Mutagenesis of p22phox Histidine 94

A HISTIDINE IN THIS POSITION IS NOT REQUIRED FOR FLAVOCYTOCHROME b558 FUNCTION

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The NADPH oxidase is a multicomponent enzyme that transfers electrons from NADPH to O2 to generate superoxide (O2•−), the precursor of microbicidal oxygen species that play an important role in host defense. Flavocytochrome b558, a heterodimeric oxidoreductase comprised of gp91phox and p22phox subunits, contains two nonidentical, bis-histidine-ligated heme groups imbedded within the membrane. Four histidine residues that appear to serve as noncovalent axial heme ligands reside within the hydrophobic N terminus of gp91phox, but the role of p22phox in heme binding is unclear. We compared biochemical and functional features of wild type flavocytochrome b558, with those in cells co-expressing gp91phox with p22phox harboring amino acid substitutions at histidine 94, the only invariant histidine residue within the p22phox subunit. Substitution with leucine, tyrosine, or methionine did not affect heterodimer formation or flavocytochrome b558 function. The heme spectrum in purified preparations of flavocytochrome b558 containing the p22phox derivative was unaffected. In contrast, substitution of histidine 94 with arginine appeared to disrupt the intrinsic stability of p22phox and, secondarily, the stability of mature gp91phox and abrogated O2•− production. These findings demonstrate that His94p22phox is not required for heme binding or function of flavocytochrome b558 in the NADPH oxidase.

The NADPH oxidase is a multicomponent enzyme that transfers electrons from NADPH to O2 to generate superoxide (O2•−), the precursor of microbicidal oxygen species such as hydrogen peroxide and hypochlorous acid that play an important role in host defense. Flavocytochrome b558, a heterodimeric oxidoreductase comprised of gp91phox and p22phox subunits, contains two nonidentical, bis-histidine-ligated heme groups imbedded within the membrane. Four histidine residues that appear to serve as noncovalent axial heme ligands reside within the hydrophobic N terminus of gp91phox, but the role of p22phox in heme binding is unclear. We compared biochemical and functional features of wild type flavocytochrome b558, with those in cells co-expressing gp91phox with p22phox harboring amino acid substitutions at histidine 94, the only invariant histidine residue within the p22phox subunit. Substitution with leucine, tyrosine, or methionine did not affect heterodimer formation or flavocytochrome b558 function. The heme spectrum in purified preparations of flavocytochrome b558 containing the p22phox derivative was unaffected. In contrast, substitution of histidine 94 with arginine appeared to disrupt the intrinsic stability of p22phox and, secondarily, the stability of mature gp91phox and abrogated O2•− production. These findings demonstrate that His94p22phox is not required for heme binding or function of flavocytochrome b558 in the NADPH oxidase.

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The abbreviations used are: CGD, chronic granulomatous disease; mAb, monoclonal antibody; INT, iodonitrotetrazolium; CHO, Chinese hamster ovary; RT, reverse transcription; HPRT, hypoxanthine phosphoribosyltransferase.

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rattus (19) and lies within a region with sequence similarity to cytochrome c oxidase and myoglobin (20). Quinn et al. (12) reported that gp91phox and p22phox each bind heme based on heme staining detected at ~22 kDa after electrophoresis of flavocytochrome b558 on lithium dodecylsulfate polyacrylamide gels. Indirect evidence of a role for p22phox His94 in heme binding came from the identification of a CGD patient with a p22phox H94R point mutation that resulted in undetectable levels of flavocytochrome b558 (21). However, we previously reported evidence indicating that both of the two heme groups of flavocytochrome b558 are entirely located with gp91phox based on analysis of membranes prepared from a Cos7 cell line expressing transgenic gp91phox in the absence of p22phox (22). More recently, four histidines that appear to be involved in axial heme ligation were identified within gp91phox. In vitro investigated the basis whereby arginine substitution of p22phox His94 results in absence of flavocytochrome b558 expression and to definitively determine whether or not His94 is involved in heme binding and flavocytochrome b558 function, we exploited nonphagocytic cell lines that are void of endogenous flavocytochrome b558, we exploited nonphagocytic cell lines that are void of endoge-

