PHAGOCYTES

Functional Analysis of NADPH Oxidase in Granulocytic Cells Expressing a Δ488-497 gp91phox Deletion Mutant

By Lixin Yu, Andrew R. Cross, Ling Zhen, and Mary C. Dinauer

Chronic granulomatous disease (CGD) is a group of inherited disorders in which phagocytes are unable to generate superoxide (O$_2^-$) due to genetic defects in any 1 of 4 essential NADPH oxidase components. One of the most important enzymes in producing microbicidal oxidants is the superoxide (O$_2^-$)-generating NADPH oxidase. The NADPH oxidase is a multicomponent enzyme complex whose redox center is a membrane-associated flavocytochrome $b_{558}$ heterodimer, account for the majority of CGD. An X-CGD patient in which a splice junction mutation results in an in-frame deletion of 30 nucleotides encoding amino acids 488 to 497 of gp91phox (Δ488-497 gp91phox) has previously been reported. In this study, we generated myeloid PLB-985 cells expressing the mutant Δ488-497 gp91phox to further characterize its functional properties. These cells mimicked the phenotype of the patient's neutrophils with normal expression of a nonfunctional Δ488-497 gp91phox flavocytochrome. Translocation of p47phox and p67phox to Δ488-497 gp91phox PLB-985 plasma membranes was not affected, as determined both in activated intact cells and in the cell-free system. Furthermore, a synthetic peptide corresponding to residues 488-497 of gp91phox was relatively ineffective in inhibiting O$_2^-$ production in the cell-free oxidase assay ($IC_{50}$ = 500 µmol/L), suggesting that residues 488-497 of gp91phox are not directly involved in oxidase assembly. Mutant Δ488-497 gp91phox flavocytochrome failed to support iodonitrotetrazolium (INT) reduction, showing a disruption of electron transfer from NADPH to the FAD center of gp91phox. However, the FAD binding capacity of the mutant flavocytochrome was normal, as measured by equilibrium dialysis. Taken together, these results suggest that the Δ488-497 deletion in gp91phox disrupts electron transfer to FAD, either due to a defect in NADPH binding or to impaired delivery of electrons from NADPH.

PHAGOCYTES PLAY a critical role in host defense by producing reactive oxygen species against invading microorganisms. One of the most important enzymes in producing microbicidal oxidants is the superoxide (O$_2^-$)-generating NADPH oxidase. The NADPH oxidase is a multicomponent enzyme complex whose redox center is a membrane-associated flavocytochrome $b_{558}$ heterodimer composed of gp91phox and p22phox. In addition, three cytosolic oxidase subunits, p47phox, p67phox, and a low molecular weight GTP binding protein Rac, are required for high level production of O$_2^-$. In resting phagocytes, the dormant oxidase is unassembled. However, upon phagocyte activation, the active oxidase complex is rapidly formed by translocation of the cytosolic oxidase components to the plasma membrane via interactions with the cytochrome. Subsequently, electrons are transferred from cytosolic NADPH to molecular oxygen (O$_2$) at the external face of the membrane to generate O$_2^-$.

Genetic deficiency of NADPH oxidase activity results in chronic granulomatous disease (CGD), a rare inherited disorder of host defense. Patients with CGD develop recurrent, often life-threatening bacterial and fungal infections due to impaired microbicidal oxidant generation by the patient's phagocytes. CGD is caused by genetic defects in any 1 of the 4 oxidase components, p47phox, p67phox, gp91phox, and p22phox. Mutations in the X-linked gene for gp91phox account for approximately two thirds of CGD, with the remaining cases due to autosomal recessive mutations in the genes encoding p22phox, p47phox, or p67phox.1

The NADPH oxidase catalyzes the transfer of electrons from the substrate NADPH to O$_2$, via intermediate flavin (FAD) and heme prosthetic groups, to produce O$_2^-$. The gp91phox polypeptide appears to be the oxidase subunit responsible for mediating electron transfer. We recently have shown that the 2 heme groups incorporated into the cytochrome heterodimer are located within gp91phox.5 The carboxyl terminus of gp91phox contains homologies to consensus FAD and NADPH binding domains of members of ferredoxin-NADP$^+$ reductase (FNR) family, although p67phox has also been reported recently to contain a functional NADPH-binding site and may also participate in the NADPH binding. Coexpression of both gp91phox and p22phox subunits are required to assemble a functional flavocytochrome capable of supporting O$_2^-$ production. In addition, expression of gp91phox in phagocytes is stabilized by association with its partner p22phox.11,12

