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Change in the Conformation of $p47^{phox}$ by Sodium Dodecyl Sulfate, an Activator of the Leukocyte NADPH Oxidase

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The leukocyte NADPH oxidase of neutrophils is a membrane-bound enzyme that catalyzes the production of O_2^- from oxygen using NADPH as an electron donor. Dormant in resting neutrophils, the enzyme acquires catalytic activity when the cells are exposed to appropriate stimuli. During activation, the cytosolic oxidase components $p47^{phox}$ and $p67^{phox}$ migrate to the plasma membrane, where they associate with cytochrome b_{558}, a membrane-bound flavohemoprotein, to assemble the active oxidase. The oxidase can be activated in a cell-free system; the activating agent usually employed is an anionic amphiphile such as sodium dodecyl sulfate (SDS). Because $p47^{phox}$ can translocate by itself during activation, the conformational change in $p47^{phox}$ may be responsible for the activation of NADPH oxidase. We show here that the treatment of $p47^{phox}$ with SDS leads to an increase in the reactivity of the sufhydryl group of cysteines toward N-ethylmaleimide, indicating that the conformational change occurs when $p47^{phox}$ is exposed to SDS. We propose that this change in conformation results in the appearance of a binding site through which $p47^{phox}$ interacts with cytochrome b_{558} during the activation process.

Keywords: Conformational change, NADPH oxidase, N-ethylmaleimide, $p47^{phox}$.

Introduction

The NADPH oxidase of phagocytes (the leukocyte NADPH oxidase), an important element of host defense against microbial infection, catalyzes the reduction of oxygen to O_2^- using NADPH as the electron donor (Babior, 1992). The oxidase is dormant in resting neutrophils, but acquires catalytic activity when cells are exposed to appropriate stimuli. Oxidase activity is located in the plasma membrane, but it is known that in resting cells the oxidase components are distributed between a membrane fraction and the cytosol, and that when activation takes place either in intact cells or in a cell-free system, the oxidase components $p47^{phox}$ and $p67^{phox}$, which exist in the cytosol as a ~240 kDa complex (Clark *et al.*, 1987; Park *et al.*, 1992; 1994), and the small guanine nucleotide-binding protein *rac2*, which also participates in oxidase activation (Knaus *et al.*, 1991; 1992), migrate to the membrane, where the $p47^{phox}$.$p67^{phox}$ complex associates with the membrane cytochrome b_{558} to assemble the functioning oxidase (Heyworth *et al.*, 1991; Park *et al.*, 1992; Tyagi *et al.*, 1992).

It has been known that in a cell-free system containing neutrophil membranes and cytosol, the NADPH oxidase can be activated by an anionic amphiphile such as sodium dodecyl sulfate (SDS) or arachidonate (Bromberg and Pick, 1984; Heyneman and Vercauteren, 1984; Curnutte, 1985; McPhail *et al.*, 1985). In an earlier study using the cell-free system, we found that the activation of the leukocyte NADPH oxidase by SDS was associated with the appearance of a membrane binding site on a cytosolic oxidase component (Park *et al.*, 1992), presumably $p47^{phox}$, since $p47^{phox}$ can translocate by itself whereas $p67^{phox}$ cannot translocate without $p47^{phox}$ (Heyworth *et al.*, 1991). This result suggested that oxidase activation involves a conformational change affecting the cytosolic components. Therefore, it is plausible to assume that SDS could lead to a change in the conformation of $p47^{phox}$ that causes the translocation of $p47^{phox}$ to the membrane during the activation of the leukocyte NADPH oxidase. We have recently developed a method to covalently label $p47^{phox}$ to assess changes in the conformation of $p47^{phox}$ that occur during activation (Park and Babior, 1997). The approach we used was based on the idea that such a conformational change may alter the accessibility of one or more of the

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four cysteine residues of $p47^{phox}$ (C98, C111, C196, and/or C378) to -SH group specific reagents. We describe here that the exposure of $p47^{phox}$ to SDS appears to involve a conformational change that is reflected in a change in the susceptibility of -SH groups of $p47^{phox}$ to alkylation by the -SH reagent N-ethylmaleimide (NEM).

