Role of Putative Second Transmembrane Region of Nox2 Protein in the Structural Stability and Electron Transfer of the Phagocytic NADPH Oxidase*

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Flavocytochrome b_{558} (cytb) of phagocytes is a heterodimeric integral membrane protein composed of two subunits, p22_{phox} and gp91_{phox}. The latter subunit, also known as Nox2, has a cytosolic C-terminal “dehydrogenase domain” containing FAD/NADPH-binding sites. The N-terminal half of Nox2 contains six predicted transmembrane α-helices coordinating two hemes. We studied the role of the second transmembrane α-helix, which contains a “hot spot” for mutations found in rare X+ and X− chronic granulomatous disease. By site-directed mutagenesis and transfection in X-CGD PLB-985 cells, we examined the functional and structural impact of seven missense mutations affecting five residues. P56L and C59F mutations drastically influence the level of Nox2 expression indicating that these residues are important for the structural stability of Nox2. A53D, R54G, R54M, and R54S mutations do not affect spectral properties of oxidized/reduced cytb, oxidase complex assembly, FAD binding, nor iodonitrotetrazolium (INT) reductase (diaphorase) activity but inhibit superoxide production. This suggests that Ala-53 and Arg-54 are essential in control of electron transfer from FAD. Surprisingly, the A57E mutation partially inhibits FAD binding, diaphorase activity, and oxidase assembly and affects the affinity of immunopurified A57E cytochrome b_{558} for p67_{phox}. By competition experiments, we demonstrated that the second transmembrane helix impacts on the function of the first intracytosolic B-loop in the control of diaphorase activity of Nox2. Finally, by comparing INT reductase activity of immunopurified mutated and wild type cytb under anaerobiosis versus aerobiciosis, we showed that INT reduction reflects the electron transfer from NADPH to FAD only in the absence of superoxide production.

Phagocytic cells such as neutrophils, monocytes, macrophages, or eosinophils possess an essential NADPH oxidase in their plasma membranes that produces microbicidal oxidants to kill pathogens during an infection. The integral membrane proteins gp91_{phox} or Nox2 and p22_{phox} form heterodimers called flavocytochrome b (cytb), the core electron transferer of the oxidase. The phagocyte NADPH oxidase is dormant in resting cells and becomes activated during phagocytosis to produce superoxide, a precursor for a variety of reactive oxygen species (ROS). Indeed, after stimulus-dependent activation, cytosolic factors such as p67_{phox}, p47_{phox}, p40_{phox}, and Rac2 migrate to the plasma or phagosomal membranes and assemble with cytb to form an activated NADPH oxidase complex (1). In the past few years, several proteins homologous to Nox2 were discovered to be superoxide/peroxide-producing NADPH oxidases (the Nox family of NADPH oxidases, Nox1, Nox3, Nox4, and Nox5) (2, 3). The activation mechanism and tissue distribution of the various members of the family are markedly different from Nox2. In addition, the physiological functions of the Nox family members include not only host defense but also cellular signaling, cell differentiation, and gene regulation. They are widely involved in a range of pathological processes such as cardiovascular diseases and neurological disorders (4). However, these proteins share a similar structural organization and the capacity to transport electrons across the plasma membrane and to generate superoxide and other down-stream ROS. Nox2 is the prototype NADPH oxidase, and its biochemical features have been extensively studied. Elucidating the function of Nox2 will allow a better understanding of the entire family of Nox proteins. Indeed, because of the involvement of ROS-producing Nox in various disease processes, these enzymes are potentially prime drug targets (5). Thus, it is essential to fully understand the mechanism of electron transfer in this protein family to gain control of its function.

Homology modeling of the cytoplasmic C-terminal domain part of the Nox family of proteins with members of the ferre-

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4 The abbreviations used are: cytb, flavocytochrome b_{558}; ROS, reactive oxygen species; CGD, chronic granulomatous disease; PMA, phorbol 12-myristate 13-acetate; DMF, dimethylformamide; DPI, diphenylene iodonium; INT, iodonitrotetrazolium; SOD, superoxide dismutase; Nox, NADPH oxidase; GTP, guanosine 5'-O-(3-thiotriphosphate).
doxin-NADP⁺ reductase family suggests the presence of FAD- and NADPH-binding sites (6–8). The functioning of the cytosolic “dehydrogenase domain” of Nox2 has been extensively studied. Iodonitrotetrazolium reductase (INT) activity reflects the electron transfer from NADPH to FAD (9). However, its interpretation is controversial (10). Molecular analysis of the role of the dehydrogenase domain of Nox2, using the three-dimensional model proposed by Taylor et al. (11), is supported by results from previous studies (12–16) and by the recent structure determination of the NADPH binding domain (Protein Data Bank code 3A1F).

According to the hydrophobicity pattern of the Nox amino acid sequences, the N-terminal half of the protein appears to be embedded in the plasma membrane and structured into six potential α-helices. This domain of the Nox proteins contains two nonidentical hemes coordinated by four histidine residues in the third and fifth transmembrane passages (17), three glycosylated asparagines in two external-loops (18), and the B- and D-intracellular-loops (see model Fig. 1A) (12, 20). The B-loop has been demonstrated to be involved in NADPH oxidase assembly during activation (19). Recently, a new role for the D-loop of Nox4 in folding and interaction with p22phox has been proposed (20). The polybasic B-loops of Nox2 and Nox4 appear to mediate binding between the dehydrogenase domain and the heme-containing transmembrane domains of Nox enzymes thus controlling the functioning of Noxes (21). However, the role of the putative second transmembrane α-helix and its inter-relationship with other functional domains have not yet been examined.

