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ACTIVATION OF RESPIRATORY BURST OXIDASE IS ACCOMPANIED BY DESENSITIZATION OF p47*phox* IN NUCLEOSIDE-TRIPHOSPHATE BINDING ALONG WITH ITS TRANSLOCATION TO CELL MEMBRANE

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Summary: A cytosolic component of human neutrophils, p47*phox*, potentiates respiratory burst oxidase translocating from cytosol to membrane upon cell stimulation. In this study, the nucleotide-binding ability of p47*phox* was examined using [^{32}P]GTP dialdehyde (oGTP), [^{32}P]oATP, and [^{32}P]oNADPH. p47*phox* showed affinities for both oGTP and oATP that were 14 times higher than that for oNADPH, suggesting that it is a nucleoside triphosphate (NTP)-binding protein rather than an NADPH-binding protein. Binding analysis of p47*phox* using either [^{32}P]oGTP or [^{32}P]oATP revealed an apparent binding constant for each individual NTP analogue and the same maximum binding value, which suggests that both NTPs share a common specific binding site. Stimulation of neutrophils with phorbol myristate acetate (PMA) resulted in enhancement of the oxidase activity to generate O_2^- anion and was accompanied by substantial translocation of p47*phox* to membrane. However, p47*phox* derived from the stimulated cell membrane had lost its NTP-binding ability, unlike that from the resting cytosol. These results suggest that the binding of NTP to p47*phox* may be involved in the process that activates the oxidase and is desensitized in translocated p47*phox*.

Professional phagocytes, such as neutrophils and monocytes, reduce molecular oxygen by one electron to superoxide anion within seconds in response to various stimuli, a process referred to as the respiratory burst (see review, 1-4). This process, which contributes greatly to the killing of microbes, is caused by activation of a membrane-bound multicomponent enzyme, an NADPH oxidase system, whose components are distributed in cell membrane and cytosol in the resting state (5, 6). The significance of this NADPH oxidase is exemplified by chronic granulomatous disease (CGD), a genetically heterogeneous group of disorders characterized by recurrent infections due to a defective O_2^- generation. Membrane-component cytochrome b_{558}, a low redox-potential heme

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Abbreviations: CGD, chronic granulomatous disease; HTG, n-heptyl-β-D-thioglucopyranoside; NTP, nucleoside triphosphate; oATP/oGTP/oNADPH/oNTP, 2', 3'-dialdehyde derivatives of ATP/GTP/NADPH/NTP; PMA, phorbol myristate acetate; PSL, photostimulative luminescence; PVDF, polyvinylidene difluoride microporous membrane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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protein composed of large and small subunits, and two cytosolic components, p47^{phox} and p67^{phox}, are defective in the more common X-linked form and in a rare form of CGD, respectively (1-4). Recently, *rac* p21s, which are regulated to form the interconvertible GDP-bound inactive and GTP-bound active forms by their stimulator and inhibitor for GDP dissociation, were proposed as regulatory components in this NADPH oxidase system (7-9).

p47^{phox} is phosphorylated in multiple sites and translocates from cytosol to cell membrane during cell stimulation (10). Failure of p47^{phox} translocation and its incomplete phosphorylation occur in neutrophils from cytochrome b_{558}-deficient CGD patients (11, 12). In addition, whereas p47^{phox} translocates to cell membrane in neutrophils with p67^{phox}-deficient CGD, the reverse never happens (12). Further, inhibition of cell-free oxidase activation with synthetic peptides for the carboxyl-terminal domains of the large and small cytochrome b_{558} subunits (13, 14) and binding analysis using [^{35}S]methionine-labeled recombinant p47^{phox} (15), have also demonstrated the interaction between p47^{phox} and cytochrome b_{558} in a more direct manner. These observations led to the notion that p47^{phox} is first anchored to the carboxyl-terminal of cytochrome b_{558}, and subsequently draws p67^{phox}. Duplicate motifs of a *src* homology (SH)3 domain in both p47^{phox} and p67^{phox} (16, 17), which is important for protein-protein interaction, may participate in this assembly. However, aside from this fairly well-characterized organizational interaction of p47^{phox} with the other oxidase components, little is known about its biochemical features except for the aforementioned phosphorylation.