Materials and Methods

Materials Chemicals, enzymes, and molecular biology reagents were obtained from the following sources: pGEX-1λT vector, dextran, Ficoll-Hypaque (Pharmacia, Uppsala, USA); NADPH, ferricytochrome c, bovine erythrocyte superoxide dismutase (SOD), NEM, ampicillin, glutathione (GSH), GSH-agarose, phenylmethylsulfonyl fluoride (PMSF), thrombin, carboxymethyl (CM)-Sepharose (Sigma, St. Louis, USA); N-[2-^{3}H]ethylmaleimide ([^{3}H]NEM; specific activity, 50.08 Ci/mmol), 1.0 mCi/ml (Dupont-New England Nuclear, Boston, USA); Amplify (Amersham, Arlington Heights, USA); restriction enzymes (New England BioLabs, Beverly, USA); *Eco*RI linker (Stratagene, La Jolla, USA); Bio-Rad protein assay kit, electrophoresis, and immunoblotting reagents (Bio-Rad, Hercules, USA).

Preparation of neutrophil fractions Neutrophil cytosol and membrane were prepared as described previously (Park and Babior, 1992). Briefly, neutrophils were obtained from normal subjects by dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn citrate-coagulated blood. The neutrophils were suspended at a concentration of 10^8 cells/ml in a modified relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES buffer, pH 7.3), and plasma membrane and cytosol were prepared by nitrogen cavitation and centrifugation through Percoll by the method of Borregaard (Borregaard *et al.*, 1983). Both the cytosol and membrane were divided into aliquots and stored at −70°C until use.

Preparation of wild-type and mutant recombinant $p47^{phox}$ proteins Amino acid substitution to Ala at C98, C111, C196, C378 of $p47^{phox}$ was produced by site-specific mutagenesis as previously described (Kunkel, 1985). Full details of the construction of mutated genes will be reported elsewhere. The transformed *Escherichia coli* containing the pGEX-1λT plasmid, with an insert of the wild-type or mutated $p47^{phox}$ cDNA (Fig. 1), were grown, and the glutathione S-transferase–$p47^{phox}$ fusion protein purified on GSH-agarose as previously described (Park, 1996). The fusion protein was cleaved by treatment with thrombin (10 U/ml) in elution buffer containing 150 mM NaCl and 2.5 mM $CaCl_2$ for 2 h at room temperature. The protein mixture was further purified on a CM-Sepharose column equilibrated with the 5 mM phosphate buffer, pH 7.0, containing 0.1 mM PMSF and eluted with a 40-ml gradient of 0–0.4 M NaCl in the same buffer. The fractions containing $p47^{phox}$ were concentrated to 1 mg/ml and stored at −70°C. The concentration of proteins was determined with the Bio-Rad assay kit using bovine serum albumin as a standard.

Labeling of $p47^{phox}$ by [^{3}H]NEM Preparations of $p47^{phox}$ (1 μg) were treated at room temperature with 2 μl (2 μCi) of [^{3}H]NEM in a total volume of 0.1 ml. After incubation for the indicated time, the sample was placed on ice for 5 min and the labeled protein was precipitated by adding 50% (w/v) trichloroacetic acid to a final concentration of 10%. Transfer RNA (250 μg) was also added as a carrier. After a further 10 min on ice, the proteins were pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C. The precipitate was suspended in 100 μl of H_2O, mixed with water-compatible scintillation fluid (Ready Safe®, Beckman), and assayed for radioactivity in a liquid scintillation counter. For SDS-PAGE, the labeled samples were precipitated with acetone for 1 h at −20°C and the precipitates isolated by centrifugation for 20 min at 13,000 × *g* (4°C). For analysis, the precipitates were dissolved in Laemmli sample buffer.

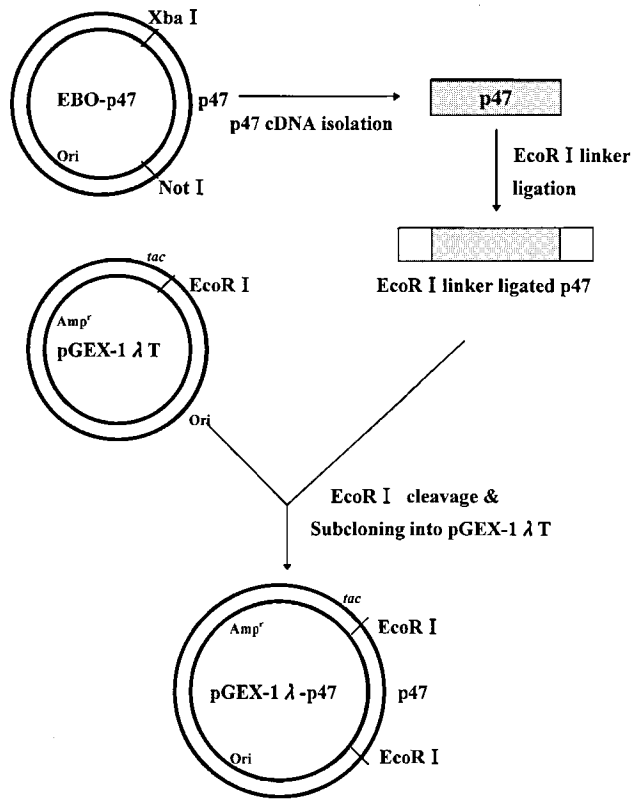

Fig. 1. Construction of the expression plasmid for the $p47^{phox}$ fusion protein.

