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Purification of the 260 kDa cytosolic complex involved in the superoxide production of guinea pig neutrophils

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A 260 kDa cytosolic complex (SP-1) was purified from guinea pig neutrophils. SP-1 was composed of 63 kDa, 47 kDa and 39 kDa proteins. The 63 kDa and 47 kDa proteins proved to correspond to human $p67^{phox}$ and $p47^{phox}$ by Western blot analysis, whereas Western blot and amino acid sequence analyses revealed that the 39 kDa protein was a novel protein. The 47 kDa protein was separated from the 63 kDa and 39 kDa proteins by dithiothreitol (DTT)-treatment. On the other hand, the 63 kDa and 39 kDa proteins were not separated with DTT, detergent and ethanol treatment. These results suggest that the 39 kDa protein tightly associates with the 63 kDa protein and may regulate the function of the 63 kDa protein.

Superoxide production; NADPH oxidase; Cytosolic factor; Neutrophil; Cell-free system

1. INTRODUCTION

The production of superoxide (O_2^-), which is catalyzed by NADPH oxidase, is essential for microbicidal and cytotoxic activities of neutrophils and other professional phagocytic cells [1]. NADPH oxidase is a multicomponent enzyme consisting of a plasma membrane-bound cytochrome b_{558} [2–6] and cytosolic proteins, such as $p47^{phox}$ and $p67^{phox}$ [7–12]. $p47^{phox}$ and $p67^{phox}$ are translocated to the membrane when the oxidase is activated [13,14]. Recently, small GTP-binding proteins (rac1, rac2 and Krev1) have been reported to be the cytosolic components which participate in the oxidase activation [15–17].

Large molecular cytosolic complexes (> 100 kDa) which are involved in superoxide production have been obtained from cytosol by gel filtration chromatography [18,19] or ammonium sulfate precipitation [20]. Recently, a 250 kDa cytosolic complex has been isolated from human neutrophils and proved to contain both $p47^{phox}$ and $p67^{phox}$ [14], suggesting that these proteins may exist as a complex in neutrophils. We have separated three cytosolic factors (SP-1, QA-1 and QA-2) from guinea pig neutrophils using ion-exchange columns, and have showed that SP-1 but not QA-1 and QA-2 activates NADPH oxidase by itself. SP-1 was a 260 kDa complex with a few components, including a 47 kDa protein [21]. Therefore, in the present study, we attempted to purify SP-1, to elucidate its activation mechanisms of NADPH oxidase.

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Abbreviations: $p47^{phox}$, 47 kDa cytosolic phagocyte oxidase component; $p67^{phox}$, 67 kDa cytosolic phagocyte oxidase component; PMSF, phenylmethanesulfonyl fluoride.

2. MATERIALS AND METHODS

2.1. *Preparation of neutrophils*

Guinea pig neutrophils were isolated from the peritoneal cavity 13–15 h after an intraperitoneal injection of 0.17% glycogen as previously described [22].

2.2. *Preparation of the cytosol and membrane fractions*

Neutrophils (2×10^8 cells/ml) were pretreated with 5 mM diisopropyl fluorophosphate 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 resuspended in ice-cold 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, and 50 μg/ml leupeptin, pH 7.0) at 2×10^8 cells/ml, and disrupted by sonication on ice using a sonicator. The sonicate was centrifuged at 8,500 × *g* for 15 min at 4°C, and the resulting supernatant was further centrifuged at 260,000 × *g* for 1 h at 4°C. The obtained supernatant, i.e. the cytosol fraction, was used as starting material for the purification of cytosolic factors. The pellet was resuspended in 0.34 M sucrose buffer and used as the membrane fraction [21]. The cytosol and the membrane fractions were stored at −80°C until use.