The majority of missense mutations or in-frame deletions identified in X-CGD result in apparent instability of the gp91phox polypeptide, with either absent or markedly reduced expression of the mutant flavocytochrome $b_{558}$. Rare mutations in which expression of flavocytochrome $b_{558}$ is preserved have been informative in identifying important structural-function relationships of the cytochrome. An X-CGD patient in which a splice junction mutation results in an in-frame deletion of 30 nucleotides encoding amino acids 488 to 497 of gp91phox (Δ488-497 gp91phox) has previously been reported. A detailed functional analysis of the mutant cytochrome could not be performed due to the death of the patient. In this study, we stably transfected the Δ488-497 gp91phox cDNA into X-CGD PLB-985 cells, which lack endogenous gp91phox expression due to gene targeting,13 to create a cell line expressing the mutant.

From the Department of Pediatrics (Hematology-Oncology) and Medical and Molecular Genetics, Herman B Wells Center for Pediatric Research, Riley Hospital for Children, Indiana University School of Medicine, Indianapolis, IN; and the Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA.

Submitted March 18, 1999; accepted May 28, 1999.

Supported in part by Grants No. RO1 HL45635 and PO1 HL 353586 to M.C.D. and AI-24838 to A.R.C.

Address reprint requests to Mary C. Dinauer, MD, PhD, Wells Center for Pediatric Research, Cancer Research Bldg, Room 466, 1044 Walnut St, Indianapolis, IN 46202; e-mail: mdinauer@iupui.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1999 by The American Society of Hematology.

0006-4971/99/9407-0012$3.00/0

Blood, Vol 94, No 7 (October 1), 1999: pp 2497-2504
Δ488-497 gp91<sub>phox</sub> flavocytochrome. This approach allowed us to perform functional studies on Δ488-497 gp91<sub>phox</sub> PLB-985 cells to further characterize the defect resulting in failure of O<sub>2</sub> production. We found that deletion of gp91<sub>phox</sub> residues 488-497 did not affect translocation of the cytosolic subunits p47<sub>phox</sub> and p67<sub>phox</sub> to plasma membranes of activated Δ488-497 gp91<sub>phox</sub> PLB-985 cells. However, mutant Δ488-497 gp91<sub>phox</sub> flavocytochrome failed to support iodonitrotetrazolium (INT) reduction, showing a defect of the proximal electron transfer pathway from NADPH to the FAD center of gp91<sub>phox</sub>. Partially purified Δ488-497 gp91<sub>phox</sub> had a normal capacity for FAD binding as determined by equilibrium dialysis against FAD. These results suggest that the Δ488-497 deletion disrupted electron transfer from NADPH to FAD, either due to a defect in NADPH binding or to impaired electron delivery from NADPH.

**MATERIALS AND METHODS**

In vitro mutagenesis and expression of recombinant gp91<sub>phox</sub> in promyelocytic PLB-985 cells. The Δ488-497 gp91<sub>phox</sub> cDNA, which has an in-frame deletion of 30 nucleotides, was generated by oligonucleotide-directed mutagenesis using the Scalpor in vitro mutagenesis kit (Amersham, Arlington Heights, IL). The mutant cDNA was verified by dideoxynucleotide sequencing and then subcloned into the Not I site of the mammalian expression vector, pEF-PGKpac. The pEF-PGKpac vector contains a mammalian EF-1α promoter to drive constitutive gp91<sub>phox</sub> expression and a linker expression cassette for puromycin-N-acetyltransferase. In parallel, the vector containing the full-length human wild-type (WT) gp91<sub>phox</sub> cDNA was also constructed. The WT or the Δ488-497 gp91<sub>phox</sub>-containing vectors were transfected by electroporation into X-CGD PLB-985 cells. Clones were selected by limiting dilution in the presence of puromycin (1 µg/mL). To minimize any possible clone-to-clone variation in recombinant gp91<sub>phox</sub> expression or NADPH oxidase activity, 3 independent clones determined to express relatively higher levels of recombinant gp91<sub>phox</sub> were pooled and used for subsequent analysis.

