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Purification of the 28.5 kDa cytosolic protein involved in the activation of NADPH oxidase from guinea pig neutrophils

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We tried to purify a new protein component required for the activation of NADPH oxidase from the guinea pig neutrophil cytosolic fraction which did not contain $p47^{phox}$ and $p67^{phox}$, using HAC-5CP, IEC-QA and Superose 12HR columns. The NADPH oxidase-activating activity was separated into three fractions on IEC-QA anion-exchange HPLC. However, when each of the fractions was purified by Superose 12HR gel filtration, the active fraction eluted at the same position, and was found to contain a common protein with a molecular weight of 28.5 kDa on SDS-PAGE. These results suggest that the 28.5 kDa protein is a novel NADPH oxidase activating protein.

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

1. INTRODUCTION

The superoxide (O_2^-) is essential for microbicidal and cytotoxic activities of neutrophils and other professional phagocytic cells [1]. O_2^- is produced by the activation of plasma membrane-bound NADPH oxidase, and the studies of O_2^- production using cell-free systems have suggested that multiple cytosolic components are required for the activation of NADPH oxidase [5–10]. The $p47^{phox}$ and the $p67^{phox}$ are well known as the cytosolic components of human neutrophils [6,11–13]. Recently, it has been reported that a cytosolic GTP-binding protein is involved in the regulation of the activation of NADPH oxidase [14,15].

On the other hand, we have separated three cytosolic factors (SP-1, QA-1 and QA-2) from guinea pig neutrophils using ion-exchange chromatography, i.e. SP-1 was adsorbed on a cation-exchange column, and QA-1 and QA-2 were adsorbed on an anion-exchange column, and we found that the combination of these factors fully activated the NADPH oxidase [10]. Using immunoblot analysis, we have found that SP-1 contains both the 47 kDa and the 63 kDa proteins which corresponds to human $p47^{phox}$ and $p67^{phox}$, respectively [16]. On the other hand, in preliminary experiments we have observed that QA-2 contains the 63 kDa protein, whereas QA-1 does not contain the proteins corresponding to $p47^{phox}$ and $p67^{phox}$. Therefore, in this study, we have tried the purification of a new protein from QA-1.

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

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2. MATERIALS AND METHODS

2.1. *Preparation of neutrophils*

Guinea pig neutrophils were isolated from exudates 13–15 h after an intraperitoneal injection of 0.17% glycogen in 0.9% saline as previously described [17]; the purity of the cells was >96%.

2.2. *Preparation of 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 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. The cells were disrupted by sonication in ice, and the cytosol and membrane fractions were prepared as described previously [10]. These fractions were stored at −80°C until use.

2.3. *Purification of cytosolic component*

To change the buffer, 80 ml of the cytosolic fraction (containing 650 mg protein) was applied to a Sephadex G-25 column (Pharmacia LKB Biotechnology, Sweden) equilibrated with 20 mM HEPES, pH 7.5, containing 0.5 mM EGTA and 0.5 mM PMSF (buffer A), and the void fraction was applied to a Q-Sepharose Fast-Flow column (26 × 120 mm, Pharmacia LKB Biotechnology) equilibrated with buffer A. After washing with buffer A, the column was eluted with a 0–500 mM NaCl gradient at a flow rate of 4.0 ml/min. The active fraction (a total volume of 108 ml) obtained from the Q-Sepharose Fast-Flow chromatography was concentrated using an ultrafilter UK-10 with a molecular mass cut-off of 10 kDa (Toyo Roshi Kaisha, Ltd., Japan) to a volume of 20 ml. The concentrated sample was mixed with the two volumes of 10 mM sodium phosphate buffer, pH 7.0, containing 0.17 M sucrose, and was applied to a Biofine HAC-5CP hydroxyapatite column (7.5 × 75 mm, JASCO-JAPAN Spectroscopic Co., Ltd., Japan) equilibrated with 10 mM sodium phosphate buffer, pH 7.0, containing 0.17 M sucrose. After washing with the equilibration

