Crucial Role of Two Potential Cytosolic Regions of Nox2, 191TSSTKTIRRS\(^ {200}\) and 484DESQANHFAVHHDEEKD\(^ {500}\), on NADPH Oxidase Activation*

Received for publication, July 7, 2005, and in revised form, January 27, 2005
Published, JBC Papers in Press, January 31, 2005, DOI 10.1074/jbc.M500226200

Xing Jun Li‡, Didier Grunwald§, Jacques Mathieu¶, Françoise Morel†, and Marie-José Stasia¶¶

From the ³Groupe de Recherche et d’Etude du Processus Inflammatoire EA 2938 Université Joseph Fourier, Laboratoire Enzymyle, Centre Hospitalier Universitaire, 38043 Grenoble cedex 9, the ²Département Réponse et Dynamique Cellulaire/Commissariat à l’Energie Atomique, 17 rue des Martyrs, 38054 Grenoble cedex 9, and the 4Département de Radiobiologie, Centre de Recherches du Service de Santé des Armées, 38702 La Tronche cedex, France

Assembly of cytosolic factors p67\(^ {\text{phox}}\) and p47\(^ {\text{phox}}\) with cytochrome b\(_{558}\) is one of the crucial keys for NADPH oxidase activation. Certain sequences of Nox2 appear to be involved in a cytosolic factor interaction. The role of the D-loop 191TSSTKTIRRS\(^ {200}\) and the C-terminal 484DESQANHFAVHHDEEKD\(^ {500}\) of Nox2 on oxidase activity and assembly was investigated. Charged amino acids were mutated to neutral or reverse charge by directed mutagenesis to generate 21 mutants. Recombinant wild-type or mutant Nox2 were expressed in the X-CGD PLB-985 cell model. K195A/E, R198E, R199E, and RR198199QQ/AA mutations in the D-loop of Nox2 totally abolished oxidase activity. However, these D-loop mutants demonstrated normal p47\(^ {\text{phox}}\) translocation and iodonitrotetrazolium (INT) reductase activity, suggesting that charged amino acids of this region are essential for electron transfer from FAD to oxygen. Replacement of Nox2 D-loop with its homolog of Nox1, Nox3, or Nox4 was fully functional. In addition, FMLP (formylmethionine- 

Baldridge and Gerard (1, 2) discovered the respiratory burst in which dramatic oxygen consumption occurred in neutrophils during bacteria phagocytosis. Subsequent research showed that the NADPH oxidase complex of phagocytes has a direct interaction between the respiratory burst oxidase, consisting of a large glycoprotein gp91\(^ {\text{phox}}\) or Nox2 and a small protein p22\(^ {\text{phox}}\) (12). Nox2 is the catalytic center that contains flavocytochrome b\(_{558}\), cytosolic proteins p47\(^ {\text{phox}}\), p67\(^ {\text{phox}}\), and p40\(^ {\text{phox}}\), and two small GTPases, Rac2 and Rap1A. Cytochrome b\(_{558}\), the redox core of the respiratory burst oxidase, contains a large glycoprotein gp91\(^ {\text{phox}}\) or Nox2 and a small protein p22\(^ {\text{phox}}\) (12). Nox2 is the catalytic center that transfers the electrons from intracellular NADPH to extracellular O\(_2\). It contains two nonidentical hemes and consensus-binding sequences for FAD and NADPH (13–16). NADPH oxidase is a multicomponent complex, composed of a heterodimeric transmembrane protein known as flavocytochrome b\(_{558}\), cytosolic proteins p47\(^ {\text{phox}}\), p67\(^ {\text{phox}}\), and p40\(^ {\text{phox}}\), and two small GTPases, Rac2 and Rap1A. Cytochrome b\(_{558}\), the redox core of the respiratory burst oxidase, contains a large glycoprotein gp91\(^ {\text{phox}}\) or Nox2 and a small protein p22\(^ {\text{phox}}\) (12). Nox2 is the catalytic center that transfers the electrons from intracellular NADPH to extracellular O\(_2\). It contains two nonidentical hemes and consensus-binding sequences for FAD and NADPH (13–16). NADPH oxidase becomes activated and generates O\(_2^\cdot\) after cytosolic factor interaction. The role of certain sequences of Nox2 appear to be involved in the correct assembly of the NADPH oxidase complex occurring during activation, permitting cytosolic factor translocation and electron transfer from NADPH to FAD.

* This work was supported by grants from the Université Joseph Fourier, Faculté de Médecine; the Région Rhône-Alpes, programme Emergence and programme concerté de recherche Tempra/Mira 2001; the Ministère de l’Education et de la Recherche; and the Direction de la Recherche Régionale Clinique, Laboratoire Merck-Sharp and Dohme-Chibret. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 33-4-7676-5483; Fax: 33-4-7676-5608; E-mail: MJStasia@chu-grenoble.fr.