**Cell culture and granulocytic differentiation.** X-CGD PLB-985 cells (X-CGD), transfected PLB-985 cells expressing WT, or the deletion mutant (Δ488-497) gp91<sub>phox</sub> were maintained in RPMI 1640 medium containing 10% fetal calf serum and 2 mmol/L L-glutamine. To induce expression of endogenous NADPH oxidase subunits, cells were differentiated for 5 days by exposure to 0.5% dimethylformamide (DMF). Under these conditions, more than 80% of the cells had undergone granulocytic differentiation as determined by observation of morphological changes and nitroblue tetrazolium (NBT) test.

**Immunoblot and confocal microscopy analysis of recombinant gp91<sub>phox</sub> expression.** To evaluate cell surface expression of flavocytochrome b<sub>558</sub>, immunostaining with the 7D5 monoclonal antibody was performed as described. After staining, 3,000 or 5,000 cells were deposited on glass slides by centrifugation at 450 rpm for 5 minutes and were observed by confocal microscopy. Expression of recombinant gp91<sub>phox</sub> and p22<sub>phox</sub> was also determined by immunoblotting as described previously.

**Translocation analysis of cytosolic oxidase components.** Granulocyte-differentiated cells (1 × 10<sup>5</sup>) were collected, washed, and then resuspended in 1 mL of relaxation buffer consisting of 10 mmol/L PIPES, pH 7.3, 100 mmol/L KCl, 3.5 mmol/L MgCl<sub>2</sub>, 3 mmol/L NaCl, and 1 mmol/L EGTA. To activate the NADPH oxidase assembly, cells were treated with either phorbol 12-myristate 13-acetate (PMA) or dimethyl sulfoxide (DMSO) vehicle control at a final concentration of 500 ng/mL for 10 minutes at 37°C. After the addition of 12 mL of cold phosphate-buffered saline (PBS) to stop the activation, cells were pelleted, resuspended in 1.5 mL of the relaxation buffer, and disrupted by sonication for 3 times at 6 seconds each at 20% power at 4°C. Subsequently, the sonicates were spun for 8 minutes at 500 g, followed by 10 minutes at 2,000 g. A total of 0.75 mL of the resulting supernatant was loaded on a discontinuous sucrose gradient (1.5 mL of 20% over 1.5 mL of 38%) and centrifuged at 41,000 rpm (204,275 g) in an SW55 rotor (Beckman Instruments, Fullerton, CA) for 40 minutes at 4°C. After centrifugation, the 0.6 mL fraction of the top of the gradient was collected as cytosol, and a distinct band located in the interface of the 20% and 38% sucrose gradient was collected as the plasma membranes (~0.6 mL). To remove sucrose, the membranes were mixed with 3.5 mL of cold PBS and centrifuged at 55,000 rpm (368,000g) for 30 minutes, and the resulting pellets were resuspended in 100 µL of relaxation buffer. Translocation of cytosolic oxidase components p47<sub>phox</sub> and p67<sub>phox</sub> to plasma membrane was detected by immunoblotting as described previously. Translocation assay was also performed in a cell-free system. Briefly, 100 µg of cellular membranes was mixed with 300 µg of cytosol isolated from granulocyte-differentiated cells indicated in the figure legends in 0.5 mL of relaxation buffer containing 10 µmol/L GTPγS. After the addition of 100 µmol/L sodium dodecyl sulfate (SDS), the mixture was incubated for 5 minutes at 37°C and then centrifuged for 30 minutes at 55,000 rpm (368,000g) at 4°C. The resulting pellets were collected and used for immunoblotting.

**Measurement of NADPH oxidase activity.** O<sub>2</sub> production by granulocyte-differentiated cells was measured both in whole cells and in the cell-free oxidase assay by monitoring the reduction of cytochrome c at 550 nm using a Thermomax microplate reader. In the assay using intact cells, PMA at a final concentration of 0.1 µg/mL was used to activate the NADPH oxidase of granulocyte-differentiated cells (a total of 2.5 × 10<sup>5</sup> cells in 200 µL of volume in a well). The cell-free assays using SDS as activator were performed as described previously, using flavocytochrome b<sub>558</sub> partially purified from membranes of the PLB-985 cell lines and cytosol from neutrophil or granulocytedifferentiated PLB-985 cells. Membrane and cytosolic fractions were prepared by continuous centrifugation followed by cell disruption by sonication. Michaelis-Menten kinetics were analyzed using GraphPad Prism (San Diego, CA). For peptide inhibition assays, peptides were dissolved in assay buffer and added to the reaction mixture before the addition of SDS (100 µmol/L). Protein concentration was determined by BCA assay (Pierce, Rockford, IL). INT reductase activity was measured as described previously.

