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Genetics

Point mutation in the cytoplasmic domain of the neutrophil p22-*phox* cytochrome *b* subunit is associated with a nonfunctional NADPH oxidase and chronic granulomatous disease

(respiratory burst/superoxide/phagocyte/polymerase chain reaction)

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ABSTRACT **Chronic granulomatous disease (CGD) is a congenital disorder in which phagocytes cannot generate superoxide (O_2^-) and other microbial oxidants due to mutations in any one of four components of the O_2^--generating complex, NADPH oxidase. We report here a female CGD patient in whom a missense mutation in one of these components, the p22-*phox* subunit of the neutrophil membrane cytochrome *b* [where *phox* indicates *ph*agocyte *ox*idase (used to designate protein components of the phagocyte NADPH oxidase)] results in a nonfunctional oxidase and failure of neutrophils to produce O_2^- in response to phorbol 12-myristrate 13-acetate. Cytochrome *b* in the patient's neutrophils was normal in appearance and abundance as determined by visible spectroscopy and by immunoblots of the gp91 and p22 subunits. However, the neutrophil plasma membranes were devoid of activity in the cell-free oxidase activation system, whereas the cytosol functioned normally. We postulated that the patient was homozygous for a mutation in p22 that results in the synthesis of normal levels of a nonfunctional cytochrome *b*. A single-base substitution (C → A) was found in the patient's mononuclear cell p22-*phox* cDNA that predicts a nonconservative Pro → Gln substitution at residue 156. The same mutation was also identified in all clones sequenced from patient genomic DNA, demonstrating homozygosity for the mutant allele. An antipeptide antibody against p22 residues 153–164 was found to bind only to permeabilized neutrophils, indicating that the mutation occurs in a cytoplasmic domain. These studies establish that this domain of p22-*phox* is cytoplasmic and that mutations in this region can have profound effects on cytochrome *b* function.**

Chronic granulomatous disease (CGD) is an inherited disorder in which phagocytes cannot generate superoxide (O_2^-) and other microbial oxidants due to mutations in any one of four components of the O_2^--generating enzyme complex referred to as NADPH oxidase (1). The classic X chromosome-linked form of the disease results from defects in the gene encoding a 91-kDa membrane glycoprotein [gp91-*phox*, where *phox* indicates *ph*agocyte *ox*idase (used to designate protein components of the phagocyte NADPH oxidase)] (2–6) that is the larger subunit of a phagocyte-specific cytochrome *b* (7). This cytochrome has an unusually low midpoint potential and is believed to serve as the terminal redox carrier in the transfer of electrons from NADPH to oxygen (8). Mutations in the gene for the 22-kDa subunit of the cytochrome (p22-*phox*) account for a rare subgroup of autosomal recessive CGD (9). However, autosomal recessive CGD is more commonly due to deficiencies in one of two cytosolic oxidase components (p47-*phox*, p67-*phox*) (10–16). These cytosolic proteins, whose functions are uncertain, are required for activity of the otherwise dormant oxidase (10, 12, 17, 18) and translocate to the membrane with activation by opsonized microorganisms or other stimuli (19, 20).

The primary amino acid sequences of the cytochrome *b* subunits, as deduced from their corresponding cDNAs, have no obvious homology to known proteins, including other cytochromes (21), and their relative functions have not yet been defined. The underlying gene defects in gp91-*phox* and p22-*phox* are heterogeneous and include single-base substitutions and deletions (2, 5, 6, 9, 22, 23). Interestingly, virtually all patients with cytochrome *b* defects lack the gp91-*phox* and p22-*phox* chains, regardless of which subunit is affected by the mutation (7, 24–26). This observation suggests that intracellular stability of gp91-*phox* and p22-*phox* may be dependent on the formation of the cytochrome *b* heterodimer (25).

Here we report a missense mutation in p22-*phox* that is associated with a nonfunctional cytochrome *b* and autosomal recessive CGD. The domain in which the mutation resides was shown to lie on the cytoplasmic face of the membrane and thus may play a role in the interaction of cytochrome *b* with cytosolic oxidase components.