p47^{phox} was first identified as a protein that was retained on a GTP-agarose affinity column (18). The nucleotide sequence of the cDNA and the derived amino acid sequence of p47^{phox} suggested that it contained a potential nucleotide-binding site (16). Nonetheless, studies using nucleotide-affinity matrices have only indicated that p47^{phox} shows ambiguous specificity with regard to GTP, ATP, and NADPH (18, 19). Therefore, in the present study, we synthesized [^{32}P]oGTP, [^{32}P]oATP, and [^{32}P]oNADPH to quantitatively compare the ability of p47^{phox} to bind GTP, ATP, and NADPH, respectively. Subsequently, the NTP-binding ability of membrane-derived p47^{phox} from stimulated cells was compared with that of cytosol-derived p47^{phox} from resting cells .

Materials and Methods

Isolation of neutrophils and subcellular fractionation- After informed consent was obtained, human neutrophils from normal subjects and autosomal recessive CGD patient were isolated and disrupted by sonication in buffer A: 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, and 10 mM Pipes (pH 7.3) containing 10 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride as previously described (20, 21). The sonicate was centrifuged at 500 x g for 5 min, and the postnuclear supernatant was then separated into membrane and cytosol fractions by centrifugation at 200,000 x g for 20 min at 4 °C. The membrane fraction was resuspended in an initial volume of buffer A containing the above protease inhibitors. Resting cytosol was fractionated as above from neutrophils that had not been treated with any stimulants. To prepare PMA-stimulated and resting membrane fractions, neutrophils (2 x 10^6 cells) were stimulated for 5 min at 37 °C with 100 ng PMA and an equivalent concentration of dimethyl sulfoxide vehicle, respectively. After being washed, the cells were subjected to the same procedure as above. When stated, PMA-stimulated membranes were solubilized with a 0.4 % (w/v) sodium deoxycholate-0.2 % (v/v) Triton X-100 mixture by stirring 4 mg of membrane protein/ml of saline-20 mM Hepes buffer (pH 7.4) containing 30 % (w/v) glycerol and 20 μg Nα-*p*-tosyl-L-lysine chloromethyl ketone for 30 min in an ice-water

bath. The lysate was then separated into soluble and insoluble membrane fractions by centrifugation at 200,000 x g for 30 min at 4 °C. The soluble fraction was used as a source of membrane-derived p47^{phox} as described later. The amount of protein was determined by the method of Bradford (22).

Affinity labeling of cytosol and membrane fractions with [^{32}P]nucleotide dialdehydes- [^{32}P]oGTP and [^{32}P]oATP were synthesized from [α-^{32}P]GTP and [α-^{32}P]ATP by oxidation with $NaIO_4$ as described previously (20). [^{32}P]oNADPH was prepared from NAD^+ and [γ-^{32}P]ATP as detailed before (20). Aliquots of cytosol and membrane fractions were incubated with 0.1 mM of [^{32}P]oGTP, [^{32}P]oATP, or [^{32}P]oNADPH in 30 mM triethanolamine HCl buffer (pH 8.0) for 30 min, supplemented with 10 mM $NaCNBH_3$, and allowed to continue incubating overnight on ice.