**Purification, relipidation, and reflavination of flavocytochrome b<sub>558</sub>.** Flavocytochrome b<sub>558</sub> was partially purified from 3 × 10<sup>6</sup> cell equivalents of salt-washed PLB-985 cell membranes using the method described previously for flavocytochrome b<sub>558</sub> purification from neutrophil membranes. This method uses mixed-bed (carboxymethyl [CM], diethyl aminoethyl [DEAE] Sepharose CL-6B, amion-ocyl agarose) and heparin chromatography. Relipidation and reflavination of the partially purified flavocytochrome b<sub>558</sub> was also performed as described previously using phosphatidylcholine (type IIS; Sigma, St Louis, MO). For the determination of FAD binding constants, portions of the partially purified cytochrome were relipidated in the absence of FAD.

**Spectroscopy.** Reduced minus oxidized difference spectra of detergent-solubilized membranes and partially purified flavocytochrome b<sub>558</sub> were recorded as described previously using a Perkin-Elmer Lambda 18 spectrophotometer (Perkin-Elmer, Norwalk, CT).

**Determination of the affinity of flavocytochrome b<sub>558</sub> for FAD.** The dissociation constant for FAD binding by the flavocytochrome b<sub>558</sub> preparations was determined by equilibrium dialysis using Sialomed equilibrium dialyzers (AmiKa Corp, Columbus, MD). One-hundredmicroliter aliquots (130 to 150 nmol/L in concentration) of partially purified flavocytochrome b<sub>558</sub> samples that had been relipidated (but not reflavinated) were placed in one side of the dialysis chamber and dialyzed against 100 µL of the same buffer containing 100 mM/L FAD. FAD standards were made by serial dilution of a freshly prepared stock solution of FAD. The concentration of the stock solution was deter-
mined from the absorbance at 450 nm using an extinction coefficient of 11.3 mmol/L⁻¹ cm⁻¹. All FAD-containing solutions were protected from light. The concentration of flavocytochrome b₅₅₈ was determined spectrophotometrically using an extinction coefficient of 21.6 mmol/L⁻¹ cm⁻¹ at 559 nm for the reduced-minus oxidized heme (10.8 mmol/L⁻¹ per mole flavocytochrome b₅₅₈). Samples were dialyzed on ice for 4 hours, and the contents of each chamber were removed for FAD analysis. All samples and FAD standards were heated in a 100°C water bath for 3 minutes to release enzyme-bound FAD and centrifuged to remove denatured protein.

FAD was estimated using a modification of the method of Hinkkanen and Decker. The assay mixture consisted of 80 to 100 µL of sample (or FAD standard), 20 mmol/L 3, 5-dichlorobenzene sulfonic acid, 200 µmol/L 4-amino antipyrene, 4 µL of horseradish peroxidase, 0.2 U/mL apo-D-amino acid oxidase, and 35 mmol/L D-proline in a total volume of 250 µL. 100 mmol/L Tris, pH 8.6. The assay was performed at 37°C and the rate of formation of N-(4-antipyryl)-3-chloro-5-sulfonate-p-benzoquinone monoimine was observed for 60 minutes by measuring the increase in absorbance at 512 nm. The FAD concentration of the samples was calculated from a standard curve of 0 to 200 nmol/L FAD plotted against maximum rate of A₅₁₂. The Kₘ (FAD) for each sample was calculated from the final concentrations of FAD in the sample and buffer compartments, and the concentration of flavocytochrome b₅₅₈ was added.

RESULTS AND DISCUSSION

D₄₈₈-₄₉₇ gp91phox PLB-985 cells mimic the phenotype of X-CGD D₄₈₈-₄₉₇ gp91phox neutrophils. Stable expression of recombinant D₄₈₈-₄₉₇ gp91phox in X-CGD PLB-985 cells mimicked the phenotype originally reported for neutrophils isolated from an X-CGD patient with the same D₄₈₈-₄₉₇ deletion in gp91phox. Expression of D₄₈₈-₄₉₇ gp91phox in transfected PLB-985 cells was examined by immunoblotting (Fig 1B). The level of recombinant D₄₈₈-₄₉₇ gp91phox was similar to that of recombinant WT gp91phox expressed in PLB-985 cells. A marked increase in expression of p2₂phox was seen in both transgenic D₄₈₈-₄₉₇ gp91phox and WT gp91phox PLB-985 cells (Fig 1B). This is consistent with previous observations indicating that coexpression of both gp91phox and p2₂phox and subsequent heterodimer formation is important for stable expression of each flavocytochrome b₅₅₈ subunit. We have previously shown that the transgenically expressed recombinant WT gp91phox is processed and targeted normally into the plasma membrane in promyelocytic PLB-985 cells. To determine whether D₄₈₈-₄₉₇ gp91phox was expressed in the plasma membranes, transfected cells were stained with 7D5, a monoclonal antibody that interacts with an extracellular epitope of gp91phox, and examined by confocal microscopy. As shown in Fig 1A, membrane surface staining was present in both D₄₈₈-₄₉₇ gp91phox as well as WT gp91phox PLB-985 cells. No positive signal above the background was obtained in X-CGD PLB-985 cells, consistent with the absence of gp91phox in the cells (Fig 1B).

