Phage Display Epitope Mapping of Human Neutrophil Flavocytochrome \( b_{558} \)

IDENTIFICATION OF TWO JUXTAPOSED EXTRACELLULAR DOMAINS*

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Despite extensive experimental and clinical evidence demonstrating the critical role of flavocytochrome \( b_{558} \) (Cyt \( b \)) in the NADPH-dependent oxidase, there is a paucity of direct structural data defining its topology in the phagocyte membrane. Unlike other Cyt \( b \)-specific monoclonal antibodies, 7D5 binds exclusively to an extracellular domain, and identification of its epitope should provide novel insight into the membrane topology of Cyt \( b \). To that end, we examined biochemical features of 7D5-Cyt \( b \) binding and used the J404 phage display nonapeptide library to identify the bound epitope. 7D5 precipitated only heterodimeric gp91-p22\(^{phox}\) and not individual or denatured Cyt \( b \) subunits from detergent extracts of human neutrophils and promyelocytic leukemia cells (gp91-PLB). Moreover, 7D5 precipitated precursor gp65-p22\(^{phox}\) complexes from detergent extracts of the biosynthetically active gp91-PLB cells, demonstrating that complex carbohydrates were not required for epitope recognition. Epitope mimetics selected from the J404 phage display library by 7D5 demonstrated that \( ^{228} \text{RIVRG}^{230} \) and \( ^{160} \text{IKNP}^{163} \) regions of gp91\(^{phox}\) were both bound by 7D5. These studies reveal specific information about Cyt \( b \) membrane topology and structure, namely that gp91\(^{phox}\) residues \( ^{228} \text{RIVRG}^{230} \) and \( ^{160} \text{IKNP}^{163} \) are closely juxtaposed on extracytoplasmic domains and that predicted helices containing residues Gly\(^{146}-\text{Ile}^{149}\) and Ser\(^{200}-\text{Glu}^{205}\) are adjacent to each other in the membrane.

The phagocyte NADPH oxidase is a plasma membrane redox system that produces superoxide anion \( (O_2^-) \), an essential precursor for other reactive oxygen metabolites critical for oxygen-dependent microbicidal activity (1–3). A genetic lesion affecting one or more of the four oxidase components, gp91\(^{phox}\), p22\(^{phox}\), p47\(^{phox}\), or p67\(^{phox}\), results in defective oxidase activity and the inability of phagocytes to kill pathogenic microorganisms, a disorder clinically recognized as chronic granulomatous disease (CGD)\(^1\) (2, 4–9). Human neutrophil flavocytochrome \( b_{558} \) (Cyt \( b \)) is a heme-containing, heterodimeric integral membrane protein composed of subunits gp91\(^{phox}\) and p22\(^{phox}\) (10). Cyt \( b \) is the electron transferase of the NADPH oxidase, relaying electrons from bound NADPH within the cell to an oxygen acceptor region of Cyt \( b \) on the exterior aspect of the cell membrane where \( O_2 \) is formed. In this functional capacity, Cyt \( b \) has been established as an essential component of the respiratory burst oxidase, although little published experimental data describe its topology in the membrane.

Determination of structural and functional aspects of epitopes bound by specific antibodies can provide information about the protein against which the antibody is directed (11). Antipeptide and antisubunit polyclonal antibodies against regions of Cyt \( b \) have been used to locate the corresponding epitopes (12, 13) and information derived from identification of epitope mimetics has led to the description of anti-Cyt \( b \) “antibody imprints” (14). We continue to elucidate the structure of Cyt \( b \) domains recognized by monoclonal antibodies to better define its transmembrane topology and gain insight into its functional organization.

