Probing the Role of the Carboxyl Terminal of the gp91\textsuperscript{phox} Subunit of Neutrophil Flavocytochrome \textit{b}_{558} using Site-directed Mutagenesis\textsuperscript{*}

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Site-directed mutagenesis was used to generate a series of substitutions and deletions in the carboxyl-terminal 11 residues of gp91\textsuperscript{phox}, the 91-kDa subunit of the phagocyte NADPH oxidase flavocytochrome \textit{b}_{558}. This region encompasses \textit{559RGVHFIF565}, implicated as a contact point for the cytosolic oxidase subunit p47\textsuperscript{phox} during oxidase activation, and a carboxyl-terminal phenylalanine (Phe\textsuperscript{570}), which corresponds in position to a highly conserved aromatic residue that interacts with the flavin group in the ferredoxin-NADP\textsuperscript{+} reductase flavoenzyme family, of which gp91\textsuperscript{phox} is a member. Mutant proteins were expressed in human myeloid leukemia cells which lack expression of endogenous gp91\textsuperscript{phox} due to targeted disruption of the X-linked gp91\textsuperscript{phox} gene. Although specific residues within \textit{559RGVHFIF565} had previously been identified by alanine scanning as essential for peptide inhibition of oxidase activity in a cell-free assay, comparable substitutions in the gp91\textsuperscript{phox} polypeptide had either no or only a modest effect on oxidase activity in whole cells. Replacement of nonpolar with polar or charged residues had greater effects on oxidase activity, but were also associated with decreased gp91\textsuperscript{phox} expression, suggesting that overall protein structure was perturbed. No stable gp91\textsuperscript{phox} protein was detected upon deletion of the terminal 11 amino acids. Alanine substitution or deletion of the carboxyl-terminal Phe\textsuperscript{570} in gp91\textsuperscript{phox} resulted in a 2-fold reduction in superoxide production. This contrasts with a \textit{300–800-fold} reduction reported for comparable mutations in \textit{pea ferredoxin-NADP}+ reductase, which suggests that structural or functional differences exist between the carboxyl terminus of gp91\textsuperscript{phox} and other ferredoxin-NADP+ reductases.

Neutrophils and other phagocytic leukocytes possess an NADPH oxidase (respiratory burst oxidase) that generates large quantities of superoxide during the respiratory burst (1). Upon phagocyte activation by opsonized bacteria or other inflammatory stimuli, the active NADPH oxidase complex is rapidly assembled at the plasma membrane from cytosolic and membrane components to catalyze the transfer of electrons from NADPH to molecular oxygen. Oxidase subunits include two polypeptides, gp91\textsuperscript{phox} and p22\textsuperscript{phox}, which form a membrane-bound phagocyte flavocytochrome \textit{b}_{558} heterodimer, and three cytosolic proteins, p47\textsuperscript{phox}, p67\textsuperscript{phox}, and a low molecular weight GTP-binding protein, Rac (1–3). A fourth cytosolic protein, p40\textsuperscript{phox}, is present as a complex with p67\textsuperscript{phox} in resting neutrophil cytosol (4, 5), but does not appear to play a direct role in superoxide production. Superoxide and its derivatives are essential for normal microbialidal activity, and genetic defects in the NADPH oxidase result in chronic granulomatous disease (CGD),\textsuperscript{1} a syndrome characterized by life-threatening fungal and bacterial infections (1). Mutations in the X-linked gene for gp91\textsuperscript{phox} account for the majority of cases of CGD, and the p22\textsuperscript{phox} subunit is defective in an uncommon autosomal recessive subgroup of CGD (1). The remaining cases of autosomal recessive CGD result from mutations in the genes encoding p47\textsuperscript{phox} or p67\textsuperscript{phox} (1).

Current evidence suggests that flavocytochrome \textit{b}_{558} functions as the redox center of the NADPH oxidase, and is regulated by the cytosolic oxidase subunits p47\textsuperscript{phox}, p67\textsuperscript{phox}, and Rac, which translocate to the cell membrane upon neutrophil activation (6–13). The p47\textsuperscript{phox} subunit appears to mediate the first steps of interaction with the flavocytochrome in assembling the active oxidase complex (6, 7, 14), although it is not required in vitro if high levels of p67\textsuperscript{phox} and Rac are supplied (15, 16). Sites within flavocytochrome \textit{b}_{558} that function as contact points with p47\textsuperscript{phox} have been identified in both the gp91\textsuperscript{phox} and p22\textsuperscript{phox} subunits (2, 3). The cytochrome has an NADPH-binding site and bears a flavin group that acts as the initial acceptor of a pair of electrons from NADPH (17–23), although recently p67\textsuperscript{phox} has also been reported to contain a functional NADPH-binding site (24). The subsequent one-electron transfer to molecular oxygen is mediated by a pair of heme groups in the flavocytochrome heterodimer that are embedded within the membrane (25–28).

