, and eluted with a 0–0.3 M sodium phosphate (pH 7.0). The active fractions containing the complex were mixed with 5 vols. of buffer A, applied to a Cosmogel CM column (Nacalai Tesque Inc.) equilibrated with Buffer A, and eluted with a 0–0.5 M NaCl gradient. The purified complex was essentially free of contaminating proteins by SDS-PAGE analysis with silver staining, and was stored at −80°C until use.

2.4. Chemical synthesis of peptides

The peptides, DIAVEEDLSSTPP (peptide 1) and HITQQDNYSVYNTTPSATQ (peptide 2), which had been identified by the amino acid sequence analysis of p40-phox [23], and TNISQYHTQTAPVSQDTNY (peptide 3; the scrambled form of peptide 2) were synthesized on a model 430A peptide synthesizer (Perkin Elmer Applied Biosystems Division, USA) according to the F-moc method [29]. The peptides were released from the solid-phase resin with trifluoroacetic acid containing 1 M trifluormethanesulfonic acid and 5% 1,2-ethanedithiol. The crude peptides were purified by reverse phase high performance liquid chromatography. Synthetic peptides were stored as lyophilized powders at −80°C.

2.5. Preparation of antiserum and affinity purification of antibody

Antiserum was raised in rabbits using the synthetic peptide 1 covalently coupled to KLH by the method of Liu et al. [30]. The 200 μg of KLH-conjugated peptide was homogenized in Freund's complete adjuvant and injected subcutaneously into rabbits, followed by 4 boosts (every week) of conjugated peptide (100 μg) in Freund's incomplete adjuvant.

Antibody was purified by affinity chromatography. Peptide-conjugated beads were prepared by coupling of peptide to Epoxy-activated Sepharose 6B (Pharmacia LKB Biotechnology) as described by the manufacturer. After washing with water, the beads were resuspended in TBS (20 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl). Antiserum was applied to the peptide-conjugated beads equilibrated with TBS, and stood for 15 min at 4°C. After sequentially washing with TBS supplemented with 0.5% Triton X-100 and 0.15 M NaCl, the bound IgG was eluted using 0.1 M glycine-HCl (pH 2.5). IgG solution was neutralized with the addition of 1/20 volumes of 1 M Tris, and was stored at −80°C until use. Purified antibody was used in immunoprecipitation experiment as described below.

2.6. Assay for translocation of cytosolic components and NADPH oxidase activity in a cell-free system

The mixtures containing 60 μg of membrane, 120 ng of the cytosolic p47-phox/p67-phox/p40-phox complex, 10 μM GTP-γ-s and 5 mM $MgCl_2$ were preincubated at 25°C for 5 min, and then added with various concentration of arachidonic acid in a total volume of 0.4 ml relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$ 1.25 mM EGTA, 10 mM PIPES, pH 7.4). After activation with arachidonic acid for 5 min at 25°C, the mixtures (0.4 ml) were layered onto a discontinuous sucrose gradient composed of 0.4 ml of 15% (w/v) sucrose over 0.2 ml of 50% (w/v) sucrose in relaxation buffer, and centrifuged at 105 000 × *g* for 30 min at 20°C. After centrifugation, 0.6-ml fractions were withdrawn from the top, and used as the 'supernatant'. Residual fractions (0.4 ml) containing the interface of the 15/50% sucrose layers were collected and used as the 'membrane'. The 'supernatant' was treated with ice-cold trichloroacetic acid (10%) for 15 min on ice, and centrifuged (12 000 × *g*, 10 min). The protein pellets were resuspended in SDS-PAGE sample buffer. The 'membrane' was diluted in relaxation buffer, centrifuged (200 000 × *g*, 1 h), and resuspended in relaxation buffer. The membrane and the supernatant were subjected to SDS-PAGE/Western blotting.

When the membrane was omitted from the activation mixture, p40-phox, p47-phox and p67-phox were not recovered from the interface of the 15/50% sucrose layers. Therefore, arachidonic acid is unlikely to cause the formation of large protein complexes of cytosolic components in our system (data not shown).

