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Actin Enhances the Activation of Human Neutrophil NADPH Oxidase in a Cell-free System

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The cell-free activation of human neutrophil NADPH oxidase (O_2^- generating enzyme) was enhanced by exogenously added G-actin (actin monomer). When cytosol, a constituent of the system, was pretreated with DNase I, which may bind to G-actin (endogenous) to block polymerization, the activation of NADPH oxidase was significantly suppressed. The activation was also impaired when cytosol G-actin was removed by DNase I-linked resin, being completely restored by the addition of G-actin. These results suggest a role of actin and its polymerization in the activation of NADPH oxidase of human neutrophils. © 1997 Academic Press

Superoxide (O_2^-) generation by phagocytic cells such as neutrophils exerts an important role in host defence against microbial infection (1). The enzyme that is responsible for O_2^- generation is called NADPH oxidase (or respiratory burst oxidase). The enzyme is dormant in resting cells and becomes active upon phagocytosis or agonist-induced activation (2).

The oxidase can be activated in a cell-free system consisting of plasma membrane and cytosol in the presence of anionic amphifile (2). NADPH oxidase is thought to be a multi-component enzyme, the subunits of which are located in the plasma membrane and cytosol. Recent studies have shown that membrane bound cytochrome b_{558} and three cytosolic factors (p47phox, p67phox, and rac) are essential for the activation (3) and upon activation they are believed to become associated to form an active complex. However, the mechanism for the activation of NADPH oxidase as well as its regulation has not been fully understood (3–5).

Neutrophils contain substantial amount of actin, a major component of cytoskeleton, as with other non-muscle cells. Upon cell activation, G-actin (actin monomer) polymerizes to F-actin (actin polymer), resulting in the organization of cytoskeleton. The relationship between actin cytoskeleton and NADPH oxidase has been suggested. Quinn et al. (6) reported that oxidase activity from PMA (phorbol myristate acetate)-stimulated neutrophils cosediments with heavy plasma membrane fraction, which includes actin and fodrin, another cytoskeletal protein. Babior's group found that the oxidase activity induced by PMA is restricted to the membrane cytoskeleton (7), and the three cytosolic factors are transferred to the cytoskeleton during activation (8).

In the previous papers, we demonstrated that the oxidase activity from cell or cell-free activation is extremely labile and remarkably stabilized by cross-linkers (9,10). However, the stabilized activity could not be extracted by detergents such as Triton X-100, suggesting that the enzyme complex is linked to the cytoskeleton.

In spite of the reports mentioned above, there has been no direct evidence for the involvement of actin in the oxidase activation or regulation, and it remains unclear if actin and its polymerization is involved in the oxidase activation. In the present study we examined the effect of actin on the activation of NADPH oxidase in a cell-free system and found that actin is required for the maximal activation of the oxidase. Based on the results, we suggest that endogenous actin may play a role in the activation of NADPH oxidase *in vivo.*

EXPERIMENTAL PROCEDURES

Materials. Actin (rabbit muscle) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). DNase I (bovine pancreas, Type II) and rabbit antibody against actin (C-terminal 11mer peptide) were purchased from Sigma Chemical Co.(St.Louis, MO, USA). Affigel-15 is a product of Bio-Rad Laboratories, Inc. (Hercules, CA, USA) and *trans*-epoxysuccinyl-leucylamido (4-guanidino) butane (E64) was ob-

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Abbreviations: G-actin, actin monomer; F-actin, actin polymer; PMA, phorbol myristate acetate; DNase I, deoxyribonuclease I; CA, carbonic anhydrase; PMSF, phenylmethansulfonyl fluoride; E64, *trans*-epoxysuccinyl-leucylamido (4-guanidino) butane; SDS, sodium dodecyl sulfate.

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tained from Genosys Biotechnologies Inc. All other reagents were of the best grade commercially available.

Neutrophil preparation and subcellular fractionation. Isolation of human neutrophils and subcellular fractionation of the cells were carried out as described previously (10) with minor modifications. Cells were suspended in Buffer C (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1mM ATP, 10 mM potassium phosphate buffer, pH 7.3) and subjected to N_2 cavitation. After sucrose gradient separation, the membrane fraction was supple-mented with 1 vol. of Buffer B (100 mM KCl, 3 mM NaCl, 4 mM $MgCl_2$, 1 mM EGTA, and 10 mM PIPES, pH7.0), sedimented by centrifugation at 200,000 × g for 1 hr, and resuspended in half concentration of Buffer B plus 0.34 M sucrose.