We next performed reduced minus oxidized difference spectroscopy on transgenically expressed flavocytochrome b₅₅₈ partially purified from membranes isolated from D₄₈₈-₄₉₇ gp91phox, WT gp91phox, and X-CGD PLB-985 cells. Virtually identical spectra characteristic of flavocytochrome b₅₅₈ were seen for both the D₄₈₈-₄₉₇ gp91phox and WT gp91phox flavocytochrome preparations, demonstrating the normal incorporation of heme groups in the gp91phox deletion mutant (Fig 2). As expected, X-CGD samples lacked specific absorption at 558 nm (Fig 2).

NADPH oxidase activity in D₄₈₈-₄₉₇ gp91phox PLB-985 cells was determined in both intact cells and in the cell-free oxidase assay. After granulocytic differentiation for 5 days to induce the expression of the endogenous p4₇phox and p₆₇phox oxidase subunits, WT gp91phox PLB-985 cells produced O₂⁻ after stimulation with PMA, as expected (Table 1). In contrast, D₄₈₈-₄₉₇ gp91phox PLB-985 cells were unable to generate O₂⁻ (Table 1). To confirm that the cellular defect in the NADPH oxidase in D₄₈₈-₄₉₇ gp91phox PLB-985 cells was related to the mutation in gp91phox, cell-free oxidase assays were performed.
using combinations of cytosol and membranes prepared from WT gp91<sup>phox</sup> and Δ488-497 gp91<sup>phox</sup> PLB-985 cells. As shown in Table 2, membranes isolated from Δ488-497 gp91<sup>phox</sup> PLB-985 cells failed to support O<sub>2</sub><sup>-</sup> generation in combination with cytosol from either WT gp91<sup>phox</sup> or Δ488-497 gp91<sup>phox</sup> PLB-985 cells, demonstrating that absence of NADPH oxidase activity resulted from a defect in the cellular membranes containing the mutant flavocytochrome b<sub>558</sub>.

Assembly of the NADPH oxidase by translocation of p47<sup>D</sup> and p67<sup>D</sup> to the plasma membrane is not affected by the Δ488-497 deletion in gp91<sup>phox</sup>. Upon phagocyte activation, cytosolic oxidase components translocate to the plasma membrane to assemble the functional NADPH oxidase. Multiple contact points between p47<sup>D</sup> and the gp91<sup>phox</sup> and p22<sup>phox</sup> subunits of flavocytochrome b<sub>558</sub> have been described previously.<sup>14,25-29</sup> Among these, a missense mutation predicting an Asp-Gly substitution at residue 500 of gp91<sup>phox</sup> has been reported to lead to defective translocation of p47<sup>D</sup> and p67<sup>D</sup> to the plasma membrane during oxidase assembly. The proximity of the Δ488-497 deletion in gp91<sup>phox</sup> to Asp500 prompted us to test whether translocation of p47<sup>D</sup> and p67<sup>D</sup> to the plasma membrane was affected by this deletion. As shown in Fig 3A, PMA-stimulated translocation of p47<sup>D</sup> and p67<sup>D</sup> to the plasma membrane in intact Δ488-497 gp91<sup>phox</sup> PLB-985 cells was similar to that seen for WT gp91<sup>phox</sup> PLB-985 cells. A similar result was obtained in a cell-free oxidase reconstitution system using SDS for activation (Fig 3B). The band seen below the 68 kD marker protein is the high mannose 65-kD precursor of gp91<sup>phox</sup>.<sup>24</sup> We have previously shown that this species is localized in the ER, as determined by cell fractionation using a 10% to 60% continuous sucrose gradient.<sup>24</sup> The membranes isolated by discontinuous sucrose gradient (20% and 38%) in the current experiment may be contaminated with intracellular membranes including ER, which may account for the presence of the precursor in the preparation (Figs 1B and 3B). A small amount of p47<sup>D</sup> was seen in WT and Δ488-497 gp91<sup>phox</sup>-transfected PLB-985 granulocytes even in the absence of PMA, as well as in X-CGD PLB-985 cells (Fig 3A and B). This likely reflects nonspecific binding of p47<sup>D</sup> to membranes, which has also been observed by others.<sup>30</sup> Alternatively, an unexpected priming of PLB-985 granulocytes during culture at 37°C may cause the translocation of p47<sup>D</sup> to the membrane, which is flavocytochrome-independent.