1 The abbreviations used are: CGD, chronic granulomatous disease; PMA, phorbol 12-myristate 13-acetate; DMLP, formylmethionineleucylleucylphenylalanine; DMP, dimethylformamide; GTP-\(^\gamma\)S, \(5\’-\)\(^{\text{3}}\)-O-(thio)triphosphate; BCS, broken cell system; WT, wild type; FACS, fluorescence-activated cell sorting; INT, iodonitrotetrazolium.
Role of Nox2 Cytosolic Regions on NADPH Oxidase Activation

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate (PMA), dimethylformamide (DMF), diisopropylfluorophosphate, and horseradish peroxidase, cytochrome c (horse heart, type VI), and latex beads were obtained from Sigma. TaqDNA polymerase, ATP, GTP-S, and NADPH were from New England Biolabs. N-ethylmaleimide was purchased from Sigma. Anti-p67phox and anti-p47phox antibodies were obtained from Upstate Biotechnology, Inc. Monoclonal antibody specific for gp91phox, D55 was kindly provided by Dr. M. Nakamura (Nagasaki University, Nagasaki, Japan). Fetal bovine serum and RPMI 1640 were from Invitrogen.

Cell Culture and Granulocyte Differentiation—WT, X-CGD, and transfected PLB-985 cells expressing WT or the mutant were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine at 37 °C in a 5% CO2 atmosphere. After selection, 0.5 mg/ml G418 was added to maintain the selection pressure. PLB-985 cells (5 × 105 cells/ml) were exposed to 0.5% DMF for 5–7 days, providing granulocytic differentiation. Analysis of Nox2 Protein Expression—PLB-985 cells (5 × 105) were incubated with 10 μg/ml of mAb 7D5 directed against Nox2 or control monoclonal IgG1 (Immunotech, Marseille, France). The WT or mutant Nox2 cDNA was subcloned into the mammalian expression vector pEF-PGKneo as previously described (37). The pEF-PGKneo constructs were electroporated into X-CGD PLB-985 cells in which the gp91phox gene was disrupted by gene targeting, resulting in the absence of Nox2 expression and NADPH oxidase activity and assembly described previously (37, 38). Clones were selected by limiting dilution in 1.5 mg/ml G418.

Site-directed Mutagenesis and Expression of Recombinant gp91phox in X-CGD PLB-985 Cell Line—Mutations were introduced into the wild-type (WT) Nox2 cDNA in pBluescript II KS(+) vector using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The sequence of WT and the mutated gp91phox cDNA were verified by diodeoxyxucleotide sequencing (Genome Express, Grenoble, France).

Site-directed Mutagenesis—The R199Q and D-loop mutations optimize the NADPH oxidase activation process. They found that two Arg at positions 91 and 92 were essential for NADPH oxidase activity and assembly. However, the role of the second intracytosolic polybasic loop of Nox2, the D-loop, on oxidase activity and assembly has not been fully elucidated.

Materials—Phorbol 12-myristate 13-acetate (PMA), dimethylformamide (DMF), diisopropylfluorophosphate, and horseradish peroxidase, cytochrome c (horse heart, type VI), and latex beads were obtained from Sigma. TaqDNA polymerase, ATP, GTP-S, and NADPH were from New England Biolabs. N-ethylmaleimide was purchased from Sigma. Anti-p67phox and anti-p47phox antibodies were obtained from Upstate Biotechnology, Inc. Monoclonal antibody specific for gp91phox, D55 was kindly provided by Dr. M. Nakamura (Nagasaki University, Nagasaki, Japan). Fetal bovine serum and RPMI 1640 were from Invitrogen.

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The aim of this work was to investigate the role of two regions of Nox2, the intracytosolic D-loop 191TSSTKIRR200 and the C-terminal α-helical loop 484DEESQANHFAVHDEEKD500, on oxidase activity and assembly by means of the mutagenesis approach in the X-CGD PLB-985 cell model (37). Results suggest that these two regions are critical for NADPH oxidase activity, especially the charged amino acids Arg195, Arg198, Arg209, Asp244, and Asp250. The D-loop of Nox2 is probably involved in electron transfer from FAD to oxygen independently of cytosolic factor translocation, whereas the C-terminal α-helical loop is crucial for the assembly of oxidase and electron transfer from NADPH to FAD. Chimeric Nox2 proteins containing the D-loop of Nox1/3/4 support NADPH oxidase activity, suggesting that this region should play a similar role on Nox analog activation. Finally, two “super-mutants” of the D-loop of Nox2, with a significant increase in oxidase activity, were obtained, suggesting that the R199Q and D-loopNox4 mutations optimize the NADPH oxidase activation process.

Materials—Phorbol 12-myristate 13-acetate (PMA), dimethylformamide (DMF), diisopropylfluorophosphate, and horseradish peroxidase, cytochrome c (horse heart, type VI), and latex beads were obtained from Sigma. TaqDNA polymerase, ATP, GTP-S, and NADPH were from New England Biolabs. N-ethylmaleimide was purchased from Sigma. Anti-p67phox and anti-p47phox antibodies were obtained from Upstate Biotechnology, Inc. Monoclonal antibody specific for gp91phox, D55 was kindly provided by Dr. M. Nakamura (Nagasaki University, Nagasaki, Japan). Fetal bovine serum and RPMI 1640 were from Invitrogen.