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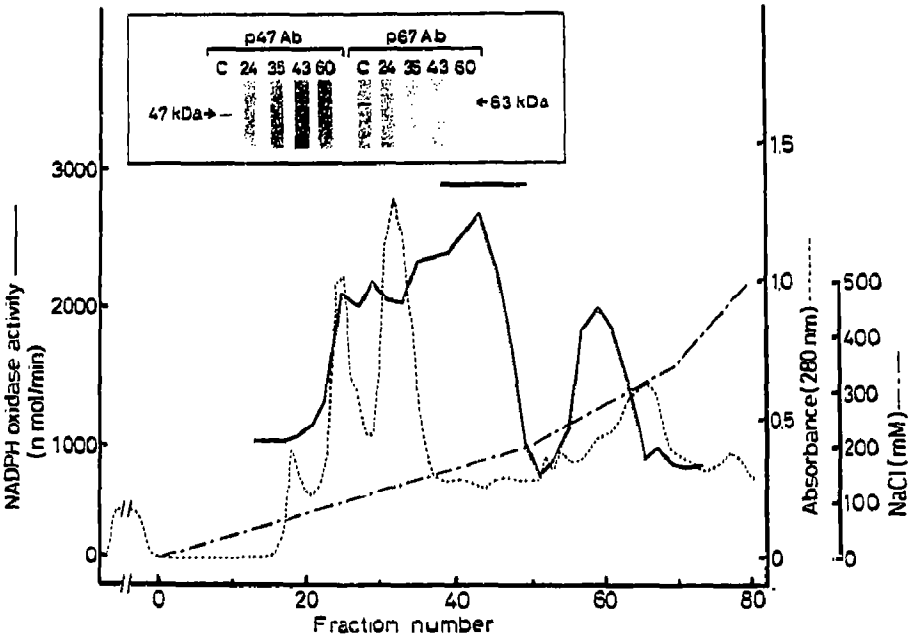

Fig. 1 Q-Sepharose Fast-Flow chromatography of the guinea pig neutrophil cytosol fraction. Cytosol fraction (650 mg protein) was applied to a Q-Sepharose Fast-Flow column, and eluted with a 0–500 mM NaCl gradient with a fraction size of 12 ml. Hundred microliter aliquots were assayed for the NADPH oxidase-activating activity. The fractions 40–48 indicated by the bar, were used for further purification. Insert, immunoblot analysis with anti-human $p47^{phox}$ (p47 Ab) or anti-human $p67^{phox}$ (p67 Ab) antibodies. C and numbers (24, 35, 43 and 60) indicate the crude cytosol and the fraction numbers, respectively.

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 was mixed with 10 volumes of distilled water, and applied to a Biofine IEC-QA anion-exchange column (7.5 × 75 mm, JASCO) equilibrated with buffer A. The column was eluted with a 0–200 mM NaCl gradient at a flow rate of 1.0 ml/min. The active fractions were concentrated using an ultrafilter UK-10 to a volume of 0.6 ml, and applied to a Superose 12HR gel filtration column (10 × 300 mm, Pharmacia LKB Biotechnology) equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 0.13 M NaCl, 0.5 mM PMSF, and 1 mM EGTA at a flow rate of 0.5 ml/min.

2.4. *Assay of the NADPH oxidase-activating activity*

Assay of the NADPH oxidase was performed in a cell-free system using an arachidonic acid as a stimulant [10].

The activity of QA-1 was assayed in the presence of SP-1, because QA-1 needed SP-1 to show activity [10]. SP-1 was prepared from 100 mg cytosol fraction by the separation procedure described previously [10], and a threshold amount of SP-1 (about 12 μg) was added in the assay mixture. The assay mixture consisted of 200 μM NADPH, 80 μM cytochrome *c*, 5 mM $MgCl_2$, 10 μM FAD, 20 μM arachidonic acid, 15 μg membrane fraction, SP-1 (15–25 μl) and each fraction from chromatography in the total volume of 0.5 ml of 65 mM sodium phosphate buffer (pH 7.0) containing 0.17 M sucrose. The assay was performed at 37°C in an Hitachi 557 dual-wavelength spectrophotometer (Hitachi, Ltd., Japan) and the absorbance difference at 550–540 nm was followed continuously. The activity was calculated using an extinction coefficient of 2.1×10^4 $M^{-1} \cdot cm^{-1}$.

2.5. *Polyacrylamide gel electrophoresis*

SDS-PAGE was carried out with a 8–16% linear gradient gel in the presence of 0.1% SDS by the method of Laemmli and Favere [18]. Samples were treated with 10 mM dithiothreitol at 100°C for 3 min. The gel was silver-stained using a commercially available reagent kit (Daiichi Pure Chemicals Co., Japan).

2.6. *Immunoblot analysis*

Proteins in each fraction were subjected to SDS-PAGE (8–16% gel) and were electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated with a Block Ace (Dainippon Pharmaceutical Co. Ltd., Japan), and reacted with a 1:2000 dilution of mouse anti-human $p47^{phox}$ or anti-human $p67^{phox}$ monoclonal antibody, or with a 1:400 dilution of mouse anti-myosin light-chain monoclonal antibody (Sigma Chemical Co., USA). The proteins were detected by the incubation with horseradish peroxidase-conjugated goat anti-mouse IgG followed by development with diformazan using the POD Immunostain Set (Wako Pure Chemical Industries Ltd., Japan).