2.3. *Purification of the SP-1 cytosolic complex*

To change the buffer, the cytosol fraction (70 ml, containing 500 mg protein) was filtered through a Sephadex G-25 column (2.6 × 100 cm, Pharmacia LKB Biotechnology, Sweden) equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EGTA and 0.5 mM PMSF (Buffer A). Almost all activity was recovered in a void fraction. The void fraction was applied to a S-Sepharose Fast-Flow column (26 × 40 mm, Pharmacia LKB Biotechnology) equilibrated with Buffer A, and the column was eluted with a linear 0–300 mM NaCl gradient at a flow rate of 3.0 ml/min. The active fraction, SP-1, (total volume of 78 ml) obtained from the S-Sepharose Fast-Flow chromatography was dialyzed against a 10 mM sodium phosphate buffer (pH 7.0), and concentrated using PM10 diafro ultrafilters with

a molecular mass cut-off of 10 kDa (Amicon Co., Ireland). Concentrated SP-1 (about 50 ml) was applied to a Biofine HAC-5CP hydroxyapatite column (7.5 × 75 mm, JASCO-JAPAN Spectroscopic Co., Japan) equilibrated with a 10 mM sodium phosphate buffer, pH 7.0, containing 0.17 M sucrose. After washing with equilibration buffer, the column was eluted with a linear gradient of 10–300 mM sodium phosphate at a flow rate of 1.0 ml/min. The active fraction (about 7 ml) was mixed with 5 volumes of Buffer A to decrease the salt concentration, and was applied to a Cosmogel CM column (8 × 75 mm, Nacalai tesque Inc., Japan) equilibrated with Buffer A. The column was eluted with a linear gradient of 0–500 mM NaCl at a flow rate of 1.0 ml/min. The active fraction (about 5 ml) was concentrated using PM10 diafro ultrafilters to a volume of 2 ml, and 400 μl was injected to a Superose 12HR gel filtration column (10 × 300 mm, Pharmacia LKB Biotechnology) equilibrated with a 20 mM sodium phosphate buffer, pH 7.0, containing 0.13 M NaCl, 0.5 mM PMSF, and 1 mM EGTA. The flow rate was 0.5 ml/min.

2.4. *Assay of NADPH oxidase activity in a cell-free system*

Assay of NADPH oxidase was performed in a cell-free system as described previously [21].

The assay mixture (0.5 ml) consisted of 80 μM cytochrome *c*, 5 mM $MgCl_2$, 10 μM FAD, 20 μM arachidonic acid, 15 μg membrane fraction and each fraction from HPLC in 65 mM sodium phosphate buffer, pH 7.0, containing 0.17 M sucrose. After a 40 s incubation at 37°C, the reaction was started by the addition of 200 μM NADPH. The reduction of cytochrome *c* was measured at 550–540 nm continuously.

2.5. *Hydrophobic chromatography of purified SP-1*

Purified SP-1 was mixed with three volumes of 100 mM sodium phosphate buffer, pH 7.0, containing 2.5 mM dithiothreitol (DTT) and 5 mM EGTA. After stirring for 30 min at 4°C, 5 M ammonium sulfate solution (adjusted to pH 7.0 with ammonium hydroxide) was added to the mixture to reach a final concentration of approximately 1.0 M. The mixture was stirred for 2 h at 4°C, and was centrifuged at 48,000 × *g* for 20 min at 4°C. The obtained supernatant was applied to a Phenyl-TOYOPEARL 650 column (4 × 50 mm, TOSOH Corp., Japan) equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 1.0 M ammonium sulfate, 500 μM DTT and 1 mM EGTA. The column was eluted with a 1.0–0 M ammonium sulfate gradient at a flow rate of 0.7 ml/min.

2.6. *Polyacrylamide gel electrophoresis*

SDS-PAGE was carried out with a 5–13% linear gradient gel in 0.1% SDS by the method of Laemmli and Favere [23]. Samples were treated with 5% 2-mercaptoethanol at 100°C for 3 min under standard conditions. The gel was silver-stained using a commercially available reagent kit (Daiichi Pure Chemicals Co., Japan).