The gp91\textsuperscript{phox} subunit of flavocytochrome \textit{b}_{558} is a 570-amino acid membrane glycoprotein with multiple hydrophobic domains in the amino-terminal half followed by a hydrophilic carboxyl terminus (1). The extreme carboxyl terminus of gp91\textsuperscript{phox}, which resides at the cytoplasmic face of the membrane (29, 30), has been implicated as both a docking site for the cytosolic oxidase subunit p47\textsuperscript{phox} and as a participant in NADPH binding. A variety of approaches have suggested that gp91\textsuperscript{phox} residues \textit{559RGVHFIF565} interact with p47\textsuperscript{phox} at an early step of oxidase assembly (14, 29, 31–34). Residues \textit{400–570} of gp91\textsuperscript{phox} also contain regions with sequence homologies

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\textsuperscript{‡}The abbreviations used are: CGD, chronic granulomatous disease; MLF, formyl-methionyl-leucyl-phenylalanine; PIPES, 1,4-piperazinediethanesulfonic acid.

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to the NADP⁺-binding site of ferrodoxin-NADP⁺ reductase flavoenzymes (17–20, 22). An aromatic amino acid at or near the carboxyl terminus is highly conserved among members of the ferrodoxin-NADP⁺ reductase flavoprotein family, including gp91phox which has a carboxyl-terminal phenylalanine (Phe³⁷⁷). Crystallographic analysis of the spinach ferrodoxin-NADP⁺ reductase has localized the carboxyl-terminal aromatic side chain to the NADP⁺-binding pocket, where it may interact with the isoalloxazine ring of FAD in the absence of NADP⁺ (35). Altering the aromatic character or deleting this conserved residue in the pea ferrodoxin-NADP⁺ reductase leads to major impairments in catalytic efficiency of the enzyme (36).

Although the majority of missense mutations in gp91phox identified in patients with X-linked CGD result in apparent structural instability of the protein, in rare cases, gp91phox expression is preserved but the mutant polypeptide forms a non-functional flavocytochrome b₅₅₆ (37, 38). These latter mutations have been informative in identifying residues critical for gp91phox function. The expression and analysis of mutant gp91phox polypeptides generated by site-directed mutagenesis has not been previously reported, at least in part due to the lack of systems in which adequate levels of functional recombinant gp91phox can be readily expressed. To undertake a more systematic analysis of structure-function relationships in gp91phox using this approach, we developed a human myeloid leukemia cell line that lacks endogenous gp91phox after targeted disruption of the X-linked gp91phox gene (39). This “X-CGD” cell line has been a valuable tool for expression of recombinant gp91phox, which, in the case of wild-type recombinant gp91phox, assembles with p22phox to form a functional flavocytochrome b₅₅₆ heterodimer (39–41).

In the present study, we have used site-directed mutagenesis to probe the role of the carboxyl terminus of gp91phox in NADPH oxidase activity. We examined both the requirements for an intact 5⁵⁵⁶RGVHFIF⁶⁵⁶ sequence and for a carboxyl-terminal aromatic residue in supporting superoxide production by intact granulocytic cells. Mutant gp91phox cDNAs encoding polypeptides with amino acid substitutions or deletions in the carboxyl terminus were transfected into the X-CGD myeloid cell line for analysis of expression and function. The results suggest that the distal carboxyl terminus is an important determinant for gp91phox stability, but that neither an intact 5⁵⁵⁶RGVHFIF⁶⁵⁶ sequence nor a carboxyl-terminal aromatic residue are absolutely essential for NADPH oxidase activity in intact granulocytic cells. Alanine substitutions had either no or only a modest effect on NADPH oxidase activity. The greatest effects on superoxide production were seen with substitution of polar or charged residues for hydrophobic amino acids in 5⁵⁵⁶RGVHFIF⁶⁵⁶, but these were also associated with reduced expression of gp91phox, suggesting that the overall protein structure was perturbed.