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Detection of NADPH Oxidase Activity in a Broken Cell System—NADPH oxidase activity in vitro was measured in a homologous BCS using previously described protocols (52). Briefly, plasma membranes obtained from transgenic PLB-985 cells and cytosol of control human neutrophils were added to a reaction mixture containing 20 mM glucose, 20 μM GTPγS, 5 mM MgCl₂, and arachidonic acid in a final volume of 100 μl. After incubation for 10 min at 25 °C, the oxidase activation was initiated in the presence of 100 μM cytochrome c and 150 μM NADPH. The specificity of the O₂⁻ production was checked by adding 50 μg/ml superoxide dismutase to stop the kinetic reduction.

Iodonitrotetrazolium Reductase Activity—Diaphorase activity was measured under the same BCS assay conditions, except that the 100 μM cytochrome c was replaced by 50 μM INT (53).

Analysis of in Vivo p47phox Translocation—In vivo p47phox translocation was detected by confocal microscopy analysis according to (38) with little modified. The 5 × 10⁵ differentiated PLB-985 cells deposited on coverslips were incubated with human neutrophil cytosol (1,000 μg) for 2 min at 30 °C and activated with (+) or without 100 μM SDS and 20 μM GTPγS for 15 min at 30 °C. Membranes were collected between the 45 and 20% (w/v) sucrose layers after centrifugation (30,000 rpm × 1 h in an SW41 rotor (Beckman) at 18 °C) and analyzed using immunoblotting with anti-peptide polyclonal antibody directed against p47phox (24).

Analysis of Translocation of p47phox in Vitro—In vitro p47phox translocation to the plasma membrane was measured using a classic protocol (37). Briefly, membranes purified from PLB-985 cells (100 μg) were preincubated with human neutrophil cytosol (1,000 μg) for 2 min at 30 °C and activated with (+) or without 100 μM SDS and 20 μM GTPγS for 15 min at 30 °C. Membranes were collected between the 45 and 20% (w/v) sucrose layers after centrifugation (30,000 rpm × 1 h in an SW41 rotor (Beckman) at 18 °C) and analyzed using immunoblotting with anti-peptide polyclonal antibody directed against p47phox (24).

Protein Determination—Protein content was estimated using the Bradford assay (54) or the Pierce® method (55).

RESULTS

To further understand the role of the D-loop (191TSSTKIRR₂₀₀) and the C-terminal (484DESQANHFAVHDEEKD) of Nox2 in the ferrodoxin NADP⁺ family, positive charged amino acid residues are shown in the light shaded column, Asp residues are shown in the dark shaded column.

FIG. 1. Amino acid sequence alignment of the D-loop (191TSSTKIRR₂₀₀) and the C-terminal (484DESQANHFAVHDEEKD) of Nox2 in the ferrodoxin NADP⁺ family.
two regions (Fig. 2). To further elucidate the function of the D-loop in the Nox family, the D-loop of Nox2 was replaced with the same region of their homologs Nox1, Nox3, and Nox4. Twelve mutants of the D-loop (Fig. 2A) and eight of the α-helical loop in the C-terminal of Nox2 (Fig. 2B) were generated by directed mutagenesis and stably transfected in X-CGD PLB-985 cells, which lack endogenous Nox2 expression because of gene targeting (56). We also produced two mutants known to disturb cytosolic factor translocation to the plasma membrane, the D500G mutant, which had reproduced a human X-CGD case (30), and the RR9192EE mutant of the B loop of Nox2 previously studied by Biberstine-Kindade et al. (35). These two mutants served as controls to validate NADPH oxidase activity and assembly experiments.

The WT and mutated Nox2 cDNA subcloned into the pEF-PGKneo vector were verified by sequence analysis and purified before transfection in X-CGD PLB-985 cells, as described under “Experimental Procedures.” Nox2 expression was studied by FACS using the monoclonal antibody 7D5, and IgG1 were used as negative control to test the specificity of Nox2 binding (38). For some mutants, FACS was also used to sort a highly Nox2-expressing population. This was done for K195A, RR198199QQ (data not shown), and K195E mutant cells (Fig. 3A). We detected 12–20 different clones for each mutant and to minimize clone-to-clone variation in NADPH oxidase activity, three to four independent clones of each highly expressing Nox2 mutant were used for subsequent analysis (data not shown). As expected, Nox2 was not detected in X-CGD PLB-985 cells or in empty vector transfected PLB-985 cells. Expression of WT or mutant Nox2 was also assessed by semiquantitative immunoblotting of soluble extracts from differentiated WT and transfected X-CGD PLB-985 cells using the monoclonal antibody 48 (38). Similar Nox2 expression was seen in all differentiated PLB-985 cells (Fig. 3B). Identical differential spectrum characteristics of flavocytochrome $b_{558}$ were observed in all mutants compared with WT Nox2-transfected PLB cells or WT PLB-985 cells (data not shown). The amount of cytochrome $b_{558}$ was equivalent in all the transfected X-CGD PLB-985 cells, confirming results previously obtained by FACS and Western blot analysis (Table I). This demonstrated correct heme incorporation in recombinant Nox2. No differential spectra were detected in X-CGD PLB-985 cells or empty vector-transfected cells. In conclusion, high, stable, and similar amounts of recombinant WT or mutant Nox2 proteins were expressed in transfected X-CGD PLB-985 cells. This was crucial for comparing the impact of each mutation on NADPH oxidase functions in such cells.