## EXPERIMENTAL PROCEDURES

### Materials—

Fetal bovine serum was purchased from Hyclone Laboratories (South Logan, UT). Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium, F12K medium, and RPMI 1640 tissue culture medium were purchased from Invitrogen. Monoclonal antibodies 449 and 48 specific for p22phox and gp91phox, respectively, were kindly provided by A. Verhoeven and D. Roos (Central Laboratory of the Netherlands Blood Transfusion Service, University of Amsterdam, Amsterdam, the Netherlands). The polyclonal anti-p47phox and polyclonal anti-p67phox antibodies were kindly provided by D. Lambeth (Emory University, Atlanta, GA). Monoclonal anti-Rac-1 antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). All of the other reagents were purchased from Sigma unless otherwise specified.

### Cell Lines—

The monkey Cos7 cell line expressing transgenic human gp91phox, Cos 91, was made previously by stable transfection of the full-length gp91phox cDNA into parental Cos7 wild type cells and was maintained as described (22). The NIH3T3 cell line was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and was stably transduced with an MFG retroviral vector containing the gp91phox cDNA (vector kindly provided by Adrienne Thrasher, University College, London, UK). The CHO K1 line was maintained in F12K medium supplemented with 10% fetal calf serum and sodium bicarbonate (Invitrogen) and was stably transduced with a full-length gp91phox cDNA cloned into the NotI site of pEF pac (23) using LipofectAMINE Plus (Invitrogen) and into CHO 91 cells using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. All of the preparations and manipulations of plasmids were performed using standard protocols (24). Following transfection, the clones were selected by limiting dilution in 1.6 mg/ml G418 (Invitrogen) and were screened for p22phox expression by immunoblot. To minimize any clone to clone variation in recombinant p22phox expression or NADPH oxidase activity, three or four independent clones determined to express transgenic p22phox were pooled and used for subsequent analysis. In some experiments, wild type p22phox or H94R p22phox cDNA constructs were harvested 18 h post-transfection and analyzed for transient expression of p22phox.

For transient expression in selected cell lines, p47phox cloned into the NotI site of the pRK5 expression vector and p67phox cloned into the pCDNA3.1 expression vector (kindly obtained from David Lambeth, Emory University, Atlanta, GA) were transfected into CHO derivatives using LipofectAMINE 2000 (Invitrogen). The cells were harvested for further analysis 18 h post-transfection.

### Protein Expression and Immunoblot Analysis—

Proteins were extracted as described previously (14) and analyzed by SDS-PAGE. Following transfer to nitrocellulose membranes, the blots were probed with monoclonal antibodies 449 and 449 for expression of recombinant gp91phox and p22phox, respectively (25). In some experiments the blots were also analysed with a polyclonal anti-p22phox antibody, which recognizes both murine and human p22phox (18).
labeled full-length human p22<sup>phox</sup> cDNA and exposed to film for various amounts of time before developing in an X-OMat processor. The membranes were stripped and reprobed with cDNA for β-actin to control for equal loading.

**RT-PCR**—RT-PCR was performed on total RNA extracted as described above using a kit obtained from Promega (Madison, WI) according to the manufacturer’s instructions with primers specific for murine p22<sup>phox</sup> cDNA or with primers specific for a sequence of p22<sup>phox</sup> common to both murine and human. RT-PCR was repeated with primers for HPRT as a control for RNA quality and equal loading.

**Measurement of NADPH Oxidase Activity—Superoxide dismutase-inhibitable NADPH oxidase activity** was measured in transgenic cell lines following stimulation with arachidonic acid as described previously (26). The assays were performed at 37 °C using a Thermomax microplate reader (Molecular Devices, Inc., Sunnyvale, CA) to measure the reduction of cytochrome c resulting from the production of O₂⁻. An extinction co-efficient of 21.1 mM⁻¹ cm⁻¹ for the reduced form of cytochrome c was used to calculate the amount of O₂⁻ generated by the transgenic cells. The data were analyzed using SOFTMAX version 2.02, and the V<sub>max</sub> was calculated over a 6-min interval. Statistical analyses were performed using GraphPad Instat, version 2.0 (GraphPad Software Inc., San Diego, CA). Alternatively, qualitative assessment of NADPH oxidase activity in single cells was performed using the nitro blue tetrazolium (INT) violet diaphorase assay as described (27). For the cell-free analysis of NADPH oxidase activity, the superoxide dismutase-inhibitable cytochrome c reduction was measured in triplicate in 96-well microtiter plates as described previously (28). Neutrophil cytosol was employed as a source of cytosolic oxidase proteins in combination with membranes prepared from transgenic Cos7 cell lines as described previously (29) or partially purified and relipidated flavocytochrome b<sub>558</sub> from CHO cell lines, as described previously (22, 30). Protein concentration was determined with BCA reagents (Pierce) according to the manufacturer’s instructions.