EXPERIMENTAL PROCEDURES

Plasmids—A full-length wild-type gp91phox cDNA, extending from 12 nucleotides upstream of the initiator ATG to a SacI site in the 3’-untranslated region (39), was cloned into the NotI site in the multiple cloning site of pBluescript II KS (Stratagene). This construct was used as a phagemid to produce single-stranded DNA for oligonucleotide-directed mutagenesis of specific codons. Mutations were introduced into the carboxy-terminal region of the gp91phox cDNA using the Sculptor in vitro mutagenesis system (Amersham), and verified by dye oligonucleotide sequencing. Deletion mutants were made by digestion of nucleotides with a premature stop codon. Mutant gp91phox cDNAs were subcloned into the NotI site of the pEF-PGKneo mammalian expression vector (39), or a related vector, pEF-PGKpac, which contains a linked expression cassette for puromycin-N-acetyltransferase instead of neomycin phosphotransferase. The mutated gp91phox cDNA expression constructs were resequenced to confirm the mutations and were linearized with KpnI prior to electroporation into a human myeloid leukemia cell line (see below). Preparation and other manipulations of plasmid DNAs were performed by standard protocols (42). Restriction enzymes and other reagents for molecular biology were obtained from Promega, Boehringer Mannheim, New England Biolabs, and U.S. Biochemical Corp.

Cell Lines—Wild-type PLB-985 cells (43), a human myeloid leukemia cell line, and a derivative line in which the X-linked gp91phox gene has been disrupted by gene targeting (X-CGD PLB-985 cells) were maintained as described (39). X-CGD PLB-985 cells do not express gp91phox protein and lack NADPH oxidase activity. After electroporation of expression constructs into X-CGD PLB-985 cells, clones were selected by limiting dilution in either 1.5 mg/ml G418 or 3 μg/ml puromycin (39). Since up to one-third of clones selected for resistance to the linked antibiotic marker will not have a full-length transgene and therefore fail to express any transgenic gp91phox mRNA, clones were analyzed for recombinant gp91phox expression by either Northern blot and/or immunoblot with gp91phox-specific probes. For subsequent analysis, three to five independent clones determined to express transgenic gp91phox were pooled to minimize potential clone-to-clone variation in recombinant gp91phox expression and NADPH oxidase activity. PLB-985 and derivative cell lines were differentiated into granulocytes with dimethylfor-
prepared at positions 561, 563, and 565. Two mutants with double substitutions (R559A/V561A and I564T/F565V) were also generated. A mutant gp91phox with an alanine-substituted His562 residue was also prepared, which was predicted to have no impact on oxidase activity based on peptide inhibition studies (33). To investigate the requirement for a carboxyl-terminal aromatic residue for gp91phox function, the phenylalanine at position 570 was substituted with an alanine (F570A) or deleted (F570Δ) by insertion of a premature stop codon at this position. Finally, the effect of entirely deleting the last 10 residues (Δ560–570) by introduction of a premature stop codon at position 560 was examined.

The mutated gp91phox cDNAs were subcloned into mammalian expression vectors under control of the EF1-α promoter, and the expression of mutant polypeptides was evaluated by immunoblot analysis (Fig. 2) after stable transfection of a derivative of human myeloid leukemia PLB-985 cells which lack endogenous gp91phox due to targeted disruption of the gp91phox gene (X-CGD PLB-985 cells) (39). Note that the wild-type gp91phox protein migrates as a diffuse band centered at ~91 kDa (Fig. 2), and the heterogeneity in size of immunoreactive species most likely reflects variations in glycosylation. Two related vectors were used in these studies, pEF-PGKneo and pEF-PGKpac, that differ only in the selectable marker gene upstream of the EF1-α promoter/gp91phox cassette. Using the pEF-PGKneo vector, which has a linked neomycin phosphotransferase binding site, expression of recombinant wild-type gp91phox cDNA is ~10–20% of the endogenous gp91phox level in granulocyte-induced wild-type PLB-985 cells (39). During the course of these studies, we observed that higher levels of expression of recombinant wild-type gp91phox (25–50% of wild-type PLB-985) were obtainable using pEF-PGKpac, for reasons which remain unclear. The majority of gp91phox mutants reported here were expressed using the pEF-PGKneo vector, and their expression compared with X-CGD PLB-985 cell lines transfected with a pEF-PGKneo vector containing the wild-type gp91phox cDNA (Fig. 2, upper two panels). Several mutants prepared later during the course of this study utilized pEF-PGKpac, and expression was compared with wild-type gp91phox expressed using pEF-PGKpac (Fig. 2, lower panel). One mutant, F570Δ, was expressed in both vector backgrounds, but most mutants were not re-cloned into pEF-PGKpac as it was felt that the conclusions would not be substantially altered.