Immunoprecipitation of cytosolic and membrane-derived p47^{phox}- To immunoprecipitate p47^{phox} in resting cytosol, rabbit antiserum raised against synthetic peptide corresponding to the carboxyl-terminal region of p47^{phox} was added in a sufficient amount (5~10 µl) to the immunoprecipitation buffer as previously reported (20). p47^{phox} that was conveyed to membrane by PMA-stimulation underwent the same process after being affinity labeled, solubilized with detergent, and ultracentrifuged as described above. After incubation with antiserum at 4 °C for 1.5 h, 4 mg of Protein A-Sepharose CL-4B was added and the mixture was rotated for an additional 1 h. Protein A-Sepharose beads washed as previously reported (20) were heated in the sample buffer and the eluted proteins were subjected to SDS-PAGE, stained with Coomassie Brilliant Blue, dried, and then exposed to an imaging plate at room temperature, followed by development in a bioimage analyzer (Fuji BAS 2000, Fuji Photofilm Corp., Tokyo). The radioactivity in the imaging plate was expressed as photostimulative luminescence (PSL) count or the amount of nucleotide bound to the p47^{phox} band. For the latter, known quantities of [^{32}P]nucleotide dialdehydes of internal controls were always exposed in parallel with test samples.

Immunoblotting- Subcellular fractions were subjected to SDS-PAGE and separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore Corp.) using a semi-dry-type electroblotting apparatus (AE-6675, Atto Corp., Tokyo). The protein-blotted PVDF sheet was probed with a mouse monoclonal antibody (only in Fig. 5) or the foregoing rabbit antibody against p47^{phox}. In the former, the immunoreactive band was visualized by color development of horseradish peroxidase-conjugated secondary anti-mouse Ig with *o*-dianisidine *plus* hydrogen peroxide. In the latter, a ^{125}I-labeled F(ab')$_2$ fragment against rabbit Ig was used as a secondary antibody and the PVDF sheet was exposed to either Kodak XAR-5 film at -80 °C or the imaging plate. For quantitative analysis of the translocation of p47^{phox} from cytosol to membrane, areas of the PVDF sheet which corresponded to the radioactive bands developed on film were excised, and ^{125}I radioactivities of the pieces were counted in a gamma counter (ARC-1000, Aloka, Tokyo).

Superoxide generation- The NADPH oxidase activity of membrane fractions was determined by the rate of superoxide dismutase-inhibitable cytochrome *c* reduction with a dual wavelength spectrophotometer (Hitachi 557) as reported previously (20).

Results

Nucleotide-binding ability of cytosolic p47^{phox} from resting cells- Earlier studies using nucleotide-affinity matrices suggested that p47^{phox} displayed ambiguous specificity regarding nucleotide recognition (18, 19). Therefore, p47^{phox} was quantitatively assessed here for its ability to bind GTP, ATP, and NADPH. Affinity labeling of cytosol derived from resting cells was carried out using one of the synthesized [^{32}P]nucleotide dialdehydes. In Fig. 1, the high binding ability of p47^{phox} to both [^{32}P]oGTP and [^{32}P]oATP was demonstrated by image-analyzing of an SDS-PAGE gel after immunoprecipitation with antiserum against p47^{phox}. The integrity of these [^{32}P]oNTPs in binding was verified by the competitive inhibition of their bindings to p47^{phox} with the unlabeled NTP (Fig. 1). The complete absence of a radioactive band in the cytosol from neutrophils with p47^{phox}-deficient autosomal recessive CGD further confirmed the specific binding of [^{32}P]oNTP to p47^{phox} (Fig. 1). p47^{phox} also bound [^{32}P]oNADPH as suggested by its