To provide additional evidence that gp91<sup>phox</sup> residues 488-497 are not essential for oxidase assembly, we synthesized a peptide corresponding to residues 488-497 and tested its ability to inhibit O<sub>2</sub><sup>-</sup> production in the cell-free oxidase assay using membrane and cytosol isolated from normal neutrophils. As shown in Fig 4, peptide 488-497 inhibited oxidase activity only at high concentrations (IC<sub>50</sub>, ~500 µmol/L), whereas a peptide derived from residues 86-102 of gp91<sup>phox</sup> (containing a probable p47<sup>D</sup> binding motif<sup>28,29</sup>) had an IC<sub>50</sub> of 2 µmol/L (Fig 4). The 488-497 peptide was also much less potent at inhibiting O<sub>2</sub><sup>-</sup> production compared with other peptides derived from gp91<sup>phox</sup> domains proposed as binding sites for cytosolic oxidase components, including peptide 491-504 containing Asp500 (IC<sub>50</sub>, 10 µmol/L),<sup>14</sup> peptide 559-570 (IC<sub>50</sub>, 28 µmol/L),<sup>31</sup> and peptide 550-569 (IC<sub>50</sub>, 4 µmol/L).<sup>32</sup> Consistent with our results, Kanegasaki’s group has reported that IC<sub>50</sub> of peptide 484-502 was greater than 300 µmol/L.<sup>32</sup>

<table>
<thead>
<tr>
<th>Source of Membranes</th>
<th>Δ488-497 gp91&lt;sup&gt;phox&lt;/sup&gt; PLB-985</th>
<th>WT gp91&lt;sup&gt;phox&lt;/sup&gt; PLB-985</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>183.4 ± 16.8*</td>
<td>165.6 ± 20.3</td>
</tr>
<tr>
<td>After 5 days of induction with DMF (0.5%), the cytosolic and membrane fractions were separated from the indicated cells and used in the cell-free oxidase reconstitution assay. Ten micrograms of membranes and 20 µg of cytosol were used in the assay. The data represent the mean ± SD of 3 separate experiments.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Units: nmol/min/mg membrane proteins.

### Table 1. Superoxide Production in Transgenic PLB-985 Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>O&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt; Production (nmol/min/10&lt;sup&gt;7&lt;/sup&gt; cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT gp91&lt;sup&gt;phox&lt;/sup&gt; PLB-985</td>
<td>55.8 ± 8.8</td>
</tr>
<tr>
<td>Δ488-497 gp91&lt;sup&gt;phox&lt;/sup&gt; PLB-985</td>
<td>0</td>
</tr>
<tr>
<td>X-CGD PLB-985</td>
<td>0</td>
</tr>
</tbody>
</table>

The indicated cells were induced for granulocytic differentiation for 5 days with DMF (0.5%), and O<sub>2</sub><sup>-</sup> production by intact cells was measured by cytochrome <i>c</i> reduction assay. The data represent mean ± SD of 3 separate experiments.
efficiently as flavocytochrome b_{558} purified from neutrophils (not shown). In contrast, flavocytochrome b_{558} from Δ488-497 gp91_{phox} PLB-985 cells was incapable of INT reductase activity, suggesting that either flavin or NADPH binding is affected in this mutant. As expected, the equivalent purification fraction from X-CGD PLB-985 cells also had no activity (Fig 5).

