Deletion Mutagenesis of p22phox Subunit of Flavocytochrome b558

IDENTIFICATION OF REGIONS CRITICAL FOR gp91phox MATURATION AND NADPH OXIDASE ACTIVITY*

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Yanmin Zhu‡1, Christophe C. Marchal†1, Amy-Jo Casbon†, Natalie Stull†, Katharina von Löhneysen†, Ulla G. Knaus†, Algirdas J. Jesaitis†, Sally McCormick**, William M. Nauseef**, and Mary C. Dinuere‡2

From the †Herman B. Wells Center for Pediatric Research, Department of Pediatrics (Hematology/Oncology), Microbiology/Immunology, and Medical and Molecular Genetics, James Whitcomb Riley Hospital for Children, Indianapolis, Indiana 46202, the ‡Tree Fruit Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Wenatchee, Washington 98801, the †Department of Immunology, The Scripps Research Institute, La Jolla, California 92037, the ‡Department of Microbiology, Montana State University, Bozeman, Montana 59717, and the **Inflammation Program, Department of Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa and Veterans Affairs Medical Center, Iowa City, Iowa 52241.

The heterodimeric flavocytochrome b558, comprised of the two integral membrane proteins p22phox and gp91phox, mediates the transfer of electrons from NADPH to molecular oxygen in the phagocyte NADPH oxidase to generate the superoxide precursor of microbicidal oxidants. This study uses deletion mutagenesis to identify regions of p22phox required for maturation of gp91phox and for NADPH oxidase activity. N-terminal, C-terminal, or internal deletions of human p22phox were generated and expressed in Chinese hamster ovary cells with transgenes for gp91phox and two other NADPH oxidase subunits, p47phox, and p67phox. The results demonstrate that p22phox-dependent maturation of gp91phox carbohydrate, cell surface expression of gp91phox, and the enzymatic function of flavocytochrome b558 are closely correlated. Whereas the 5 N-terminal and 25 C-terminal amino acids are dispensable for these functions, the N-terminal 11 amino acids of p22phox are required, as is a hydrophilic region between amino acids 65 and 90. Upon deletion of 54 residues at the C terminus of p22phox (amino acids 142–195), maturation and cell surface expression of gp91phox was still preserved, although NADPH oxidase activity was absent, as expected, due to removal of a proline-rich domain between amino acids 151–160 that is required for recruitment of p47phox. Antibody binding studies indicate that the extreme N terminus of p22phox is inaccessible in the absence of cell permeabilization, supporting a model in which both the N- and C-terminal domains of p22phox extend into the cytoplasm, anchored by two membrane-embedded regions.

The phagocyte NADPH oxidase is a multicomponent enzyme that catalyzes the transfer of electrons from NADPH to generate the superoxide radical (O2·−), the precursor to additional reactive oxidants with potent microbicidal properties. The importance of this enzyme in innate immunity and inflammation is illustrated by chronic granulomatous disease (CGD),3 a syndrome characterized by absent NADPH oxidase activity, recurrent bacterial and fungal infections, and chronic inflammatory granulomas (1). The focal point for oxidase assembly is the phagocyte-specific flavocytochrome b558, a heterodimer comprised of two integral membrane proteins, gp91phox, a 91-kDa glycoprotein encoded by an X-linked gene that is the site of mutations in X-linked CGD, and p22phox, a non-glycosylated peptide derived from an autosomal CGD locus (1–3). Initiation of electron transport through flavocytochrome b to produce O2·− at plasma or phagosome membranes requires activation and translocation of Rac-GTP and two cytosolic phox subunits, p47phox and p67phox, which are affected in two other autosomal recessive forms of CGD.

The gp91phox and p22phox subunits of the flavocytochrome b heterodimer are closely associated and, in phagocytic cells, copurify unless denatured (4) and cross-link with small homo- or heterobifunctional cross-linking agents (5). Both the flavin and heme redox centers that mediate the transfer of electrons from NADPH to molecular oxygen reside within gp91phox (3, 6). The hydrophilic C-terminal portion of this 570 amino acid subunit contains an NADPH-binding site and bears a flavin group that acts as the initial acceptor of a pair of electrons from NADPH (7–10). The N terminus of gp91phox is intracellular and followed by six membrane-spanning domains, which contain two heme groups that lie embedded within the membrane and ligated non-covalently by four histidine residues (3, 6, 11–15).

The topology and functional domains within the p22phox

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* The abbreviations used are: CGD, chronic granulomatous disease; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PB5G, phosphate-buffered saline with glucose; phox, phagocyte oxidase; PRR, proline-rich region; mAb, monoclonal antibody; ER, endoplasmic reticulum; CHO, Chinese hamster ovary.

1 These authors contributed equally to this manuscript.

2 To whom correspondence should be addressed: Cancer Research Institute, Indiana University School of Medicine, 1044 West Walnut St., R4 402C, Indianapolis, IN 46202. Tel.: 317-274-8645; Fax: 317-274-8679; E-mail: mdinauer@iupui.edu.