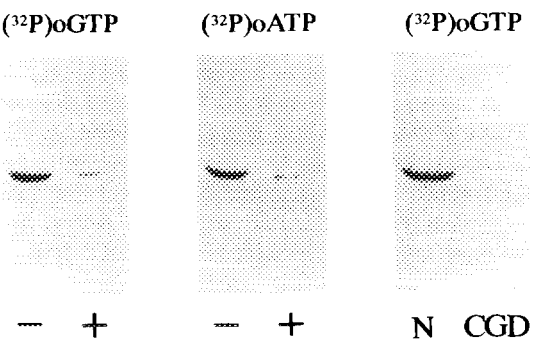

Fig. 1. Immunoprecipitation of [^{32}P]oNTP-bound cytosolic p47^{phox}. Aliquots of resting cytosol (1.5 x 10^7 cell equiv.) were preincubated without (-) or with (+) 15 mM unlabeled NTP for 2 h, and affinity labeled with 0.1 mM of each corresponding [^{32}P]oNTP overnight on ice. After immunoprecipitation with rabbit anti-p47^{phox} serum *plus* Protein A-Sepharose CL-4B, the proteins were subjected to SDS-PAGE (10 %), exposed to an imaging plate, and image-analyzed. N and CGD denote experiments performed using cytosol from normal and p47^{phox}-deficient CGD subjects, respectively.

ambiguous nature in nucleotide recognition (Table I) (18, 19). However, the incorporated count of [^{32}P]oNADPH was about 14-fold less than those of [^{32}P]oNTPs. In porcine neutrophils, dialysis of the cytosol did not dramatically augment the [^{32}P]oNADPH binding, which was thus still far less than the [^{32}P]oNTP binding (20). These results provide direct evidence that p47^{phox} is an NTP-binding protein intended for GTP and ATP rather than for NADPH.

Apparent affinity of cytosolic p47^{phox} to GTP and ATP- We attempted to clarify the NTP-binding property of p47^{phox} by determining the binding constant. After extensive dialysis against buffer A, an aliquot of resting cytosol was affinity labeled with an indicated concentration of either [^{32}P]oNTP. The amount of [^{32}P]oNTP bound to p47^{phox} was then estimated after having been immunoprecipitated by anti-p47^{phox} serum. A single class of saturable binding sites was

Table I
Binding activities of resting cytosolic p47^{phox} as to [^{32}P]nucleotide dialdehydes

Nucleotide analogues	Binding (pmol/10^7 cell equiv.)	Kd (μM)	Maximum binding (pmol/10^7 cell equiv.)
(dialysis)	(-)	(+)	(+)
[^{32}P]oGTP	0.88 ± 0.08	50	2.15
[^{32}P]oATP	0.86 ± 0.10	100	2.15
[^{32}P]oNADPH	0.06 ± 0.02	ND	ND

Aliquots of cytosol (1.5 x 10^7 cell equiv.) (-) from resting cells were affinity labeled with 0.1 mM of [^{32}P]oGTP, [^{32}P]oATP, or [^{32}P]oNADPH. For double reciprocal plots, aliquots of cytosol (1.5 x 10^7 cell equiv.) dialyzed extensively against buffer A (+) was also affinity labeled with various concentrations of either [^{32}P]oGTP or [^{32}P]oATP. Radiolabeled p47^{phox} was immunoprecipitated with rabbit anti-p47^{phox} serum *plus* Protein A-Sepharose CL-4B and analyzed by SDS-PAGE (10 %). The dried gel was image-analyzed and the radioactivity of each band was expressed as the amount of bound nucleotide to p47^{phox} using internal controls (see *Materials and Methods*). Data show means ± SD (n=3) performed in duplicate and representative one of three experiments, performed in duplicate, respectively. ND denotes not done.