METHODS

Clinical History. The patient is a now 22-year-old Caucasian female offspring of consanguineous parents. She is HLA-identical with her mother and has a 46 XX karyotype. The patient has mental retardation, cortical atrophy, and a long history of recurrent infections typical of CGD, which include bronchopneumonias, interstitial pulmonary fibrosis, and recurrent perianal fistulae. The diagnosis of CGD was established at age 18 based on absent O_2^- production and O_2 consumption by her neutrophils in response to phorbol 12-myristrate 13-acetate (PMA). Similarly, her nitroblue tetrazolium (NBT) test revealed no positive cells following PMA stimulation. Neutrophils obtained from the mother were normal in these studies. Based on these findings, the patient

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Abbreviations: CGD, chronic granulomatous disease; FCS, fetal calf serum; KLH, keyhole limpet hemocyanin; NBT, nitroblue tetrazolium; O_2^-, superoxide; *phox*, *ph*agocyte *ox*idase (used to designate protein components of the phagocyte NADPH oxidase); PMA, phorbol 12-myristrate 13-acetate.

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was diagnosed as having autosomal recessive CGD. The patient has been on trimethoprim/sulfamethoxazole prophylaxis since diagnosis and recombinant human interferon γ (50 $\mu g/m^2$ per dose subcutaneously three times per week) since age 20. Her O_2^- and NBT test results with PMA have not improved while on interferon.

Determination of Cytochrome *b* Visible Absorbance Spectra. Visible absorption difference spectra (400–600 nm) of neutrophil subcellular fractions were determined by computer-subtracting the spectra of the oxidized from the dithionite-reduced samples as described (27).

Immunoblot Analysis of Oxidase Proteins. Rabbit antisera were raised against the following keyhole limpet hemocyanin (KLH)-conjugated peptides derived from oxidase component sequences: p47-*phox* amino acid residues 340–355 (RPGPQSPGSPLEEERQ), p67-*phox* residues 437–450 (DEPKESEKADANNQ), gp91-*phox* residues 537–556 (CGPEALAETLSKQSISNSES), p22-*phox* residues 153–170 (SNPPPRPPAEARKKPSEE), and p22-*phox* residues 153–164 (SNPPPRPPAEAR) (designated as antibody p22-153). Affinity purification of antibody p22-153 was performed using a column prepared by coupling the peptide to Affi-Gel 10 (Bio-Rad) (3), whereas the antibodies against p47-*phox* and gp91-*phox* were purified using peptides immobilized on an AminoLink column (Pierce). Immunoblots of neutrophil membranes and cytosol were performed as described (28) using Zeta-Probe membranes (Bio-Rad).

Cell-Free Assay of NADPH Oxidase Activation. Neutrophils were obtained from whole blood (in the case of the patient) or by leukapheresis (for normal controls) as described (29, 30) after obtaining informed consent. Cytosol and plasma membrane fractions were prepared by disruption of unstimulated neutrophils as described (27). The kinetics of activation of NADPH oxidase were studied in a cell-free system using the method reported earlier (31) in which cytosol [0–1.62 $\times$ 10^6 cell equivalents (0–40 μg of protein)] and deoxycholate-solubilized membranes (29) [0–2.5 $\times$ 10^6 cell equivalents (0–6.3 μg of protein)] are activated with SDS (40 μM) in 96-well microtitration plates (0.15-ml total volume per reaction) and the rate of O_2^- production is measured in a ThermoMax kinetic microplate reader equipped with a 550 $\pm$ 1 nm filter (Molecular Devices, Palo Alto, CA).

Isolation and Analysis of RNA and Genomic DNA. Peripheral blood mononuclear cells were used to isolate RNA by guanidine isothiocyanate precipitation and $CsCl_2$ ultracentrifugation (32). Genomic DNA was isolated from peripheral blood cell nuclei or from frozen neutrophil pellets (33). Northern blots of gp91-*phox* and p22-*phox* RNA were performed as described (9). Amplification of the p22-*phox* sequences from mononuclear cell-derived cDNA or from genomic DNA was also performed as described using specific primers (9). For DNA sequence analysis, amplified DNA fragments were subcloned into M13 derivatives using standard techniques (9) and multiple independent clones were sequenced by the dideoxynucleotide chain-termination method.