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1 The abbreviations used are: CGD, chronic granulomatous disease; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PB5G, phosphate-buffered saline with glucose; phox, phagocyte oxidase; PRR, proline-rich region; mAb, monoclonal antibody; ER, endoplasmic reticulum; CHO, Chinese hamster ovary.
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FIGURE 1. p22phox deletion mutants. Schematic diagram showing deletion mutants of p22phox used in this study. Putative transmembrane domains are filled in gray in the top diagram. Black horizontal lines show sequences present in the deletion mutants. Dashed vertical lines are included to facilitate comparison of deleted with wild-type p22phox sequences.

The deletion subunit, which has 190 (murine)-195 (human) amino acids (see Ref. 16 and Fig. 1), are less well characterized. There are multiple hydrophobic regions in p22phox, including two in the N-terminal portion of the polypeptide, that could potentially form up to four membrane spanning domains (e.g. Refs. 6 and 17). However, the combined evidence from previous antibody and proteolysis experiments, computational predictions and newly described p22phox monoclonal antibodies (mAbs) suggests that extensive N-terminal regions of p22phox are intracytoplasmic, with two membrane-spanning domains located between amino acids 90 and 127 (18). The remaining C-terminal portion of p22phox, which is localized to the cytoplasmic face of the membrane (19–22), is hydrophilic and contains multiple proline residues, including a proline-rich region (PRR, amino acids 151–160) containing a proline II helix. The PRR and an adjacent α-helical region extending through amino acid 164 provide a high affinity binding site for tandem SH3 domains in p47phox, which is essential for recruitment of the cytosolic phox subunits and oxidase activity (6, 17, 19, 23–30). The p23phox subunit also contains a single invariant histidine, His38, which is dispensable for heme binding and cytochrome b558 function (31).

Additional studies indicate that the biosynthesis and assembly of the flavocytochrome b subunits are closely linked. Heterodimer formation and heme incorporation are important for stable expression of gp91phox and p22phox in phagocytes, and both polypeptides are absent from CGD patients with null mutations in either flavocytochrome b subunit (1). gp91phox is cotranslationally glycosylated and first detected as a high mannose 65-kDa monomer in the endoplasmic reticulum (ER). Full maturation of gp91phox requires sequential incorporation of heme into the 65-kDa precursor, heterodimerization with p22phox, which occurs in the late ER, and addition of N-linked oligosaccharides in the Golgi prior to transport to the plasma membrane, and, in neutrophils, specific granules (14, 21, 32, 33). The apparent size of the mature glycoprotein during SDS-PAGE, where it migrates as a broad band, is ~91 kDa (4, 32–34). Note that neither gp91phox nor p22phox has a cleaved signal peptide (4, 19, 35), and inhibitor studies demonstrate that glycosylation of gp91phox is not required for heterodimer formation or function (32, 33). Although in phagocytic leukocytes, gp91phox/p22phox heterodimer formation is essential to evade degradation by the cytosolic proteosome before exit from the ER (33), unassembled p22phox or gp91phox each is relatively more stable in heterologous cell lines such as COS7 monkey kidney epithelial cells, murine 3T3 fibroblasts, and Chinese Hamster Ovary (CHO) cells (31, 32, 34). In addition, co-transfection of p47phox and p67phox in COS7 or CHO cells expressing flavocytochrome b via gp91phox and p22phox transgenes enables these non-phagocytic cells to assemble a functional NADPH oxidase upon stimulation with either arachidonic acid or phorbol myristate acetate (31, 36), two pharmacologic agonists frequently used to activate the oxidase in phagocytes.

Despite recent progress, additional work is needed to augment our understanding of the overall domain structure of p22phox and associated functions in flavocytochrome b558 biosynthesis and in NADPH oxidase activity. In this study we analyze the properties of N-terminal, C-terminal or internal deletion mutants of human p22phox, taking advantage of heterologous CHO cells engineered to express wild-type or mutant p22phox in the presence of wild-type gp91phox, p47phox, and p67phox.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Bovine growth serum (BGS) was purchased from Hyclone Laboratories (South Logan, UT). Ham’s F12K medium was purchased from Invitrogen (Carlsbad, CA). mAbs 54.1 and 7D5 against gp91phox and 44.1, CS9, NS2, and NS5 against p22phox were previously described (18, 21, 37). A polyclonal anti-p22phox generated in rabbits was also used (19). Anti-Myc mAb was from Upstate (Lake Placid, NY). Alexa fluor 488 (goat anti-mouse IgG) was from Molecular Probes/Invitrogen (Eugene, OR). All other reagents were from Sigma unless otherwise specified.

Construction of p22phox Deletion Mutants—Deletion mutants of p23phox were generated by PCR amplification from a full-length human p22phox cDNA as template in the presence of oligonucleotide primers (position and sequence available upon request) and Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) used according to the manufacturer’s instructions. In addition, to generate constructs with deletions at both the N-terminal and C-terminal ends of p22phox, a p22phox cDNA already deleted for base pairs encoding the first 5 amino acids was used as a PCR template to place a premature stop codon. cDNAs were subcloned into the mammalian expression vectors pcDNA3.1/Zeo (Invitrogen) or, to place a c-Myc-derived epitope tag on the N terminus, into pCMV-Myc (BD Clontech, Palo Alto, CA) (which results in the addition of 14 amino acids corresponding to the Myc epitope and 9 amino acids encoded by polylinker sequence prior to the start of the p22phox sequences). The N-terminal Myc tag was also placed on wild-
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type p22\textsuperscript{phox} (Myc-p22\textsuperscript{phox}) by subcloning into pCMV-Myc. To prepare p22\textsuperscript{phox} cDNAs with a C-terminal Myc tag, a synthetic oligonucleotide encoding 14 amino acids for the c-Myc epitope was ligated in-frame to the 3’ end of the p22\textsuperscript{phox} cDNAs prior to subcloning into pcDNA3.1/Zeo. The modified p22\textsuperscript{phox} cDNA sequences were verified by dideoxynucleotide sequencing on both strands. Preparation and manipulation of plasmids were performed using standard protocols (38).