Cell-free activation of the NADPH oxidase O_2^- production by SDS-activated NADPH oxidase was measured as the superoxide dismutase-inhibitable reduction of ferricytochrome c by NADPH (Park *et al.*, 1992). Assay mixtures contained 0.1 mM cytochrome c, 90 μM SDS, 0.16 mM NADPH, 1.6×10^6 cell equivalent of membrane, 10^6 or 10^7 cell equivalent of cytosol as indicated, and relaxation buffer to a final volume of 0.75 ml. All components except NADPH were mixed in the cuvette and equilibrated at 25°C for 1 min. Reactions were then started by adding the NADPH in 25 μl relaxation buffer, and cytochrome c reduction was followed at 550 nm, read against a reference containing the same components plus 45 μg of superoxide dismutase.

Electrophoresis and immunoblotting Protein samples were subjected to SDS-PAGE on 8% polyacrylamide gels using the Laemmli buffer system (Laemmli, 1970). The separated proteins were electrophoretically transferred onto a nitrocellulose sheet (Towbin *et al.*, 1979), which was blocked with dried milk, and then probed with partially purified rabbit polyclonal antibodies raised against synthetic peptide from p47^{phox} used at a dilution of 1:10,000, and finally detected with a 1:2000 dilution of alkaline phosphatase-labeled goat anti-rabbit Ig antibody (Sigma) using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as substrate (Bio-Rad).

Autoradiography of polyacrylamide gels The detection of radiolabeled p47^{phox} on Laemmli gels was accomplished by soaking the gels in Amplify (Amersham) with agitation for 30 min to increase the signal, followed by drying and then finally placing the dried gels in direct contact with X-ray film and an intensifying screen at −70°C. For a more accurate quantitation, the regions of the gels containing the radioactive proteins were excised using an autoradiogram as a template, and the gel slices were placed in counting vials and dissolved by incubating overnight at room temperature with gentle agitation in a freshly prepared mixture of 150 μl of ammonium hydroxide (20–22%), 1 ml of NCS solublizer (Amersham), and 10 ml of scintillation fluid, as described above. The resulting solutions were assayed by liquid scintillation counting. Background counts were determined by the same procedure using a piece of gel of similar size excised from a tritium-free region of the gel.

Replicates Each result described in this paper is representative of at least three separate experiments.

Results and Discussion

Addition of 90 μM SDS to the mixture of the membrane fraction and the cytosol fraction, that was derived from unstimulated human neutrophils, induced NADPH-dependent O_2^- formation (Fig. 2A). Only minimal amounts of O_2^- were generated in the absence of SDS. The activity of the recombinant p47^{phox} protein was evaluated by its ability to support SDS-dependent O_2^- generation by the cell-free system using a suboptimal amount of cytosol (10^6 cell eq.), which is approximately 10% of the cytosol used for a standard cell-free assay, to supply the other cytosolic oxidase components. The enhancement of oxidase activity by supplementing the recombinant p47^{phox} (Fig. 2B) indicates that the structure and function of recombinant p47^{phox} is the same as that of endogenous p47^{phox}.

In order to evaluate the degree of labeling in the individual cysteine residue in p47^{phox} with [^{3}H]NEM, the wild-type and the cysteine mutants were expressed in *E. coli*, purified as a GST-protein, and subsequently cleaved by thrombin and repurified p47^{phox} by CM-sepharose. Immunoblotting confirms that wild-type and mutant proteins cross-reacted with antipeptide antibody against p47^{phox} (data not shown).