Reported epitope mapping data for monoclonal antibodies (mAbs) specific for Cyt \( b \) indicate that they bind cystolic aspects of the protein (15–19). However, it has been reported that mAb 7D5 (19) binds an extracellular Cyt \( b \) epitope on intact neutrophils derived from normal but not CGD patients that lack Cyt \( b \) (20). Thus, 7D5 has proven useful in the determination of Cyt \( b \) up-regulation as an indicator of neutrophil activation and granule exocytosis (21) and for the identification of individuals deficient in Cyt \( b \) (20, 22, 23). However, neither the subunit location nor chemical nature of the 7D5 epitope has been elucidated.

In our current studies, we have used phage display and immunological analyses to identify the 7D5 epitope on Cyt \( b \). Although we confirmed the inability of 7D5 to recognize Cyt \( b \) on immunoblots, we found that 7D5 immunoprecipitated detergent-solubilized Cyt \( b \) heterodimer containing its fully processed 91kDa form of gp91\(^{phox}\) and its 65-kDa precursor. Additionally, it precipitated the deglycosylated gp91\(^{phox}\) core protein, suggesting that neither the mature nor high man-
Identification of Two Extracellular Domains of Cytochrome b

The presence of carbohydrate contributes significantly to the epitope. Furthermore, our results indicate that the 226RIVRG230 and 166IKTP163 segments of gp91phox form the 7D5 epitope and therefore must be exposed on the cell surface. These sequences of gp91phox were not bound by 7D5 in the absence of p22phox nor under conditions that disrupted the heterodimer, suggesting that, although 7D5 binding is confined to nonlinear but contiguous regions of gp91phox, it depends on associated p22phox for its conformational integrity. In combination, these data provide direct evidence for the identity of two adjacent transmembrane helices in gp91phox.

EXPERIMENTAL PROCEDURES

Chemicals, Reagents, and Materials—Prestained protein molecular weight standards were purchased from Life Technologies, Inc. Reagents for buffers and bacteriological media and Nunc Maxisorb flat bottom plates for ELISA were purchased from Fisher. Cyagen bromide-activated Sepharose CL-4B and GammaBind Sepharose were purchased from Amersham Pharmacia Biotech. Sequencing data were obtained using a Sequenase version 2.0 sequencing kit purchased from U.S. Biochemical Corp. Unless specified, all other reagents were purchased from Sigma.

Neutrophil Isolation and Flow Cytometry—Heparinized, venous blood was obtained from healthy individuals (or from patients with CGD) in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa, and neutrophils were isolated as described previously using Hypaque-Ficoll gradients after dextran sedimentation (24). Genetic analyses of the individuals with either X-linked deficiency of gp91phox or autosomal deficiency of p22phox were performed by Paul G. Heyworth (The Scripps Research Institute, La Jolla, CA) and John T. Curtinette (Genentech, Inc.). The individual with autosomal deficiency of p22phox (A22+) had a nucleotide replacement at position C304 (C304 → A), which resulted in the replacement of Ser118 with Arg. The individual with deficiency of gp91phox (X91+) had a frameshift in exon 11, resulting in the replacement of nine nucleotides with eight. Surface-expressed Cyt b was detected using 7D5 by flow cytometry as described previously (21). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) at the University of Iowa Core Flow Cytometry facility. For most experiments, unfixed neutrophils were used, and a single live gate eliminated debris and contaminating cells. However, when patient cells were shipped overnight, it was sometimes necessary to use propidium iodide staining in combination with an additional gate to further exclude dead cells from the analysis.