We next measured $\mathrm{H}_2\mathrm{O}_2$ production in transfected X-CGD PLB-985 cells stimulated by 80 ng/ml PMA using luminol-amplified CL. The RR9192EE Nox2 transfected PLB-985 cells exhibited no oxidase activity, whereas its cytochrome $b_{558}$ expression was comparable to the WT Nox2-transfected PLB-985 cells (Table I). This was consistent with previously published results (35) and allowed us to validate our methodology.
FIG. 3. Expression of WT and mutated Nox2 in transfected X-CGD PLB-985 cells. A, $5 \times 10^5$ transfected X-CGD PLB-985 cells were incubated with the gp91\textsuperscript{phox} monoclonal antibody 7D5, combined with a phycoerythrin-conjugated goat-anti-IgG (H+L) as described under "Experimental Procedures." Mouse IgG1 isotype was used as an irrelevant monoclonal antibody. B, immunodetection of gp91\textsuperscript{phox} subunits of cytochrome $b_{558}$ (Nox2) was performed with 50 $\mu$g of 1% Triton X-100 soluble extracts from WT or transfected X-CGD PLB-985 cells, subjected to SDS-PAGE (10% acrylamide gel), blotted onto a nitrocellulose sheet, and revealed with mAb 48 monoclonal antibodies. Results are from one experiment representative of three.
Role of Nox2 Cytosolic Regions on NADPH Oxidase Activation

NADPH oxidase activity was completely abolished in cells expressing K195A/E, R198E, R199E, and RR198199QQ mutations of the D-loop of Nox2. The RR198199AA mutation had the same inhibitory effect on oxidase activity as the RR198199QQ mutation (data not shown). However, cells expressing R198Q-Nox2 and RR198199EE-Nox2 mutants exhibited almost 60% of NADPH oxidase activity measured in WT PLB-985 cells. Surprisingly, transfected R199Q and D-loop_{Nox4/Nox 2} mutant PLB-985 cells stimulated with PMA had an increase of 1.3- to 1.6-fold in H₂O₂ production compared with the WT PLB-985 cells (Table I). In the C-terminal tail of Nox2, changing Asp^{484} to a neutral amino acid (D484T) inhibited the NADPH oxidase activity, whereas replacing it with a positive charge (D484H) had little effect (79% of control). In addition, the D496H mutation had no effect on oxidase activity. However, cells expressing H490A-Nox2 exhibited normal oxidase activity, whereas the H490D mutation destroyed most of the NADPH oxidase activity (10% of control) (Table I). In conclusion, the effect of D484 and H490 charge changes had different effects on oxidase activity depending on the type of amino acid replacement. Meanwhile, the disappearance of the negative charge supported by Asp^{490} in the D500A, D500R, and H490A mutations had a definitive inhibitory effect on NADPH oxidase activity (Table I). It should be noted that the D500G Nox2-transfected cells mimicked the phenotype of a previously described X-CGD case (30).

To improve our knowledge of H₂O₂ production in DMF-differentiated transfected X-CGD PLB-985 cells, PMA and fMLP stimulation were compared (Fig. 4A). All the experiments were done in triplicate and reproduced twice. Inhibitory effects of some mutations on oxidase activity, observed previously in PMA-stimulated PLB-985 cells, were also obtained in fMLP-activated mutant cells. The increasing effect on NADPH oxidase activity of the R199Q mutation and the replacement of the Nox2 D-loop with the same from Nox1/3/4, was more evident using fMLP stimulation. Indeed in the R199Q and the D-loop_{Nox4} mutant cells, H₂O₂ production was 7.9 and 4.6 times higher than in the WT PLB-985 cells, respectively (Fig. 4A). The kinetics of H₂O₂ production was not the same when WT or WT Nox2-transfected PLB-985 cells were activated by PMA or fMLP (Fig. 4B). The maximum of H₂O₂ production (V_max) was obtained in 10–13 min (T_max) for PMA activation, whereas for fMLP, the T_max was reached in roughly 4 min (Table II). Interestingly, the V_max for the D-loop_{Nox4}-Nox2 super-mutant cells activated with PMA was obtained in 4–5 min versus 10–13 min in WT PLB-985 cells. However, when these cells were stimulated with fMLP, the kinetics of H₂O₂ production remained unchanged, whereas the V_max was higher than in the WT PLB-985 cells (Fig. 4B and Table II). The same kinetic changes in H₂O₂ production were also observed in the R199Q mutant cells (data not shown).

