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COMBINATION OF ARACHIDONIC ACID AND GUANOSINE 5'-O-(3-THIOTRIPHOSPHATE) INDUCE TRANSLOCATION OF rac p21s TO MEMBRANE AND ACTIVATION OF NADPH OXIDASE IN A CELL-FREE SYSTEM

Tohru Sawai[1], Makoto Asada, Hiroyuki Nunoi[#2], Ichiro Matsuda[#], Satoshi Ando[*], Takuya Sasaki[*], Kozo Kaibuchi[*], Yoshimi Takai[*], and Kouichi Katayama

Department of Biochemistry, Tsukuba Research Laboratories, Eisai Co. Ltd., Tsukuba, Ibaraki 300-26, JAPAN

[#]Department of Pediatrics, Kumamoto University School of Medicine, Kumamoto 860, JAPAN

[*]Department of Biochemistry, Kobe University School of Medicine, Kobe 650, JAPAN

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Summary: The superoxide-generating NADPH oxidase system in phagocytes consists of membrane-associated cytochrome b558 and three cytosolic components named p67-phox, p47-phox, and rac p21s. In a cell-free system consisting of membrane and cytosol, the oxidase can be activated with guanosine 5'-O-(3-thiotriphosphate) (GTPγS) and an unsaturated fatty acid such as arachidonic acid (AA). Incubation of cytosol and membrane with AA alone caused clear translocation of p47-phox and p67-phox to the membrane, but only slight translocation of rac p21s. GTPγS alone did not significantly induce the translocation of rac p21s. However, GTPγS in combination with AA markedly augmented rac p21s translocation to the membrane. The translocation of rac p21s is not induced by GDP or GDPβS. These results indicate that the GTP-bound active form of rac p21s is the entity that is translocated to the membrane by the action of AA.

The production of superoxide radicals (O_2^-) by phagocytes such as neutrophils and macrophages, which play a key role in killing invading microorganisms, is catalyzed by the NADPH oxidase system (for reviews, see ref. 1). This NADPH oxidase system consists of membrane-associated cytochrome b558 and two cytosolic components, p47-phox and p67-phox (2-4). In addition, the augmentation of O_2^- generation by guanine nucleotides (5) implies involvement of a GTP-binding protein (G protein). G protein responsible for the oxidase activation was recently reported to be a member of the rac p21 family, which consists of rac1 and rac2 p21s (6-8). Subsequently, Ando et al. reported that both recombinant rac1 and rac2 p21s have an ability to stimulate the NADPH oxidase activity (9), and proposed that G protein

[1] To whom correspondence shoud be addressed.

[2] Present address: Department of Bacterial Infection, Tokyo University Institute of Medical Science, Tokyo 108, JAPAN.

Abbreviations used are: AA, arachidonic acid; G protein, GTP-binding protein; GDPβS, guanosine 5'-O-(2-thiodiphosphate); GTPγS, guanosine 5'-O-(3-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis; p-APMSF, 4-amidinophenylmethanesulfonyl fluoride; SOD, superoxide dismutase.

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implicated in the NADPH oxidase reaction includes both rac p21s. In resting cells, membrane-associated cytochrome b558 is located separately from the cytosolic components, while in activated cells, it is known that the catalytic activity of the oxidase is located only in the membrane fraction (10). This observation suggests that in the course of enzyme activation, the cytosolic components are translocated to the membrane. Evidence has recently accumulated indicating that p47-phox and p67-phox are translocated to the membrane during the oxidase activation (11-13). However, translocation of rac p21s remains to be clarified.

Small G proteins have interconvertible GDP-bound inactive and GTP-bound active forms (14). It has been reported that the NADPH oxidase activity was stimulated and inhibited by smg GDS (GDP dissociation stimulator) and rho GDI (GDP dissocation inhibitor), two different types of GTP/GDP exchange protein, respectively, through rac2 p21 (8). Moreover, several reports have suggested that small G proteins are translocated to the membrane in association with interchange to the GTP-bound active forms (15,16), but direct evidence for this translocation using specific antibodies against the proteins has not yet been obtained.