2.7. *Immunoblot analysis*

The purified SP-1 was subjected to SDS-PAGE and was electroblotted onto a nitrocellulose membrane according to the method of Towbin et al. [24]. The membrane was incubated with a Block Ace (Dainippon Pharmaceutical Co. Ltd., Japan), and then reacted with a 1 : 2000 dilution of anti-human $p47^{phox}$ or anti-human $p67^{phox}$ monoclonal antibody. The proteins were finally detected with a 1 : 1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG followed by development with diformazan using the POD Immunostain Set (Wako Pure Chemical Industries Ltd., Japan).

2.8. *Analysis of amino acid sequence*

The purified SP-1 (about 100 pmol) was subjected to SDS-PAGE and was electroblotted onto a polyvinylidine fluoride (PVDF) membrane (Millipore Co., Bedford) as described previously [25]. The PVDF membrane was stained with Ponceau S, and then the band (39 kDa) was excised and used for the analysis. After the treatment of reduction and *S*-carboxymethylation of PVDF-blotted protein, in situ digestion of the *S*-carboxymethylated 39 kDa protein was performed by the method of Iwamatsu [26]. The membrane was treated with 0.5% polyvinylpyrrolidone (PVP-40) at 37°C for 30 min and cut into small pieces, and then the protein on the membrane was digested with lysyl endopeptidase (Wako Pure Chemical Industries Ltd., Japan) at 35°C for 16 h in 50 mM Tris-HCl, pH 9.0, containing 9% acetonitrile at an enzyme-to-substrate ration of 1 : 100 (mol/mol). Two major peaks were separated by HPLC using a μ-Bondasphere 5 μC8-300A (2.1 × 150 mm, Millipore Co.) with a gradient of 2–70% acetonitrile