We also examined the in vitro NADPH oxidase activity of transfected X-CGD PLB-985 cells using a broken cell system (BCS). To compare only the effect of mutations on Nox2 of the plasma membranes purified from transfected PLB-985 cells, cytosol from human neutrophils was used as the source of NADPH oxidase cytosolic components. The in vitro oxidase activity was totally restored in X-CGD PLB-985 transfected with WT Nox2 cDNA compared with WT PLB-985 cells (Table I). For almost all the studied mutations, the results obtained in BCS were correlated with those obtained in intact cells, although residual oxidase activity (~20% of the original WT PLB-985 cells) was observed in some mutants that had no oxidase activity in vivo. In addition, the highly increased NADPH oxidase activity measured in intact R199Q and in the D-loop_{Nox4}-Nox2 mutant cells activated with fMLP was not reconstituted in vitro.

As demonstrated above, mutations within these two cytosolic regions of Nox2 (K195A/E, R198E, R199E, RR198199QQ, D484T, and D500A/R/G) completely abolished the NADPH ox-
idase activity. To investigate the effect of these mutations on oxidase complex assembly, we used the confocal microscopy method, which allowed us to follow p47phox translocation to the plasma membrane in intact cells after stimulation, and in some experiments Nox2 localization was visualized by 7D5 recognition. To fully express p47phox, p67phox, p40phox, and gp91phox.

**FIG. 4.** H$_2$O$_2$ production in WT and in transfected X-CGD PLB-985 cells after PMA and fMLP activation. A, total H$_2$O$_2$ production was measured by luminol-amplified CL from $5 \times 10^5$ intact WT or transfected X-CGD PLB-985 cells differentiated with 0.5% DMF for 6 days and stimulated with 80 ng/ml PMA or $4 \times 10^{-7}$ M fMLP. Results are expressed in a percentage by dividing the total RLU value obtained for each type of cells by the value of WT PLB-985 cells. Values in this figure represent the mean ± S.D. of triplicate determinations obtained the same day. The same experiment was done twice on 2 different days. *, p < 0.05; **, p < 0.01. B, kinetics of H$_2$O$_2$ production in $5 \times 10^5$ intact WT, or WT Nox2, or D-loop-Nox2 transfected X-CGD PLB-985 cells stimulated either by 80 ng/ml PMA or $4 \times 10^{-7}$ M fMLP.

|          | WT PLB-985 cells | WT Nox2 cells | D-loop of NOX 4 |
|----------|-----------------|---------------|-----------------|
| **PMA** |                 |               |                 |
| Total H$_2$O$_2$ production (RLU) | 397.75 ± 5.07 | 453.02 ± 4.19 | 646.93 ± 11.11 |
| $T_{\text{max}}$ (min) | 13.17 ± 1.44 | 10.83 ± 0.58 | 4.67 ± 0.29 |
| $V_{\text{max}}$ (RLU) | 5.94 ± 0.18 | 9.06 ± 0.17 | 38.9 ± 0.97 |
| **fMLP** |                 |               |                 |
| Total H$_2$O$_2$ production (RLU) | 4.65 ± 0.26 | 5.32 ± 0.15 | 22.79 ± 1.98 |
| $T_{\text{max}}$ (min) | 4.00 ± 0.00 | 3.50 ± 0.00 | 2.83 ± 0.29 |
| $V_{\text{max}}$ (RLU) | 0.40 ± 0.03 | 0.66 ± 0.04 | 3.07 ± 0.57 |