A summary of the relative levels of expression of recombinant gp91phox derivatives in granulocyte-induced PLB-985 cells is shown in Table I. Several of the gp91phox mutants appeared to be unstable, with deletion of the carboxyl-terminal 11 amino acids having the greatest impact on protein expression. No gp91phox was detected in immunoblots of cell extracts prepared from either undifferentiated (not shown) or granulocyte-induced cell lines transfected with the Δ560–570 construct, using either the monoclonal antibody MoAb48 (Fig. 2A), directed against an uncharacterized epitope on gp91phox, or two different gp91phox polyclonal antisera (not shown). In cells expressing F563T, F565D, or F564/F565V gp91phox mutants, very small but detectable amounts of the gp91phox polypeptide were present. More modest reductions in the relative level of gp91phox expression were seen for several other mutants (V561A, V561T, V561E, F563A, F565A, and F570Δ). The relative levels of the remaining mutants were not significantly different from wild-type recombinant gp91phox as expressed with the corresponding pEF-PGKneo or pEF-PGKpac vector. The amount of recombinant protein detected was unrelated to the abundance of the transgene-derived gp91phox mRNAs, which were all similar (not shown). As was previously observed with expression of wild-type recombinant gp91phox (39), expression of mutant derivatives of gp91phox rescued expression of the cytochrome p22phox subunit in X-CGD PLB-985 cells, in proportion to the relative abundance of the mutant gp91phox subunit (not shown).

To provide evidence that the wild-type and mutant gp91phox are successfully delivered to the plasma membrane when transgenically expressed in X-CGD PLB-985 cells, we stained the transfected and parental X-CGD cells with a monoclonal antibody, 7D5, and examined cell surface expression of flavocytochrome b2phox. The 7D5 antibody reacts with an extracellular epitope on flavocytochrome b2phox (49). Since the mutant gp91phox proteins were expressed at variable levels, we chose F570A as an example of a mutant that was expressed at relatively high levels, comparable to the wild-type recombinant gp91phox, and F563T as an example of poorly expressed gp91phox mutant. As seen in

FIG. 1. Mutagenesis of the carboxyl terminus of gp91phox. The wild-type amino acid sequence from residues 552 through 570 is shown in the top line. The RGVHFVFIFNKENF sequence identified as a p47phox binding site is shown in bold, and critical residues identified by peptide alanine scanning are marked with asterisks. Also shown in bold is the carboxyl-terminal phenylalanine residue, which is highly conserved among ferredoxin-NADPH reductase family members. The gp91phox mutants generated in this study are listed below the wild-type sequence, with point substitutions or deletions as indicated by the shaded boxes.

FIG. 2. Immunoblot analysis of gp91phox expression in PLB-985 cell lines. Cell extracts were prepared from granulocyte-differentiated PLB-985 cells and derivatives, and analyzed for gp91phox expression by immunoblotting. PLB-985, wild-type PLB-985 cells; X-CGD, X-CGD PLB-985 cells; X-CGD cells transfected with either wild-type gp91phox (WT) or mutant cDNAs are as indicated in italics. The upper two panels show results obtained using the pEF-PGKneo vector for transfection, and the bottom panel shows results obtained from pEF-PGKpac transfectants (asterisks). Blots were probed with a gp91phox monoclonal antibody, MoAb48. Twenty μg of protein was loaded in each lane, except for wild-type PLB-985 cells, for which 5 μg was loaded. In most cases, two independently prepared representative samples from mutant gp91phox transfectants are shown.
Superoxide generation by granulocyte-induced PLB-985 cells expressing wild-type or mutant gp91<sub>phox</sub> subunits of cytochrome b

Mutagenesis of Phagocyte Flavocytochrome b<sub>558</sub> gp91<sub>phox</sub> Subunit