The nomenclature of the p22\textsuperscript{phox} mutants (Fig. 1) is based on the amino acids that are deleted, and the absence or presence of a Myc tag, as follows. Four N-terminal deletions, p22\textsuperscript{phox}-(1–5), p22\textsuperscript{phox}-(1–11), p22\textsuperscript{phox}-(1–31), and p22\textsuperscript{phox}-(1–61), lack the first 5, 11, 31, or 61 N-terminal residues of p22\textsuperscript{phox}, respectively. p22\textsuperscript{phox} constructs with either an N- or C-terminal c-Myc epitope tag use the same nomenclature, but with inclusion of “Myc” at the beginning or end, respectively (e.g. Myc-p22\textsuperscript{phox}-(1–5), which has a N-terminal Myc tag). Note that for non-Myc-tagged N-terminal deletions, a new in-frame 5’-ATG encoding a methionine is present. Two internal deletions, p22\textsuperscript{phox}-(67–72) and p22\textsuperscript{phox}-(66–90), lack portions of a hydrophilic region spanning amino acids 53–91. Progressive C-terminal deletions were also prepared (p22\textsuperscript{phox}-(172–195), p22\textsuperscript{phox}-(149–195), and p22\textsuperscript{phox}-(131–195)), without or with N-terminal Myc tags, along with several mutants with a C-terminal Myc tag (p22\textsuperscript{phox}-(172–195)-Myc, p22\textsuperscript{phox}-(142–195)-Myc, and p22\textsuperscript{phox}-(132–195)-Myc). Finally, two constructs for p22\textsuperscript{phox} derivatives deleted at both the N- and C terminus (p22\textsuperscript{phox}-(1–5, 172–195) and p22\textsuperscript{phox}-(1–5, 149–195)) were prepared.

**Cell Lines**—CHO K1 cells were obtained from American Type Culture Collection (Manassas, VA) (CRL-1651) and maintained in F12K medium, supplemented with 10% heat-inactivated BGS and 0.15% sodium bicarbonate, 50 units/ml penicillin, and 50 mg/ml streptomycin (Invitrogen). Adherent CHO cells were harvested by incubating with trypsin/EDTA for 1 mg/ml puromycin. For CHO 91-22-47-67 and 91-47-67 expresses gp91\textsuperscript{phox} (kindly provided by H. Malech, National Institutes of Health), following packaging using the Pantropic Retroviral Expression System (BD Clontech, Palo Alto, CA). Individual clones were screened by immunoblotting for expression of desired phox components.

pCMV-Myc and pcDNA3.1/Zeo plasmids for transient expression of wild-type p22\textsuperscript{phox} or p22\textsuperscript{phox} deletions were transfected into CHO 91-47-67 cells using Lipofectamine 2000 (Invitrogen). 10 μg of plasmid DNA was used for each 60-mm plate. Cells were analyzed 21 to 42 h after transfection. Transient transfection efficiency was assayed using flow cytometry to monitor expression of GFP in cells transfected in parallel with pIRE52-EGFP (BD Clontech). Transfection efficiency averaged 30–40%.

**Immunoblot Analysis**—Cells extracts were prepared for SDS-PAGE and immunoblotting as described (31, 36). Signal from horseradish peroxidase-conjugated secondary anti-immunoglobulin G was visualized by enhanced chemiluminescence (ECL) (Amersham Biosciences or Pierce). The films were scanned using a HP Scan Jet 5300c scanner and the HP Precision Scan Pro software at a resolution of 400 dpi. Densitometry of bands corresponding to gp91\textsuperscript{phox}, p22\textsuperscript{phox}, and β-actin was obtained using the Scion Image 1.63 software (Frederick, MD).