**TABLE II**

Kinetics of H$_2$O$_2$ production in WT and in X-CGD transfected PLB-985 cells

H$_2$O$_2$ production in intact $5 \times 10^5$ differentiated WT or transfected X-CGD PLB-985 cells was measured using the chemiluminescence method after activation by 80 ng/ml PMA or $4 \times 10^{-7}$ M fMLP for 90 min. Values represent the mean ± S.D. of triplicate determinations. $V_{\text{max}}$ indicates the maximum of H$_2$O$_2$ production obtained. $T_{\text{max}}$ indicates the time to $V_{\text{max}}$, which was measured as the elapsed time from the start of the assay until the maximum H$_2$O$_2$ production was obtained.
PLB-985 WT or mutated cells were differentiated by 0.5% DMF for 6 days before PMA-treated latex-bead stimulation, as described under “Experimental Procedures.” For each translocation experiment, NADPH oxidase activity was measured the same day. The deformation of the TO-PRO 3 iodine-labeled nuclei allowed us to ascertain that the latex beads were indeed in the cell. As seen in Fig. 5, the phagocytosis of latex beads occurred in WT PLB-985 cells as in empty-vector transfected X-CGD PLB-985 cells and in X/H11001 CGD PLB-985 cells (D500G mutant) independently of oxidase activity. As expected, p47phox protein was present in cytosol from all the tested PLB-985 cells. No fluorescence was observed in WT PLB-985 cells when primary antibodies were omitted. The Red and Green fluorescence colors representing Nox2 and p47phox, respectively, surrounded the phagosome membranes around the latex beads only in intact WT PLB-985 cells (Fig. 5). A yellow merged image indicated the co-localization of p47phox and Nox2 in phagosome membranes. However, the D500G-Nox2 mutation disrupted p47phox membrane translocation. Nox2 was localized in phagosome and plasma membranes, whereas p47phox was uniformly distributed in the cytosol, confirming previously published results (30) and confirming this method. In K195A/E, R198E, R199E, and RR198199QQ mutations, as the RR198199AA mutation (data not shown) in the D-loop of Nox2, which totally inhibited NADPH oxidase activity, a strong green fluorescence was seen surrounding phagocytosed latex particles (Fig. 6A), suggesting that the p47phox protein translocated normally to the phagosome membranes. Other mutations generated in the D-loop of Nox2 had no influence on NADPH oxidase assembly (data not shown). In contrast, in some mutants of the α-helical loop of the C-terminal of Nox2 (D484T, D500A, and D500R) with no oxidase activity, the p47phox membrane translocation
was totally disrupted, as in the RR9192EE mutant of the B loop of Nox2, previously studied by Biberstine-Kankade et al. (35). However, in the H490D-Nox2-transfected PLB-985 cells that had 10% activity of original WT PLB-985 cells, a normal p47phox translocation occurred as in WT Nox2-transfected PLB-985 cells (Fig. 6B). Similar results were obtained from in vitro translocation experiments using purified plasma membranes from transfected X-CGD PLB-985 cells after SDS and GTPγS stimulation (Fig. 7). The in vitro p47phox translocation was normal for K195A/E, R198E, R199E, and RR198199QQ/AA-Nox2-transfected PLB-985 cells similar to WT Nox2- and RR9192EE-Nox2 mutant PLB-985 cells (35), whereas WT Nox2-transfected X-CGD PLB-985 cells were used as a positive control of p47phox translocation.

**DISCUSSION**

The NADPH oxidase of phagocytic cells is an enzymatic complex assembled from membranous cytochrome b558 and cytosolic components, p67phox, p47phox, p40phox, and Rac2, upon cellular activation by microorganisms or inflammatorv stimuli. The objective of this study was to examine the role of two cytosolic domains of Nox2 encompassing residues 191–200 (D-loop) and residues 484–500 (C-terminal) of Nox2 in NADPH oxidase function and assembly (30, 34). The D-loop has never been studied, whereas the C-terminal region of Nox2 was previously identified, by a predicted three-dimensional structure of Nox2, as an α-helical loop constituting a potential binding site for cytosolic factors (35). A point mutation D500G in this region in an X−CGD case led to inhibition of cytosolic factor translocation to the plasma membrane of patient's neutrophils, confirming the previous hypothesis. However, in a second case...
of X*CGD caused by a Δ488–497 gp91(phox) deletion in the α-helical loop, p47(phox) and p67(phox) translocation was normal, whereas electron transfer from NADPH to FAD was disturbed (40). In this study, we used site-directed mutagenesis to probe the role of specifically charged amino acid residues within the D-loop and the C-terminal α-helical loop of Nox2, hypothesizing that electrostatic interactions between charged residues of Nox2 and cytosolic factors may be important for assembly of the active NADPH oxidase complex. To study the functional effect of these mutations we chose the granulocytic cell line PLB-985 as the cellular model, so as to be close to physiological events occurring in phagocytic cells.

We found that the D-loop and the C-terminal α-helical loop of Nox2 played a crucial role in oxidase activity. We demonstrated that the positive charge of Lys195 was essential, because reversal or cancellation of it inhibited the NADPH oxidase activity (Table I). In addition, reversal of the electrostatic charge of Arg198 or Arg199 induced more inhibitory effects on NADPH oxidase activity than its cancellation. In contrast, when these two arginines were mutated simultaneously by opposite residues (RR198199EE), more than half of the oxidase activity was restored (49). Therefore, we hypothesized that the mutations lead to a conformational change in Nox2 maturation and stability because in all the transfected PLB-985 cells mutant Nox2 expression was normal (Fig. 3).