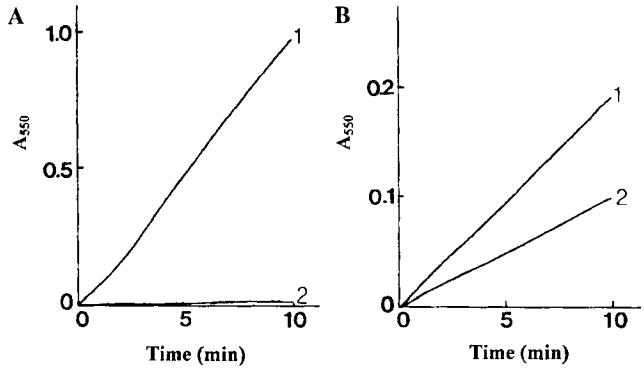

Fig. 2. A. Time courses of SDS-induced formation in the cell-free system. The O_2^- formation was measured by determining the rate of SOD-inhibitable cytochrome c reduction at 25°C. 1, Complete assay mixture; 2, an assay mixture lacks of SDS. B. Ability of recombinant p47^{phox} to enhance O_2^- production in the cell-free oxidase activating system in the presence of SDS. The assay mixture receiving cytosolic oxidase components in the form of a suboptimal amount of cytosol (10^6 cell eq.). 1, An assay mixture containing 10 μg recombinant p47^{phox}; 2, an assay mixture lacking recombinant p47^{phox}.

The oxidase subunit p47^{phox}, which consists of 390 amino acid residues, contains four cysteine residues: C98, C111, and C196 in the N-terminal half of the molecule, and C378 in a location very close to the C-terminus. As shown in Fig. 3, when the wild-type and mutant p47^{phox} proteins, in which the individual cysteine was replaced by Ala, were labeled with [^{3}H]NEM, the extent of labeling of protein was significantly decreased in the C378A mutant protein. In contrast, the labeling of other mutant proteins with [^{3}H]NEM was affected to a much lesser degree. This result indicates that C378 contains the most reactive -SH to NEM, which is consistent with the result obtained with the CNBr cleavage of [^{3}H]NEM labeled p47^{phox} and following

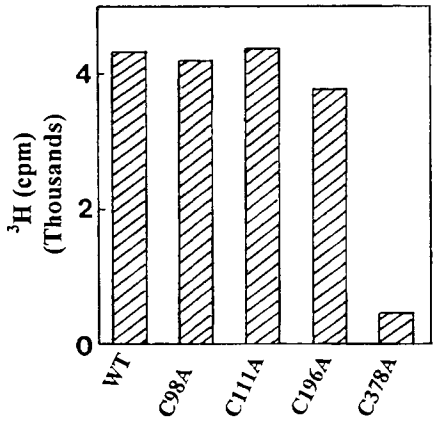

Fig. 3. Labeling of p47^{phox} and four cysteine mutant p47^{phox} by [^{3}H]NEM. Labeling of recombinant proteins was measured by liquid scintillation counting.

Tris-tricine gel electrophoresis (Park and Babior, 1997). It was also confirmed that little radioactivity was found from the deletion mutant $p47^{phox}\Delta348$ (Park *et al.*, 1997), which lacks the 43 C-terminal amino acids including C378 (Fig. 4).

As shown in Fig. 5A, $p47^{phox}$ treated with SDS resulted in a considerable increase in the rate of alkylation of the protein by [^{3}H]NEM as measured by liquid scintillation counting. These findings imply that, as a result of activation, a change occurs in the conformation of $p47^{phox}$ that changes the access of alkylating agent to the cysteine residue of $p47^{phox}$. The increase of labeling was also observed (Fig. 5B) when untreated and SDS-treated recombinant $p47^{phox}$ were incubated for 5 min with [^{3}H]NEM, as determined by SDS-PAGE and autoradiography.

Although $p47^{phox}$ is known as an essential component of the NADPH oxidase activation, the molecular function of this cytosolic subunit has not been elucidated clearly. *In vivo* (Heyworth *et al.*, 1991) and *in vitro* (Park *et al.*, 1992) results indicate that the only known function of $p47^{phox}$ so far is as a mediator of translocation of whole cytosolic components of NADPH oxidase. It is a consensus opinion that $p47^{phox}$ can migrate on its own from the cytosol to the membrane during oxidase activation. Other cytosolic factors such as $p67^{phox}$ and *ras* related G-protein *rac 2* can only migrate to the membrane in the presence of $p47^{phox}$ (Heyworth *et al.*, 1991; Park *et al.*, 1992).

Earlier studies have suggested that a conformational change involving the leukocyte NADPH oxidase subunit $p47^{phox}$ participates in the activation of the enzyme. These studies showed (1) that exposure of neutrophil cytosol to SDS at a concentration that activates the enzyme in the cell-free system results in the development of a membrane binding site on the $p47^{phox} \cdot p67^{phox}$ complex (Park *et al.*, 1992) and (2) that SH3 domains that are occluded in resting $p47^{phox}$ by a proline-rich region near the C-terminus of the molecule are exposed by arachidonic acid, another oxidase-activating lipid (Sumitomo *et al.*, 1994). We show here that the treatment of $p47^{phox}$ with SDS caused a change in the conformation of the protein which is reflected by a change in the accessibility of a specific modification reagent to the -SH group of cysteine.