The Δ488-497 mutant flavocytochrome b_{558} has a normal affinity for FAD. To evaluate the capacity of the Δ488-497 gp91_{phox} mutant to bind FAD, we measured the affinity of the partially purified flavocytochrome b_{558} preparations for FAD by equilibrium dialysis as described in the Materials and Methods. Both WT gp91_{phox} and Δ488-497 gp91_{phox} flavocytochrome preparations had virtually identical affinities for FAD of approximately 66 nmol/L (Table 3). These values are consistent with the literature values of 20 to 85 nmol/L.\textsuperscript{33-35} An equivalent volume of the X-CGD PLB fraction eluted from the heparin column that corresponded to the peak fractions of the Δ488-497 gp91_{phox} and WT gp91_{phox} flavocytochrome b_{558} showed no ability to bind FAD. In all cases, the recovery of FAD from each equilibrium dialysis experiment (sample + buffer) was 100% ± 4%.

Analysis of NADPH binding in the Δ488-497 gp91_{phox} flavocytochrome b_{558}. Residues 488-497 have been postulated to lie near the NADPH binding domain of gp91_{phox}.\textsuperscript{36} To address whether the Δ488-497 mutation alters the affinity of flavocytochrome b_{558} for NADPH, we measured the \( K_m \) for NADPH on

![Fig 3. Translocation of p47_{phox} and p67_{phox} to plasma membrane in intact cells activated with PMA and in the cell-free oxidase reconstitution assay stimulated with SDS. (A) The indicated cells undergone granulocytic differentiation for 5 days were stimulated with (+) or without (−) PMA (500 ng/mL) for 10 minutes at 37°C, and the plasma membranes were prepared on discontinuous sucrose gradients and analyzed for translocation of p47_{phox} and p67_{phox} by immunoblot analysis using p47_{phox} and p67_{phox} antibodies (left panel). After stripping, the blots were reprobed with gp91_{phox} and p22_{phox} monoclonal antibodies to show an equal loading (right panel). Each lane was loaded with 5 μg of proteins. (B) Membranes separated from the indicated cells were mixed with 3-fold of neutrophil cytosol in the cell-free oxidase reconstitution assay. After 10 minutes of incubation at 25°C in the presence (+) or absence (−) of 100 μmol/L SDS, the membranes were reisolated by ultracentrifugation and detected for the translocation of p47_{phox} and p67_{phox} (left panel) by immunoblot analysis as described in (A). Each lane was loaded with 5 μg of proteins.](image)

![Fig 4. Effect of peptide 488-497 on NADPH oxidase activity in the cell-free oxidase assay. Plasma membrane (8 μg) and cytosolic fractions (20 μg) separated from normal neutrophils were used in the cell-free assay. Peptide 488-497 of gp91_{phox} (●) as well as a control peptide corresponding to residues 86-102 of gp91_{phox} containing a putative p47_{phox} binding site 86-93 (■) were added to the assay before the addition of SDS, and \( \Delta O_2^- \) generation was measured. The activity of superoxide production in the absence of peptides was 216 ± 22 nmol/min/mg of membrane proteins. The data represent the mean ± SD of 3 separate experiments.](image)
mutant flavocytochrome partially purified from membranes of Δ488-497 gp91<sub>phox</sub> PLB-985 cells. The NADPH oxidase has the ability to use NADPH or NADH as substrate, although the K<sub>m</sub> for NADPH is approximately 10-fold lower (<sup>37</sup>39). The K<sub>m</sub> for NADPH of the enzyme in the cell-free system using neutrophil membranes was found to be 57.1 ± 1.8 µmol/L (<i>n</i> = 22), and the K<sub>m</sub> of purified neutrophil flavocytochrome b<sub>558</sub> was 27.5 ± 1.3 µmol/L (<i>n</i> = 17; A. Cross, unpublished data). The K<sub>m</sub> of the flavocytochrome purified from membranes of WT gp91<sub>phox</sub> PLB-985 cells was found to be 30.4 ± 1.5 µmol/L (not shown). No activity was evident in the cytochrome purified from the Δ488-497 mutant and neither did the addition of 4.9 mmol/L NADH (Fig 6). This suggests that either the Δ488-497 gp91<sub>phox</sub> cannot bind the substrate NADPH or it cannot support electron transfer from NADPH to FAD.