**Metabolic Labeling and Immunoprecipitation of Flavocytochrome b Subunits**—CHO cell lines were grown to ~70% confluence and then washed before adding 5 ml of methionine-free RPMI containing 10% dialyzed fetal calf serum and incubating for 1 h. [\textsuperscript{35}S]methionine (25 μCi/ml) was then added and the cells labeled for 2 h prior to harvesting, or in the case of pulse-chase experiments, supplementing with unlabeled methionine (final concentration 1 mM) and culturing an additional 20 h. Adherent cells were recovered by trypsin treatment, washed in PBS and cell pellets resuspended in 1.5 ml of solubilization buffer (10 mM NaCl, 100 mM KCl, in 10 mM HEPES at pH 7.4, containing 1 mM EDTA, 10 μg/ml chymostatin, 100 μM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a 1:1,000 dilution of the P8340 protease inhibitor mixture mix). Triton X-100 (final concentration 1%) was added to the cell suspension prior to sonication on ice (5 s × 3, with 1 min rest between each sonication). The cell sonicate was then adjusted to 1% deoxycholate, 1% Nonidet P-40 and 0.05% SDS and was stirred overnight at 4 °C prior to clarification by centrifugation (55,000 rpm × 30 min at 4 °C in TLA 120.2 rotor, using a Beckman TL-100 tabletop ultracentrifuge). mAbs against gp91\textsuperscript{phox} or p22\textsuperscript{phox} were added to the clarified supernatant, and the samples tumbled at 4 °C for 4 h. To recover immune complexes either protein A (for 44.1) or protein G (for 54.1 and CS9) coupled to Sepharose (30 ml/500 ml sample) was added and samples tumbled at 4 °C for 1 h. Sepharose beads were recovered by centrifugation and the pellets washed five times with 900 ml of ice-cold washing solution (1% deoxycholate, 1% Nonidet P-40, 1% Triton X-100, and 0.05% SDS in 10 mM NaCl, 100 mM KCl, in 10 mM HEPES at pH 7.4, containing 1 mM EDTA). Immune complexes were released from the Sepharose beads by heating (90 °C × 3 min) in SDS sample buffer without reducing agents. After centrifugation, the supernatant was collected, adjusted to
10 mM dithiothreitol and incubated at 37 °C before separating by SDS-PAGE on a 5–20% SDS gradient gel.

**Flow Cytometric Analysis of gp91phox Cell Surface Localization**—CHO 91-47-67 cells were transiently transfected with either wild type or p22

phox

mutants as described above. Cells were trypsinized 21–42 h after transfection, collected by centrifugation and washed once with PBS. Following blocking in 0.1% bovine serum albumin and 1% normal goat serum in PBS for 30 min on ice, cells were stained with the anti-gp91

phox

7D5 mAb, without permeabilization. After washing twice with PBS, cells were then stained with Alexa Fluor 488-conjugated goat anti-mouse antibody IgG, and fluorescence measured by FACScan (Becton Dickinson, San Jose, CA). Mouse IgG1 was used as an isotype control. In some experiments, stained cells were fixed with 1% paraformaldehyde and analyzed by FACScan the following morning, with results the same as those obtained using freshly stained, unfixed cells.

**Measurement of NADPH Oxidase Activity**—Following stimulation with 200 µM arachidonic acid, the production of reactive oxidants by the NADPH oxidase was measured in phox-expressing CHO cell lines using isoluminol chemiluminescence (40). Although oxidant production in these cells is stimulated by either arachidonic acid or phorbol myristate acetate, the former gives a more robust response (Ref. 31, and was thus used for NADPH oxidase assays. For the isoluminol assay, cells were removed by brief trypsinization as described (36), and arachidonic acid-stimulated chemiluminescence detected as relative luminescence units by fast kinetic mode and integrated over 100 s using an Lmax microplate luminometer and SoftMax Pro software (Molecular Devices, Sunnyvale, CA). Under these conditions, 97.5% of chemiluminescence was inhibited by superoxide dismutase.

**Confocal Microscopy**—CHO 91 cells transiently transfected for expression of wild-type p22

phox

tagged at the N terminus with Myc or parental CHO cells were plated on gelatin-coated glass coverslips and grown to 50–60% confluence. Cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice with PBS, and permeabilized in 0.02% saponin in PBS with 0.1% bovine serum albumin for 30 min on ice. In some cases, permeabilization was omitted, and fixation was performed after blocking and staining. Cells were incubated with 2% bovine serum albumin with 1% goat serum and 0.1% fish skin gelatin overnight. Cells were then stained for 1 h with anti-Myc or 7D5 mAb, followed by 1 h of incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG. Cells were washed three times with 0.1% bovine serum albumin in PBS, briefly rinsed in water and mounted with coverslips. Images were scanned with the multitracking mode on a Zeiss LSM 510 using the 488 laser line. Controls were performed with no primary antibody. Images were processed with Photoshop (Adobe). All imaging studies were repeated in 2–3 independent experiments.

**p22

phox

Sequence Analysis**—The following public domain software was used for analysis of the p22

phox

protein sequence: PROSITE program on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics; MOTIF pattern search service and PSORT for signal peptides search; TMHMM for predicting transmembrane helices in proteins (CBS; Denmark). The alignment of different mammalian p22

phox

sequences utilized ClustalW, a multiple sequence alignment program, which calculates the best match for the selected sequences.

4 M. Dinauer, unpublished observations.
RESULTS

Alignment and Analysis of p22phox Amino Acid Sequences—Published p22phox sequences from nine mammalian species were retrieved from UniProt Knowledgebase (Swiss-Prot/TrEMBL) protein data base and aligned using ClustalW. As previously described in analyses of smaller data sets (16, 41), multiple sequence alignment reveals a high degree of conservation throughout the protein sequence with the exception of the C-terminal region (amino acids 170 through 190–195, depending on the species) (Fig. 2), with an overall similarity across species from rat to human to dolphin of ~86%. Several protein sequence analysis tools were used to search for conserved functional motifs in the p22phox protein family. Four hydrophobic regions were identified that could be membrane-spanning segments of p22phox. However, recent studies suggest that only the two of the hydrophobic domains (domains 3 and 4) are integrated within the membrane (18). A known SH3-binding motif was identified in the proline-rich region (PRR) in the C terminus of p22phox, which in combination with an adjacent α-helical region, provides a high affinity site for binding of a pair of SH3 domains in p47phox during NADPH oxidase assembly (6, 19, 26, 28, 29). No other functional motifs were identified in p22phox. 