Interestingly, our data show the difference in the NADPH oxidase activity measured in some mutant-transfected X*CGD PLB-985 cells depending on the different type of stimulus employed to activate the enzyme. The slight H₂O₂ overproduction observed in PMA-stimulated mutant PLB-985 cells expressing the R199Q and the D-loopNox4-Nox2 proteins (super-mutant cells) was particularly exacerbated when these cells were activated with FMLP (Fig. 4A). In addition, the activation kinetics of the NADPH oxidase in WT PLB-985 cells, WT Nox2 PLB-985 cells, and D-loopNox4-Nox2 mutant PLB-985 cells activated with FMLP was more rapid and transient than with PMA. However, the H₂O₂ production kinetics was not changed in all the FMLP-activated PLB-985 cells, whereas this production was more rapid in PMA-activated D-loopNox4-Nox2 mutant PLB-985 cells (Fig. 4B and Table II) and in R199Q mutant cells only (data not shown). It is known that the kinetics of oxidase activation is not the same when human neutrophils are stimulated by FMLP or by PMA, because the signaling cascades triggered by these two agents are different. The high production of H₂O₂ in the D-loopNox4 and the R199Q-Nox2 mutant PLB-985 cells activated by PMA or FMLP may be due to a conformational change of the mutated Nox2 in the assembly of the NADPH oxidase complex, promoting a more efficient electron transfer to reduce molecular oxygen. If we admit that the H₂O₂ production is the result of an equilibrium between an active and inactive state of the NADPH oxidase, we can speculate that the mutations lead to a conformational change in favor of the active state of the enzyme. The difference in the activation level of the oxidase complex in the super-mutant PLB-985 cells stimulated by either PMA or FMLP can be due to different phosphorylation states of the cytosolic factors induced by these stimuli (8), leading to a more or less efficient interaction between them and the D-loopNox4 and R199Q-Nox2 proteins. This hypothesis is supported by the fact that the oxidase activity of these two super-mutants in the reconstituted system (BCS), where no phosphorylation occurs, is not higher than in the WT-PLB-985 cells (Table I). The same difference between oxidase activity measured in intact PLB-985

![Fig. 8](image-url)
cells and the in vitro reconstituted oxidase activity was previously observed in the study of the R91T/R92A mutant of the B loop of Nox2. Indeed the different kinetics of O$_2$ production in this mutant activated by opsonized zymosan and PMA could be explained by different phosphorylation states of p47phox (35). In conclusion, the phosphorylation state of the cytosolic factors occurring in vivo may be essential to generate a “super-efficient” oxidase complex, including the D-loop of Nox2 or the R199Q-Nox2 proteins. However, it does not mean that the D-loop of Nox2 is directly involved in cytosolic factor binding. We should note that only mutations in the D-loop produce super-mutant PLB-985 cells, pointing out a crucial role of this region on NADPH oxidase activation. In addition, only two X-CGD mutations in the D-loop of Nox2 were described. The first case was a deletion in a codon corresponding to Arg$^{199}$ leading to the creation of a premature stop codon at position 213 (59). The second one was a X-CGD case originated from a S193F mis-sense mutation, suggesting that this region is possibly involved in the conformational stability of cytochrome $b_{558}$ (60).

To elucidate the reason why the NADPH oxidase activity was abolished in some mutant PLB-985 cells, assembly of the oxidase complex was studied. Translocation of p47$^{phox}$ was followed by confocal microscopy in intact differentiated PLB-985 cells during latex-bead activation (Figs. 5 and 6). Experiments were conducted in all the mutant PLB-985 cells, and the data obtained were confirmed by the in vitro translocation experiment (Fig. 7). We previously described the same type of approach using opsonized zymosan particles (38). Using latex beads opsonized with human IgG or serum AB, we found a significant background in nonphagocytosed particles due to unspecific recognition by the fluorescent secondary antibody. We decided to use the latex particle method coated with PMA, as previously described to purify phagosome membranes (61, 62). Although K195A, K195E, R199E, R199E, and RR198199 QQ/AA mutations in the D-loop of Nox2 led to total oxidase inhibition, the p47$^{phox}$ translocation process was normal, suggesting that basic amino acids of this region do not participate in the direct binding of oxidase cytosolic factors (Fig. 6A). This suggests that translocation of p47$^{phox}$ and oxidase activation are two dissociated processes. However, mutant PLB-985 cells of the C-terminal of Nox2 (D484T, D500A/RG mutations) with no oxidase activity had a p47$^{phox}$ translocation defect (Fig. 6B). This result is consistent with the hypothesis that when the oxidase is in a resting state, the potential α-helical loop of 20 amino acid residues (Asp$^{484}$_Gly$^{504}$) lies over the NADPH binding cleft. During oxidase activation, this loop is thought to move aside to allow NADPH to reach its binding site and deliver electrons to FAD. In this activated conformation, this loop is able to bind cytosolic factors. Yet we cannot speculate on the chronology of such events on the oxidase activation process. This result is also consistent with the assembly defect observed in an X-CGD patient’s neutrophils with a D500G missense mutation in Nox2 (30). However, it was reported that the deletion of Nox2 residues 488–497 did not affect translocation of p47$^{phox}$ and p67$^{phox}$ but only electron transfer from NADPH to FAD (40). Meanwhile, in this last case, deletion of the small region encompassing residues 488–497 conserves the acidic amino acids Asp$^{484}$ and Asp$^{500}$, which seems to be essential to maintain the oxidase activity and the complex assembly. Asp$^{500}$ of Nox2 is probably a crucial charged amino acid because whatever mutation was introduced, the oxidase activity and the p47$^{phox}$ translocation were inhibited.