Over the past 10 years, several groups have tried to identify the NADPH binding component of the oxidase complex by labeling with <sup>32</sup>P- or <sup>3</sup>H-labeled NADPH analogues. However, different results have been reported by different groups, including a 66-kD cytosolic protein,<sup>40</sup> an approximately 32-kD cytosolic protein,<sup>41</sup> a 52-kD membrane-associated protein,<sup>42</sup> and p67<sub>phox</sub>,<sup>9</sup> therefore leaving the issue still uncertain. By comparison with known NADPH binding regions of members of Ferredoxin-NADP<sup>1</sup> Reductase family, Segal’s group,<sup>6</sup> Rotrosen et al,<sup>8</sup> and Sumimoto et al<sup>7</sup> have proposed that the apparent NADPH binding pocket resides in the carboxyl terminal portion of gp91<sub>phox</sub>. This postulation has subsequently been strongly supported by the experiments showing that reflavinated and repalidated membrane fractions isolated from normal neutrophils are capable of supporting O<sub>2</sub><sup>−</sup> generation in the absence of any cytosolic proteins.<sup>43,44</sup>

We attempted to analyze the ability of the Δ488-497 mutant flavocytochrome b<sub>558</sub> to bind NADPH by affinity labeling using the photoaffinity label [4-N-(4-azido-2-nitrophenyl) aminobutyryl] NAD<sup>[32</sup>P].<sup>45</sup> Despite using a number of different experimental conditions, it was not possible to convincingly, or reproducibly, label WT gp91<sub>phox</sub> from either neutrophils or WT

![Source of flavocytochrome](image)

**Table 3. FAD Binding of WT and Δ488-497 gp91<sub>phox</sub> Flavocytochrome b<sub>558</sub>**

<table>
<thead>
<tr>
<th>Source of Flavocytochrome b&lt;sub&gt;558&lt;/sub&gt;</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (FAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT gp91&lt;sub&gt;phox&lt;/sub&gt; PLB-985</td>
<td>66.9 ± 1.7 nmol/L</td>
</tr>
<tr>
<td>Δ488-497 gp91&lt;sub&gt;phox&lt;/sub&gt; PLB-985</td>
<td>65.3 ± 2.2 nmol/L</td>
</tr>
<tr>
<td>X-CGD PLB-985*</td>
<td>No binding</td>
</tr>
</tbody>
</table>

*An equivalent volume of the X-CGD PLB-985 cell fractions eluted from the heparin column that corresponding to the peak fractions of the Δ488-497 gp91<sub>phox</sub> and WT gp91<sub>phox</sub> flavocytochrome b<sub>558</sub> was used. The concentration of FAD was 51.3 nmol/L in the buffer chamber and 53 nmol/L in the sample chamber after dialysis.

![Source of flavocytochrome](image)
The authors thank Drs Dirk Roos and Arthur J. Verhoeven (Central Laboratory of The Netherlands Blood Transfusion Service, Amsterdam, The Netherlands) for kindly providing anti-gp91\(^{\text{phox}}\) and anti-p22\(^{\text{phox}}\) monoclonal antibodies 48 and 449, respectively. In addition, Dr David Lambeth (Emory University, Atlanta, GA) provided polyclonal anti-p47\(^{\text{phox}}\) and Dr Paul Heyworth (The Scripps Research Institute, La Jolla, CA) provided polyclonal anti-p67\(^{\text{phox}}\) antibodies. The anti-flavocytochrome \(b_{558}\) monoclonal antibody 7DS was a generous gift from Dr Michio Nakamura (Nagasaki University, Nagasaki, Japan).

REFERENCES


5. Yu L, Quinn M, Cross A, Dinauer M: Gp91\(^{\text{phox}}\) is the heme binding subunit of the superoxide-generating NADPH oxidase. Proc Natl Acad Sci USA 95:7993, 1998


27. Leto T, Adams A, De Mendez I: Assembly of the phagocyte NADPH oxidase: Binding of Src homology 3 domains to proline-rich targets. Proc Natl Acad Sci USA 91:10650, 1994
32. Park M, Imajoh-Ohmi S, Nunoi H, Kanegasaki S: Synthetic peptides corresponding to various hydrophilic regions of the large subunit of cytochrome b558 inhibit superoxide generation in a cell-free system from neutrophils. Biochem Biophys Res Commun 234:531, 1997
33. Doussiere J, Buzenet G, Vignais P: Photoaffinity labeling and photoinactivation of the O2− generating oxidase of neutrophils by an azido derivative of FAD. Biochemistry 34:1760, 1995
34. Koshkin V: Aerobic and anaerobic functioning of superoxide-producing cytochrome b559 reconstituted with phospholipids. Biochim Biophys Acta 1232:225, 1995
Functional Analysis of NADPH Oxidase in Granulocytic Cells Expressing a ?488-497 gp91phox Deletion Mutant

Lixin Yu, Andrew R. Cross, Ling Zhen and Mary C. Dinauer