**Analysis of Heme Spectrum**—The membranes were harvested, and flavocytochrome b<sub>558</sub> was partially purified and relipidated as described previously (22, 30). Dithionite-reduced minus oxidized difference spectroscopy was performed using a PerkinElmer Lambda 18 spectrophotometer. Flavocytochrome b<sub>558</sub> heme content was estimated using an extinction co-efficient of 21.6 mM⁻¹ cm⁻¹ at 559 nm, as described previously (31).

**RESULTS**

**Arginine, but Not Other Amino Acid Substitutions of p22<sup>phox</sup> His<sup>94</sup>, Affects the Stable Expression of Flavocytochrome b<sub>558</sub> in Cos7 Cells.** To investigate the role of p22<sup>phox</sup> His<sup>94</sup> in flavocytochrome b<sub>558</sub> function, we first utilized a p22<sup>phox</sup>-deficient Cos7 cell line that was identified in a previous study (22) to express engineered mutant derivatives of p22<sup>phox</sup> in a null background. We used site-directed mutagenesis to introduce point substitutions into p22<sup>phox</sup> cDNA at codon 94, the only invariant histidine residue within p22<sup>phox</sup>. His<sup>94</sup> was mutated...
Mutagenesis of \(p22^{\text{phox}}\) His\(^{94}\)

TABLE I

| Sample          | Heme* | INT Rate | Electron turnover* | Rate | Electron turnover* |
|-----------------|-------|----------|--------------------|------|--------------------|
| CHO             | <5    | <2       |                    | <4   |                    |
| CHO 22          | <5    | <2       |                    | <4   |                    |
| CHO 91.22       | 165 ± 10 | 251 ± 20 | 25.3 ± 2          | 728 ± 30 | 73.5 ± 3          |
| CHO 91.22 H94L  | 100.5 ± 3 | 136 ± 18 | 22.6 ± 3          | 359 ± 48 | 59.5 ± 8          |

Typical values for PMN flavocytochrome \(b_{558}\) are as follows: heme, \(~400 \text{ pmol/mg}\); INT, 20–30 e\(^{-}/\text{heme/s}\); \(O_2\), 60–120 e\(^{-}/\text{heme/s}\).

* The values are the means ± S.D. of two independent experiments, each performed in triplicate.

† The activity is normalized for the flavocytochrome \(b_{558}\) content.

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We examined the expression of recombinant \(p22^{\text{phox}}\) in stable transfections of wild type or mutant \(p22^{\text{phox}}\) cDNA constructs into the Cos7 cell line in the absence of gp91\(^{phox}\). We had previously observed that the individual flavocytochrome subunits can be stably expressed in the absence of the other in this heterologous cell line (22), in contrast to phagocytes. Protein levels of the \(p22^{\text{phox}}\) mutants H94L and H94Y were similar to wild type \(p22^{\text{phox}}\) as determined by Western blot analysis (Fig. 1A), whereas levels of the \(p22^{\text{phox}}\) mutant H94M were moderately decreased as compared with wild type \(p22^{\text{phox}}\) (Fig. 1A). However, substitution of the His\(^{94}\) with arginine resulted in an instability and, secondarily, an instability of the gp91\(^{phox}\) heterodimer. However, an arginine substitution at position 94 does not appear to affect maturation of gp91\(^{phox}\) from its 65-kDa precursor to its fully glycosylated \(~91\text{-kDa}\) form (33). As in the \(p22^{\text{phox}}\)-transfected Cos7 cells, Western blot analysis showed that protein levels of the \(p22^{\text{phox}}\) mutants H94Y (Fig. 1B), H94L, or H94M (data not shown) were similar to wild type \(p22^{\text{phox}}\) when expressed in Cos 91 cells, but the H94R substitution dramatically decreased the level of \(p22^{\text{phox}}\) protein expression (Fig. 1B). Furthermore, in contrast to Cos 91 cells expressing \(p22^{\text{phox}}\) in Cos 91 cells and CHO 91.22 H94L failed to increase maturation of transgenic gp91\(^{phox}\) from its 65-kDa precursor to its fully glycosylated 91-kDa form, as shown by Western blot (Fig. 1B). This carbohydrate maturation step occurs in the endoplasmic reticulum and Golgi compartments of granulocytes after association of \(p22^{\text{phox}}\) with the 65-kDa high mannose precursor of gp91\(^{phox}\) (33). Taken together, these data suggest that a histidine at position 94 does not appear to be required for \(p22^{\text{phox}}\) to interact with gp91\(^{phox}\). However, an arginine substitution at position 94 results in intrinsic \(p22^{\text{phox}}\) instability and, secondarily, an instability of gp91\(^{phox}\).