NADPH oxidase activity of the membrane was measured by a spectrophotometric assay of cytochrome *c* reduction [23,28]. The assay mixtures

consisted of 10 μg of activated membrane (as described above), 80 μM cytochrome *c*, 10 μM FAD, and 200 μM NADPH in the total volume of 0.5 ml relaxation buffer, and the assay was performed at 37°C in a Hitachi 557 dual-wavelength spectrophotometer (Hitachi, Ltd., Japan). Cytochrome *c* reduction was calculated from the linear portion of the increase in the absorbance difference at 550–540 nm by using an absorption coefficient of 21000 M^{-1} cm^{-1}.

2.7. Immunoprecipitation

The cytosolic p47-phox/p67-phox/p40-phox complex (200 ng) was incubated with or without 100 μM arachidonic acid for 5 min at 20°C in the total volume of 0.4 ml relaxation buffer, and then affinity-purified anti-p40-phox rabbit polyclonal antibody (10 μg) was added. After incubation for 2 h at 4°C, protein G Sepharose beads (10 μl; Pharmacia LKB Biotechnology) were added, and rotated for 1.5 h at 4°C. The beads were washed four times by relaxation buffer, and suspended in SDS-PAGE sample buffer. The cytosolic components were subjected to SDS-PAGE/Western blotting.

2.8. Effects of synthetic peptides on translocation of cytosolic components and NADPH oxidase activity

The mixtures containing 60 μg of membrane, 120 ng of the cytosolic p47-phox/p67-phox/p40-phox complex, 10 μM GTP-γ-s and 5 mM $MgCl_2$ were preincubated with various concentrations of the synthetic peptides at 25°C for 5 min, and then added with 100 μM arachidonic acid in a total volume of 0.4 ml relaxation buffer. After 5 min at 25°C, the membrane was isolated by sucrose gradient centrifugation. As described in Section 2.6, the membrane was subjected to SDS-PAGE/Western blotting for evaluation of translocation of cytosolic components, and NADPH oxidase activity was measured by spectrophotometric assay.

2.9. SDS-PAGE and Western blotting

The membrane and the supernatant prepared in the above sections were subjected to SDS-PAGE on 13% polyacrylamide gels based on the method of Laemmli and Faver [31]. The resolved proteins were electrophoretically transferred to nitrocellulose filters. The blots were blocked in Block Ace (Dainippon Pharmaceutical Co. Ltd., Japan) containing 10 mg/ml BSA. Then, the blots were incubated 15 h at 4°C with the anti-p47-phox and anti-p67-phox mouse monoclonal antibodies (diluted 5000-fold in 10% Block Ace), or anti-p40-phox rabbit polyclonal antibody (diluted 1000-fold in 10% Block Ace containing 1% goat