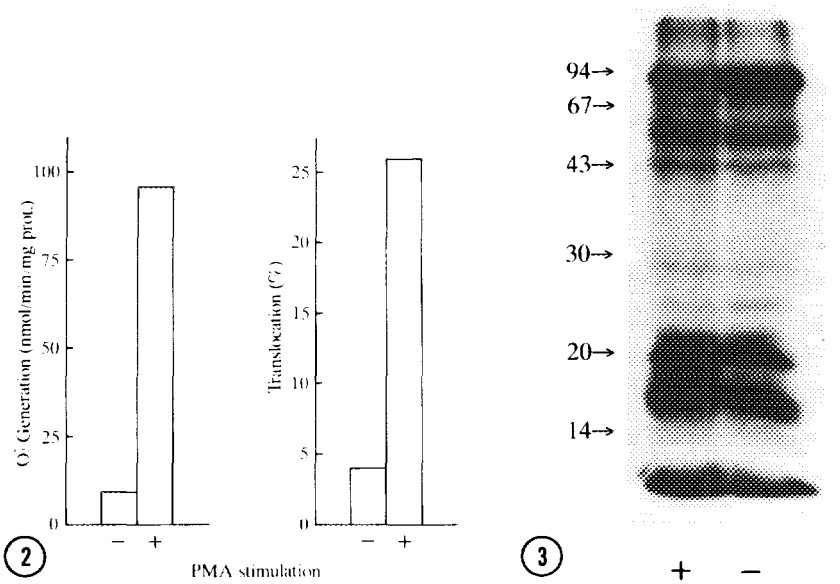

Fig. 2. Relationship between PMA stimulation and both p47^{phox} translocation and $O_2^{\cdot-}$-generating activity. Membrane and corresponding cytosol were fractionated from resting (-) and PMA-stimulated (+) cells. $O_2^{\cdot-}$ generation was initiated by adding 0.2 mM NADPH to 10 μg membrane protein. Data are means of one experiment performed in duplicate. Percent translocation of p47^{phox} was determined by immunoblotting of both fractions (2 x 10^6 cell equiv.) with rabbit anti-p47^{phox} serum *plus* a secondary ^{125}I-labeled F(ab')$_2$ fragment on a PVDF sheet (for details, see *Materials and Methods*). Results are expressed as the ratio of the count for the membrane fraction to the total count.

Fig. 3. Autoradiograph of [^{32}P]oGTP-binding proteins in resting and PMA-stimulated membranes. Both membrane fractions (5 x 10^6 cell equiv.) were affinity labeled with 0.1 mM [^{32}P]oGTP, analyzed by SDS-PAGE (13.5 %), and image-analyzed. PMA-stimulated (+) and resting (-) membranes exhibited the $O_2^{\cdot-}$-generating activities of 137.6 and 7.4 nmol/min/10^7 cell equiv., respectively.

obtained for each analogue: a Kd of 50 (100) μM and a maximum binding of 2.15 (2.15) pmol/10^7 cell equiv. for [^{32}P]oGTP (values in parenthesis are for [^{32}P]oATP) (Table I). A strict analysis would require that the values were under conditions of reversible binding using isolated p47^{phox} and native NTP instead of NTP dialdehyde. However, at least, the existence of these binding constants removes any apprehension that p47^{phox} may bind to NTP-agarose electrostatically, but not through an affinity for an NTP portion.

Affinity labeling of GTP-binding sites in membranes- Prior to affinity labeling of p47^{phox} with [^{32}P]oGTP in the stimulated membranes, we first assessed the degree of p47^{phox} translocation. As shown in Fig. 2, stimulation with PMA resulted in substantial translocation of p47^{phox} to the membrane fraction. After 5 min of incubation with PMA, 26 % of the total had become membrane-associated, along with an increase in $O_2^{\cdot-}$-generating activity, which is consistent with a previous report (5). Subsequently, the ability of p47^{phox} to bind [^{32}P]oGTP was compared between resting and PMA-stimulated membranes (Fig. 3). Bands at molecular masses near 20 kDa and 85 kDa were found to be labeled as in previous reports (23, 24), in which only these predominant small GTP-binding (~ 20 kDa) and 85 kDa proteins could be detected by either [α-

^{32}P]GTP or [^{35}S]GTPγS binding assay on nitrocellulose blot. On the other hand, in the present study, covalent radioactivity of [^{32}P]oGTP revealed additional GTP-binding proteins besides the two mentioned above even after separation by SDS-PAGE (Fig. 3). A band at 43 kDa may represent the binding to the α subunit of heterotrimetric G protein. Although broad tailing bands caused by glycosylation of membrane proteins, or some unknown mechanism, prevented their clear separation, there seemed to be no significant increment in radioactivity at 47 kDa in the PMA-stimulated membrane.