The sensitivity of the anti-myosin light chain monoclonal antibody was examined using partially purified guinea pig macrophage $p21^{rac1}$, and 1 pmol of $p21^{rac1}$ was detected by the antibody.

3. RESULTS AND DISCUSSION

As shown in Fig. 1, two peaks of the activities appeared on a Q-Sepharose Fast-Flow anion-exchange chromatography. One activity was eluted as a broad peak over a range from 100 to 190 mM NaCl, and another activity was eluted at about 250 mM NaCl. The proteins in these fractions seemed to be anionic judging by their adsorption behavior on anion-exchange chromatography. Furthermore, judging from the elution pattern the first peak and the second peak seemed to be QA_1 and QA-2, respectively, as described previously [10]. QA-2 (fraction 60) contained a 63 kDa protein reacting with anti-human $p67^{phox}$ antibody, but did not contain protein(s) reacting with anti-human $p47^{phox}$ antibody (Fig. 1, insert). These results indicate that guinea

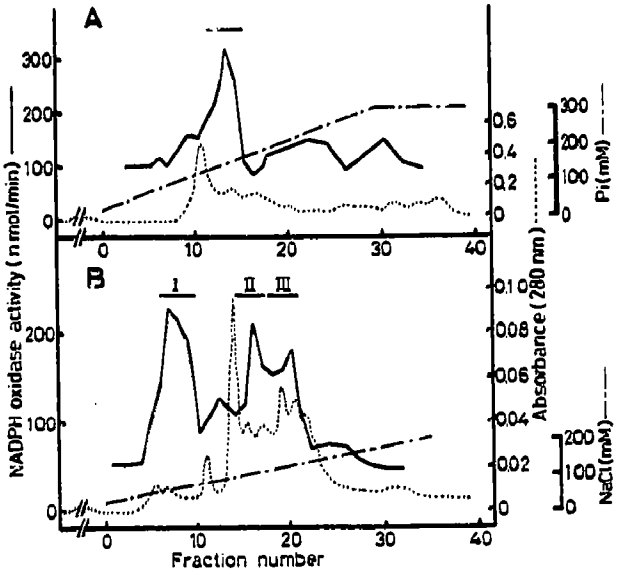

Fig. 2. HAC-5CP and IEC-QA chromatographies of the cytosolic component. (A) The active fraction from Q-Sepharose Fast-Flow chromatography was applied to a HAC-5CP column, and eluted with a sodium phosphate gradient with a fraction size of 1 ml. Hundred microliter aliquots were assayed for the NADPH oxidase-activating activity. The bars indicate the fractions that were used for further purification. (B) Fractions 12–15 obtained from the HAC-5CP HPLC were applied to a IEC-QA column, and eluted with a 0–200 mM NaCl gradient with a fraction size of 1 ml. Hundred microliter aliquots were assayed for the NADPH oxidase-activating activity. Fractions 8–10, fractions 16–18 and fractions 19–21 were designated as Peak I, Peak II and Peak III, respectively.