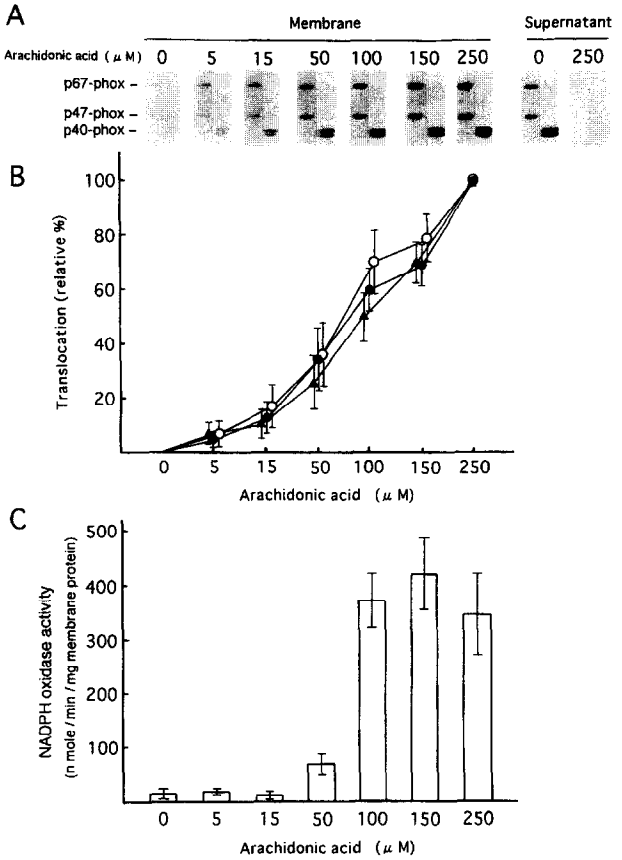

Fig. 1. Dose-dependency of arachidonic acid on translocation of cytosolic components and NADPH oxidase activity. The mixtures containing the membrane and the cytosolic p47-phox/p67-phox/p40-phox complex were stimulated by various concentrations of arachidonic acid. After 5 min, the membrane and the supernatant were isolated from each mixture by sucrose gradient centrifugation. (A) The membrane and the supernatant were analyzed by SDS-PAGE/Western blotting. (B) The translocated p40-phox (●), p47-phox (○) and p67-phox (▲) to the membrane were quantified by scanning. Translocation is expressed as relative % compared with the amounts of cytosolic components stimulated by 250 μM arachidonic acid. (C) NADPH oxidase activities of the membrane. The results shown are the mean ± S.E. of six separate experiments.

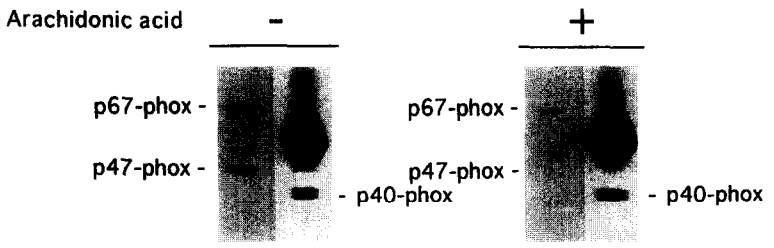

Fig. 2. Immunoprecipitation analysis using anti-p40-phox peptide antibody. p47-phox/p67-phox/p40-phox complex was incubated without (−) or with (+) arachidonic acid, then immunoprecipitation was performed using anti-p40-phox antibody. The 45–65 kDa intense bands were the heavy chains of rabbit IgG contained in anti-p40-phox rabbit antibody. The results are representative of five separate experiments.

whole serum). The cytosolic components were detected using horseradish peroxidase-conjugated goat anti-mouse IgG/IgM (5000-fold diluted; Kirnegaad and Petty, USA) or goat anti-rabbit IgG (10000-fold diluted; Organon Teknika Co., USA) with ECL Western blotting system (Amersham International plc, UK). We confirmed that anti-p40-phox, anti-p47-phox and anti-p67-phox antibodies specifically reacted with p40-phox, p47-phox and p67-phox, respectively. To measure the relative amounts of the translocated cytosolic components to the membrane, the bands detected by ECL were scanned using MasterScan™ System (Scanalytics, USA).

In this study, p40-phox band detected was doublet. Analysis of partial amino acid sequences of these two proteins revealed that the sequences of the two proteins were identical (data not shown). Therefore, the low molecular weight protein reacting with anti-p40-phox antibody is likely a partially degraded protein of p40-phox.

3. Results

3.1. Translocation of p40-phox to the membrane

To elucidate the role of p40-phox in activation of NADPH oxidase, we first examined the translocation of p40-phox to the membrane by stimulation with various concentrations of arachidonic acid using the cytosolic p47-phox/p67-phox/p40-phox complex. As shown in Fig. 1A,B, p40-phox, p47-phox and p67-phox were translocated to membrane, dependent on the concentrations of arachidonic acid. The translocation pattern of p40-phox was similar to those of p47-phox and p67-phox. Fig. 1C shows a dose-dependency of NADPH oxidase activity on arachidonic acid. NADPH oxidase activity was maximally activated by 100–150 μM arachidonic acid, whereas the oxidase was little activated below 50 μM arachidonic acid.