Chronic granulomatous disease (CGD) is a rare genetic disorder (frequency 1 in 200,000) characterized by severe and recurrent infections in childhood caused by deficient superoxide production by the phagocytic NADPH oxidase. The most abundant form involves X-linked transmission (X-CGD) (22). Sometimes missense mutations in the CYBB gene encoding Nox2 lead to extremely rare CGD variants termed X⁺ or X⁻-CGD and are characterized by normal or low Nox2 expression, respectively, and are associated with no activity or a faint NADPH oxidase activity (23). The preponderance of mutations in the N-terminal region of Nox2 appears to occur in a “hot spot” of five residues from Ala-53 to Cys-59, located in the putative second transmembrane α-helix (Fig. 2) (23, 24). However, the functional consequences of such mutations have only been studied in the neutrophils of one patient (25). In that study, the authors found that the R54S mutation affects the function of the heme moiety of cytochrome b₅₅₈ and the electron transfer from the FAD moiety to heme.

The aim of this study was to investigate the role of the putative second transmembrane domain of Nox2, containing the sequence 45LLGSALALARAPAACLNFNCMLILL69, on Nox2 expression, INT reductase, and NADPH oxidase activities, as
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well as FAD binding and NADPH oxidase complex assembly. We approached this study employing site-directed mutagenesis in the X-CGD PLB-985 cell model (26) and by affinity purification of the expressed mutated cytb using the CS9 anti-p22phox antibody (27). We demonstrated the importance of the second N-terminal putative transmembrane region of Nox2, not studied before, in the structural stability and the control of electron transfer in the phagocytic NADPH oxidase.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—Phorbol 12-myristate 13-acetate (PMA), dimethylformamide (DMF), cytochrome c (horse heart, type VI), diisopropyl fluorophosphate, horseradish peroxidase (HRP), Nα-tosyl-l-lysyl-chloromethyl ketone, latex beads, polystyrene (3 μm), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT), luminol, Triton X-100, sodium dithionite, FAD, deoxyribonucleic acid, ribonucleic acid, superoxide dismutase, phosphatidylcholine type XVI-E, and mammalian protease inhibitor mixture P8340 were obtained from Sigma. GTPγS, reduced nicotinamide adenine dinucleotide (NADPH), leupeptin, and pepstatin were from Roche Applied Science. Fetal bovine serum, RPMI 1640 medium, geneticin G418, Alexa Fluor 488 goat-F(ab′)2 fragment antirabbit IgG1 (H + L), and Alexa Fluor 546 donkey-F(ab′)2 fragment anti-goat IgG1 (H + L) were from Invitrogen. Monoclonal antibody specific for Nox2 7D5 was purchased from MBL Medical and Biological Laboratories (Naka-ku Nagoya, Japan). Polyclonal antibodies anti-p47phox and p67phox were purchased from Upstate Biotechnologies, Inc. (New York) and Tebu-Bio (Le Perray en Yvelines, France), respectively. Endo-free plasmid purification kits were purchased from Qiagen (Courtaboeuf, France). Octyl glucose and paraformaldehyde was purchased from Pierce Thermo Scientific (Rockford, IL). The Sephaglas kit and molecular weight markers (Page Ruler®) were obtained from Amersham Biosciences and Fermentas (Burlington, Canada) respectively. Nitrocellulose sheets for Western blotting were purchased from Bio-Rad. Phosphatidic acid was purchased from Coger (Paris, France). Peptides (WT and mutated 51ALARAPACLNFN93 and 51ALARAPAECLNFN93, respectively, and 72CRNLSSLRGGSSACSTRVRQRQDLRLNT99) were synthesized by Proteognix (Oberhausenberg, France). Sequence and purity of the peptides (>95%) were checked by mass spectrometry.

In Vitro Mutagenesis and Expression of Recombinant Nox2 in Transfected PLB-985 Cells—Mutations were introduced into the wild type (WT) Nox2 cDNA in a pBlueScript II KS(+) vector using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla) according to the manufacturer’s recommendations. The mutated sequence of Nox2 cDNA was verified by sequencing (Cogenics, Grenoble, France). The WT or mutant Nox2 cDNA were subcloned into the BamHI site of the mammalian expression vector pEF-pGKNeo as described previously (12). The constructs were transfected into the X-CGD PLB-985 cell line (a generous gift of Professor M. Dinauer, Indiana University) by electroporation at 250 V (one pulse of 20 ms). X-CGD PLB-985 cells correspond to CYBB knock-out PLB-985 cells as described by Dinauer and co-workers (26). Clones were selected by limiting dilution in the presence of 1.5 mg/ml geneticin. Positive clones expressing WT or mutated Nox2 were selected by flow cytometry using monoclonal antibody 7D5 directed against Nox2 as described previously (28). In this way, we selected three positive clones of each mutation from 15 to 20 tested clones that were stored in liquid nitrogen.