Cell-free activation and assay for O_2^- generation. Cytosol (74 ~ 97 μg) and plasma membrane (2.5 ~ 2.7 μg) were mixed per 50 μl of Buffer A (8 mM $MgCl_2$, 20 mM potassium phosphate buffer, pH 7.0), supplemented with sodium dodecyl sulfate (SDS) (final 240 μM), and incubated for 10 min at 25°C. An aliquot (10 μl) of the mixture was mixed with 240 μl of Buffer A containing 80 μM cytochrome *c* and 200 μM NADPH and transferred into sample cuvet. The reference cuvet contained the same content plus superoxide dismutase (200 μg/ml). Superoxide dismutase-inhibitable cytochrome *c* reduction was monitored by following the absorbance change at 550 nm. O_2^- generating activity was expressed as nmol of O_2^- formed per min per mg of plasma membrane protein.

Treatment of DNase I with protease inhibitors. To eliminate the activities of proteases contaminated in DNase I preparation commercially available, DNase I was treated with several protease inhibitors (5 mM diisopropyl fluorophosphate, 1 mM PMSF, 10 μM E64, and 1 mM EGTA) at 0°C for 10 min and dialyzed against 20 mM potassium phosphate buffer, pH 7.0 containing 1 mM EGTA for 2 hr and additional 2 hr against the same buffer without EGTA.

Preparation of DNase I-coupled resin. DNase I-resin was prepared using Affi-gel 15 resin (reactive agarose beads) following the method described by Ebisawa et al. (11) with some modifications. Prior to the coupling reaction, DNase I was treated with protease inhibitors as above and mixed with Affi-gel 15 resin in 0.1 M MOPS (pH 7.5) for 2 hr at 4 °C, followed by addition of 1M ethanolamine-HCl (pH 8.0). The coupled resin was then washed with 20 mM potassium phosphate buffer (pH 7.0). Carbonic anhydrase (CA)-resin was prepared by the same conditions except that CA was added instead of DNase I. The resins were stored at 4°C until use. These resins were referred to as "DNase I-resin" and "CA-resin", respectively.

DNase I-resin treatment of cytosol. Cytosol (130 μl, 7.2 mg/ml) was mixed rotately with DNase I-resin (130 μl slurry), which had been equilibrated with Buffer C, for 1 hr at 5°C and the mixture was centrifuged at 1,200 xg for 10 min. The supernatant was taken up and referred to as "DNase I-resin-teated cytosol". "CA-resin-treated cytosol" was prepared under the same conditions as above except that CA-resin was used. As a control, cytosol was rotated without resins and centrifuged as above.

Immunoblotting and densitometric analysis. After SDS-polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, BA85) and the blots were proved with antibodies against actin, p47phox, p67phox or rac 1. Antibodies against p47phox (C-terminal 13mer peptide), p67phox (fusion protein with β-Gal), and rac 1(C189S) were generous gifts from David Lambeth and David Uhlinger (Emory University) (12). The antibody against rac 1(C189S) was shown to recognize both rac 1 and rac 2. The blots were detected with a goat antirabbit IgG conjugated with peroxidase. After color development with 4-chloro-1-naphtol, the immunoblots were subjected to densitometric analysis with a gel scanning densitometer (Shimadzu CS-9000).