In a cell-free system consisting of membrane and cytosolic fraction, unsaturated fatty acids such as arachidonic acid (AA) are well known to activate O_2^- generation (17). Such a cell-free system could be valuable for studies on the mechanism for the NADPH oxidase activation. We therefore examined the translocation of rac p21s in a cell-free system by using anti-rac p21s antibody as a probe. This paper presents for the first time evidence that rac p21s are translocated to the membrane fraction only in the presence of both AA and GTPγS.

Materials and Methods

Materials——Mouse anti-p47-phox and anti-p67-phox monoclonal antibodies were prepared as described previously (18). Rabbit anti-rac p21s antiserum was raised against the peptide ^{123}KDTIEKLKEKKLT PITY, this sequence being common to rac1 and rac2 p21s except for ^{135}Thr, which replaces ^{135}Ala in rac2 p21. The antiserum obtained by immunization with this peptide recognizes both rac1 and rac2 p21s. All other chemicals were purchased from Sigma.

Fractionation of membrane and cytosol of differentiated HL-60 cells——HL-60 cells were differentiated into neutrophil-like cells by treatment with 100 nM retinoic acid for 5 days. The cytosol and membrane fractions of the differentiated HL-60 cells were prepared as described previously (19) with some modifications. All preparation procedures were carried out at 4°C. The cells (5 x10^9 cells) were suspended in 25 ml of relaxation buffer (10 mM KH_2PO_4/K_2HPO_4 at pH 7.2, 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 20 μM 4-amidinophenylmethanesulfonyl fluoride (p-APMSF), and 15 μg/ml leupeptin) and disrupted by 10-strokes of a Teflon/glass homogenizer. After removal of unbroken cells and nuclei by centrifugation at 800 x g for 10 min, the cell lysates were layered on 25 ml of 15% (w/v) sucrose and centrifuged at 200,000 x g for 1 h. The resulting supernatant, floated on the 15% sucrose cushion, was further centrifuged at 200,000 x g for 1 h to remove residual membranes and stored at -80°C until use as the cytosol fraction. The pelleted fraction, passed through the 15% sucrose layer, was suspended in 10 ml of the relaxation buffer and centrifuged at 200,000 x g for 1 h on a discontinuous gradient of 10 ml of 15% sucrose on 10 ml of 40% sucrose. The fraction at the 15/40% sucrose interface was collected, diluted 3-fold with the relaxation buffer, and again centrifuged at 200,000 x g for 1 h. The resulting pellet was resuspended to a concentration of 5 x10^8 cell equivalents/ml in the relaxation buffer and stored at -80°C until use as the membrane fraction.

Cell-free NADPH oxidase assay——The cell-free NADPH oxidase activity was assayed by measuring the arachidonic acid-elicited superoxide generation, which was determined by measuring the SOD-inhibitable cytochrome c reduction (20).

Translocation assay——Cytosol thawed from -80°C was centrifuged at 200,000 x g for 30 min to remove aggregated proteins. Reaction mixtures (800 μl) contained centrifuged cytosol (100 μg of protein), membrane (8 μg of protein), 10 μM GTPγS, 20 μM p-APMSF, and 15 μg/ml leupeptin in the oxidase buffer. After a 5-min preincubation at 25°C, the reaction

was initiated by the addition of 25 μM AA. Following incubation for a further 20 min, the reaction mixtures were carefully layered onto 4 ml of 15% sucrose, and centrifuged at 200,000 x g for 1 h at 4°C to re-isolate the membrane fraction. The pelleted membranes were suspended in SDS-sample buffer and resolved by SDS-PAGE as described below. In some experiments, the re-isolated membranes were tested for residual oxidase activity in the cell-free system where no cytosol was added.

Electrophoresis and immunoblotting----The re-isolated membranes in the translocation experiments were subjected to SDS-PAGE on 10-20% polyacrylamide gradient gels using the Laemmli buffer system (21). The separated proteins were electrophoretically transferred onto a nitrocellulose sheet, probed with either mouse anti-p47-phox and p67-phox antibody (1:4000 dilution) or rabbit anti-rac p21s antiserum (1:100 dilution), further probed with either horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin antibodies (1:4000 and 1:8000 dilution, respectively), and finally detected by enhanced chemiluminescence with Amersham's western blotting detection system.