Allele-specific hybridization was performed essentially as described (5) to identify single-base substitutions in exon 6 of the p22-*phox* gene that was amplified from genomic DNA samples. Oligonucleotide probes used for hybridizations were specific for the wild-type Pro-156 allele (5′ AGC AAC CCC CCG CCG CG 3′) and for the Gln-156 mutation (5′ AGC AAC CCC CAG CCG CG 3′).

Binding of p22-*phox* Antibody to Intact and Permeabilized Cells. Freshly isolated neutrophils (1 $\times$ 10^6 per ml) were fixed by incubation for 15 min on ice in a freshly made solution of 0.5% (vol/vol) paraformaldehyde in phosphate-buffered saline (PBS). For preparation of permeabilized cells, the paraformaldehyde solution also contained 0.05% (wt/vol) saponin. All subsequent steps were also performed on ice. After fixation, cells were washed four times in Hepes-buffered saline (HBSS; 124 mM NaCl/5.8 mM KCl/10 mM dextrose/20 mM Hepes, pH 7.4) containing 1% (vol/vol) fetal calf serum (FCS) and incubated for 30 min with affinity-purified anti-p22 (antibody p22-153) at 30 μg/ml in PBS/1% FCS supplemented with 0.05% (wt/vol) sodium azide. For competition experiments, the cognate peptide was added at 30 μg/ml. Rabbit IgG (Jackson ImmunoResearch) was used at 30 μg/ml as a nonimmune control. After incubation with the primary antibody, cells were washed three times with HBSS/1% FCS and resuspended in PBS/1% FCS/0.05% sodium azide containing affinity-purified fluorescein-conjugated goat anti-rabbit $F(ab')_2$ (Cappel Laboratories) and 8% (vol/vol) human cord serum. After incubation for 30 min on ice, cells were washed three times with HBSS/1% FCS. Fluorescence was measured with an Ortho Diagnostic Systems cytofluorograph.

RESULTS

Analysis of Oxidase Components and Localization of the Defect to the Membrane Fraction. The patient was diagnosed as having autosomal recessive CGD based on the absence of O_2^- production by her PMA-stimulated neutrophils, her sex (confirmed by karyotype analysis), and the normal NBT test in her mother. Neutrophil membrane and cytosol fractions from the patient were analyzed on immunoblots using antipeptide antibodies to the four known oxidase components. As shown in Fig. 1*A*, patient membranes contained normal amounts of the two cytochrome *b* subunits, gp91-*phox* and p22-*phox*. Separate experiments using a different antipeptide antibody to p22-*phox* (antibody p22-153) confirmed that normal levels of this component were present in the patient's neutrophil membranes (Fig. 1*B*). The quantities of the two cytosolic oxidase components, p47-*phox* and p67-*phox*, also appeared to be normal (Fig. 1 *C* and *D*, respectively). This was surprising since >85% of autosomal recessive CGD patients are severely deficient in either p47-*phox* or p67-*phox* (16). In light of these findings, it was necessary to evaluate patient cytosol and membrane fractions in the cell-free oxidase activation system to determine the subcellular localization of the functional lesion. Patient cytosol was able to support normal oxidase activation when assayed in the presence of control neutrophil membranes (Fig. 2*A*). In contrast, oxidase activation did not occur when patient membranes were used, even in the presence of normal cytosol (Fig. 2*B*). Thus, it appeared that a defect in either cytochrome *b* or some as yet undiscovered membrane oxidase component was responsible for the respiratory burst defect in this patient. We next examined the spectral properties of the patient's cytochrome *b*. As shown in Fig. 3, the dithionite-reduced minus oxidized difference spectrum of the neutrophil membranes from the patient was identical to that of control membranes (Fig. 3), with characteristic cytochrome *b* absorption peaks at 427 and 558 nm. The calculated cytochrome *b* content in the patient membranes of 38.3 pmol per 10^7 cell equivalents was also normal. Similarly, the cytochrome *b* spectra and levels in whole neutrophils were normal in the patient (103.7 pmol per 10^7 cells) and in her mother (88.2 pmol per 10^7 cells; control, 84.4 pmol per 10^7 cells) (data not shown).