To determine whether the inhibition of oxidase activity and/or the defect of complex assembly in mutant PLB-985 cells were associated with the electron transfer process from NADPH to FAD, we measured the INT reductase activity in purified plasma membranes from all the mutant transfected X-CGD PLB-985 cells (Fig. 8). This activity was abolished only in mutant PLB-985 cells of the α-helical loop 484DESFQAN-HFAVHDEEDK$^{500}$ of Nox2, whereas a p47$^{phox}$ translocation defect was also demonstrated. This suggests that the assembly of the NADPH oxidase complex is closely related to the electron transfer process from NADPH to FAD. Asp$^{500}$ is possibly involved in the direct binding with cytosolic factors, resulting in the liberation of the NADPH binding site, as proposed by Taylor et al. (28). In addition, this C-terminal region of Nox2 is near the potential binding site for the adenine unit of NADPH ($^{634}$GLKQ$^{507}$). However, the electron transfer process can be dissociated from the assembly of the enzyme. Indeed in a study of an X-CGD case, which was induced by a mutation that converted Glu$^{507}$_Lys$^{508}$_Thr$^{509}$ into His-Ile-Trp-Ala in a region near the potential binding site of NADPH, the translocation of both p47$^{phox}$ and p67$^{phox}$ to the membrane fractions of the patient’s neutrophils was normal, indicating that this region is important for a correct electron transfer but not for the assembly of the oxidase complex (63). In addition, in the X-CGD case resulting from a deletion of residues 488–497 in Nox2 reproduced in the X-CGD PLB-985 cells, cytosolic factor translocation occurred normally, whereas electron transfer from NADPH to FAD was defective (40). In the mutants of the D-loop (K195E/A, R198E, R199E, and RR198199QQ/AA), although the assembly of the oxidase complex and the electron transfer from NADPH to FAD were normal, the NADPH oxidase activity was totally abolished. The D-loop is localized close to the V transmembranous passage in the N-terminal part of Nox2, which has been proposed as being involved in heme binding. We speculate that this region might be involved in electron transfer from the FAD to the hemes. This hypothesis is currently under investigation in our laboratory.

Finally, we can conclude that charged amino acids of the D-loop (Lys$^{196}$, Arg$^{198}$, and Arg$^{296}$) and of the C-terminal region encompassing residues 484–500 (Asp$^{484}$, His$^{496}$, and Asp$^{500}$) are essential to maintain the NADPH oxidase activity in phagocytic cells. The D-loop of Nox1, -2, -3, and -4 is probably an important functional domain for the active conformational structure of Nox2, leading the electron transfer from FAD to oxygen, whereas the potential α-helical loop of the C-terminal tail of Nox2 is involved in the binding of cytosolic factors and in the electron transfer process from NADPH to FAD. The study of the relationship between these two events will allow us to better understand the molecular mechanisms of oxidase assembly.

Acknowledgments—We are grateful to Dr. Mary C. Dinauer for the generous gift of X-CGD PLB-985 cells. The 7D5 was kindly provided by Dr. Michio Nakamura. The antibodies 449 and 48 were generous gifts from Dr. D. Roos. We thank Pr. Philippe Gaudin for financial support and Michelle Guillot for technical assistance.

REFERENCES

1. Baldrige, C. W., and Gerard, R. W. (1933) Am. J. Physiol. 103, 235–236
2. Cross, A. R., and Segal, A. W. (2004) Biochim. Biophys. Acta 1637, 1–22
3. Sbarra, A. J., and Karnovsky, M. L. (1959) J. Biol. Chem. 234, 1355–1362
4. Mandelli, G. L. (1974) Infect. Immun. 9, 337–341
5. Babior, B. M., Curnutte, J. T., and Kipnes, R. S. (1975) J. Lab. Clin. Med. 85, 235–244
6. Babior, B. M., Kipnes, R. S., and Curnutte, J. T. (1973) J. Clin. Invest. 52, 741–744
7. Dinauer, M. C. (1993) Crit. Rev. Clin. Lab. Soc. 30, 329–369
8. Babior, B. M. (1990) Blood 85, 1464–1476
9. Goldblatt, D., and Thrasher, A. J. (2000) Clin. Exp. Immunol. 122, 1–9
10. Heyworth, P. G., Cross, A. R., and Curnutte, J. T. (2003) Curr. Opin. Immunol. 15, 578–584
11. Reeves, E. P., Lu, H., Jacobs, H. L., Messina, C. G., Bolovero, S., Gabella, G., Potma, E. O., Warley, A., Roes, J., and Segal, A. W. (2002) Nature 416, 291–297
12. Vignais, P. V. (2002) Cell. Mol. Life Sci. 59, 1428–1459
13. Segal, A. W., West, I., Wijetjes, F., Nugent, J. H., Chavan, A. J., Haley, B., Garcia, R. C., Rosen, H., and Scrace, G. (1992) Biochem. J. 284, 781–788
14. Rotrosen, D., Yeung, C. L., Leto, T. L., Malech, H. L., and Kwong, C. H. (1992)