Results

Using the specific antibodies against each cytosolic component of NADPH oxidase, we examined the translocation of these proteins in a cell-free system. In dormant HL-60 cells differentiated into neutrophil-like cells, neither p67-phox nor rac p21s were detected in the membrane fraction, though p47-phox was weakly detected (Fig. 1, Memb). No change in the distribution of cytosolic components was found during the translocation experiments in the absence of any stimulation. When the cytosol and membrane fractions were incubated with 25 μM AA, p47-phox and p67-phox were translocated to the membrane fraction. On the other hand, only small amounts of rac p21s were found in the membrane fraction. The translocation of p67-phox and p47-phox to the membrane fraction was not due to a nonspecific precipitation of the proteins by AA as previously reported (12), since neither of the proteins precipitated in the absence of the membranes (data not shown). GTPγS at 10 μM alone did not significantly stimulate the translocation of rac p21s to the membrane; however, GTPγS in combination with AA markedly augmented the translocation. The rac p21s translocation to the membrane was also not due to a nonspecific precipitation of the protein by AA and GTPγS (data not shown). GTPγS had no effect on the behavior of p67-phox and p47-phox, indicating that both cytosolic

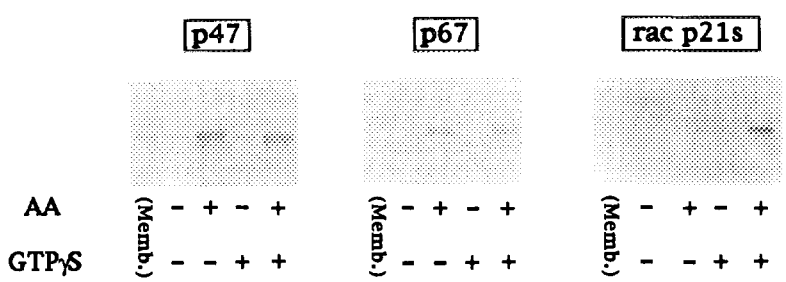

Fig. 1. **Translocation of cytosolic NADPH oxidase components to the membrane upon activation with AA and/or GTPγS.** Mixtures of cytosol (100 μg protein) and membrane fractions (8 μg) were preincubated for 5 min at 25°C with or without 10 μM GTPγS and further incubated for 20 min with the vehicle or 25 μM AA (as indicated by +/- at the bottom of each lane). The membranes were then re-isolated by centrifugation, and subsequently analyzed by SDS-PAGE and immunoblotting as described under "Materials and Methods". Native membranes from differentiated HL-60 cells (8 μg) were also analyzed by SDS-PAGE and immunoblotting (Memb). Immunoblots are representative of three similar experiments.

Table I
NADPH oxidase activity of re-isolated membrane fractions from the cell-free translocation system

Treatment	NADPH oxidase activity (nmol superoxide /min /mg)
None	N.D.[a]
AA	0.88 ± 0.04
GTPγS	N.D.[a]
AA/GTPγS	9.79 ± 0.50
without re-isolation [b]	11.70 ± 0.62

[a] Not detected. [b] NADPH oxidase activity was directly tested in a cell-free system without re-isolation of cytosol and membrane.

After membrane fractions had been re-isolated as described in the legend to Fig. 1, the NADPH oxidase activity of the membranes was measured as described under "Materials and Methods". The values shown are means ± S.E. for triplicate experiments.

proteins could be fully translocated to the membrane by AA alone. These results of translocation experiments are completely consistent with those of NADPH oxidase activity of the re-isolated membranes from the cell-free translocation system (Table I). When the cytosol and membrane fractions were treated with both GTPγS and AA, 84% of the oxidase activity was recovered in the re-isolated membrane fraction. On the other hand, little or no O_2^- was generated by the membranes re-isolated from translocation assay mixtures that lacked AA, GTPγS, or both.