Deletion Mutagenesis of p22phox—Other than the above-mentioned region in the C terminus of p22phox that interacts with p47phox, little is known about the function of other regions of the p22phox subunit. As an approach to identify additional domains in p22phox important for superoxide production and for flavocytochrome b biosynthesis, a series of p22phox deletion mutants were generated (Fig. 1). Four N-terminal deletions were designed to remove the first 5, 11, 31, or 61 N-terminal residues of p22phox. Amino acids 1–11 are hydrophilic residues whereas amino acids 12–59 include two hydrophobic regions that might be inserted in or span the membrane (Fig. 1). Two internal deletions, p22phox-(65–72) and p22phox-(66–90), remove portions of a hydrophilic region between amino acids 53–91. Progressive C-terminal deletions were also generated including two, which also lacked the first 5 N-terminal amino acids (Fig. 1). The p22phox-(172–195) derivatives retain the C-terminal PRR (amino acids 151–160) and an adjacent α-helical region (amino acids 161–164) for binding of p47phox, whereas this region is absent in the other C-terminal deletions. Because available p22phox antibodies might not detect some of the mutant proteins, many of the p22phox derivatives were also prepared with an N-terminal Myc tag, and, after initial studies indicated that an N-terminal Myc interfered with p22phox function in some cases (see below), selected deletion mutants were also generated with a C-terminal Myc tag (Fig. 1). 

CHO cells do not express endogenous phox subunits (31), and thus are a useful system in which to evaluate the behavior of recombinant phox proteins expressed from transgenes. When gp91phox is expressed in the absence of p22phox (CHO 91 cells), maturation of gp91phox from its gp65 form is very inefficient, and only very low levels of the mature ~91-kDa species are detected (Fig. 3A), consistent with previous studies (31, 32). Co-expression of wild type p22phox with gp91phox (CHO 91-22 cells) results in markedly increased amounts of the ~91 kDa form of gp91phox (Fig. 3A). Previous studies in PLB-985 promyelocytes and COS7 cells suggest that gp91phox maturation is tightly correlated with heterodimerization of p22phox and gp91phox during flavocytochrome b biosynthesis (32, 33). The association between gp91phox and p22phox in CHO cells was confirmed by co-immunoprecipitation studies in metabolically labeled cells. After 2 h of labeling with [35S]methionine, only the 65 kDa form of gp91phox was recovered from CHO 91 and CHO 91-22 cells following immunoprecipitation with the gp91phox 54.1 mAb (Fig. 3B). After a 20-hour chase with unlabeled amino acids, the ~91-kDa form of gp91phox was immunoprecipitated along with p22phox in CHO 91-22 cells by mAb54.1, whereas little gp65 remained in either CHO 91 or CHO 91-22 cells (Fig. 3B). Conversely, in addition to p22phox, gp91phox was recovered using a p22phox mAb to immunoprecipitate CHO 91-22 extracts after a 20-h chase (Fig. 3B). The time lag between synthesis of the gp65 form of gp91phox, and its subsequent association with p22phox and maturation to the 91-kDa form was similar to that observed in PLB-985 promyelocytes, as was the increase in stability of the gp91phox polypeptide under heterodimer formation (33).

The effect of the p22phox deletions on flavocytochrome b biosynthesis and NADPH oxidase activity was examined following transient expression in CHO cells containing stable transgenes for wild-type gp91phox, p47phox, and p67phox (CHO 91-47-67 cells) (Fig. 3, C and D). Expression of the deleted forms of p22phox was confirmed by SDS-PAGE and immunoblot analysis. Antibodies used to detect p22phox include CS9 mAb and NS2, which bind to p22phox amino acids 165–170 and 131–139, respectively (18), and a mAb for c-Myc for detection of the Myc-tagged derivatives. Except for expression of p22Δ(132–195)-Myc, which was consistently low, all p22phox derivatives were expressed at levels similar to wild-type p22phox although there was some variability between individual experiments, most likely related to efficiency of transfection. In most cases, the truncated p22phox proteins had the expected electrophoretic mobility; several exceptions are detailed below, although these do not affect the overall interpretation of each deletion’s effects on flavocytochrome b maturation and function.

The untagged p22phox-(1–11), p22phox-(1–31), and p22phox-(1–61) DNAs yielded both a species of the expected size as well as a more rapidly migrating polypeptide (Fig. 3C). Although a Kozak sequence was placed at the new ATG in these N-terminal-deleted forms of p22phox, the smaller polypeptide product is the size expected if an internal ATG codon (encoding amino acid 65 in the wild-type protein) is utilized as the initiator methionine. The polypeptides derived from the internal ATG appear to migrate slightly faster than the p22phox-(1–61) derivative of p22phox, but we cannot rule out that p22phox-(1–61) also utilizes this alternative start codon. Another unexpected finding was that all derivatives with C-terminal truncations migrated more rapidly than predicted, for reasons that are uncertain, either in the absence or presence of the Myc tag. For example, although only lacking 24 amino acids, p22phox-(172–195) migrates faster than p22phox-(1–61), which lacks 61 amino acids. Finally, in cells transfected with the Myc-tagged form of p22phox-(1–61), two additional smaller species were detected by the anti-Myc mAb (Fig. 3D). The larger of these also reacted
with a rabbit polyclonal antibody raised to a peptide spanning the proline-rich region at amino acids 153–160 (19) (not shown), but not with CS9 (Fig. 3D). Thus these bands are likely to represent polypeptides generated by C-terminal proteolysis. More rapidly migrating species were also detected for p22phox-(67–72) and p22phox-(66–90), Myc-p22phox-(66–90), Myc-p22phox-(1–31), and both N- and C-terminally Myc-tagged p22phox-(172–195), which also likely represent products of partial proteolysis.