Solubilization of membrane-associated p47*phox*- p47*phox* that had translocated to the PMA-stimulated membranes was solubilized with a mixture of 0.4 % (w/v) sodium deoxycholate and 0.2 % (v/v) Triton X-100, as was used for rabbit neutrophils (25). After ultracentrifugation, amounts of p47*phox* in soluble and insoluble membrane fractions were determined by immunoblot analysis. Ninety-six percent of p47*phox* in the membrane fraction was recovered as a soluble membrane fraction. In contrast to the successful solubilization of O_2^--generating activity and cytochrome b_{558} of porcine neutrophil membrane with n-heptyl-β-D-thioglucopyranoside (HTG) (26), only 26.8 % of the total human neutrophil membrane-associated p47*phox* could be solubilized with 1 % (w/v) HTG. The p47*phox* standard curves for both the cytosolic and soluble membrane fractions were linear up to 80 μg protein (Fig. 4). Each immunoblot band in the inset in Fig. 4 corresponds to a plot in the standard curves.

Comparison of [^{32}P]oGTP-binding between membrane-derived p47*phox* and cytosolic p47*phox*- To overcome the obscure results using membrane particles (Fig. 3), we attempted to specifically assess the ability of p47*phox* to bind [^{32}P]oGTP. As part of this quantitative estimation, the effect of the detergent used for membrane solubilization on [^{32}P]oGTP-binding was examined using the cytosolic fraction. When the detergent was present during affinity labeling, the binding of [^{32}P]oGTP to cytosolic p47*phox* was inhibited in a dose-dependent manner (data not shown). Deformation of p47*phox*'s tertiary structure due to the surfactant action of the detergent seems to render its sound NTP-binding ability inert. Therefore, the detergent was added to the membrane fraction after affinity labeling with [^{32}P]oGTP.

Equal amounts of membranous and cytosolic p47*phox*, as estimated by the standard curves from the quantitative immunoblot (Fig. 4), were affinity labeled with [^{32}P]oGTP, treated with the detergent mixture, immunoprecipitated with rabbit anti-p47*phox* serum, and subjected to immunoblotting with a mouse monoclonal antibody. Single and equivalent bands of p47*phox* were obtained by developing both of the immunoprecipitates with *o*-dianisidine (Fig. 5, right). However, binding of cytosolic p47*phox* to [^{32}P]oGTP revealed double bands (Fig. 5, left). The higher band was superimposed on the immunoblot band. The lower band did not emerge unless the detergent was added. Therefore, [^{32}P]oGTP-bound p47*phox* may be divided into two different types due to the action of the detergent. One type represents the entire molecular mass. The other type seems to be cleaved in an amino-terminal side which includes the epitope that is recognized by the mouse monoclonal antibody, which differs from the carboxyl-terminal epitope which is involved in immunoprecipitation. However, p47*phox* that was derived from the PMA-stimulated membrane lysate exhibited far less radioactivity (10 PSL) than that from the resting cytosol (higher band, 546 PSL) (Fig. 5, left). These results demonstrate that p47*phox* in the resting cytosol is involved in the