Identification of a Mutation in the Gene for the p22-*phox* Subunit of Cytochrome *b*. We hypothesized that this patient bore a mutation in the autosomal gene for the cytochrome *b* p22-*phox* subunit that results in normal levels of a nonfunctional cytochrome. Peripheral blood mononuclear cell RNA contained ostensibly normal p22-*phox* transcripts by Northern blot analysis (not shown), a result that was anticipated based on the normal appearance of the cytochrome by spectral and immunoblot analysis. The corresponding cDNA

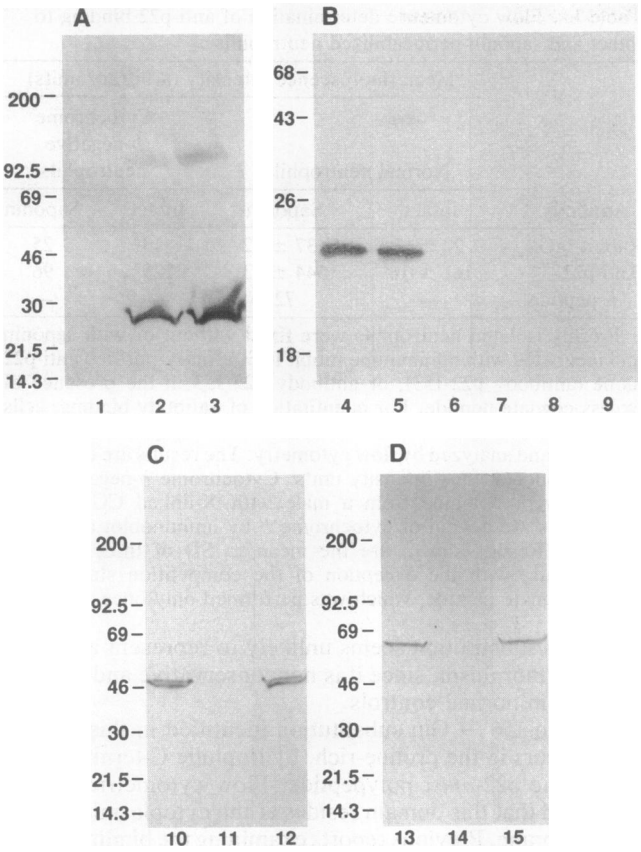

FIG. 1. Immunoblots of NADPH oxidase components in control and CGD patient neutrophils. Western blots were performed as described in the text. (*A*) Immunoblots of the gp91-*phox* and p22-*phox* cytochrome *b* subunits in the neutrophil plasma membrane fractions from a patient with X-linked/cytochrome *b*-deficient CGD (lane 1), a normal control (lane 2), and the proband (lane 3). (*B*) A different antibody to p22-*phox* (antibody p22-153) used to confirm the normal appearance of p22-*phox* in the neutrophil membranes of a normal control (lane 4) and the proband (lane 5), in contrast to the complete lack of p22-*phox* in the membranes from a patient with autosomal recessive/cytochrome *b*-deficient CGD (lane 6). Lanes 7–9 represent an identical immunoblot configuration performed in the presence of competing cognate peptide. The immunoblots for p47-*phox* (*C*) and p67-*phox* (*D*) were prepared using cytosols from normal controls (lanes 10 and 13), CGD patients known to be deficient in either p47-*phox* (lane 11) or p67-*phox* (lane 14), and the proband (lanes 12 and 15). The results shown are representative of two experiments, each performed with a different preparation of neutrophils. Molecular weights are shown as $M_r \times 10^{-3}$.