Identification of Other \(p22^{\text{phox}}\)-deficient Cell Lines—To confirm that substitution of \(p22^{\text{phox}}\) His\(^{94}\) does not necessarily affect the stable expression of flavocytochrome \(b_{558}\), we identified other nonphagocytic cell lines that were devoid of \(p22^{\text{phox}}\) expression. Northern blot analyses on NIH3T3 mouse fibroblasts and CHO cell lines established the absence of \(p22^{\text{phox}}\) mRNA (Fig. 2A). Confirmation of the absence of \(p22^{\text{phox}}\) mRNA in these two cell lines was obtained by RT-PCR using murine-specific \(p22^{\text{phox}}\) primers (Fig. 2B) and \(p22^{\text{phox}}\)-specific primers common for both human and murine \(p22^{\text{phox}}\) (data not shown) because hamster-specific \(p22^{\text{phox}}\) primers are unavailable. Equal loading and integrity of RNA samples was confirmed by RT-PCR using HPRT primers (Fig. 2B).

Transgenic wild type \(p22^{\text{phox}}\) or H94L \(p22^{\text{phox}}\) constructs were stably co-expressed with transgenic wild type gp91\(^{phox}\) in 3T3 cells and CHO cells. As in the Cos7 cell derivatives, expression levels of \(p22^{\text{phox}}\) H94L were similar to wild type \(p22^{\text{phox}}\) when expressed in 3T3 91 cells or CHO 91 cells (Fig. 3A) as determined by Western blot analysis. Likewise, expression of either H94L \(p22^{\text{phox}}\) or wild type \(p22^{\text{phox}}\) promoted maturation of transgenic gp91\(^{phox}\) from its 65-kDa precursor to its fully glycosylated 91-kDa form in both 3T3 91 and CHO 91 cells (Fig. 3A). There was a modest reduction in the amount of mature gp91\(^{phox}\) in CHO 91.22 H94L cells as compared with CHO 91.22 cells (Fig. 3A), suggesting that His\(^{94}\) may have a subtle influence on assembly of flavocytochrome \(b_{558}\) heterodimer in this cell background. However, the \(p22^{\text{phox}}\) H94L mutation does not appear to affect flavocytochrome \(b_{558}\) expression in 3T3 cells (Fig. 3A) or Cos7 cells (data not shown). We also expressed H94R \(p22^{\text{phox}}\) in the CHO cell derivatives, both in the presence and the absence of the gp91\(^{phox}\) subunit. As in the Cos7 cells, H94R \(p22^{\text{phox}}\) was expressed at a markedly decreased level in both CHO cells.

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![Superoxide Production](image-url)
and CHO 91 cells (Fig. 3B), supporting the hypothesis that arginine substitution leads to intrinsic p22\textsuperscript{phox} instability.