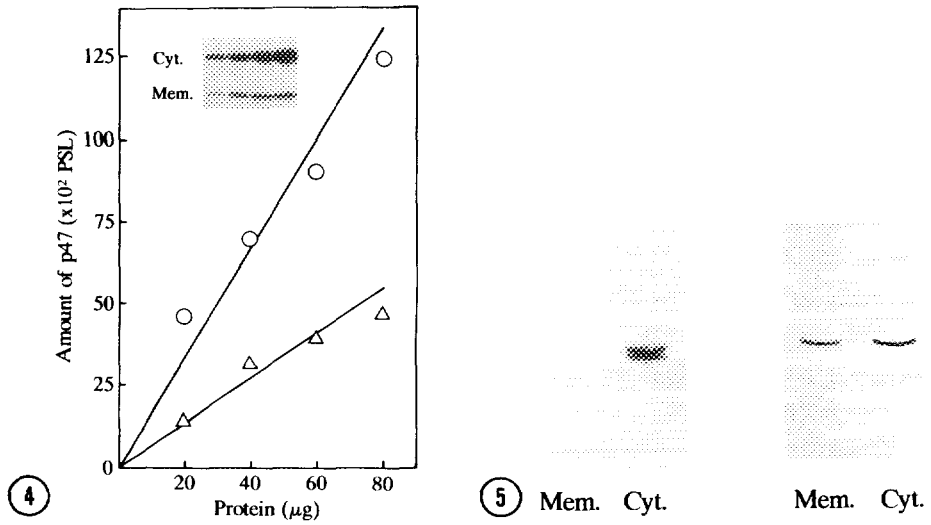

Fig. 4. Quantitative immunoblots of membrane-derived and cytosolic p47*phox*. PMA-stimulated membrane and resting cytosol (both 4 mg/ml) were incubated with a sodium deoxycholate-Triton X-100 mixture and ultracentrifuged as described in *Materials and Methods*. The amount of p47*phox* in the supernatant of either the cytosol (O) or soluble membrane (Δ) fraction was determined by an immunoblot technique. The standard curves show the amount of p47*phox* plotted as PSL count *versus* protein quantity in both of the fractions. The p47*phox* band (inset) revealed with a ^{125}I-labeled F(ab')$_2$ fragment against rabbit Ig corresponds to the amount of protein indicated in the standard curves.

Fig. 5. [^{32}P]oGTP-binding ability of membrane-derived and cytosolic p47*phox*. The amount of p47*phox* in PMA-stimulated membrane and resting cytosol was determined using the standard curves of the quantitative immunoblots as described in Fig. 4. Equal amount of membrane-derived and cytosolic p47*phox* were affinity labeled with 0.1 mM [^{32}P]oGTP, treated with a sodium deoxycholate-Triton X-100 mixture, and ultracentrifuged. The supernatants were then immunoprecipitated with rabbit anti-p47*phox* serum *plus* Protein A-Sepharose CL-4B, and subjected to SDS-PAGE (10 %). The transferred proteins were probed with a mouse monoclonal antibody against p47*phox* *plus* peroxidase-conjugated anti-mouse Ig, developed with *o*-dianisidine (right), and image-analyzed for [^{32}P]oGTP binding (left).

activation of the NADPH oxidase and loses its ability to bind NTP during translocation from cytosol to the cell membrane.

Discussion

Studies using a cell-free activation system and semipermeable neutrophils have supported the involvement of GTP-sensitive component(s) in the NADPH oxidase system. Recently, small GTP-binding proteins, *rac* 1 and *rac* 2 p21s, were proposed to be cytosolic GTP-dependent components (7-9). The superoxide-generating NADPH oxidase system seems to be regulated through the GDP/GTP exchange reaction on *rac* p21s. However, the possibility that p47*phox* is an NTP-binding component cannot be ruled out, since p47*phox* was initially found as a protein that was retained on a GTP-agarose affinity column (18). The antibody raised against this preparation contributed greatly to the identification of p47*phox* and p67*phox*, which are defective in neutrophils of patients with autosomal recessive CGD (18).