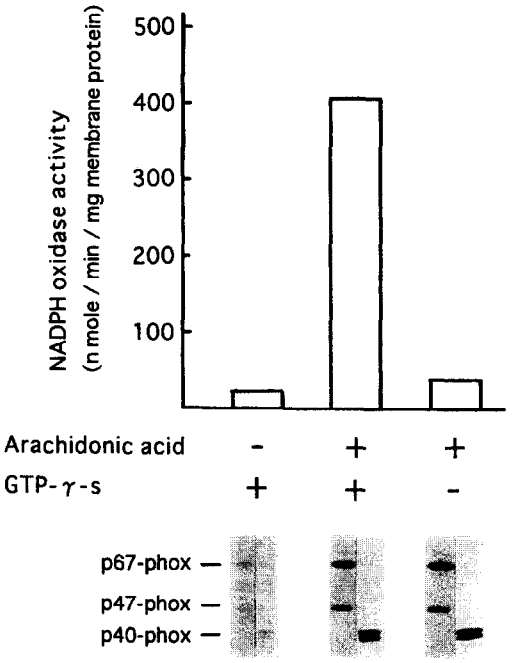

Fig. 3. Effect of GTP-γ-s on NADPH oxidase activity and translocation of cytosolic components. The mixtures containing the membrane and the cytosolic p47-phox/p67-phox/p40-phox complex were incubated with (+) or without (−) arachidonic acid (100 μM) in the presence (+) or absence (−) of 10 μM GTP-γ-s. After 5 min, the membrane was isolated from each mixture by sucrose gradient centrifugation. Upper: NADPH oxidase activity of the membrane. Lower: SDS-PAGE/Western blotting of the membrane. The results are representative of three separate experiments.

Next, to examine whether p40-phox is dissociated from p47-phox and p67-phox during activation, we performed the immunoprecipitation using p40-phox antibody. As shown in Fig. 2, p47-phox and p67-phox were recovered in the p40-phox/anti-p40-phox antibody complex without arachidonic acid-treatment (in resting state). In contrast, p47-phox and p67-phox were lost from the complex by arachidonic acid-treatment. Although we previously observed that p40-phox was tightly associated with p67-phox in resting state [23], the results of immunoprecipitation analysis suggest that p40-phox is dissociated from p67-phox during activation.

3.2. Effect of GTP-γ-s on NADPH oxidase activity and translocation of cytosolic components

We examined the effect of GTP-γ-s on NADPH oxidase activity and translocation of p40-phox, p47-phox and p67-phox. As shown in Fig. 3, activation of NADPH oxidase by arachidonic acid was dependent on GTP-γ-s. On the other hand, translocation of p40-phox, p47-phox and p67-phox to the membrane by arachidonic acid was independent of GTP-γ-s.

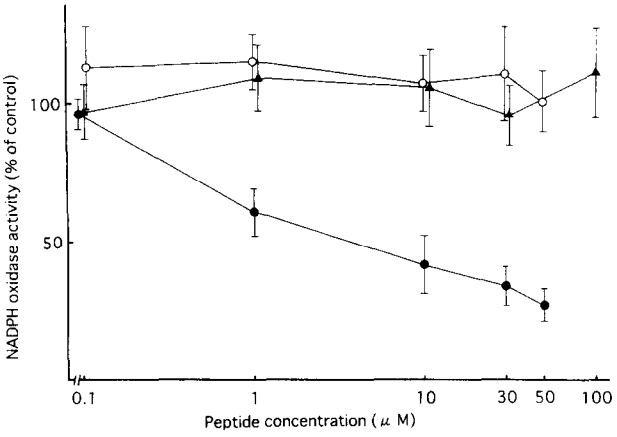

Fig. 4. Effects of p40-phox-derived peptides on NADPH oxidase activity. The mixtures containing the membrane and the cytosolic p47-phox/p67-phox/p40-phox complex were preincubated for 5 min with the indicated concentrations of peptide 1 (○), peptide 2 (●) or peptide 3 (▲). Then, the mixtures were added with arachidonic acid. After 5 min, the membrane fractions were isolated from each mixture by sucrose gradient centrifugation. Then, NADPH oxidase activity of membrane fractions were measured. NADPH oxidase activity stimulated in the absence of peptides was 325.5±47.3 nmol/min/mg membrane protein (mean±S.E.). The results shown are the mean±S.E. of three separate experiments.