Cyt b ELISA—35 μl of relax buffer (10 mM Hepes, 100 mM KCl, 10 mM NaCl, pH 7.4) containing 8 pmol of heparin-purified Cyt b (determined by spectral absorbance at 414 nm, extension coefficient = 21.6 mM−1 cm−1) (25), in 2% octyl glucoside was used to coat each well of a 96-well Corning Maxisorb ELISA plate overnight at 4 °C. Rinsing and blocking were performed as described (14), except a different blocking agent was used, 2% blocking agent made 1% SDS, heated to 100 °C for 4–5 min, and then iced immediately for 5 min. Lysate was then diluted to 1.2 ml with dilution buffer so that SDS concentration was 0.1%, and precipitation was performed as described for nondenaturing immunoprecipitations (above). Alternatively, Cyt b was precipitated from solubilized gp91-PLB cells, a described previously human promyelocytic leukemia cell line constitutively expressing gp91phox (27), using 7D5, gp91, and p22. Cyt b was precipitated from gp91-PLB A or B human promyelocytic leukemia cell line with the CYBB gene deleted (XC GD-PLB) as reported earlier (26), and denaturing conditions were as described above. Following 5–20% SDS-PAGE, immune complexes were immunoblotted using a combination of gp91 and p22. Immunoblots were developed using an enhanced chemiluminescence detection system (SuperSignal Substrate; Pierce) according to the manufacturer’s instructions.

Antibody and Phage Display Epitope Mapping—The determination of monoclonal antibody 7D5 has been described previously (19), and the production of the J404 nonapeptide phage display library was reported (28). 7D5 was purified from spent RPMI 1640 media of a hybridoma cell line using GammaBind Sepharose (Amersham Pharmacia Biotech) according to the manufacturer’s instructions, and purity was assessed by SDS-PAGE. Mapping of 7D5 with the phage display library and plaque lift analyses were carried out as described (16), except the affinity-selected clones were amplified following each round of selection by replicating as plaques on a lawn of K91 cells (instead of as K91 colonies on LB agar containing 75 μg/ml kanamycin).

Immunoblots of Phage-displayed Sequences—5 × 1010 plaque-forming units of phage produced as described above were disrupted with SDS loading buffer at 100 °C for 5 min and loaded onto a 5–20% SDS-PAGE gel (29) to separate capsid proteins. Following transfer to nitrocellulose, the immunoblot was probed with 5 μg/ml 7D5 and detected by goat anti-mouse alkaline phosphatase-conjugated secondary (Bio-Rad) in combination with chromagen reagent (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) as described (18).

RESULTS

Specificity of mAb 7D5—7D5 has been previously reported to recognize Cyt b expressed at the plasma membrane of neutrophils but not on cells from X-linked CGD individuals (19, 30). The absence of either gp91phox or p22phox in X-linked autosomal recessive CGD, respectively, results in the absence of both proteins on the neutrophil membrane (5, 31). Thus, to rule out the possibility that 7D5 binding is dependent on transcriptional regulation of only gp91phox and not p22phox, we compared its binding to cells derived from homozygous and heterozygous individuals with autosomal and X-linked inheritance of the disease. Intact neutrophils derived from individuals deficient in gp91phox (X91+) or p22phox (A22+) were probed for surface expression of Cyt b using 7D5 in flow cytometry (Fig. 1, A and B). In contrast to neutrophils from normal individuals, 7D5 did not stain the cell surface of neutrophils deficient in either p22phox or gp91phox (Fig. 1, A and B). Neutrophils from the mother and female siblings of an individual with X-linked CGD displayed bimodal fluorescence, demonstrating that they have both normal and Cyt b-deficient neutrophils. The identification of heterogeneous Cyt b expression in their neutrophils indicates that they possess one copy of a mutant CYBB allele and therefore are carriers for X-linked CGD (Fig. 1B). Approximately 87% of the combination was from the normal Cyt b gene, and 13% of the daughter shown in Fig. 1B exhibited normal nitrogen blue tetrazolium to formazan, indicating that those cells possessed normal O2−-generating capacity. A second daughter also had similar lysonization with 74% nitrogen blue tetrazolium-positive neutrophils (not shown). That three females within the same family would have similar degrees of lysonization may reflect familial nonrandom X-chromosome inactivation, which has been identified in other X-linked disorders (32–37). The percentage of their neutrophils with normal O2−-producing capability corre-
7D5, the use of 7D5 with nondenaturing conditions for immunoprecipitation of Cyt b using 7D5. Using nondenaturing conditions, we compared into the structural nature of the 7D5 epitope, we compared Cyt b-deficient individuals or those who are carriers for X-linked CGD.