Table I

| Cell line | Relative level of gp91<sub>phox</sub> | V<sub>max</sub> | Cumulative O<sub>2</sub> | Relative rate of O<sub>2</sub> formation |
|-----------|-------------------------------------|---------------|------------------------|--------------------------------------|
|           | %                                   | nmol O<sub>2</sub> min<sup>−1</sup> cells | nmol O<sub>2</sub> min<sup>−1</sup> 30 min cells | %                                   |
| Wild-type | 100 ± 18                            | 53 ± 6        | 670 ± 10               | 100 ± 11                             |
| X-CGD     | 0                                   | 0             | 0                      | 0                                    |
| X-CGD + recombinant gp91<sub>phox</sub> |                          |               |                        |                                      |
| Wild-type (neo) | 21 ± 5                          | 48 ± 9        | 698 ± 33               | 89 ± 17                              |
| Wild-type (pac) | 37 ± 5                          | 50 ± 17       | 695 ± 66               | 94 ± 34                              |
| Δ560–570 | 0                                  | 0             | 0                      | 0                                    |
| R559A     | 13 ± 1                              | 43 ± 7        | 676 ± 69               | 81 ± 14                              |
| R559A/V561A | 25 ± 9                          | 37 ± 10       | 499 ± 131              | 70 ± 19                              |
| V561A     | 26 ± 3                              | 26 ± 6        | 444 ± 98               | 49 ± 16<sup>a</sup>                   |
| V561T     | 7 ± 1<sup>b</sup>                  | 29 ± 6        | 569 ± 100              | 55 ± 12<sup>a</sup>                   |
| V561E     | 5 ± 1<sup>b</sup>                  | 11 ± 2        | 232 ± 48               | 21 ± 5<sup>a</sup>                    |
| H562A     | 16 ± 1                              | 51 ± 17       | 702 ± 20               | 95 ± 32                              |
| F563A(pac) | 24 ± 2<sup>b</sup>                | 48 ± 8        | 720 ± 24               | 87 ± 15                              |
| F563T(pac) | 1.0 ± 1<sup>b</sup>                | 2 ± 1         | 12 ± 7                 | 4 ± 2<sup>b</sup>                     |
| I564A     | 15 ± 6.5                            | 32 ± 7        | 515 ± 175              | 60 ± 13<sup>a</sup>                   |
| I564T/F565V(pac) | 1.0 ± 1<sup>b</sup> | <1.0         | <10                    | <1<sup>o</sup>                         |
| F565A     | 8 ± 1<sup>b</sup>                  | 18 ± 4        | 375 ± 70               | 34 ± 7<sup>a</sup>                    |
| F565D     | 1.0 ± 1<sup>b</sup>                | 3 ± 0.4       | 67 ± 27                | 6 ± 1<sup>a</sup>                     |
| F570A     | 16 ± 5                              | 25 ± 3        | 460 ± 67               | 47 ± 5<sup>a</sup>                    |
| F570Δ     | 6 ± 2<sup>b</sup>                  | 7 ± 2         | 147 ± 56               | 13 ± 4<sup>a</sup>                    |
| F570Δ(pac) | 17 ± 7<sup>b</sup>                | 33 ± 6        | 640 ± 85               | 62 ± 11<sup>a</sup>                   |

<sup>a</sup> p < 0.05 (wild-type PLB-985 versus transfected X-CGD PLB-985 expressing recombinant gp91<sub>phox</sub>), using the unpaired t test.
<sup>b</sup> p < 0.05 (wild-type recombinant gp91<sub>phox</sub> versus mutant gp91<sub>phox</sub> expressed using the same vector), using the unpaired t test.

Fig. 3, the 7D5 antibody produced a complete shift in fluorescence intensity in flow cytometry of cells transfected with wild-type gp91<sub>phox</sub>, and confocal microscopy showed immunofluorescent staining on the cell surface. Similar results were obtained for wild-type PLB-985 granulocytes (not shown), whereas no staining was seen for X-CGD PLB-985 cells (Fig. 3). Immunofluorescent staining of cells expressing recombinant F570Δ gp91<sub>phox</sub> was similar to that seen for cells expressing recombinant wild-type gp91<sub>phox</sub>. However, only weak staining of F563T gp91<sub>phox</sub>-expressing cells was seen, consistent with the results seen by immunoblotting (Fig. 2). Taken together, these observations demonstrate that the transgenic expression of wild-type and mutant gp91<sub>phox</sub> in X-CGD PLB-985 cells results in the plasma membrane expression of flavocytochrome b<sub>558</sub>, at levels that correlate with the amount of gp91<sub>phox</sub> seen in immunoblots of cell extracts.

We next examined the impact of carboxyl-terminal mutations in gp91<sub>phox</sub> on NADPH oxidase activity in intact cells after granulocyte differentiation (Table I). A continuous cytotoxic c reduction assay was used to quantitate superoxide formation after NADPH oxidase activation by phorbol myristate acetate. X-CGD PLB-985 cells expressing wild-type recombinant gp91<sub>phox</sub> at only 21–37% of that seen in wild-type PLB-985 granulocytes exhibited NADPH oxidase activity that was not significantly different from wild-type PLB-985 granulocytes (Table I). This is consistent with our previous studies which have shown that expression of wild-type recombinant gp91<sub>phox</sub> at ≥10% of wild-type levels fully reconstitutes NADPH oxidase activity, and that cells expressing as little as ~5% of wild-type gp91<sub>phox</sub> levels exhibit 40–60% of wild-type activity (39–41, 50). These observations have suggested that gp91<sub>phox</sub> and flavocytochrome b<sub>558</sub> are normally present in excess and not rate-limiting for superoxide formation in wild-type neutrophils or PLB-985 granulocytes.