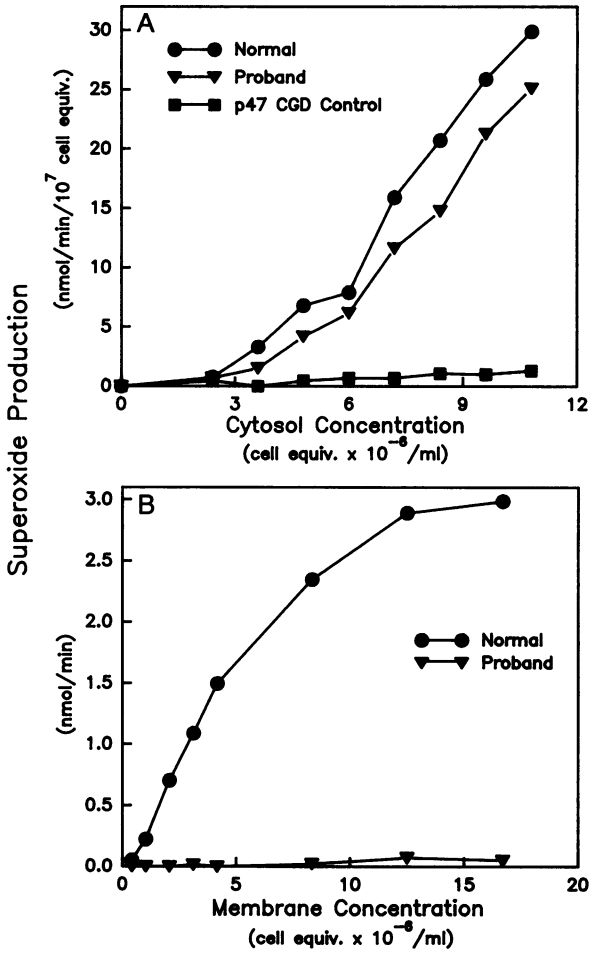

FIG. 2. NADPH oxidase activation as a function of cytosol and membrane concentration in the patient. Cell-free activation of NADPH oxidase was performed as described in the text. (*A*) Effect of increasing cytosol concentration on the extent of oxidase activation (measured by O_2^- production) using cytosol from a normal control (●), the proband (▼), and an autosomal recessive/cytochrome *b*-positive CGD patient known to be deficient in p47-*phox* (■). (*B*) Effect on oxidase activation of increasing concentration of membranes from a normal control (●) or from the proband (▼). The results are representative of four experiments performed with fractions from two different neutrophil preparations.

was amplified by the polymerase chain reaction (PCR) using p22-*phox*-specific primers and subcloned, and individual subclones were sequenced. All clones contained a single nucleotide alteration, a C → A transversion, that predicts the nonconservative replacement of a proline with a glutamine residue at position 156 (Fig. 4). This finding was confirmed by amplification and sequencing of the corresponding exon (exon 6 of the p22-*phox* gene) using genomic DNA isolated from the patient. No clones for the wild-type sequence were identified, demonstrating homozygosity for the mutant allele. The parents were unavailable for study. Allele-specific oligonucleotide probes were used to examine the occurrence of the C → A substitution in a sample of 20 unrelated normal controls after amplification of the genomic sequence by PCR; the substitution was unique to the patient (data not shown).

Cellular Localization of the Pro-156 → Gln Substitution. A peptide sequence derived from the proline-rich C-terminal region of p22-*phox*, which contains the Pro-156 residue affected in this patient, was used to raise a rabbit polyclonal antiserum, designated p22-153. The antibody was purified by affinity chromatography using the p22 peptide and was found to react specifically with p22-*phox* in immunoblots of solubilized normal neutrophil extracts (see Fig. 1*B*). Neutrophil labeling with affinity-purified antibody was studied by flow cytometry. There was some binding of the p22-153 antibody to intact neutrophils relative to nonimmune rabbit IgG, but this level of binding was also detected in neutrophils obtained from a patient with classic X-linked/cytochrome-negative CGD (Table 1). This suggests that the binding observed in intact cells does not involve the cytochrome subunit. Permeabilization of the plasma membrane with saponin markedly increased the binding of the p22-153 antibody to normal neutrophils but not to those from the cytochrome-negative patient (Table 1). All binding to permeabilized cells was abolished by competition with the cognate peptide. These results indicate that the p22-*phox*-153 antibody binds to a cytoplasmic epitope of p22-*phox*.