Cell Culture and Granulocyte Differentiation—WT, X-CGD PLB-985 cells, and transfected X-CGD PLB-985 cells were maintained in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mm l-glutamine at 37 °C in a 5% CO2 humidified atmosphere. After selection of transfected X-CGD PLB-985 cells, 0.5 mg/ml geneticin was added to maintain the selection pressure. To induce granulocyte differentiation and expression of endogenous NADPH oxidase components, PLB-985 cells (2 × 10⁶ cells/ml) were differentiated for 6 days with 0.5% (v/v) DMF.

Measurement of O2− Production by Chemiluminescence—O2− production of intact differentiated PLB-985 cells (5 × 10⁶ cells) in PBS containing 0.9 mM CaCl2, 0.5 mM MgCl2, 20 mM glucose, 20 μM luminol, and 10 units/ml HRP was measured after PMA stimulation (77 ng/ml). Relative luminescence units were recorded at 37 °C over a time course of 60 min in a luminoscan luminometer (Labsystems, Helsinki, Finland) connected to a computer. Results were expressed as the sum of relative luminescence detected during the time course (28).

Detection of p47phox and p67phox Translocation in Transfected PLB-985 Cells by Confocal Microscopy—5×10⁶ differentiated PLB-985 cells were activated by PMA-treated latex beads for 15 min at 37 °C on a glass coverslip. Polyclonal anti-p47phox and anti-p67phox antibodies were used as primary stain after Triton X-100 solubilization. After fluorescent second antibodies treatment as described previously (28), cells were examined with a confocal laser scanning microscope and analyzed using Leica confocal software. Cellular nuclei were visualized by Hoechst 33258 staining.

Preparation of Membranes and Cytosol Fractions—After treatment with 3 mM diisopropyl fluorophosphate in ice for 15 min, differentiated transfected PLB-985 cells were resuspended at a concentration of 5×10⁶ cells/ml in PBS containing 1.5 μM pepstatin, 2 μM leupeptin, and 12 μM Nα-tosyl-l-lysyl-chloromethyl ketone. Cells were disrupted by sonication (three times for 10 s at 40 watts), and the homogenate was centrifuged at 1000 × g for 15 min at 4 °C. The supernatant was centrifuged at 200 000 × g for 1 h at 4 °C. The high speed supernatant was referred to as the cytosol, and the pellet consisting of crude membranes was resuspended in the same buffer at a protein concentration of 3–5 mg/ml (29).

Detection of NADPH Oxidase and Diaphorase Activities in a Cell-free System Assay—in vitro NADPH oxidase activity was measured using plasma membranes (100 μg) obtained from differentiated transfected PLB-985 cells and cytosol (300 μg) from human neutrophils in a reaction mixture containing 20 μM GTPγS, 5 mM MgCl2, and an optimal amount of archidonic acid in a final volume of 100 μl. Superoxide dismutase-sensitive oxidase activity was measured in the presence of 100 μM cytochrome c as described previously (29). INT reductase (or diaphorase) activity was assayed in the same conditions, except that cytochrome c was replaced with 100 μM iodonitro-
tetrazolium (INT) (9). Peptide inhibition experiments were conducted by incubating purified plasma membranes with WT or mutated peptides (51ALARAPACLNFN63 and 51ALARAP-EACLNFN63 or 72CRNLLSFRLGSSACCSTRVRRQLD-RNLNT99) at the indicated concentrations in the reaction mixture (Fig. 8) in the presence of recombinant p47phox, p67phox, and Rac proteins. A negative control was performed by incubating with DMSO. Arachidonic acid was used to induce NADPH oxidase activation (30). In some experiments, plasma membranes of PLB-985 cells and cytosol from neutrophils were replaced by purified cytochrome b558 (2.5 nM), purified recombinant proteins p67phox, p47phox, and Rac1 preloaded with GTPγS (1 μM of each). We added 10 μM FAD to the reaction medium when purified cytochrome b558 was used (28). In this case, arachidonic acid was replaced by phosphatidic acid, 10:0 (2.5 μM), to induce NADPH oxidase assembly as described previously (27). In some experiments, in vitro INT reductase activities were assayed in a temperature-regulated glove box at 20 °C and around 2 ppm of oxygen (Jacomex). For quantitation, an ε value of 21.1 mM cm⁻¹ was taken for cytochrome c and 11 mM cm⁻¹ for INT.

Detection of FAD Incorporation into Cytochrome b558—The FAD content of membranes was determined using 5·10⁸ PLB-985 transfected cells. Assays were performed from resulting plasma membranes (0.7–1 mg) incubated at 100 °C for 15 min. After centrifugation at 20,000 × g for 15 min, supernatants were saved for measurement of noncovalently bound flavin by a fluorometric method (31). The emission of FAD was measured at 535 nm after excitation at 450 nm using a Varioskan (Thermo Scientific) fluorescence spectrophotometer against a FAD standard scale (50–1000 pmol/well). The amount of FAD present in membranes from PLB-985 KO cells was subtracted.