All mutants within the 558<sup>RGVHFIF</sup>565 with detectable gp91<sub>phox</sub> expression were capable of supporting at least a small amount of superoxide generation (Table I). The lag time between stimulation of cells with phorbol myristate acetate and the onset of superoxide generation (≥2 min) was the same for cells expressing mutant gp91<sub>phox</sub> derivatives as for wild-type gp91<sub>phox</sub> (not shown). Alanine substitutions at Arg<sup>559</sup>, His<sup>562</sup>, or F563A did not affect NADPH oxidase activity significantly, as was also seen for the double R559A/V561A substitution. Point substitutions at Val<sup>563</sup>, Ile<sup>564</sup>, and Phe<sup>565</sup> resulted in a 2–5-fold reduction in the rate of superoxide formation relative to wild-type PLB-985 cells; the greatest reduction seen in the V561E mutant, which was only ~40% as active as the V561T mutant despite similar levels of gp91<sub>phox</sub> expression. Superoxide formation observed with the F565D gp91<sub>phox</sub> derivative, which was expressed at very low levels, was 20-fold less than wild-type. In cells transfected with the F563T or I564T/F565V mutants, in which expression was also very low, NADPH oxidase activity appeared to be even further reduced but was difficult to measure reliably using the cytochrome c assay. Note that the above comparisons do not take into account variations in recombinant gp91<sub>phox</sub> expression levels, and therefore the relative function of gp91<sub>phox</sub> mutants expressed in reduced amounts may be an underestimate. All of the above gp91<sub>phox</sub> mutants supported the reduction of nitro blue tetrazolium dye, with the intensity of resultant formazan staining proportional to enzyme activity measured by the cytochrome c reduction assay. However, NADPH oxidase activity was undetectable by either the cytochrome c or nitro blue tetrazolium assay in cells transfected with the Δ560–570 mutant, which did not express detectable ~91 kDa gp91<sub>phox</sub> protein. Superoxide generation in response to fMLP was studied for several mutants (R559A, V561A, V561E, V561T, and F565A) (not shown). The kinetics of superoxide formation were unaffected, and the rank order of the relative magnitude of the response corresponded to that seen for phorbol myristate acetate.
Mutagenesis of Phagocyte Flavocytochrome b558 gp91phox Subunit

FIG. 3. Cell surface expression of wild-type and mutant gp91phox in transgenic X-CGD PLB-985 cell lines. Flow cytometry and confocal microscopy were performed using the 7D5 monoclonal antibody to stain X-CGD PLB-985 cells and derivative lines obtained after stable transfection with pEF-PGKpac vectors for expression of either wild-type (WT) gp91phox or the F570Δ or F563T mutants, as indicated. A, cells were labeled with 7D5 antibody (solid lines) or a mouse IgG1 as an isotype control (dotted lines) and analyzed by flow cytometry. B, representative images obtained using confocal microscopy after cell staining with the 7D5 antibody (×730). Insets show results obtained after cells were stained with the mouse IgG1 (×340); no cells are visible due to a lack of staining.

Mutant derivatives of gp91phox in which the final carboxyl-terminal residue, Phe570, was substituted with an alanine or deleted entirely (F570Δ) were studied to test the hypothesis that an aromatic amino acid at this position is critical for NADPH oxidase enzymatic activity, as observed for pea ferredoxin-NADP+ reductase (36), another member of the ferredoxin-NADP+ reductase family. Although the F570A mutant was expressed at levels comparable to wild-type recombinant gp91phox, the rate of superoxide formation was reduced by ∼2-fold (Table I). Deletion of the carboxyl-terminal residue (F570Δ) resulted in a ∼4-fold reduction in expression of recombinant gp91phox and an almost 10-fold reduction in NADPH oxidase activity compared with wild-type cells (Table I). When the relative level of recombinant F570Δ protein was increased by using pEF-PGKpac for expression, an almost 2-fold reduction in NADPH-oxidase activity relative to wild-type cells was still observed (Table I).

DISCUSSION

The phagocyte flavocytochrome b558 is a plasma membrane-associated heterodimer that is the electron transfer center of the superoxide-generating NADPH oxidase. The objective of this study was to use site-directed mutagenesis to probe the role of the distal carboxyl terminus of the gp91phox subunit, which contains domains implicated in the binding of NADPH and in a critical interaction with p47phox during assembly of the active oxidase complex. This is the first reported study using site-directed mutagenesis to investigate structure-function relationships in gp91phox, and utilized an X-CGD myeloid cell line for expression of mutant gp91phox polypeptides. For functional analysis, we specifically focused on analysis of NADPH oxidase activity in intact granulocytic cells, since the protein-protein interactions required for oxidase assembly and function in the cell-free assay do not always reflect those relevant in whole cells. For example, phosphorylation of p47phox is required for NADPH oxidase activity in neutrophils (12, 51, 52), but not in the cell-free system (53). As another example, the p67phox subunit contains two SRC homology 3-binding domains that are essential for oxidase function in whole cells, but are dispensable in the cell-free system (54).