GTP-binding proteins possess at least three consensus elements, GXXXXGK(S/T) (G-1 region), DXXG (G-3 region), and (N/T)(K/Q)XD (G-4 region) (27). The sequence accumulation

of GTP-binding proteins has provided additional G-2 and G-5 regions as consensus elements (28, 29). p47^{phox} contains some regions if not all of 5 G-regions. The AGGSSGKT sequence motif (residue 262-269), which matches the less common A type of the consensus site, (G/A)XGAAGK(S/T) (G-1 region) (16), appears with variations in many NTP-ulilizing enzymes (28, 29). The sequence motif DITG (residues 151-154) (G-3 region) is conserved in all GTPases (29). However, since p47^{phox} does not contain all of the 5 G-regions, there is some doubt regarding its capacity as a GTP-binding protein, and even as an NTP-binding protein (30). However, previous studies using nucleotide-affinity matrices such as 2', 5'-ADP-, GTP-, and ATP-agarose have suggested that p47^{phox} could bind to NTP, although its specificity regarding nucleotide recognition is ambiguous (18, 19). In the present study, we have provided the direct evidence that p47^{phox} has higher binding affinities for GTP and ATP than that for NADPH (Table I). This suggests that p47^{phox} is intended to bind to GTP and ATP, but not to NADPH, which is consistent with the previous finding that cytosolic p47^{phox} is not an NADPH-binding site of the oxidase system (21, 31). The integrity of the synthesized oNTPs was confirmed by the competitive inhibition of [^{32}P]oNTPs binding to p47^{phox} by native NTPs (Fig. 1), as well as by their comparable abilities to force cytosol in a cell-free activation system (20).

A recent study (30) demonstrated that recombinant p47^{phox} could be completely retained on GTP-agarose, whereas recombinant p67^{phox} could not. However, when these recombinant proteins were combined, recombinant p67^{phox} was also retained by an association with recombinant p47^{phox}, which suggests that p47^{phox} interacts directly with GTP-agarose. These findings suggest two possibilities: 1) p47^{phox} possesses an affinity for NTPs, yet its specificity in nucleotide recognition is ambiguous; or 2) p47^{phox} binds to the columns simply by electrostatic action. To evaluate the latter possibility, we performed an experiment to determine whether or not p47^{phox} has binding constants for GTP and ATP (Table I). Although the values obtained under the present semi-reversible conditions do not denote true binding constants, p47^{phox} showed affinities for both GTP and ATP, which suggests that it contains specific binding sites. Since binding by p47^{phox} was affected by ionic strength, (the removal of 0.15 NaCl from the elution buffer decreased p47^{phox} recovery and the addition of the salt to the loading buffer partially inhibited p47^{phox} binding), the authors in Ref. 30 supposed that p47^{phox} bound electrostatically to the GTP-agarose matrix. However, once p47^{phox} binds to the GTP portion of an affinity column, it also interacts electrostatically with other parts of the matrix. Therefore, in general, dissociation becomes more difficult than association. Prior addition of salts may inhibit the specific interaction between p47^{phox} and a GTP portion due to high ionic strength.

In previous studies to identify an NADPH-binding component of the NADPH oxidase system, we attempted to affinity label the stimulated membranes with [^{32}P]oNADPH, in which cytosolic components had become membrane-associated. However, no radioactive band was observed at the positions which corresponded to p47^{phox} (21) or p49^{phox} (the porcine counterpart) (20), despite the fact that they showed [^{32}P]oNADPH-binding activity in resting cytosol due to their ambiguous property in nucleotide recognition. This observation predicted us that p47^{phox} which has translocated to the stimulated membrane may have lost the ability to bind NTP. If this is the case, then it would support the notion that p47^{phox} actually uses NTP for oxidase activation.

[^{32}P]oGTP-affinity labeling of membrane-derived p47^{phox} has clearly demonstrated this possibility. Whereas the NTP-binding site of cytosolic p47^{phox} was available for binding, it had failed when translocated to the stimulated membrane. p47^{phox} may have been occupied by NTP during cell stimulation. The biochemical significance of p47^{phox}'s NTP-binding nature and its multirecognition between GTP and ATP remains to be resolved.

Acknowledgments

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