Purification and Relipidation of Cytochrome b558 from Transfected PLB-985 Cells—Cytochrome b558 was purified from 1·10¹⁰ transfected PLB-985 cells as described previously (27). Purified cytochrome b558 was relipidated with 1-α-phosphati-
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**TABLE 1**
NADPH oxidase and INT reductase activities in the membranes of transfected X-CGD PLB-985 cells

O₂⁻ production was measured by luminol-amplified chemiluminescence in 5 × 10⁵ differentiated X-CGD PLB-985 cells after PMA stimulation. Results are presented as total activity calculated from the integrated sum of luminescence measured over 60 min in relative luminescence units (RLU). In vitro NADPH oxidase was reconstituted in a cell-free system with purified plasma membranes from transfected X-CGD PLB-985 cells in the presence of human neutrophil cytosol and activated with GTP-γ-S and arachidonic acid as described under “Experimental Procedures.” INT reductase activity was determined in the same conditions as described above, except that cytochrome c was replaced by INT. Activities are expressed as percentage of activity compared with the WT-Nox2 PLB-985 cell line. The data represent an average ± S.D. of at least three independent experiments.

| n = 3–5 | CGD types | Transgenic PLB-985 cells | Ex vivo | In vitro | INT reductase activity, in vitro |
|---------|-----------|--------------------------|---------|----------|---------------------------------|
| Controls | WT-Nox2 cells | 350 ± 7 | 100 | 101 ± 11 | 100 |
| Mutants | A53D | 1.17 ± 0.30 | 2 | 5.40 ± 4.40 | 0 | 80.1 ± 4.8 |
| X⁻ | R54G | 53.3 ± 5.2 | 15 | 3.35 ± 0.58 | 0 | 78.3 ± 8.8 |
| X⁻ | R54M | 0.35 ± 0.03 | 3 | 1.33 ± 1.70 | 0 | 77.0 ± 0.8 |
| X⁻ | 56L | 11.1 ± 0.9 | 3 | 1.18 ± 0.15 | 0 | 94.7 ± 6.1 |
| X⁻ | A57E | 0.57 ± 0.11 | 0 | 3.33 ± 0.58 | 0 | 28.6 ± 3.7 |
| X⁻ | C59F | 0 | 0 | 0 | 0 |

**TABLE 2**
Quantification of cytochrome b₅₅₈ and FAD contents in membranes of transfected X-CGD PLB-985 cells

Cytochrome b₅₅₈ was quantified by reduced minus oxidized absorbance difference spectroscopy using absorbation at 558 nm. FAD content was measured by fluorimetry as described under “Experimental Procedures.” The amounts of cytochrome b₅₅₈ expressed in picomoles of cytb/mg of protein and of FAD expressed in picomoles/mg of protein represent the mean ± S.D. of three separate experiments. Ratios between cytb and FAD were calculated by dividing the corresponding values. Percentages were calculated based on the cytb and FAD content of the WT Nox2 membranes. ND means not determined.

| n = 3–5 | CGD types | Transgenic PLB-985 cells | Cytochrome b₅₅₈ | FAD |
|---------|-----------|--------------------------|----------------|-----|
| Controls | WT-Nox2 cells | 24.4 ± 2.3 | 36.6 ± 5.8 | 0 |
| Mutants | A53D | 18.3 ± 3.7 | 23.7 ± 2.4 | 0 |
| X⁻ | R54G | 22.5 ± 3.0 | 30 ± 5.7 | 0 |
| X⁻ | R54M | 23.3 ± 1.5 | 29.9 ± 2.8 | 0 |
| X⁻ | R54S | 26.9 ± 3.1 | 7.4 ± 4.6 | ND |
| X⁻ | P56L | Traces | ND | ND |
| X⁻ | A57E | 21.8 ± 0.9 | 12.2 ± 2.1 | 33 |
| X⁻ | C59F | 0 ± 0 | 0 ± 0 | ND |

aTrace means that we found the presence of cytochrome b₅₅₈ but were unable to quantify it because of the limitation of the sensitivity of the method.

dycholine XVI-E and 18:1 phosphatidic acid (DOPA). Cytochrome b₅₅₈ purity was checked by silver-stained SDS-PAGE and Western blotting using the monoclonal antibodies 54.1 and 44.1 directed against Nox2 and p22^phox, respectively (32). Cytochrome b₅₅₈ was quantified by reduced minus oxidized difference spectroscopy using band absorbation at 558 nm (7). Lipoosomes were stored at −80 °C until used.

**RESULTS**

Phenotypic Characterization of the Nox2 CGD Mutants in the Region 45LLGSALARAPACNLFNCMLILL₆⁹—Seven missense mutations affecting five residues of the putative second transmembrane α-helix of Nox2 were successfully reproduced in X-CGD PLB-985 cells. A53D, R54G, R54M, R54S, and A57E mutations led to an X⁺CGD phenotype characterized by normal Nox2 expression (Fig. 3) but a drastic decrease (A53D, R54G, and R54S) or a total inhibition (R54M and A57E) of NADPH oxidase activity (Table 1). However, the P56L mutation exhibited a decrease of Nox2 expression and proportional reduction in NADPH oxidase activity as typically observed in X⁻CGD phenotypes (Fig. 3 and Table 1). In addition, in vitro reconstituted NADPH oxidase activity was abolished for all the tested mutants (Table 1). The C59F mutation in Nox2 leads to the abolation of oxidase activity and Nox2 expression as seen in classical X⁰CGD (Fig. 3 and Table 1). The level of Nox2 expression in all the mutants was confirmed by the amount of cytochrome b₅₅₈ measured by differential absorbance spectroscopy (Table 2). These data suggest that residues Ala-53, Arg-54, and Ala-57, which are highly conserved in the Nox family members (Fig. 2), are necessary to the functioning of
NADPH oxidase activity, whereas Pro-56 and Cys-59 are residues essential for the integrity of the structure of Nox2 and cytochrome \( b_{558} \).