To demonstrate further the specificity of immunoreactivity of 7D5 to Cyt b, human neutrophil Cyt b was purified from cell membranes as described (38) and bound to 96-well plates. Probing the immobilized Cyt b with 7D5, anti-p22phox mAb 44.1 (ap22) (14), anti-gp91phox mAb 54.1 (apgp91) (14), and an irrelevant mAb suggested that the epitope bound by 7D5 was intact on the detergent-solubilized protein (Fig. 2). Although the activity of 7D5 in the ELISA was less than that of ap22 or apgp91, all three mAbs specifically recognized Cyt b. It is possible that the binding of mAb 7D5 to Cyt b requires a more native or membrane-resident conformation of the protein than does either ap22 or apgp91, although we were unable to test this directly using our ELISA.

7D5 Recognizes Native Cyt b Heterodimers—To gain insight into the structural nature of the 7D5 epitope, we compared denaturing with denaturing conditions for immunoprecipitation of Cyt b using 7D5. Using nondenaturing conditions, 7D5, apgp91, and ap22 precipitated both gp91phox and p22phox from human neutrophil detergent lysates (Fig. 3A). When the lysates were denatured with heat and 1% SDS, apgp91 and ap22, binding linear regions of the protein, precipitated their respective subunits alone. However, 7D5 precipitated neither subunit after denaturation (Fig. 3A).

To determine whether 7D5 bound complex carbohydrates on gp91phox, we examined the ability of 7D5 to precipitate Cyt b using biosynthetically active gp91-PLB cells as a source of both precursor and mature Cyt b (Fig. 3B). Using non-denaturing conditions, 7D5 precipitated gp91phox, p22phox, and, a small amount of gp65, the gp91phox precursor that has exclusively high mannose oligosaccharides (Fig. 3B). Since 7D5 precipitated gp65, the complex carbohydrates of gp91phox were not required for 7D5 binding to Cyt b. These findings were confirmed by the ability of 7D5 to precipitate the heterodimeric complex consisting of 55–58-kDa core gp91phox protein-p22phox synthesized in the presence of tunicamycin2 and also bind heterodimer following digestion with PNGase F (data not shown). Consistent with the neutrophil experiments, when gp91-PLB lysates were denatured with 1% SDS and heat, 7D5 precipitated neither mature subunit nor gp65. In contrast, apgp91 and ap22 precipitated their respective subunits following denaturation (Fig. 3B). These findings suggest that 7D5 recognized the Cyt b peptide backbone in its native form only but do not elucidate whether the antibody bound to an epitope shared by both subunits or if it associated with individual gp91phox or p22phox subunits in their native conformation.

Our previous studies on the biosynthesis of Cyt b demonstrated that pools of uncomplexed gp65 and p22phox accumulate for a limited time following synthesis in gp91-PLB cells (26). Therefore, these cells provide a source of native, monomeric Cyt b subunits from which to test whether 7D5 recognizes

![Log Fluorescence](image1.png)

**Fig. 1.** Identification of female carriers of gp91phox deficiency or individuals deficient in gp91phox or p22phox by flow cytometry. A, neutrophils (10^6) from healthy subjects (father, mother, and normal daughter as indicated) or from an individual with autosomal deficiency of p22phox (A22p) were probed with 7D5 and analyzed by flow cytometry as described under “Experimental Procedures.” B, a similar analysis was performed on neutrophils from an individual with X-linked CGD (X91) or from the mother and female sibling of an individual with X-linked CGD (mother and daughter carriers as indicated) and were compared with a healthy individual (normal as indicated). Dashed histograms represent staining with IgG1, an isotype control antibody.