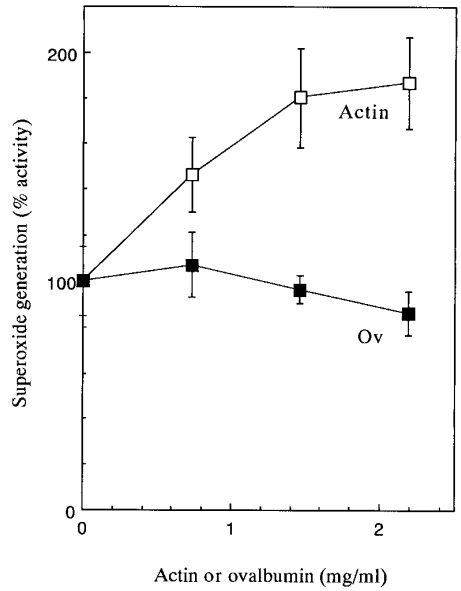

FIG. 1. Effect of actin on the cell-free activation of neutrophil NADPH oxidase. Cytosol (181 μg) was incubated with actin (70–210 μg) in 75 μl of 20 mM potassium phosphate buffer (pH 7.0) for 1 min at 0°C, and then the mixture was supplemented with plasma membrane (5.3 μg) and SDS (final 240 μM) in Buffer A (100 μl). The mixture was incubated for 10 min at 25°C. An aliquot (10 μl) of the mixture was taken and assayed for O_2^- generating activity as described under Experimental Procedures. The data points are the mean ± SE for three determinations.

RESULTS

Figure 1 shows the effect of G-actin on the cell-free activation of neutrophil NADPH oxidase. The control activity was 549 ± 81 (nmol O_2^- / min / mg of plasma membrane protein). When actin was added to the system, the activity increased about 2-fold. The concentration at the half maximal effect (EC_{50}) was 650 μg / ml of the activaton mixture (15.5 μM). When ovalbumin was added to the system as a control protein, no stimulating effect was observed, suggesting that the actin effect is not a general protein effect but a specific one. Also the addition of ATP, which was included in G-actin preparation for stabilization, had no effect on the activation. Besides when actin was added after the activation, it showed no effect on the activation.

Neutrophil cytosol contains substantial amount of actin, thus we examined the role of endogenous actin in the cell-free activation. Cytosol was treated with DNase I, which is well-known to form an 1 : 1 complex with actin (13) and interfere with its polymerization (14). The treatment of cytosol with DNase I apparently decreased its ability of activation of the oxidase (Fig. 2). The inhibitory effect was concentration-dependent and IC_{50} was around 1.0 mg/ml of the activation mixture (32 μM). When DNase I was treated with actin before adding to the system, the inhibitory effect of

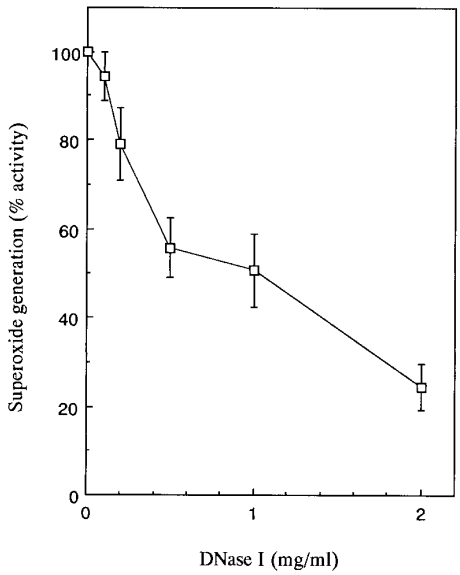

FIG. 2. Effect of DNase I on the cell-free activation of NADPH oxidase. DNase I, pretreated with protease inhibitors, was added to cytosol (74 μg) in 50 μl of 20 mM potassium phosphate buffer, pH 7.0. After incubation at 0°C for 1 min, the mixture was supplemented with plasma membrane (2.5 μg), $MgCl_2$ (final 8 mM), and SDS (final 240 μM). An aliquot of the mixture was assayed for O_2^- generating activity as described under Experimental procedures. The activities are expressed as mean ± SE for three to four determinations. The control activity was 521.7 ± 144.3 nmol per min per mg of membrane protein.

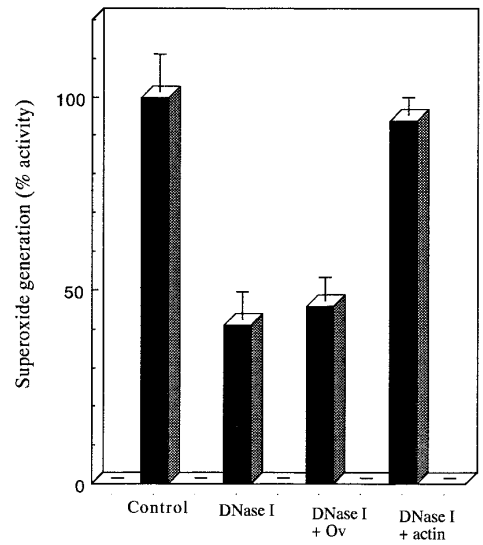

FIG. 3. Actin prevents DNase I from suppressing the oxidase activation. DNase I (75 μg) was treated with actin (110 μg) or ovalbumin (110 μg) at 4°C for 15 min, cooled on ice, and incubated with cytosol in 20 mM potassium phosphate buffer (pH 7.0) at 0°C for 1 min. Then the mixture was subjected to the cell-free activation (150 μl). Other experimental conditions are described in the legend to Fig.1. The activities are expressed as mean ± SE for three determinations. The control value is 553.4 ± 72.3 nmol per min per mg of membrane protein.