According to Cross et al. (9), INT reductase activity corresponds to diaphorase activity, which reflects the electron flow from NADPH to FAD and then to INT directly. We found the INT reductase activity in purified membranes from most of the Nox2 mutants (R54G, R54M, R54S, and A53D) to be roughly equivalent to the wild type activity. The activity of the A57E mutant, however, was reduced by 74% compared with WT-Nox2 membranes (Table 1). Table 2 shows that the FAD content of plasma membranes was normal and proportional to the amount of cytochrome \( b_{558} \) in the A53D, R54G, and R54M mutants (ratio cytb/FAD ≈1) but not in the A57E mutants.
(ratio cytb/FAD ≈ 3) (Table 2). Thus, the faint diaphorase activity of the A57E mutant could be explained by a defect of FAD binding in Nox2. The slight INT reductase activity of the P56L mutant (Table 1) could also be explained by a proportional decrease of Nox2 expression (Table 2).

According to Fig. 4, p47(phox) and p67(phox) translocation to the plasma membrane of transfected X-CGD PLB-985 cells during phagocytosis of PMA-treated latex beads was normal except for the A57E mutant where the assembly of the NADPH oxidase complex appeared reduced. This was also confirmed by a more classical translocation assay with plasma membranes from PMA-activated cells, purified on a discontinuous sucrose gradient where cytosolic factor translocation was examined by Western blot analysis (data not shown).

FIGURE 6. In vitro NADPH oxidase and INT reductase activities of purified WT-Nox2 and mutated cytochrome b. A, in vitro NADPH oxidase activities of purified WT-Nox2 and mutated cytochrome b were measured in the presence of p47(phox), p67(phox), and Rac1 as described under “Experimental Procedures.” B, effect of the presence of p47(phox), p67(phox), and Rac1 on in vitro NADPH oxidase activity of purified WT-Nox2 cyt. C, in vitro INT reductase activities of purified WT-Nox2 cytochrome b were reconstituted in the same manner except that cytochrome c was replaced by INT. D, effect of the presence of p47(phox), p67(phox), and Rac1 on in vitro INT reductase activities of purified WT-Nox2 cyt. The same results were obtained with R54M and R54S mutated cytochrome b except that the INT reductase activities of these mutants were normal and comparable with that of WT-Nox2 cyt (data not shown). The data in A and C represent the means ± S.D. of at least five independent experiments. Results in B and D are from one experiment representative of three.
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**Table 3**

**Affinity constants for p67phox and FAD and \( V_{max} \) for purified WT-Nox2 and A57E mutated cytochrome \( b_{558} \) calculated from the INT reductase activity**

In vitro INT reductase activity was reconstituted in a simplified cell-free system assay using purified WT and A57E mutated cytochrome \( b_{558} \) in the presence of recombinant purified cytosolic factors as described under “Experimental Procedures” with increasing amounts of p67phox (0–1000 nM) or FAD (0–10 nM). Kinetic parameters (both \( V_{max} \) and the EC50 for FAD) were deduced from nonlinear regression plots as shown in Fig. 8, A and C. The EC50 for p67phox was deduced from the Lineweaver-Burk plot (Fig. 8B).

|                  | \( V_{max} \) mol of electrons/mol of cyt \( b_{558} \)/s | EC50 | \( V_{max} \) mol of electrons/mol of cyt \( b_{558} \)/s | EC50 |
|------------------|---------------------------------------------------------|------|---------------------------------------------------------|------|
| WT-Nox2          | 24.4 ± 2.5                                              | 2.2 ± 0.2 | 20.7 ± 3.0                                              | 0.23 ± 0.03 |
| A57E             | 11.3 ± 1.6                                              | 7.1 ± 0.1 | 10.2 ± 2.6                                              | 0.35 ± 0.05 |