Previous studies on X-CGD patients with deletions involving the carboxyl terminus of gp91phox have suggested that this domain is required for the stability of this protein. Based on computer modeling analysis, the extreme carboxyl terminus of gp91phox has been proposed to be buried within the interior of the protein, where it interacts with the flavin group (22). A complete lack of gp91phox and flavocytochrome b558 has been reported in X-CGD when the last 40–50 amino acids of gp91phox are absent (45, 55), and a 5-fold reduction in gp91phox expression was observed with deletion of the last six residues of gp91phox (56). In this study, we found that many of the gp91phox carboxyl-terminal mutants were expressed at significantly reduced amounts in the X-CGD PLB-985 cells compared with wild-type recombinant gp91phox. Although expression of alanine-substituted derivatives were generally comparable to wild-type recombinant gp91phox, decreased amounts of gp91phox were observed when apolar residues within the 559RGVH-FIFphox sequence were replaced with polar or charged residues. This was particularly marked with polar substitutions at positions Phe563 and Phe565. A mutant gp91phox with the double replacement I564T/F565V was also expressed at very low levels. Removal of the last residue of gp91phox resulted in a modest decrease in gp91phox expression, and no recombinant gp91phox was detected at all when the last 11 residues were deleted. These observations support the concept that the carboxyl terminus of gp91phox is involved in maintaining a stable conformation of gp91phox and/or in heterodimer formation with
p29<sub>phox</sub>, which plays an important role in the intracellular stability of each cytochrome subunit (1).

There has been great interest in characterizing the specific contact sites in NADPH oxidase subunits that mediate assembly of the active oxidase complex and regulate electron transfer through flavocytochrome b<sub>558</sub> (2, 3). A number of studies have suggested that interactions between p47<sub>phox</sub> and the 559RGVHF<b>IF</b><sup>565</sup> domain encompassed within the distal carboxyl termini of gp91<sup>phox</sup> play a key role in this process. An antibody directed against the carboxyl-terminal 13 amino acids of gp91<sup>phox</sup> inhibits superoxide formation in the cell-free assay (29). Synthetic peptides derived from this region inhibit NADPH oxidase activity in permeabilized neutrophils (29, 34) and in the cell-free oxidase assay (29, 31, 57). A peptide corresponding to gp91<sup>phox</sup> residues 559RGVHF<b>IF</b><sup>565</sup> is the minimum sequence capable of oxidase inhibition (IC<sub>50</sub> = 38 μM) (33), which can be abolished by preincubation of neutrophil membranes with p47<sup>phox</sup>-containing neutrophil cytosol (14). When incubated with neutrophil cytosol, a similar carboxyl-terminal gp91<sup>phox</sup> peptide can be cross-linked to p47<sup>phox</sup> (31). Finally, a strategy using random sequence peptide bacteriophage display libraries of identified peptides with homologies to RGVHFIF as ligands of recombinant p47<sup>phox</sup> (32).

One goal of the current study was to further examine the role of the 559RGVHF<b>IF</b><sup>565</sup> domain of gp91<sup>phox</sup> with regards to NADPH oxidase activity in intact granulocytic cells. Specific residues within this sequence (Arg<sup>559</sup>, Val<sup>561</sup>, Phe<sup>563</sup>, Ile<sup>564</sup>, and Phe<sup>565</sup>) have been identified by alanine scanning as important for the function of this domain, based on the relative ability of alanine-substituted peptides to inhibit oxidase activity in a cell-free assay (33). However, we found that comparable alanine substitutions in the gp91<sup>phox</sup> polypeptide resulted in either no or only a modest reduction in NADPH oxidase activity when the mutant gp91<sup>phox</sup> was expressed in whole cells. The elimination of the positive charge at position 559 (Arg<sup>559</sup>), which had been proposed to mediate a critical ionic interaction between gp91<sup>phox</sup> and another oxidase subunit (33), had no significant impact on gp91<sup>phox</sup> expression or NADPH oxidase activity. Replacement of nonpolar residues with polar or charged residues in 559RGVHF<b>IF</b><sup>565</sup> resulted in relatively greater reductions in superoxide generation, but these mutations also were associated with apparent decreased stability of the gp91<sup>phox</sup> polypeptide, suggesting that overall protein structure was perturbed. Hence, it is not possible to rule out long-range effects on other domains within the gp91<sup>phox</sup> as the primary cause of decreased NADPH oxidase activity seen with alterations in the nonpolar character of these residues. It would also be of considerable interest to examine p47<sup>phox</sup> translocation in gp91<sup>phox</sup> mutants in cases where superoxide formation appeared to be affected, which would provide information as to whether enzyme assembly or catalytic activity was impaired as a result of the gp91<sup>phox</sup> mutation. However, as noted above, mutations in gp91<sup>phox</sup> in which NADPH oxidase activity was affected also resulted in an apparent reduction in the stability of gp91<sup>phox</sup>. Because of the low level of expression of these gp91<sup>phox</sup> mutants, quantitation of p47<sup>phox</sup> translocation could not be determined reliably.