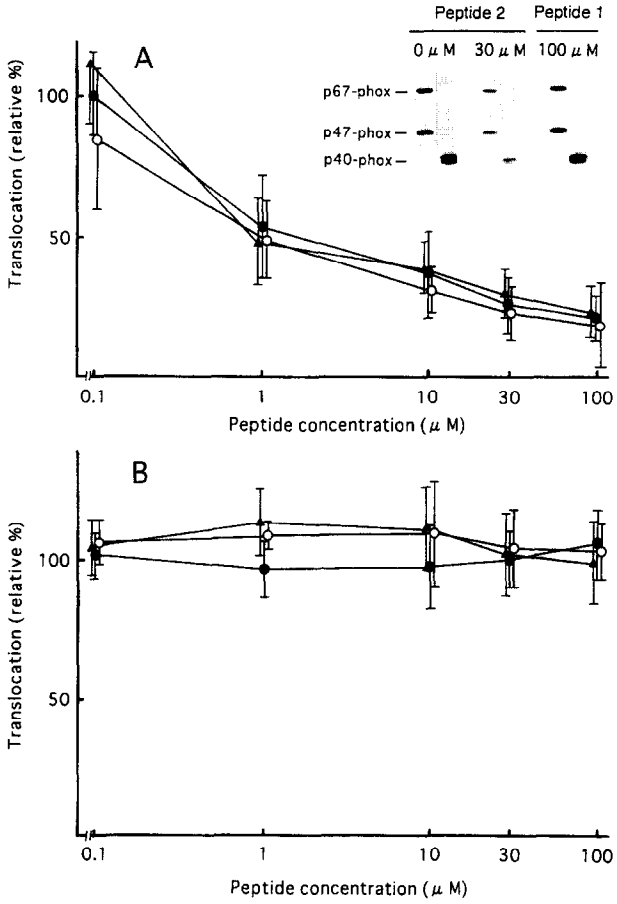

Fig. 5. Effects of p40-phox-derived peptides on translocation of cytosolic components. The mixtures containing the membrane and the cytosolic p47-phox/p67-phox/p40-phox complex were preincubated for 5 min with the indicated concentrations of peptide 2 (A) or peptide 3 (B). Then, the mixtures were added with arachidonic acid. After 5 min, the membrane fractions were isolated from each mixture by sucrose gradient centrifugation. Translocation of the cytosolic components to the membrane was analyzed by SDS-PAGE/Western blotting. The translocated p40-phox (●), p47-phox (○) and p67-phox (▲) is expressed as relative % of the amounts of cytosolic components stimulated in the absence of the peptide. The results shown are the mean±S.E. of 3–6 separate experiments. Inset: Western blotting of the translocated cytosolic components to the membrane in the presence of peptide 1 (100 μM) or peptide 2 (30 μM).

3.3. Effects of synthetic peptides on NADPH oxidase activity and translocation of cytosolic components

We previously identified the amino acid sequences of the two fragments (DIAVEEDLSSTPPF and

LHITQQDNYSVYNTTPSATQ) of guinea pig p40-phox [23] corresponding to the sequences of the residues 252-265 and the residues 325-349 (carboxyl-terminal sequence) of human p40-phox, respectively [26]. Then, we examined the effect of these synthetic peptides DIAVEEDLSSTPP (peptide 1) and HITQQDNYSVYNTTPSATQ (peptide 2) on NADPH oxidase activity. Peptide 1 had no effect on NADPH oxidase activity (Fig. 4). On the other hand, peptide 2 inhibited NADPH oxidase activity in a concentration-dependent manner (IC_{50} of 3–4 μM). The activity was reduced to about 30% of control by 50 μM peptide 2. When peptide 2 was added 5 min after arachidonic acid stimulation, no inhibitory effect was observed (data not shown). These results indicate that peptide 2 acts on the activation process of NADPH oxidase but not on the activated oxidase. Peptide 3 (TNISQYHTQTAPVSQDTNY) with the same amino acid composition as peptide 2 but with a scrambled sequence had no effect on the NADPH oxidase activity.

Furthermore, the effects of these peptides on translocation of cytosolic components to the membrane were examined. As shown in Fig. 5A, translocation of p40-phox, p47-phox and p67-phox was dose-dependently inhibited by peptide 2. IC_{50} values for three cytosolic components were 1–2 μM. On the other hand, peptides 1 and 3 did not inhibit the translocation of cytosolic components (Fig. 5A, inset and Fig. 5B).