DNase I disappeared (Fig. 3), showing that actin-binding site of DNase I is responsible for the inhibition. These result suggest that endogenous actin (and perhaps its polymerization) is necessary for the maximal activation.

To further confirm this, we tried to deplete G-actin from the cytosol. Cytosol was treated with DNase I-linked resin (agarose beads) and then the resin was removed by centrifugation. Fig. 4A shows SDS-polyacrylamide gel electrophoresis of control and DNase I-resin-treated cytosol. The band at 42 kD, prominently found in the control (lane 1), was largely disappeared by DNase I-resin treatment (lane 3), being identified as actin by immunoblotting (Fig. 4B). When cytosol was treated with carbonic anhydrase (CA)-linked resin, the actin content was hardly influenced (lane 2). The densitometric analysis of immunoblots estimated that actin content of DNase I-resin-treated or CA-resin-treated cytosol was 4% or 88% of the control, respectively.

Figure 5 shows the activities from the cell-free activation using control and treated cytosol fractions. DNase I-resin-treated cytosol showed 38% activity of control, whereas CA-resin-treated cytosol showed 95% activity. To verify that the impaired activation is due to the loss of actin, we added actin to the system including DNase I-resin-treated cytosol and assayed for the oxidase activity (Fig. 5). When added to the system, actin restored the activation to the level of control at 70μg (16 μM in the activation mixture), the activity getting higher at 140 μg (32 μM).

It is possible that DNase I-resin treatment might remove some cytosolic components from cytosol, therefore we estimated for the amount of cytosolic factors in the treated cytosol by immunoblotting and densitome-

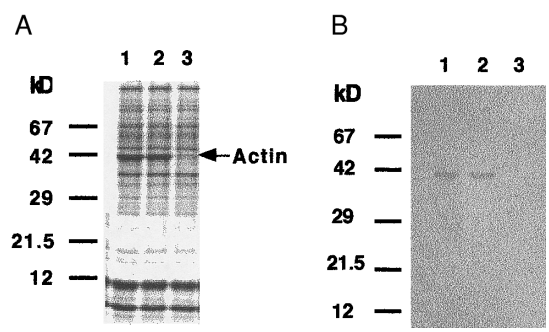

FIG. 4. Removal of actin from cytosol by DNase I- resin treatment. Cytosol was mixed with DNase I-resin (lane 3) or carbonic anhydrase (CA)-resin (lane 2) and rotated for 1 hr at 5°C. After centrifugation, the supernatant was taken up and subjected to SDS-polyacryl-amide gel electrophoresis and immunoblotting. The control cytosol was treated under the same conditions except that the resins are not included (lane 1). (**A**) SDS-polyacrylamide gel electrophoresis using 15% polyacrylamide gel. As molecular weight markers, bovine serum albumin (67kD), ovalbumin (42kD), CA (29kD), trypsin inhibitor (21.5kD), and cytochrome *c* (12kD) were used. (**B**) Immunoblotting using the antibody against actin.