Immunoblots of p22phox deletions were also probed with p22phox mAbs NS5 and 44.1 (18, 21) (not shown). Detection of p22phox derivatives was consistent with mAb epitopes identified by phage display, but suggested additional complexity to the NS5 epitope. The NS5 antibody failed to detect C-terminal truncations of p22phox at amino acids 131, 132, or 142. This was unexpected since initial observations suggested that NS5 detects an epitope comprised of amino acids 51–57 and 77–82 (18). However, additional phage display analysis indicates that amino acids 147–149 also contribute to the protein surface recognized by NS5,5 and thus explains the lack of reactivity with the above-mentioned deletions.

p22phox Regions Required for Maturation of gp91phox and Its Expression on the Cell Surface—The impact of p22phox deletions on maturation of gp91phox from 65 to ~91 kDa was evaluated by immunoblot analysis. As a positive control, wild-type p22phox, either untagged or with an N-terminal Myc tag, was transiently transfected into CHO 91 or CHO 91-22 cells, resulting in a marked increase in the abundance of the mature ~91 kDa form of gp91phox (Fig. 3, C and D). The abundance of the ~91-kDa gp91phox polypeptide following transfection of these p22phox derivatives was similar

5 A. Jesaitis, unpublished observations.
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**TABLE 1**

### p22\textsuperscript{phox} deletion mutants and effects on gp91\textsuperscript{phox} maturation and NADPH oxidase activity

| Plasmid | −Myc tag | +Myc tag* |
|---------|----------|-----------|
|         | gp91\textsuperscript{phox} maturation | O₂\textsuperscript{−} | gp91\textsuperscript{phox} maturation | O₂\textsuperscript{−} |
| Mock    | −       | −         | −       | −         |
| p22wt   | −       | −         | −       | −         |
| p22−(1–5) | +       | +         | +       | +         |
| p22−(1–11) | −       | −         | −       | −         |
| p22−(1–31) | −       | −         | −       | −         |
| p22−(1–61) | −       | −         | −       | −         |
| p22−(67–72) | −       | −         | ND\textsuperscript{a} | ND         |
| p22−(66–90) | −       | −         | −       | −         |
| p22−(131–195) | −       | −         | −       | −         |
| p22−(132–195) | ND      | ND        | ND      | ND        |
| p22−(142–195) | +       | +         | +       | +         |
| p22−(149–195) | +       | +         | +       | +         |
| p22−(172–195) | +       | +         | +       | +         |
| p22−(1–5, 172–195) | +       | ND        | ND      | ND        |
| p22−(1–5, 149–195) | +       | ND        | ND      | ND        |
| p22−(1–31) | +       | −         | +       | +         |
| p22−(1–11) | −       | −         | −       | −         |

**a** Myc tags were always located on the N terminus, with these exceptions: p22−(1–15) and p22−(1–15) mutants had a C-terminal Myc tag, and both N- and C-terminal Myc-tagged versions p22−(172–195) were studied.

**b** Absent.

**c** Present.

**d** ND, not done.

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Transfection of wild-type p22\textsuperscript{phox}, except for consistently modestly lower levels of mature gp91\textsuperscript{phox} in cells expressing p22\textsuperscript{phox}−(1–5, 172–195) (=30% lower, after normalizing to p22\textsuperscript{phox} expression; see Fig. 3C and data not shown). All other deleted variants of p22\textsuperscript{phox} fail to promote maturation of gp91\textsuperscript{phox} carbohydrate (Fig. 3, C and D; summarized in Table 1). Thus, at the N terminus, the only first 5 amino acids of p22\textsuperscript{phox} are dispensable for enhancing maturation of gp91\textsuperscript{phox}, whereas a large portion of the C terminus (amino acids 141–195) does not appear to be required for this function. It is unclear why placement of a Myc tag on the N terminus of the p22\textsuperscript{phox}−(1–5) deletion interfered with its ability to promote gp91\textsuperscript{phox} maturation, but this finding illustrates both the importance of evaluating effects of placement of molecular tags and the sensitivity of the N terminus of p22\textsuperscript{phox} to manipulation. We were unable to unambiguously assess the effect of removing amino acids 131–195 from the C terminus of p22\textsuperscript{phox} a deletion that requires an epitope tag for detection. A similar derivative with a C-terminal tag (p22Δ(132–195)-Myc) was unexpectedly poorly expressed (Fig. 3D). Although a deletion with N-terminal Myc tag was well-expressed and did not enhance gp91\textsuperscript{phox} maturation (Fig. 3D, see also Fig. 4B), the adverse effect of the N-terminal Myc tag on p22Δ(1–5) function makes it difficult to draw a firm conclusion regarding impact of deleting amino acids 131–195.