**Affinity Purification of Transmembrane Cytochrome \( b_{558} \)
Mutated in the \( ^{45}\text{LLGSALARAPAACLNFNCMLILL}^{69} \)
Region and Study of Electron Transfer—The striking point highlighted from our results obtained both in intact cells or purified plasma membranes was the unusual phenotype of the A57E mutant. Although the locus of this mutation is in the putative second transmembrane \( \alpha \)-helix, the mutant showed a partial defect of cytosolic factor translocation, FAD incorporation, and INT reductase activity. To study the kinetic parameters of the INT reductase activity of the purified A57E mutant protein and compare it with the purified WT-Nox2 cyt b and with mutated R54M and R54S cyt bs, the four cyt bs were purified nearly to homogeneity by an immunoaffinity purification method using the monoclonal antibody CS9 previously described previously (27). The pure flavocytochromes were visualized by silver staining and Western blot analysis revealed
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by the 54.1 and 44.1 monoclonal antibodies directed against both subunits of the cytb (34). A supplementary band was seen at around 40 kDa that was probably a dimerization of p22<sub>phox</sub> (Fig. 5A). Generally, from 1×10<sup>10</sup> WT-Nox2 or mutated Nox2 PLB-985 cells, we obtained 40 ± 2 μg of pure cytochrome b<sub>558</sub> as described previously (27). We first verified the heme content in the purified mutated cytochrome b<sub>558</sub>. Indeed, differential reduced minus oxidized absorbance spectra of all purified mutated cytochromes exhibited the characteristic peaks perfectly superimposable on those of purified WT-Nox2 cytb (Fig. 5B). Then cytochrome c and INT reductase activities were measured in a simplified cell-free system with purified cytochromes in the presence of recombinant p47<sub>phox</sub>, p67<sub>phox</sub>, and Rac1 proteins as described above. No NADPH oxidase activity could be reconstituted <i>in vitro</i> for the purified A57E, R54M, and R54S cytochrome b<sub>558</sub> (Fig. 6A), as found previously in cell-free system assays with purified plasma membranes from mutant cells (Table 1). Nevertheless, purified WT-Nox2 cytb was active in this system, and its function was characteristically dependent on the presence of p67<sub>phox</sub> and Rac1 (Fig. 6, A and B). INT reductase activities of the purified R54M and R54S mutated cytochromes were normal and superimposable to what was found for purified plasma membranes from the mutant PLB-985 cells, i.e. (Fig. 6C and Table 1). In conclusion, the abolition of the oxidase activity of the Arg-54 mutants was not caused by defects in oxidase assembly (Fig. 4), in FAD incorporation (Table 2), in heme incorporation (Fig. 5, Table 2), or in electron transfer from NADPH to FAD (Fig. 6C) but was most probably due to an inhibition of electron transfer from FAD to molecular oxygen.

The INT reductase activity of the purified A57E cytb was only half of the value found in the WT-Nox2 cytb as we had previously found in purified plasma membranes and was highly dependent on the presence of p67<sub>phox</sub> and Rac1 (Fig. 6, C and D). Interestingly, the presence of p47<sub>phox</sub> significantly enhanced the INT reductase activity of both WT and A57E cytochromes (Fig. 6D). To better understand the decrease of this activity compared with that of the other mutants within this region, we studied the effect of increasing amounts of p67<sub>phox</sub> and FAD on INT reductase activity of the A57E cytb. We used a simplified cell-free system assay with purified A57E cytb and recombinant cytosolic proteins and examined the effect of concentration of p67<sub>phox</sub> protein at saturating amounts of Rac1 and p47<sub>phox</sub>. Even at excessive concentrations of p67<sub>phox</sub> protein, the V<sub>max</sub> of A57E cytb never reached the V<sub>max</sub> of WT-Nox2 cytb (Fig. 7, A and B). Additionally, the EC<sub>50</sub> value of the dependence of A57E cytb-INT reductase activity on p67<sub>phox</sub> protein was slightly increased (7.1 versus 2 nm) (Table 3). We also tested the concentration dependence of FAD on the INT reductase (Fig. 7, C and D). The affinity for FAD was similar in the A57E mutant and in the WT-Nox2 cytb (0.35 ± 0.05 versus 0.23 ± 0.03 nm), and the V<sub>max</sub> was 2-fold lower in the A57E mutant than in the WT-Nox2 cytb (Table 3). Thus, the diminished amount of FAD bound (>60% lower) by this mutant was probably not due to a change of affinity for this cofactor.

In Fig. 4 we show that NADPH oxidase assembly appeared to be partially inhibited during phagocytosis of latex beads in A57E mutant PLB-985 cells. To explore the possibility that the region centered on alanine 57 serves as an anchoring site for the assembly of cytosolic factors, we examined the ability of WT (51<sup>ALARAPAACLNFN</sup> and mutated (51<sup>ALARAPAECLNFN</sup>) peptides to inhibit INT reductase activity of the plasma membranes from WT-Nox2 PLB-985 cells (Fig. 8A). No effect was observed even at high concentrations of the peptides (200 μM). This result suggests that this sequence region may not be directly involved in the binding of cytosolic factors during NADPH oxidase assembly, but an indirect interaction cannot be ruled out. Furthermore, to test whether the second transmembrane α-helix (and more specifically residue Ala-57) exerts a direct influence on the function of the B-loop, we measured the diaphorase activity of the WT-Nox2 and the A57E purified membranes in the presence of increasing amounts of a peptide mimicking the charged region of the B-loop. At a low concentration (<0.2 μM), this peptide had a drastic inhibitory effect on the diaphorase activity of the WT-Nox2 membranes (about 80% of inhibition), although it had no effect on that of the A57E membranes. In addition at higher concentrations

![FIGURE 8. A, peptide inhibition experiments were conducted by incubating purified plasma membranes from WT-Nox2 PLB-985 cells with WT (triangles) or mutated peptides (circles) 51<sup>ALARAPAACLNFN</sup> and 51<sup>ALARAPAECLNFN</sup>, respectively, at the indicated concentrations in the reaction mixture, in the presence of recombinant p47<sub>phox</sub>, p67<sub>phox</sub>, and Rac1 proteins. INT reductase activity was measured as described under "Experimental Procedures." B, same experiment was conducted with WT-Nox2 (triangles) and A57E-Nox2 (squares) purified plasma membranes, in the presence of increasing amounts of a peptide mimicking the charged sequence of the B-loop 77<sup>CRNLLSLRGGSSACCSTRVRQ</sup>DLNL<sup>165</sup>. INT reductase activity was measured as described under "Experimental Procedures." The data represent mean ± S.D. of two separate experiments.](image-url)
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A  WT-Nox2

B  R54M

C  SOD inhibition of N/T reductase activity (%)

D  mol electrons/mol cytochrome b556/sec

E  mol electrons/mol cytochrome b556/sec

WT-Nox2  A57E  R54M  R54S

- DPI  + DPI
(>20 μM), it can restore the diaphorase activity of the A57E plasma membranes that attain the activity of the WT membranes with 100 μM peptide (Fig. 8B). In conclusion, the second transmembrane helix controls the function of the B-loop and both regions are clearly involved in the electron transfer occurring in the dehydrogenase domain of Nox2.