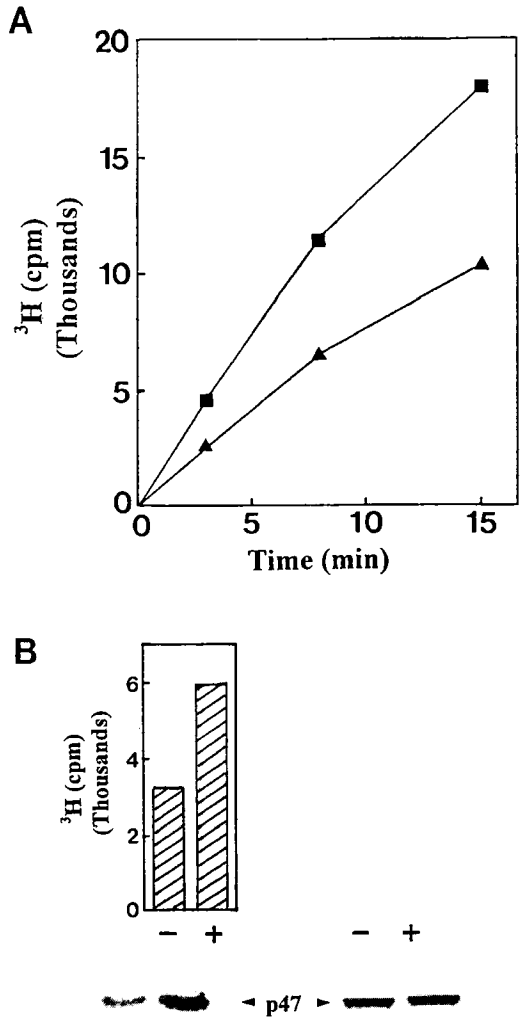

Fig. 5. Effect of SDS treatment on the labeling of recombinant $p47^{phox}$ by [^{3}H]NEM. A. Rates of labeling of recombinant $p47^{phox}$ as measured by liquid scintillation counting [(▲) no treatment; (■) SDS]. B. Left, Labeling of untreated or SDS-treated recombinant $p47^{phox}$ during a 5 min incubation with [^{3}H]NEM, as determined by SDS-PAGE and autoradiography. (−) No treatment; (+) treated with 90 μM SDS. Radioactivity in labeled bands excised from the gel and assayed by liquid scintillation counting. Right, Immunoblots of untreated and SDS-treated recombinant $p47^{phox}$. (−) No treatment; (+) treated with 90 μM SDS.

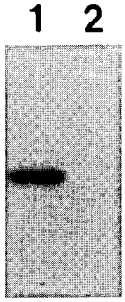

Fig. 4. Labeling of $p47^{phox}$ and $p47^{phox}\Delta348$ by [^{3}H]NEM. Recombinant $p47^{phox}$ (lane 1) and $p47^{phox}\Delta348$ (lane 2) were incubated for 5 min with [^{3}H]NEM and analyzed by SDS-PAGE followed by autoradiography.

The purpose of the conformational change is most likely to create binding sites for cytochrome b_{558} on one or more of the cytosolic oxidase components. Several stretches of sequence in both $gp91^{phox}$ and $p22^{phox}$ have been shown to interact with $p47^{phox}$ (Sumitomo *et al.*, 1994; Fuchs *et al.*, 1995). Of particular interest in connection with the present results are the findings that SH3 domains near the middle of $p47^{phox}$ interact with proline-rich domains in both $p22^{phox}$ (P151-P160) and $p47^{phox}$ itself (Q357-P364)

(Sumitomo *et al.*, 1994), and that peptides from the C-terminal quarter of p47^{phox} (A323-L332, Q334-G347, and Q357-P364) inhibit the activation of the oxidase and the transfer of p47 ^{phox} to the membrane in the cell-free oxidase activating system (Nauseef *et al.*, 1993; Finan *et al.*, 1994; DeLeo *et al.*, 1995). These results suggest that the short stretches of sequence may be of special importance for the binding of p47^{phox} to the membrane-associated components of the oxidase. These sequences are all located in the C-terminal quarter of p47^{phox}, and it is possible that the same conformational change that modulates the accessibility of NEM to C378 could lead to their appearance on the surface of the rearranged protein, where they would be available to bind to the membrane-associated components of the oxidase. However, the present results only suggest the existence of a conformational change of p47^{phox} during activation. The absolute conformational change of p47^{phox} caused by activation conditions awaits X-ray crystallographic data.

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