Although a small amount of gp91\textsuperscript{phox} is detectable on the cell surface of CHO 91 cells (Fig. 4A), co-expression of wild type gp91\textsuperscript{phox} and p22\textsuperscript{phox} in CHO cells correlated with increased cell surface staining with the 7D5 gp91\textsuperscript{phox} monoclonal antibody (Fig. 4A), which recognizes the heme-containing form of gp91\textsuperscript{phox} (14, 37, 42). These results are consistent with our previous studies on the biosynthesis of flavocytochrome b in PLB-985 and COS 7 cells, where maturation, heme incorporation, and cell surface expression of gp91\textsuperscript{phox} are closely linked (14, 33, 37). Flow cytometry analysis of CHO 91–47–67 cells following transient transfection of either wild-type or deleted forms of p22\textsuperscript{phox} correlated with gp91\textsuperscript{phox} maturation as assessed on immunoblots of cell extracts (Fig. 3, C and D). As shown in representative data displayed in Fig. 4B, cells transfected with either wild-type p22\textsuperscript{phox} (±Myc tag) or all derivatives that fail to enhance maturation of gp91\textsuperscript{phox} to its ~91-kDa form (see above) exhibited a population with increased cell surface staining with the 7D5 mAb compared with mock-transfected CHO 91–47–67 cells. In contrast, for Myc-p22\textsuperscript{phox}−(1–5), p22\textsuperscript{phox}−(1–11), Myc-p22\textsuperscript{phox}−(1–11), and all other derivatives that fail to enhance gp91\textsuperscript{phox} carbohydrate maturation, 7D5 staining was similar to mock-transfected CHO 91–47–67 cells (Fig. 4B and not shown).

p22\textsuperscript{phox} Regions Required for Superoxide Production—The impact of p22\textsuperscript{phox}− deletion mutations on NADPH oxidase activity was examined by comparing NADPH oxidase activity in CHO 91–47–67 cells transiently expressing wild-type or mutant p22\textsuperscript{phox}. Only cells expressing either wild-type p22\textsuperscript{phox} or p22\textsuperscript{phox} mutants that supported gp91\textsuperscript{phox} maturation and also retained the C-terminal p47\textsuperscript{phox} binding site (residues 151–164) exhibited NADPH oxidase activity (Table 1). Fig. 5 summarizes results from isoluminol chemiluminescence assays for p22\textsuperscript{phox} mutants functional for NADPH oxidase activity, as compared with wild-type p22\textsuperscript{phox}, Myc-p22\textsuperscript{phox}, and a p47\textsuperscript{phox} binding site-deleted mutant (p22Δ(1–5, 149–195)) that supports gp91\textsuperscript{phox} maturation (Figs. 3C and 4B) but not oxidase activity. NADPH oxidase activity following transfection of p22\textsuperscript{phox} derivatives lacking the 5 N-terminal amino acids, C-terminal 24 amino acids, or both of these regions, was similar to wild-type p22\textsuperscript{phox}. Two exceptions were Myc-22\textsuperscript{phox} and Myc-p22\textsuperscript{phox} (172–195), where oxidase activity was decreased, particularly for the latter mutant (Fig. 5). The reason for the effect of the N-terminal Myc tag on NADPH oxidase activity is unknown, and not related to expression levels of mature gp91\textsuperscript{phox} (Fig. 3D), but again illustrates the sensitivity of the N terminus of p22\textsuperscript{phox} to modification.

Location of the p22\textsuperscript{phox} N Terminal—N-terminal epitopes recognized by p22\textsuperscript{phox} mAbs 44.1 and NS5 (amino acids 29–33 and 50–57, respectively) appear to be intracytoplasmic based on their inaccessibility in permeabilized cells (18, 21). It has not been firmly established whether or not the first N-terminal amino acids of p22\textsuperscript{phox} are buried or otherwise inaccessible. We therefore examined the accessibility of the N-terminal Myc epitope following expression of Myc-p22\textsuperscript{phox}. The Myc epitope and downstream amino acids 1–25 reacted with neutrophils only after their permeabilization (18, 21).
acids encoded by the vector add an additional N-terminal 23 amino acids to p22phox. Myc-p22phox interacts with gp91phox to support flavocytochrome b maturation (Figs. 3D and 4B) and NADPH oxidase activity, although the latter is reduced by ~35% compared with wild-type p22phox (Fig. 5). In non-permeabilized CHO 91 cells transiently transfected for expression of Myc-p22phox, Myc staining was absent (Fig. 6A), in contrast to the extracellular epitope of gp91phox detected by the 7D5 mAb (Fig. 6C). However, the Myc epitope was detected intracellularly in ~40% of saponin-permeabilized cell (Fig. 6B), a frequency similar to the transfection efficiency of the Myc-p22phox expression vector. These results are consistent with the studies of Imajoh-Ohimi et al. (20), and strongly suggest that the N terminus of p22phox is on the cytoplasmic side of the plasma membrane.