Finally, we took advantage of having purified WT and R54S, and R54M mutated cytochrome b_{558} with normal INT reductase activity to clarify whether superoxide reduces INT. The first part of our experiment was under aerobic conditions where INT reduction by the WT-Nox2 cyt b was inhibited by 90% by SOD, and the INT reduction of the R54M cyt b was not or was only slightly inhibited (20%) (Fig. 9, A–C). Surprisingly in the absence of oxygen (i.e. in absence of O_{2} production by WT-Nox2 cyt b), the addition of SOD had only a small and equivalent inhibitory effect on INT reduction by the WT-Nox2 and R54M cytsb (10–20%). The same result was observed for the R54S and A57E mutants, even though the diaphorase activity of this last mutant was about 30% of normal (data not shown). In other words, INT reduction reflects the electron transfer from NADPH to FAD only in the absence of O_{2} production (in anaerobiosis for the WT-Nox2 cyt b and in aerobicosis for X^{+} CGD cytsb). The same result was obtained when INT reductase activity was initiated by adding NADPH and SOD simultaneously (Fig. 9D). Finally, under aerobic conditions, we confirmed that the electron transfer by the mutated cytsb was totally inhibited by the addition of DPI (Fig. 9E).

**DISCUSSION**

Nox2 or gp91^{phox}, the heavy chain of cyt b, is the redox element of the NADPH oxidase in which electron transfer from NADPH to molecular oxygen occurs after activation. According to the hydrophobicity profile of its sequence, the N-terminal domain of Nox2 could be structured into six α-helices embedded in the plasma membrane of phagocytic cells (Fig. 1A). However, the organization and the functioning of this region are not fully understood. The objective of this study was to examine the role of the potential second transmembrane α-helix X^{283–291}LLGSALARAPAACLNFNCMLIL (69) and, more specifically, the role of the underlined residues from Ala-53 to Cys-59, which until now have never been studied.

Most of the missense mutations leading to rare X^{+} CGD variants are located in the cytosolic C-terminal tail of Nox2 (28), with the exception of mutations of residues Arg-54 and Ala-57 (23) located in the putative second transmembrane α-helix of Nox2 according to the commonly accepted model (35). In addition only three missense mutations in this region A53D, P56L, and C59F, lead to X^{−} CGD variants (23). We used an original approach, i.e. the reproduction of seven human CGD missense mutations in the PLB-985 cell model, A53D, R54G, R54S, R54M, P56L, A57E, and C59F to study their structural and functional impact on Nox2.

We found that A53D, R54G, R54S, R54M, and A57E mutations inhibited NADPH oxidase activity and led to an X^{−} CGD phenotype, whereas mutation P56L led to an X^{−} CGD case. The most drastic effect was obtained with the change of cysteine 59 to phenylalanine, which totally abrogates the expression of Nox2. Thus a hydrophobic residue (Leu or Phe) at position 56 or 59 or of X^{283–291}LLGSALARAPAACLNFNCMLIL (69) destabilizes the conformation of the entire protein. These residues could be potential sites for anchoring p22^{phox} and/or disulfide bridge formation (Cys-59) during the maturation process of cyt b. Recently the D-loop has been described as another possible anchoring region for p22^{phox} (20).

Previously, the functional consequences of the R54S mutation were shown in neutrophils from an X^{+} CGD patient, where only a slight ROS production was observed (25). Using chemiluminescence, we confirmed the weak ROS production in the R54S mutant and in the R54G mutant but not in the R54M mutant. In this instance, the positive charge of Arg-54 appears to be essential for maintaining NADPH oxidase activity. Thus, the change to a neutral residue such as serine or glycine is likely to have a less drastic effect on NADPH oxidase activity than the introduction of a methionine residue with a nonpolar side chain that modifies the electronic environment and leads to steric clustering. However, we could detect no in vitro NADPH oxidase activity by SOD-sensitive cytochrome c reduction for the R54G and R54S mutants as well, probably because of the low sensitivity of the assay. Additionally, diaphorase activity measured by the INT reduction assay was validated in our X^{+} CGD mutants. The diaphorase activity in plasma membranes and in purified mutated cytsb was normal (R54S, R54M, and R54G) and inhibited by DPI suggesting the normal reduction of flavin in these mutants. The A53D mutant cytochrome b_{558} had roughly the same properties as the Arg-54 mutants, namely normal diaphorase activity with abolished oxidase activity.