![Monoclonal Antibody](image2.png)

**Fig. 2.** Analysis of 7D5-Cyt b interaction by ELISA. One µg of heparin-purified human neutrophil Cyt b was used to coat wells in an ELISA plate so that binding by each of three anti-Cyt b mAbs could be determined. apgp91 and ap22 were previously shown to be Cyt b-specific (14) and were used as positive controls, and the anti-rhodopsin mAb K42.41 (60) served as a negative control. The results indicate specific binding of 7D5 to the detergent-solubilized form of the protein. These data are typical of five separate analyses.
individual gp65 or p22phox subunits. Sequential immunoprecipitations were performed to determine whether 7D5 was capable of depleting lysates of Cyt b heterodimer and/or individual subunits (Fig. 4A). Although 7D5 completely removed gp91-p22phox heterodimer from lysates after three rounds of immunoprecipitation, monomeric gp65 could be subsequently precipitated from those lysates using mAb 7D5 (Fig. 4A, arrowheads). Since neither gp91phox nor p22phox coprecipitated with gp65 using mAb 7D5 following three rounds of precipitation with 7D5, gp65 was precipitated free of associated p22phox. The finding that gp65 but neither gp91phox nor p22phox coprecipitated with 7D5 following the immunodepletions with 7D5 suggests that heterodimeric gp91phox-p22phox complexes were completely removed by 7D5 (Fig. 4A). The inability to precipitate and detect by immunoblotting monomeric p22phox following the three immunodepletions with 7D5 or those with 7D5 followed by a single depletion with 7D5 may be due to the rapid degradation of uncomplexed p22phox or its rapid processing to heterodimeric form (26) (Fig. 4A). It is likely that the amount of newly synthesized, monomeric p22phox present in the depleted lysates may have been below the limits of detection by immunoblotting. Therefore, we pulse-labeled gp91-PLB cells and subsequently chased for 2 h to allow for partial processing of gp65 to gp91phox and also to allow the formation of gp91phox-p22phox complexes (Fig. 4B). As with the unlabeled immunodepletion experiment, three rounds of precipitation of radio-labeled Cyt b using 7D5 completely removed gp91phox-p22phox complexes from lysates (Fig. 4B). In contrast, following depletion with 7D5, subsequent precipitation with either mAb 7D5 or op22 revealed that both gp65 and p22phox remained in depleted lysates free of their complementary subunit (Fig. 4B). These results suggest that 7D5 precipitated only gp91phox-p22phox or gp65-p22phox heterodimers.

Epitope Mapping Using Phage Display—To identify whether the 7D5 epitope was located on gp91phox, p22phox, or both subunits, we selected phage display library clones using a 7D5 immunoaffinity Sepharose bead matrix. Limiting dilutions were performed on each of three successive eluate samples, to determine the titer and to provide isolated plaques for plaque lift analysis. A six-log increase in the number of adherent clones was observed between the first and third round of selection, suggesting strong enrichment for peptide sequences by the antibody (data not shown). About 15% of the plaques from the second round and 95% of the plaques from the third round elution gave strong signals when probed by 7D5 in a plaque lift analysis (14), compared with an irrelevant monoclonal (data not shown). Isolated plaques were then selected for nucleotide sequence analysis as described (16) (Table I).