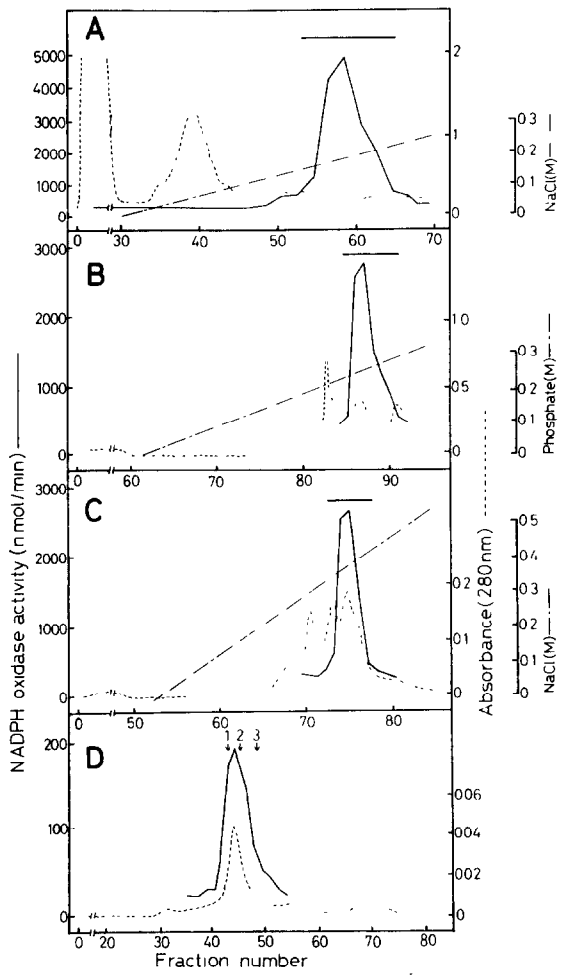

Fig. 1. Chromatographic purification of SP-1. (A) S-Sepharose Fast-Flow chromatography. The desalted cytosol fraction was applied to a S-Sepharose Fast-Flow column, and eluted with a 0–300 mM NaCl gradient with a fraction size of 6 ml. Aliquots (40 μl) were assayed for the NADPH oxidase activity. (B) HAC-5CP chromatography. The fractions indicated by the bar in the S-Sepharose Fast-Flow chromatography were applied to a HAC-5CP column, and eluted with a 10–300 mM sodium phosphate gradient with a fraction size of 1 ml. Aliquots (10 μl) were assayed for NADPH oxidase activity. (C) Cosmogel CM chromatography. The fractions indicated by the bar in the HAC-5CP chromatography were applied to a Cosmogel CM column, and eluted with a 0–500 mM NaCl gradient with a fraction size of 1 ml. Aliquots (10 μl) were assayed for the NADPH oxidase activity. (D) Superose 12HR chromatography. SP-1 fraction obtained from the Cosmogel CM chromatography indicated by the bar was applied to a Superose 12HR column. Fractions of 0.5 ml were corrected, and 30 μl aliquots were assayed for the NADPH oxidase activity. Allows show eluting position of molecular weight standards: 1, ferritin (440 kDa); 2, catalase (232 kDa); 3, bovine serum albumin (67 kDa).

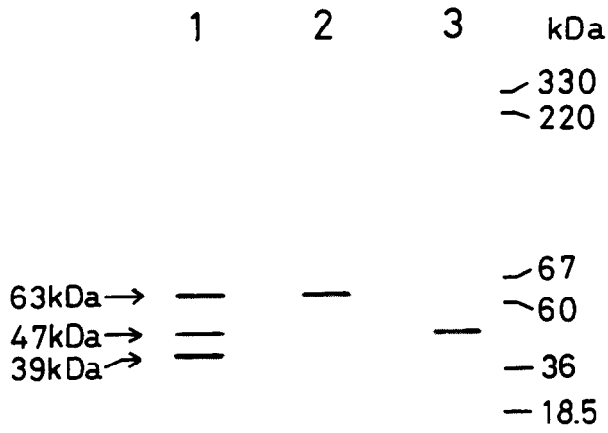

Fig. 2. SDS-PAGE and immunoblot analyses of purified SP-1. Lane 1, silver staining pattern; lane 2, immunoblot analysis with anti-human $p67^{phox}$ antibody; lane 3, immunoblot analysis with anti-human $p47^{phox}$ antibody. Molecular weight standards are indicated on the right.

in 0.05% trifluoroacetic acid. Amino acid sequences of the two peaks were analyzed on an automated Applied Biosystems gas-phase sequencer (447A) equipped with an on-line PTH-amino acid analyzer (Model 120A).

3. RESULTS AND DISCUSSION

As reported previously, we separated SP-1 using a cation-exchange column [21]. Fig. 1A shows an elution profile of cytosol fraction on a S-Sepharose Fast-Flow cation-exchange chromatography. A single peak of activity (SP-1) was eluted at about 0.18 M NaCl, and was further purified using a HAC-5CP hydroxyapatite column (Fig. 1B) followed by a Cosmogel CM cation-exchange column (Fig. 1C). SP-1 was eluted from those columns at 250 mM sodium phosphate and 380 mM NaCl, respectively.

SP-1 eluted from the Cosmogel CM HPLC was applied to a Superose 12HR column, and purified as a single protein peak with a molecular weight of 260 kDa (Fig. 1D). From 500 mg of cytosol protein, 0.12 mg of SP-1 was recovered. The yield was 17%, and the increase in the activity was 25.6-fold.