Using CS9 immunoaffinity-purified cytsb, we studied INT reduction by the R54S and R54M mutants in more depth. No superoxide production could be detected in contrast to the identically purified WT cyt b, which showed normal superoxide production. With the immunopurified cyt b_{558}, we also demonstrated that INT reduction actually reflected electron transfer from NADPH to FAD only in the absence of superoxide production. This idea was originally proposed by Poinas et al. (10) but was called into question by Cross et al. (9), who observed a 1-nm shift in the reduced minus oxidized difference spectrum of the R54G mutant at the Soret band, in a crude membrane fraction (25). Using R54S, and R54M mutant cyt b, purified almost to homogeneity (purity >95%), we found that the peak

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**FIGURE 9. Impact of superoxide anion production on in vitro INT reduction of purified WT-Nox2 and transmembrane cytochrome b_{558} mutants.** Kinetics of INT reduction of purified WT-Nox2 (A) or transmembrane R54M cytochrome b_{558} (B) were evaluated in the presence (black curve) or in the absence (gray curve) of oxygen. SOD (50 μg/ml) was added to evaluate the role of O_{2} in INT reduction. Results are from one experiment representative of three. C, data represent the percentage of SOD inhibition of INT reductase activity in aerobic or anaerobic conditions. The same results were obtained with A57E and R54S mutants. D, INT reductase activity of purified cyt b was initiated by adding either NADPH alone or NADPH and SOD at the same time. INT reductase activity was measured as described previously. The data represent mean ± S.D. of two separate experiments. E, effect of DPI on INT reductase activity of WT-Nox2 and transmembrane cytochrome b_{558} mutants. INT reductase activity was measured as described under “Experimental Procedures” in the presence (black column) or absence of DPI 5 μM (gray column).
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values at 426 and 558 nm of the reduced minus oxidized absorbance difference spectra were exactly superimposable on those of WT-Nox2 PLB-985 cells, supporting correctly incorporated hemes in the mutant Nox2. We also found no difference in the oxidized and reduced spectra of the mutated cytbs (data not shown). Finally, NADPH oxidase complex assembly during phagocytosis and the binding of FAD were normal in the Arg-54 and the Ala-53 mutant cells. In conclusion, the absence of NADPH oxidase activity in R54G, R54S, and R54M and A53E mutant cells can be explained if alanine 53 and arginine 54 are essential residues involved in the control of electron flow from FAD to molecular oxygen.

The A57E mutation, like the other X° CGD mutants of this region, totally inhibited NADPH oxidase activity, but surprisingly, diaphorase activity was 30% of control in purified plasma membranes. For purified A57E cytb, this residual INT reductase activity was dependent on the presence of p67phox and Rac. Increasing amounts of p67phox did not change the initial V\textsubscript{max} values, but we found about a 3-fold reduction in the affinity of purified A57E cytb for p67phox. Phagocytosis of latex beads in the mutated A57E PLB-985 cell line revealed a partial defect in p67phox translocation to the plasma membrane. WT and A57E mutated peptides used in competition experiments had no effect on diaphorase activity of WT Nox2 plasma membranes suggesting that the second transmembrane a-helix is not directly involved in the binding of cytosolic factors during oxidase assembly. In addition, FAD binding in the plasma membranes of the A57E mutant was diminished (about 30% of the control), but the affinity of the purified A57E cytb for FAD was only slightly affected compared with WT-Nox2. Thus, we conclude that the partial inhibition of diaphorase activity in the A57E mutant may result from a defect of FAD access to its binding site resulting from nonoptimal oxidase assembly. Mutation of alanine 57 to an acidic residue clearly modifies the net charge of the region, and directly or indirectly it disturbs the integrity of the dehydrogenase domain leading to partial inhibition of electron transfer from NADPH to FAD (Fig. 1B). This also suggests some interaction between the transmembrane domain and the cytosolic dehydrogenase domain of Nox2.

Recently, the B-loops of Nox4 and Nox2 have been proposed as an interface between the dehydrogenase domains and the transmembrane domains of Nox enzymes (21). In addition, the B-loop is involved in the oxidase assembly process of the phagocytic NADPH oxidase (19). Very recently, a charged peptide CSSTRVRLQ\textsuperscript{84} mimicking part of the B-loop sequence was demonstrated to be an isoform-specific inhibitor of Nox2 showing that the B-loop is important in maintaining the oxidase activity (36). Indeed, by using a peptide mimicking the charged sequence of the B-loop, we showed that the B-loop controls the diaphorase activity of Nox2 and that the second transmembrane a-helix of Nox2 influences the function of the B-loop. The presence of an acidic residue at position 57 would interfere with the structural organization of the B-loop thus explaining the partial oxidase assembly and inhibited diaphorase activity of this mutant. In Taylor’s model of the “dehydrogenase” domain of Nox2 (11), there is an open space above the NADPH- and FAD-binding sites. This void could provide a region for other cytoplasmic residues to be involved in the organization of the NADPH/FAD-binding site so as to control electron transfer (11, 14, 28, 37). Candidates include the C-terminal aromatic residue Phe-570, a conserved and essential residue in the whole ferredoxin-NADP\textsuperscript+ reductase family, and/or the region centered on Cys-369, which is a docking site for p67phox (28). Dang et al. (37) also proposed that p67phox had a FAD-binding site that could participate to the electron flow from the NADPH on Nox2. Crystallization and resolution of the structure of the N-terminal part of Nox2 will be of great help in confirming the organization of these different structures involved in the control of p67phox anchoring, FAD binding, and dehydrogenase activity.

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