Immuonaffinity selection of peptides presented on phage display clones produced 29 unique amino acid sequences, many of which appeared on several different phage (Table I). The first phage sequence listed in Table I showed a five-residue match to the 226RIVRG230 segment of gp91phox, a region predicted to be extracytoplasmic by hydropathy analysis and by its proximity to the Asn240 putative glycosylation site. The recovery of this 226RIVRG230 peptide thus provided important evidence supporting the 226RIVRG230 segment of gp91phox as being part of the epitope (Table I, clone A). In addition to containing similarity to the 226RIVRG230 segment of gp91phox, several other phage sequences selected by 7D5, including YKNPWPGRGM, LKNPWQRGDL, LPNPWVGDRG, and NNPWSR6GF, suggested a match to 161KNP161 of gp91phox as well
(Table I, clones B, C, D, and F). The $^{161}$KNP$^{163}$ region of gp91$^{phox}$ lies above a predicted transmembrane region 12 residues from Asn$^{149}$, another possible glycosylation site, and 63 residues from the $^{226}$RIVRG$^{230}$ segment, just above the fifth predicted transmembrane-spanning domain (12, 39). Modest matches in several other selected clones (Table I) also reflected the $^{161}$KNP$^{163}$ segment of gp91$^{phox}$. More than 50% of the selected phage peptides contained an aliphatic or hydrophobic residue, including Leu, Ile, Val, or Tyr, which when aligned with the $^{161}$KNP$^{163}$ and $^{226}$RIVRG$^{230}$ segments of gp91$^{phox}$, corresponded to Ile$^{160}$ (Table I). Most impressively, these two regions of gp91$^{phox}$ were represented by four phage peptides with five-residue identities and additional conservatively substituted or shifted residues (clones A–D, Table I). In our previous studies, mapping antibody epitopes or protein-protein interactions, such extended matches were rare (16, 40). When phage peptides were aligned with the $^{161}$KNP$^{163}$ and $^{226}$RIVRG$^{230}$ segments, Trp was represented in the same position in greater than 91% of the total number of phage isolates (Table I). Although no residue in the identified gp91$^{phox}$ sequences fits with this selected residue, Trp$^{125}$ immediately outside of the third transmembrane region of gp91$^{phox}$ is a possible candidate, since all of these transmembrane regions are likely to be in close proximity. Trp$^{251}$ could also be represented by the phage-selected sequences, yet we were unable to block the binding of 7D5 in flow cytometry with another antibody that binds this residue (data not shown). It is also possible that Trp$^{251}$ of p22$^{phox}$ contributes the tryptophan in the epitope identified in the phage display mapping and could therefore provide some rationale for the heterodimer requirement for epitope conformation. Another possibility is that the Trp selected by the phage clones represented a hydrophobic “pocket” or “spacer,” which bridged the gap between the extracellular transmembrane loops containing $^{161}$KNP$^{163}$ and $^{226}$RIVRG$^{230}$ sequences.

Several phage sequences also contained an RGD tripeptide motif, which aligned well within the gp91$^{phox}$ residues $^{226}$RIVRGQ$^{231}$, if flexibility is provided to allow for a polar residue substitution at Gln$^{231}$. The unexpected identification of RGD in several selected sequences suggested that this surface-accessible region of Cyt b might interact with integrins in an RGD-dependent manner (41), yet our attempts to confirm such an interaction were unsuccessful (data not shown).

Our mapping data indicate that the minimal epitope bound by 7D5 consists of five mapped residues in the $^{226}$RIVRG$^{230}$ segment, and four more matching the $^{160}$KNP$^{163}$ region. These two regions are likely to be extracellular based on hydropathy analysis that suggests they are located immediately adjacent to the extracellular aspect of two putative membrane-spanning helices (39) (see Fig. 6 for putative epitope location). The com-
bination of these two regions constitutes a logical target for
binding by 7D5, based on our findings from the biochemical
assays, i.e. the ability of the antibody to identify an accessible
epitope on the plasma membrane of intact neutrophils
combined with its inability to bind denatured protein.

The binding of 7D5 to the selected peptides on the denatured
pIII display protein (42) from 5 × 10^13 plaque-forming units
was examined by SDS-PAGE and immunoblotting (Fig. 5, im-
munoblot). Of the eight phage representatives tested, clones M and
G, which each denatured clone was able to represent the Cyt
protein to 7D5, whereas the ELISA promotes interactions that
showed much greater interaction with 7D5 in the ELISA. The
munoblot despite its high similarity to the putative epitope,
showed much greater interaction with 7D5 in the ELISA. The
difference in immunoreactivity between the immunoblot and
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regions outside the plasma membrane. mAb 7D5 has been previously utilized in reports describing molecular and genetic analysis of CGD and the NADPH oxidase, yet the region(s) of Cyt b bound by 7D5 have remained undefined. To provide a better view of the 7D5 epitope and to gain structural information about Cyt b membrane topology, we investigated the 7D5-Cyt b interaction to identify which regions of Cyt b are recognized by the antibody.