4. Discussion

We have recently identified a new 39 kDa cytosolic component, which is complexed with p67-phox and p47-phox, in guinea pig neutrophils [23]. Other investigators have also identified a new cytosolic component, p40-phox, in human neutrophils [26,27]. Homologies of amino acid sequences between 39 kDa protein (DIAVEEDLSSTPP and HITQQDNYSVYNTTPSATQ) and p40-phox (residues 252-265 and 325-349) are more than 85% [23,27], suggesting that guinea pig 39 kDa cytosolic component is a homologue of p40-phox [3,4]. p40-phox is assumed to play a role in stabilizing p67-phox [32], because p40-phox is tightly associated with p67-phox and is markedly reduced in chronic granulomatous disease deficient in p67-phox [26,27]. However, the role of the p40-phox in the activation of NADPH oxidase is not clear.

In this study, using guinea pig p40-phox, we have observed that p40-phox is translocated to the membrane by arachidonic acid in a dose-dependent manner. The translocation pattern of p40-phox was similar to those of p47-phox and p67-phox. Previously, we found that p40-phox was tightly associated with p67-phox in resting state. However, the immunoprecipitation experiment revealed that p40-phox was dissociated from p47-phox and p67-phox by arachidonic acid stimulation.

Translocation of p40-phox, p47-phox and p67-phox was not affected by the deletion of GTP-γ-s from the reaction mixture, suggesting these cytosolic components translocate independent of G-protein. On the other hand, the activation of NADPH oxidase was dependent on the GTP-γ-s. p21rac, one of the small G-proteins, have been known as a functional component of the oxidase [14–16]. A small amount of p21rac is associated with the membrane, and the rest of p21rac is present in the cytosol [33]. In this study, we did not add the p21rac to the activation mixture, but substantial activity of NADPH oxidase was obtained. We detected that a small amount of p21rac was associated with the resting and activated membrane by Western blot analysis (data not shown). Therefore, p21rac associated with the membrane may be enough for the activation of NADPH oxidase, as reported in the previous paper [33]. Recently, the membrane-bound small G-protein rap1 is reported to be involved in the regulation of NADPH oxidase [3,4,34]. However, a role of rap1 in the activation of NADPH oxidase is controversial [3].

Addition of synthetic peptide 2, corresponding to the carboxyl-terminus of p40-phox, inhibited the activation of NADPH oxidase. Furthermore, peptide 2 inhibited the translocation of p40-phox, p47-phox and p67-phox. On the other hand, peptide 1 and peptide 3 (with the same amino acid composition as peptide 2 but with a scrambled sequence) inhibited neither NADPH oxidase activity nor translocation of cytosolic components. Therefore, the inhibitory effects of peptide 2 seems to be sequence-specific.

Recently, Fuchs et al. [35] have suggested that p40-phox likely functions as a chaperone for p67-phox via the interaction between the carboxyl-terminus of

p40-phox and p67-phox. After stimulation, p47-phox is known to associate first with p22-phox, subunit of cytochrome b_{558}, followed by the binding with p67-phox [12,13,18,22]. Interestingly, Uhlinger et al. [36] have revealed that p67-phox enhances the binding of p47-phox to the membrane. These observations suggest that peptide 2 (corresponding to the carboxyl-terminus of p40-phox) might reduce the translocation of p47-phox and p67-phox by affecting the function of p67-phox through the competition with p40-phox for the binding to p67-phox. However, the possibility that peptide 2 might affect the interaction between p22-phox and p47-phox, and/or p47-phox and p67-phox, cannot be excluded. The detailed mechanism of the action of peptide 2 remains to be clarified.

In summary, we found that p40-phox was dissociated from p67-phox and p47-phox during activation, and translocated to the membrane independent of GTP-γ-s. Furthermore, we observed that the carboxyl-terminal peptide of p40-phox inhibited the activation of NADPH oxidase and translocation of cytosolic components, suggesting that p40-phox might be involved in the activation of NADPH oxidase.

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