Effect of Amino Acid Substitution of p22\textsuperscript{phox} His\textsuperscript{94} on Flavocytochrome b\textsubscript{558} Heme Spectrum—To determine whether the heme spectrum of recombinant flavocytochrome b\textsubscript{558} was altered by the H94L substitution in p22\textsuperscript{phox}, we analyzed the oxidation-reduction difference spectra for partially purified flavocytochrome b\textsubscript{558} derived from transgenic CHO cell lines. The membranes from CHO cells expressing wild type p22\textsuperscript{phox} and wild type gp91\textsuperscript{phox} exhibited a characteristic flavocytochrome b\textsubscript{558} spectrum with peaks at 426 and 558 nm (Fig. 3C). The heme spectrum of the partially purified gp91\textsuperscript{phox}/p22\textsuperscript{phox} H94L heterodimer was qualitatively similar to that of the wild type flavocytochrome b\textsubscript{558}, although the total heme content was modestly decreased (Fig. 3C and Table I), consistent with the decrease level of gp91\textsuperscript{phox} protein expression seen on the immunoblot (Fig. 3A).

Expression of Mutant Transgenic p22\textsuperscript{phox} Does Not Affect Superoxide Production in Heterologous Cells—A cell-free NADPH oxidase assay was performed to measure superoxide-generating activity in membranes prepared from transgenic flavocytochrome b\textsubscript{558}-expressing Cos7 cells when combined with neutrophil cytosol. Although, as previously shown, membranes from Cos 91 cells did not support \(O\textsubscript{2}\) production (22) (Fig. 4), co-expression of H94L p22\textsuperscript{phox} or H94Y p22\textsuperscript{phox} with gp91\textsuperscript{phox} supported NADPH oxidase activity at a level similar to that of membranes derived from cells expressing wild p22\textsuperscript{phox} and gp91\textsuperscript{phox} (Fig. 4). These results indicate that a histidine at position 94 in p22\textsuperscript{phox} is not required for NADPH oxidase activity in this system. Not surprisingly, membranes from cells expressing H94R p22\textsuperscript{phox} did not support \(O\textsubscript{2}\) production, consistent with the effect of this mutation on p22\textsuperscript{phox} polypeptide stability (Fig. 1) and, secondarily, gp91\textsuperscript{phox} expression (Fig. 1B). We also examined diaphorase and NADPH oxidase activity using neutrophil cytosol and purified flavocytochrome b\textsubscript{558} isolated from CHO cells expressing either wild type gp91\textsuperscript{phox}/p22\textsuperscript{phox} or gp91\textsuperscript{phox}/p22\textsuperscript{H94L}. The rate of transfer of electrons from NADPH to FAD, as determined by the INT diaphorase assay, and the formation of \(O\textsubscript{2}\) by purified flavocytochrome b\textsubscript{558} from both CHO 91.22 and CHO 91.22 H94L membranes was similar to that seen previously with purified neutrophil flavocytochrome b\textsubscript{558}. Diaphorase activity, relative to heme content, was unaffected by the H94L p22\textsuperscript{phox} mutation (Table I), as was \(O\textsubscript{2}\) production (Table I). The rate per milligram of protein was decreased, consistent with the lower level of gp91\textsuperscript{phox} protein expression in CHO91.22 H94L cells (Fig. 3A). Thus, His\textsuperscript{94} p22\textsuperscript{phox} does not appear to be directly involved in electron transport by flavocytochrome b\textsubscript{558} in the NADPH oxidase.

We also analyzed whole cell NADPH oxidase activity in CHO cells expressing recombinant flavocytochrome b\textsubscript{558}. We previously established that high level NADPH oxidase activity can be elicited by either arachidonic acid or phorbol myristate acetate in Cos7 cells expressing transgenic gp91\textsuperscript{phox}, p22\textsuperscript{phox}, p47\textsuperscript{phox}, and p67\textsuperscript{phox} (26). CHO derivatives that stably expressed transgenic wild type gp91\textsuperscript{phox} along with either transgenic wild type or H94L p22\textsuperscript{phox} were transiently transfected with vectors for expression of wild type p47\textsuperscript{phox} and wild type...
Mutagenesis of \( p_{22}^{\text{phox}} \) \( \text{His}^{94} \)