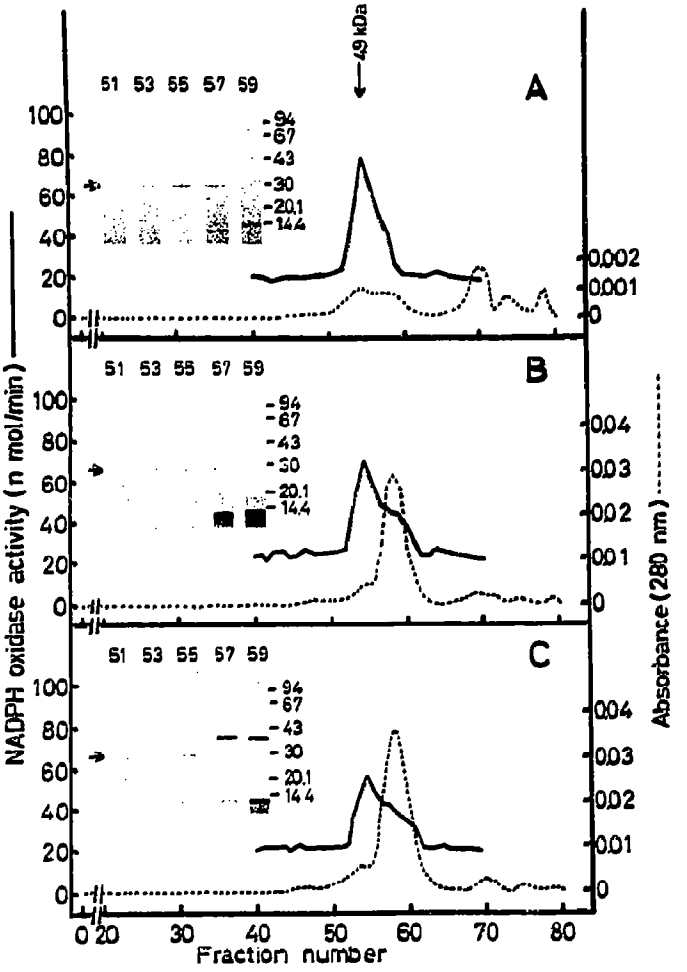

Fig. 3. Superose 12HR chromatography of Peaks I, II and III. Three active peaks obtained from IEC-QA HPLC were purified on a Superose 12HR column with a fraction size of 0.5 ml. A, B and C indicate the elution profiles of Peak I, Peak II and Peak III, respectively. One hundred and fifty aliquots were assayed for the NADPH oxidase-activating activity. Insert, SDS-PAGE analysis of the fractions 51, 53, 55, 57 and 59. Molecular weight standards: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). The arrows show the position of the 28.5 kDa protein.

pig neutrophils also contain the 63 kDa NADPH oxidase-activating protein corresponding to human p67^{phox}, as reported in bovine neutrophils [19,20]. On the other hand, QA-1 (fractions 24, 35 and 43) did not react with anti- human p47^{phox} and anti-human p67^{phox} antibodies, indicating that QA-1 is a new cytosolic factor which does not contain the protein(s) corresponding to human p47^{phox} and human p67^{phox}. The fractions 40–48 were further purified by a HAC-5CP hydroxyapatite column (Fig. 2A), because these fractions contained little contaminating protein. Then, the active fractions were applied to a IEC-QA anion-exchange column (Fig. 2B). As seen in the figure, the three active peaks were eluted at about 40 mM, 90 mM and 110 mM NaCl, and were designated as Peak I, Peak II and Peak III, respectively. Each of the peaks was applied to a Superose 12HR gel filtration column.

Peak I was eluted as a single peak with an apparent molecular weight of 49 kDa, which contained a single protein band with a molecular weight of 28.5 kDa on SDS-PAGE (Fig. 3A). Peak II (Fig. 3B) and Peak III (Fig. 3C) were also eluted with an apparent molecular weight of 49 kDa. SDS-PAGE analysis showed that these active fractions also contained the 28.5 kDa protein band, and that the density of this protein band paralleled the NADH oxidase-activating activity. These observations indicate that the 28.5 kDa protein is an essential component of QA-1.

Abo et al. have purified a third component (σ1) from guinea pig macrophages [21], which is composed of a small GTP-binding protein p21^{rac1} (22 kDa protein) and a GDP-dissociation inhibitor *rho*GDI (26 kDa protein), and suggested that p21^{rac1} is the active component of σ1, based on the results of the effect of recombinant p21^{rac1} on NADPH oxidase activity [14]. Recently, a small GTP-binding protein p21^{rac2} (22 kDa protein) has been reported to be a cytosolic regulatory component of the NADPH oxidase in human neutrophils [15]. The O_2^--producing activities of p21^{rac1} and p21^{rac2} have been reported to be dependent on GTP [14,15]. However, the NADPH oxidase-activating activity of the 28.5 kDa protein was hardly potentiated by the addition of 1–80

μM GTPγS (data not shown). Furthermore, we observed that the 28.5 kDa protein (2 pmol) did not react with the anti-myosin light chain antibody which reacted with $p21^{rac1}$ [14]. From the differences in molecular weights, the dependence of GTP and the reactivity against the antibody, the 28.5 kDa protein seems to be a new active component different from the GTP-binding proteins.

We have observed that SP-1, QA-1 and QA-2 activate the NADPH oxidase synergistically [10]. SP-1 contained the 47 kDa and the 63 kDa proteins corresponding to human $p47^{phox}$ and human $p67^{phox}$, respectively [16]. On the other hand, QA-2 contained the 63 kDa protein. Therefore, it seems to be possible that the 28.5 kDa protein, an essential component of QA-1, activates the NADPH oxidase through the interaction with the 47 kDa and/or the 63 kDa proteins. The detailed mechanism by which the 28.5 kDa protein activates the NADPH oxidase must be studied further.

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