When purified SP-1 was analyzed on SDS-PAGE, 63 kDa, 47 kDa and 39 kDa protein bands were observed (Fig. 2, lane 1). Under reducing and non-reducing conditions, the same SDS-PAGE pattern was observed (data not shown), suggesting that these three proteins were not joined by disulfide bonds. The 63 kDa and 47 kDa proteins proved to correspond to human $p67^{phox}$ and $p47^{phox}$, respectively, from the results of Western blot analysis using anti-human $p67^{phox}$ and anti-human $p47^{phox}$ antibodies (Fig. 2, lanes 2 and 3). On the other hand, the 39 kDa protein band did not react with either antibody. Furthermore, the amino acid sequences of the two peptides obtained from the lysyl endopeptidase-digested 39 kDa protein (peptide 1, LHITQQDNYSVYNTTPSATQ; peptide 2, DIAVEEDLSSTPPF) did not exhibit homology to the amino acid sequences of $p67^{phox}$, $p47^{phox}$ and small GTP-binding proteins reported so far. These results indicate that the 39 kDa protein is a novel protein different from the 63 kDa, 47 kDa and small GTP-binding proteins.

Fig. 3 illustrates a typical profile of hydrophobic interaction chromatography of SP-1. After treatment with 500 μM DTT, SP-1 was applied to a Phenyl-TOYOPEARL 650 column equilibrated with a DTT-containing buffer. Of the three proteins of SP-1, the 47 kDa protein was eluted at about 0.45 M ammonium sulfate, but the 63 kDa and 39 kDa proteins were eluted as a complex at about 0.05 M ammonium sulfate. Next, after pretreatment with DTT, detergents (3-[(3-cholamidpropyl)dimethylammonio]-1-propanesulfonate (CHAPS), *n*-octyl-β-D-glucoside or nonidet P-40) and ethanol, SP-1 was applied to a DEAE anion-exchange column equilibrated with 20 mM HEPES, pH 7.5, containing these reagents. The 47 kDa protein was not adsorbed on the DEAE column, whereas the 63 kDa and 39 kDa proteins were adsorbed on the column and eluted as a complex at 0.25 M NaCl (data not shown).

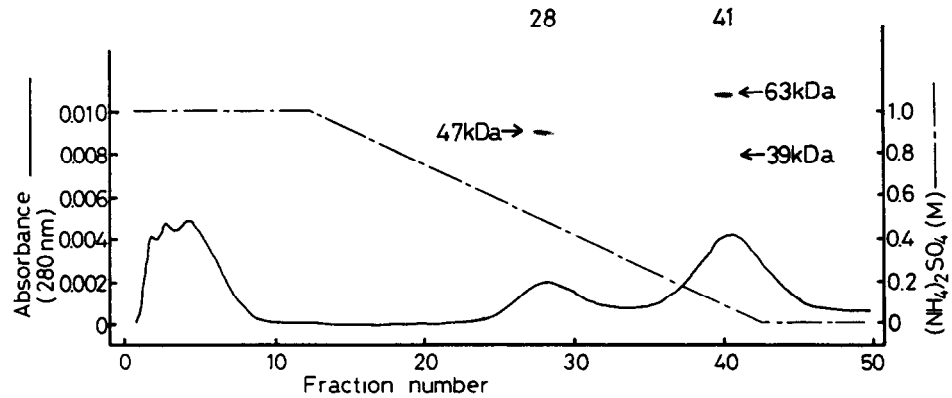

Fig. 3. Hydrophobic chromatography of purified SP-1. After pretreatment with DTT as described in section 2, SP-1 was applied to a Phenyl-TOYOPEARL 650 column, and eluted with a 1.0–0 M ammonium sulfate gradient. Fractions of 0.7 ml were collected. Insert: SDS-PAGE analysis of fractions 28 and 41 obtained from the chromatography. A front peak (fractions 1–9) did not contain protein.

Therefore, the 63 kDa and 39 kDa proteins seem to associate tightly with each other.

At this stage, a role of the 39 kDa protein in superoxide production is not clear. However, as the 39 kDa protein tightly associates with the 63 kDa protein, the 39 kDa protein seems to regulate the function of the 63 kDa protein, for example, its interaction with the 47 kDa protein.

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