30373

**Discussion**

The phagocyte NADPH oxidase plays a critical role in the innate immune response by producing \( O_2^- \), which is then converted to additional reactive oxygen species important for microbial killing. Flavocytochrome \( b_{558} \), a heterodimer comprised of two oxidase subunits, \( p_{22}^{\text{phox}} \) and \( p_{91}^{\text{phox}} \), serves as the redox center of the oxidase. The \( gp_{91}^{\text{phox}} \) subunit contains regions that are homologous to the flavin- and NADPH-binding domains of ferredoxin NADP+ reductase (34). It also contains two nonidentical heme groups with low midpoint redox potentials of \( \text{Em}_7 = -265 \text{ mV} \) and \( \text{Em}_7 = -225 \text{ mV} \), respectively (11). Raman and electron paramagnetic resonance spectroscopy predicts that the hemes reside within the membrane in a hexacoordinated state with axial imidazole or imidazolate ligands supplied by histidine residues (35–37). Currently, there are conflicting views about the distribution and the exact location and coordination of the hemes within the flavocytochrome. Quinn et al. (12) suggested that \( gp_{91}^{\text{phox}} \) and \( p_{22}^{\text{phox}} \) each bind heme and proposed that one heme binds exclusively to \( gp_{91}^{\text{phox}} \), whereas the other is shared between \( gp_{91}^{\text{phox}} \) and \( p_{22}^{\text{phox}} \). However, more recent evidence indicates that both hemes reside within \( gp_{91}^{\text{phox}} \) (14, 22). The \( p_{22}^{\text{phox}} \) subunit of flavocytochrome \( b_{558} \) has only a single invariant histidine at codon 94. Herein we provide evidence suggesting that although mutations in \( \text{His}^{94} \) of \( p_{22}^{\text{phox}} \) can affect stability of the \( p_{22}^{\text{phox}} \) polypeptide, a histidine residue is not required for flavocytochrome \( b_{558} \) heme binding or electron transfer.

We have previously observed that inhibition of heme biosynthesis with succinyl acetone results in markedly decreased expression of \( p_{22}^{\text{phox}} \) and the mature 91-kDa form of \( gp_{91}^{\text{phox}} \), but not its 65-kDa high mannose precursor, suggesting that heme incorporation is required for heterodimer formation (6, 7, 33). It is therefore likely that heme insertion may impart a structural constraint to \( gp_{91}^{\text{phox}} \) necessary for it to bind \( p_{22}^{\text{phox}} \). It has also been proposed that \( gp_{91}^{\text{phox}} \) and \( p_{22}^{\text{phox}} \) are linked directly through heme (12). We have also demonstrated that point mutation of any one of four histidine residues at positions 101, 115, 209, or 222 in \( gp_{91}^{\text{phox}} \) disrupts heterodimer formation, maturation of \( gp_{91}^{\text{phox}} \), and NADPH oxidase activity. These results strongly suggest that these four histidines are the heme-binding ligands in flavocytochrome \( b_{558} \) and that disruption of any individual heme ligand interferes with normal flavocytochrome \( b_{558} \) maturation and oxidase function. In the current study, we provide evidence that point substitution of \( \text{His}^{94} \) with leucine, methionine, or tyrosine does not affect expression of transgenic \( p_{22}^{\text{phox}} \) and promotes maturation of \( gp_{91}^{\text{phox}} \) in Cos7, 3T3, and CHO cells, which do not express endogenous \( p_{22}^{\text{phox}} \) and \( p_{22}^{\text{phox}} \) is qualitatively similar to that of the wild type flavocytochrome \( b_{558} \), thus providing direct evidence that \( p_{22}^{\text{phox}} \) \( \text{His}^{94} \) is not involved in heme binding.

NADPH oxidase-catalyzed \( O_2^- \) production involves the transfer of two electrons from NADPH to FAD, which are then transferred as single electrons to \( O_2^- \) via two nonidentical bis-histidine-ligated heme groups (11). If the binding sites for any of the redox components are disrupted, \( O_2^- \) cannot be produced. Recombinant wild type or \( H_{94L} \) \( p_{22}^{\text{phox}} \) mutant flavocytochrome \( b_{558} \) both supported INT diaphorase reduction and \( O_2^- \) production under cell-free conditions in the presence of \( gp_{91}^{\text{phox}} \), \( p_{47}^{\text{phox}} \), and \( p_{67}^{\text{phox}} \) (data not shown). Thus, the invariant histidine residue at position 94 of \( p_{22}^{\text{phox}} \) is not required for \( O_2^- \) production in whole cells, consistent with the results from cell-free assays.