To determine the specificity of guanine nucleotides, we next examined the effects of GDP and its nonhydrolyzable analogue, GDPβS, on the NADPH oxidase activity and rac p21s translocation. In the presence of AA, GTPγS caused a 6-fold increase in the NADPH oxidase activity, whereas GDP and GDPβS had no effect (Table II). In accordance with these results on the oxidase activity, translocation of rac p21s was induced by GTPγS but not by GDP in the presence of AA (Fig. 2). The effect of GDPβS was rather inhibitory to the slight translocation of rac p21s induced by AA alone. In the absence of AA, NADPH oxidase activation and rac p21s translocation scarcely occurred even in the presence of GTPγS (data not shown).

Table II
Effects of guanine nucleotides on NADPH oxidase activity

guanine nucleotide	NADPH oxidase activity (nmol superoxide/min/mg)
None	2.29 ± 0.08
GTPγS	13.55 ± 0.02
GDP	1.90 ± 0.08
GDPβS	2.08 ± 0.49

Mixtures of cytosol and membrane fractions were preincubated for 5 min at 25°C with or without 10 μM GTPγS, or GDP, or GDPβS, and then incubated with 25μM AA for 20 min. The NADPH oxidase activity was measured as described under "Materials and Methods". The values shown are means ± S.E. for triplicate experiments.

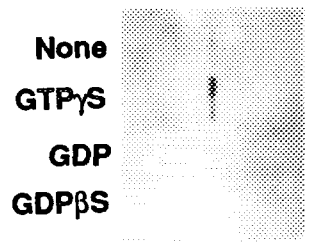

Fig. 2. **Effects of guanine nucleotides on rac p21s translocation.** The reaction conditions were identical to those given in Table II and translocation assay of rac p21s was performed as described under "Materials and Methods".

Discussion

We have shown here for the first time that rac p21s are substantially translocated to the membrane fraction only in the presence of both AA and GTPγS, whereas translocation of p47-phox and p67-phox is dependent on AA alone. This finding provides a clear explanation for the well known phenomenon that the NADPH oxidase is not significantly activated unless both AA and GTPγS are present in a cell-free system (5). The requirement of both AA and GTPγS for substantial translocation of rac p21s suggests that the GTP-bound active form of rac p21s is the entity that is translocated to the membrane by the action of AA. Slight induction of rac p21s translocation by AA alone might be due to the small population of rac p21s present as the GTP-bound form in dormant cells (22). It is conceivable from this point of view that the inhibitory effect of GDPβS on the slight rac p21s translocation induced by AA alone reflects conversion of endogenously present GTP-bound form of rac p21s to the GDP-bound form, which results in inability of rac p21s to translocate to the membrane. We further examined the specificity of guanine nucleotides for rac p21s translocation. Since the translocation is induced by GTPγS but not by GDP or GDPβS, this process appears to be a specific response to activation of rac p21s. The rac p21 family consists of rac1 and rac2 p21 and belongs to the ras p21/ras p21-like small G protein superfamily (23). rac1 and rac2 p21s share 92% amino acid identity, and only 15 amino acids of 192 amino acids differ from each other. The putative effector regions of rac1 and rac2 p21s have the same amino acid sequence. It has recently been shown that both recombinant rac1 and rac2 p21s produced by a baculovirus/insect cell system have an ability to stimulate the NADPH oxidase activity (9). In differentiated HL-60 cells, both rac p21s mRNAs are present (23), and anti-rac p21s-antibody used in this study recognizes both rac1 and rac2 p21s. It is therefore unknown at present which rac p21 mainly contributes to the NADPH oxidase activation in intact cells.

In neutrophils and macrophages, an increase in phospholipase A2 activity and subsequent release of AA are caused by stimuli which also activate the NADPH oxidase (24,25). Our findings give the first intriguing evidence that not only GTPγS, but also AA regulates the association of small G protein with its target enzyme. Considering that membrane-permeant fatty acids, such as AA, serve as a second messenger (26,27), the NADPH oxidase system may also point up the role of AA as a second messenger, which acts on small G protein. Further studies are needed to elucidate the molecular mechanism of the NADPH oxidase activation, and the present work may well pave the way for this elucidation.

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