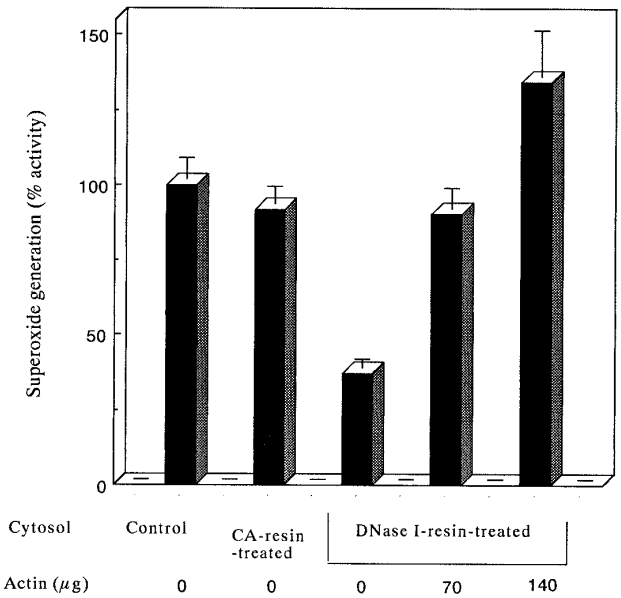

FIG. 5. Impaired activation by DNase I-resin-treated cytosol and restoration by added actin. Control or treated cytosol (194 μg) was used for the cell-free activation (100 μl) with 0–140 μg G-actin. Other experimental conditions are as described under Experimental Procedures. The activities are expressed as mean ± SE for three to four determinations. The control activity was 240.4 ± 10.1 nmol per min per mg of membrane protein.

try as above. Of three cytosolic components, p67phox and rac were almost quantitatively recovered (more than 90%), but p47phox was partially removed (61% recovery from the control). When added to the system, however, p47phox (recombinant) hardly restored the activation (data not shown). These results indicate that actin, and not p47phox, was limiting in the activation mixture consisting of DNase I-treated cytosol and that the impaired activation is due to the depletion of endogenous actin.

DISCUSSIONS

In the present study, we found that added G-actin enhances the cell-free activation of neutrophil NADPH oxidase. Depletion of endogenous actin from cytosol impaired its activating ability, and addition of G-actin completely recovered the activation. The results obtained here showed that actin is required for the maximal activation in a cell-free system although it does not seem essential for the activation.

As mentioned, neutrophils contain substantial amount of actin. According to Yassin et al. (15), human neutrophils contain actin at about 47 μM in total, and in resting cells actin is distributed between the cytosol and the cytoskeleton in the ratio of 65 : 35. The effective actin concentration observed in this study (Fig. 1 and Fig. 5) is comparable to its physiological concentration. Therefore, we consider that actin plays a role in the activation of the oxidase also *in vivo*.

The mechanism by which actin enhances the oxidase activation is not clear. When F-actin (polymerized) was added to the system, no enhancing effect was observed (data not shown). This suggests that actin polymerization should occur simultaneously with the complex formation although the possibility can not be excluded that actin functions as a monomer. It is plausible that F-actin (or actin cytoskeleton) provides a scaffold on which the oxidase components can assemble readily and the complex can be maintained stably. In fact, the activated oxidase was significantly destabilized under the conditions of actin depolymerization (unpublished result).

In our cell-free system, cytochalasin B did not inhibit the activation (data not shown). Although cytochalasin B is known to inhibit the elongation of polymerizing F-actin, it does not act on cortical F-actin, the actin filament that lies directly beneath the plasma membrane (16). We speculate that F-actin which could support the oxidase complex, would be cortical actin.

Cytosolic factors p47phox and p67phox have SH3 regions, which are considered to interact with actin cytoskeleton (17,18). These cytosolic factors are thought to occur as a 240-kD complex (19) with p40phox (20), another regulately protein which also contains SH3 region. We speculate that neutrophil actin (F-actin) may interact with either of these components. De Leo et al. (21) suggested that 240-kD complex may reorganize during the activation. The interaction of the complex with actin may facilitate the topological rearrangement of the complex.

Recently a G-protein rac, a cytosolic component of NADPH oxidase, was found to induce actin polymerization in fibroblast cells (22). It is tempting to speculate that rac facilitates the oxidase activation by inducing actin polymerization. Meanwhile, in a previous study we found that phosphatidic acid can induce NADPH oxidase activation in permeabilized human neutrophils (23). Phosphatidic acid was also reported to activate actin polymerization in fibroblast cells (24). Thus it seems possible that phosphatidic acid may enhance NADPH oxidase activation by actin polymeri-zation in neutrophils. Further study will be required to elucidate these possibilities.

Finally, what is the biological significance of the actin effect? It should be noted here that superoxide and its derivatives, which are bactericidal, are also toxic to the host cells. The complex formation of NADPH oxidase on the membrane cytoskeleton may restrict the area of respiratory burst to the phagocytic vacuole and avoid the burst at unfavorable site.

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