![Hydropathy analysis of the \( p_{22}^{\text{phox}} \) subunit of flavocytochrome \( b_{558} \)](image)

**Fig. 6.** Hydropathy analysis of the \( p_{22}^{\text{phox}} \) subunit of flavocytochrome \( b_{558} \). **A**, Kite-Doolittle plot of the \( p_{22}^{\text{phox}} \) polypeptide calculated using a window of 19 amino acids. The \( p_{22}^{\text{phox}} \) polypeptide contains three hydrophobic regions that are predicted to be transmembrane \( \alpha \) helices, indicated by boxes (for algorithms, see genome.cbs.dtu.dk). **B**, the hydropathy profiles of segments of \( p_{22}^{\text{phox}} \) from residues 91–97 with amino acid substitutions for the histidine residue at position 94, as indicated. A three-point weighted moving average was used for determination of local hydrophobic character.

\( p_{67}^{\text{phox}} \). After 18 h, the cells were harvested, and NADPH oxidase protein expression was verified by Western blotting (Fig. 5A). Arachidonic acid-elicited \( O_2^- \) production was measured by the cytochrome \( c \) reduction assay (Fig. 5B), and phorbol myristate acetate-elicited \( O_2^- \) production was assessed in the nitro blue tetrazolium assay (data not shown). As shown in Fig. 5B, CHO 91.22 H94L produced \( O_2^- \) at a rate and level that was indistinguishable from CHO 91.22 following transient transfection of \( p_{47}^{\text{phox}} \) and \( p_{67}^{\text{phox}} \). No \( O_2^- \) production was detected in wild type CHO cells transfected with \( p_{47}^{\text{phox}} \) and \( p_{67}^{\text{phox}} \) (Fig. 5B). The nitro blue tetrazolium assay confirmed that H94L \( p_{22}^{\text{phox}} \) supported whole cell NADPH oxidase activity in the presence of \( gp_{91}^{\text{phox}} \), \( p_{47}^{\text{phox}} \), and \( p_{67}^{\text{phox}} \) (data not shown). Thus, the invariant histidine residue at position 94 of \( p_{22}^{\text{phox}} \) is not required for \( O_2^- \) production in whole cells, consistent with the results from cell-free assays.
data in intact cells confirm that the histidine residue located at position His\(^{94}\) of p22\(^{phox}\) is not required for flavocytochrome \(b_{558}\) expression, heme binding, or NADPH oxidase function; we observed that substitution of His\(^{94}\) p22\(^{phox}\) with an arginine residue resulted in substantially decreased expression of recombinant p22\(^{phox}\) in either Cos7 or CHO cells. Expression of H94R p22\(^{phox}\), which mimics a mutation previously identified in a CGD patient, was also associated with decreased expression of mature gp91\(^{phox}\), and \(O_{2}^{\ast}\) production was virtually undetectable when transgenic Cos 91.22 H94R membranes were used in a cell-free assay of NADPH oxidase activity. Taken together, we infer that arginine substitution results in intrinsic p22\(^{phox}\) instability and, secondarily, instability of gp91\(^{phox}\) when expressed in human phagocytes, which leads to deficient NADPH oxidase activity and thus CGD.

The p22\(^{phox}\) polypeptide contains three hydrophobic regions that are predicted to be transmembrane \(\alpha\) helices (Fig. 6A; for algorithms, see genome.cbs.dtu.dk). The histidine residue at position 94 is adjacent to a predicted transmembrane helix that spans residues 95–117. The hydropathy profile of the His\(^{94}\)-containing segment of p22\(^{phox}\) from residues 91–97 (Fig. 6B) demonstrates that leucine and tyrosine have higher hydropathy indices than histidine; hence, substitution of His\(^{94}\) with either leucine or tyrosine may have less potential to disrupt the stability and function of the p22\(^{phox}\) polypeptide. However, when an arginine residue, which has a lower hydropathy index than histidine, is substituted for His\(^{94}\) adjacent to the predicted transmembrane \(\alpha\) helix, this region of p22\(^{phox}\) is now predicted to be hydrophilic. An arginine substitution may therefore produce more substantial conformational changes in p22\(^{phox}\), which could result in misfolding and subsequent instability of p22\(^{phox}\), leading to absent flavocytochrome \(b_{558}\) expression and CGD.

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