There are a number of possible explanations for the observed discrepancy between the 559RGVHF<b>IF</b><sup>565</sup> peptide inhibition data and the results obtained in this study. A kinetic analysis of oxidase inhibition by 559RGVHF<b>IF</b><sup>565</sup> has suggested that this peptide acts as a non-competitive inhibitor (57), which might explain many of the mutations in the gp91<sup>phox</sup> polypeptide did not have a profound effect. Alternatively, additional sites of interaction between p47<sup>phox</sup> and gp91<sup>phox</sup> may obviate the requirement for an intact 559RGVHF<b>IF</b><sup>565</sup> domain for oxidase assembly in intact cells. A proline-rich region in the cytoplasmic carboxyl terminus of p29<sup>phox</sup> is a target for SRC homology 3 domains within p47<sup>phox</sup> (47, 58–62). A P156H point mutation in this domain, identified in a CGD patient homozgyous for a mutant p29<sup>phox</sup> gene, abolishes translocation of p47<sup>phox</sup> and NADPH oxidase activity (47, 58, 59, 61). This interaction between p47<sup>phox</sup> and the cytochrome thus appears to be critical for oxidase assembly in intact granulocytes. A D500G mutation in gp91<sup>phox</sup>, identified in a patient with X-linked CGD, is also associated with virtually absent oxidase activity and deficient translocation of p47<sup>phox</sup> (63). Residues 77–93 in gp91<sup>phox</sup> are another domain that appears to interact with p47<sup>phox</sup> as peptides homologous to this sequence bind to recombinant p47<sup>phox</sup> and inhibit oxidase function in the cell-free assay (32). Finally, it is also possible that single or multiple substitutions other than those studied within the 559RGVHF<b>IF</b><sup>565</sup> sequence are required to fully inhibit oxidase assembly in whole cells. However, we found that the ability to analyze the impact of more drastic alterations was limited by a lack of mutant protein stability.

The presence of an aromatic amino acid as the final or penultimate residue is highly conserved among ferredoxin-NAD<sup>+</sup> reductase family members, including gp91<sup>phox</sup>, which contains a carboxyl-terminal phenylalanine (Phe<sup>570</sup>). The aromatic ring has been proposed to maintain enzyme structure in the absence of NADP<sup>+</sup> by acting as a pseudosubstrate (18, 22, 35). X-ray diffraction analysis of spinach ferredoxin-NAD<sup>+</sup> reductase indicates that the carboxyl-terminal 19 residues, which lie just distal to the NADP<sup>+</sup>-binding domain, form an α-helix/β-strand region that extends into the FAD site, so that the terminal tyrosine residue interacts closely with the flavin ring (35). Altering the aromatic character of the corresponding residue in gp91<sup>phox</sup>, Phe<sup>570</sup>, or deleting it entirely resulted in a ~2-fold reduction in the rate of superoxide generation relative to cells expressing similar levels of wild-type recombinant gp91<sup>phox</sup>. Alanine substitution of the other aromatic residues (Phe<sup>563</sup> and Phe<sup>565</sup>) in the carboxyl terminus of gp91<sup>phox</sup> also resulted in, at most, a 3-fold reduction in NADPH activity.

These data contrast to the ~300–850-fold reduction in catalytic activity observed when non-aromatic replacements of the carboxyl-terminal tyrosine residue or its deletion were created in spinach ferredoxin-NAD<sup>+</sup> reductase (36). Our results suggest that there are structural and/or functional differences between the extreme carboxyl-terminal domain of gp91<sup>phox</sup> and the corresponding aromatic residues in other members of the ferredoxin-NAD<sup>+</sup> reductase family.

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