DISCUSSION

This study used deletion mutagenesis of human p22phox to identify regions of this NADPH oxidase subunit that are functionally important for flavocytochrome b assembly and enzyme activity, as monitored by gp91phox maturation, cell surface expression, and NADPH oxidase activity. The results establish that regions in both the N and C terminus of p22phox contain determinants that are essential for these p22phox functions. However, the first 5 N-terminal and the final 25 C-terminal amino acids of p22phox (residues 171–195) are dispensable for heterodimer formation with gp91phox and for NADPH oxidase activity. Note that the region from amino acid 170 through the end of the C terminus is the most variable portion of p22phox between species (Fig. 2). In addition, the results confirm previ-
ous conclusions regarding the important role of the C-terminal PRR in p22phox (amino acids 151–160) for NADPH oxidase activity, although this domain is not required for interactions in flavocytochrome b biosynthesis leading to maturation of gp91phox carbohydrates and increased expression at the cell surface. The impact of some of the N- and C-terminal deletion mutations of p22phox is also influenced by the presence or absence of an N-terminal Myc epitope tag, suggesting that the N-terminal region of p22phox is highly sensitive to modification.

When expressed in CHO cells in the absence of p22phox, very little fully mature gp91phox is detected. In contrast, upon association with co-expressed p22phox, gp91phox is efficiently processed to its mature size of ~91 kDa and is significantly increased in abundance on the cell surface. These data are consistent with previous studies of flavocytochrome b biosynthesis in PLB-985 myeloid cells, where heterodimerization with p22phox is tightly coupled to the process of gp91phox maturation and transport of the flavocytochrome heterodimer into the plasma membrane (14, 32–34). With the exception of deletion mutants lacking a crucial domain for p47phox binding in the C terminus of p22phox, maturation of gp91phox also correlates positively with NADPH oxidase activity.

The sensitivity of the N-terminal region of p22phox to deletion mutagenesis suggests that it contains important signals that influence flavocytochrome b assembly. Deletions of p22phox that remove more than 5 N-terminal residues or as few as 6 amino acids within a hydrophilic internal region from 65 to 91, as well as replacing the 5 N-terminal residues with a Myc epitope tag, prevented p22phox from supporting gp91phox maturation. Although mAb epitopes suggest that N- and C-terminal domains of p22phox interact, removal of their mutual interaction per se is unlikely to account for loss of this p22phox function. Deletion of up to 54 amino acids from the C terminus, including the C-terminal portion (amino acids 183–188) of the discontinuous mAb 44.1 epitope that interacts with amino acids 29–33 (21), does not impair gp91phox maturation. p22phox-enhanced gp91phox maturation is also preserved using the p22Δ(143–195)-Myc deletion, which lacks the C-terminal portion of a second discontinuous epitope recognized by mAb NS5 (amino acids 51–57 and 77–82, and 147–149 (18). The indispensable regions of p22phox may play a direct role in heterodimerization with gp91phox, induce deleterious conformational change(s) in p22phox that inhibit heterodimerization, and/or influence interactions with molecular chaperones during flavocytochrome biosynthesis. Because deletion mutants can have long-range effects in structure that indirectly affect interactions between
p22phox and gp91phox, future studies will focus on replacement of amino acids within p22phox to more precisely identify specific residues that mediate these interactions.

Results presented above suggest that the N terminus of the p22phox polypeptide plays an important role in assembly of the flavocytochrome heterodimer. Because p22phox is an integral membrane protein, it was thus important to establish if the N terminus is on the cytoplasmic or the extracellular side of the plasma membrane. This question was addressed with N-terminally Myc-tagged p22phox and an anti-Myc antibody. The results indicate that the N terminus of p22phox is on the cytoplasmic side of the plasma membrane, supporting a previous study using antibody to residues 1–25 of p23phox (20). These data also support the model of Taylor et al. (18), which suggests that p22phox has two transmembrane segments and that the N and C termini of p22phox are located in the cytoplasm, rather than alternative models placing the N terminus at the external face of the membrane (6, 17).

The PRR of p22phox has been the subject of extensive study and functions as a critical domain in NADPH oxidase assembly via binding of tandem SH3 domains. In the current study, as expected, NADPH oxidase activity was lost upon deletion of this domain. The p22phox polypeptide has also been shown to interact functionally with the gp91phox (Nox2) homologs, Nox1, Nox3, and Nox4, which function in a closely related family of NADPH oxidases, although the PRR is only important for enzyme activity of Nox1 and Nox3, which also exhibit a requirement for the p47phox homolog, NOXO1 (27, 43–46). Kawahara et al. (27) reported that expression of C-terminal truncations of p22phox lacking residues 150–195 or 156–195 acted as competitive inhibitors with endogenous p22phox for NADPH oxidase activity of Nox1, gp91phox, or Nox3 in transfected HEK 293 cell, suggesting that these truncated p22phox species could form a complex with these Nox proteins. The current study expands upon this finding to directly show that C-terminal truncations of p22phox deleted up to amino acid 141 are able to interact with gp91phox as evidenced by matura-
gion of gp91phox carbohydrate and its enhanced expression at the cell surface.

In conclusion, these studies provide evidence that different domains within p22phox have distinct roles in dictating the fate of the gp91phox subunit following gp91phox biosynthesis and its subsequent ability to function in the production of superoxide. The C-terminal region of p22phox, which is more poorly conserved than the rest of the polypeptide, is more tolerant of deletional mutagenesis than the N-terminal or internal regions of p22phox. Additional mutagenesis and structural studies are needed to define the domain structure of p22phox more precisely and to determine the functional roles of conserved regions of p22phox.

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