Our analysis of 7D5 binding first established its specificity for the Cyt b protein. 7D5 bound neither to neutrophils from patients with autosomal deficiency of p22<sub>phox</sub> nor to those from patients with X-linked CGD. Flow cytometric 7D5 staining of neutrophils from female carriers of X-linked CGD was bimodal, patients with X-linked CGD. Flow cytometric 7D5 staining of neutrophils from female carriers of X-linked CGD was bimodal, patients with X-linked CGD.

Because 7D5 appeared to bind a polypeptide region on Cyt b, we used phage display epitope mapping to identify residues of Cyt b involved in 7D5 binding. The phage-displayed sequences show similarity to both 160IKNP163 and 226RIVRG230 regions of gp91<sub>phox</sub>, yet no matches to p22<sub>phox</sub> sequences could be identified. The diversity of the 29 selected sequences suggests that the 7D5 epitope involves a nonlinear or conformational epitope, consistent with the inability of 7D5 to recognize denatured protein on immunoblots. Several phage clones, A–D from Table I, gave five-residue identities to the discontinuous region spanning 160IKNP163 and 226RIVRG230 of gp91<sub>phox</sub>. Such strong similarity provides credible support to the identification of this region as the 7D5 epitope, especially because these epitope mimetics were recovered from a randomly generated peptide library (14, 16, 40). Moreover, clone A, one of the clones with five residues identical to the gp91<sub>phox</sub> sequence, interacted with 7D5 in the native ELISA better than all clones tested except one (Fig. 5). Tryptophan was also recovered in nearly every phage clone (Table I), although this residue does not appear in either of the two gp91<sub>phox</sub> regions identified. It is possible that this tryptophan represents a residue from another membrane-spanning helix of gp91<sub>phox</sub> or a hydrophobic pocket, potentially bridging 160IKNP163 and 226RIVRG230 of gp91<sub>phox</sub>. A subset of four phage clones (Z, AA, BB, and CC, each beginning with YPGW; Table I) are listed in the reverse orientation (carboxyl to amino, left to right, respectively) compared with the other clones in Table I. These clones are unique because 1) they fit...
Although it was not represented by selected sequences. Although trypsin represents polar residue conservation for the epitope in that position the sequences selected from the phage display library and thus accessing helices. Residues identified in exist immediately extracellular to the fourth and fifth membrane-spanning helices. These residues are predicted by hydropathy analysis of the protein to be at the extracellular surface. Our data not only extend those findings but demonstrate that gp91phox residues 160IKNP163 and 226RIVRG230 are juxtaposed on the extracytoplasmic face of the plasma membrane (Fig. 6), providing direct evidence for the identity and positioning of two previously predicted transmembrane regions (12, 39). Moreover, these results are compatible with the findings of Wallach et al., which demonstrated that gp91phox residues Asn132, Asn149, and Asn240 are glycosylated and, therefore, exposed on the cell surface (59).

Our mapping data suggest that the epitope bound by 7D5 was located entirely on gp91phox, but the presence of p22phox in the gp91-p22 heterodimer was required for 7D5 binding on human myeloid cells. It should also be noted that heme insertion is a prerequisite for heterodimer formation (26) that in turn is essential for recognition by 7D5. To the extent that heterodimer formation requires heme coordination by one or both subunits, the 7D5 epitope is at the least indirectly heme-sensitive. Thus, p22phox, possibly in combination with heme, appears to impart a structural constraint to gp91phox that is essential for recognition by 7D5. Further structural analysis will be necessary to elucidate the influence of p22phox on the membrane topology and processing of gp91phox and the possible role of heme in this process.

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Phage Display Epitope Mapping of Human Neutrophil Flavocytochromeb 558: IDENTIFICATION OF TWO JUXTAPOSED EXTRACELLULAR DOMAINS

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