DISCUSSION

We have identified a missense mutation in the gene encoding the p22-*phox* subunit of the neutrophil cytochrome *b* het-

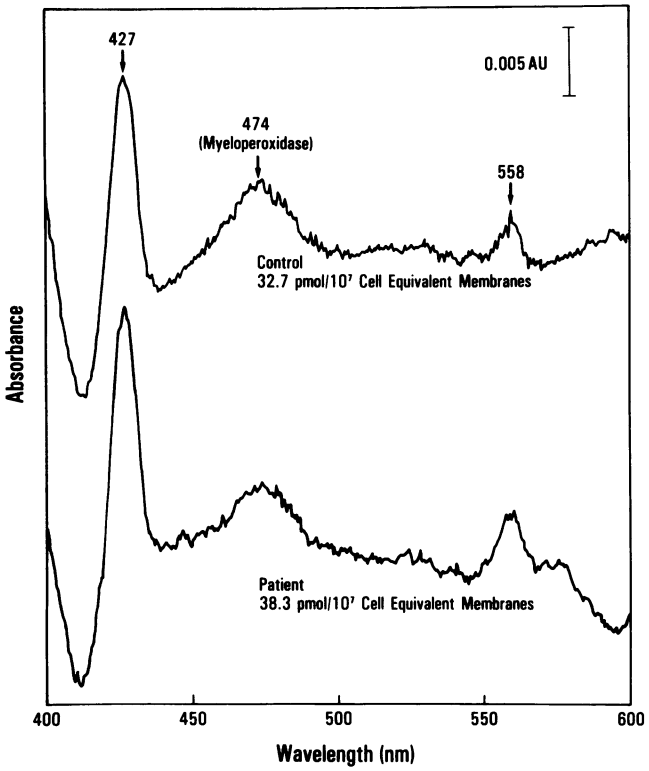

FIG. 3. Cytochrome *b* difference spectra in control and patient membranes. The visible range dithionite-reduced minus oxidized spectra of plasma membrane fractions from control (top) and patient (bottom) neutrophils were determined. Cytochrome *b* absorbance peaks are seen at 427 nm and 558 nm. The spectra shown are representative of six such measurements performed on two different preparations of patient and control membranes. AU, absorbance unit.

erodimer in a form of autosomal recessive, cytochrome-positive CGD. The gp91-*phox* and p22-*phox* subunits of cytochrome *b* are derived from loci at Xp21.1 and 16q24, respectively (2, 9). Whereas mutations in either of these two genes are typically associated with the absence of the cytochrome *b* heterodimer (25), the visible absorbance spectrum and immunoblots of the cytochrome were normal in this patient. The unexpected finding that the cellular defect in the NADPH oxidase of the patient was localized to the membrane fraction suggested that the cytochrome was functionally defective. In light of the autosomal recessive inheritance and the normal female karyotype in this case, a mutation in the gene for p22-*phox* seemed most likely. A single nucleotide alteration (C → A) in the p22-*phox* transcript was identified by DNA sequencing after amplification of p22-*phox* cDNA by the PCR. This mutation predicts the nonconservative substitution of Pro-156 with a glutamine residue. The effect of this mutation cannot be tested directly, as functional assays for cloned cytochrome cDNAs are not yet available. However, this substitution seems unlikely to represent an irrelevant polymorphism, since it is nonconservative and was not detected in normal controls.

Wild type		Asn	Pro	Pro[156]	Pro	Arg
	...	AAC	CCC	CCG	CCG	CGG ...
Patient				CAG		
				Gln[156]		

FIG. 4. Point mutation in p22-*phox*. A portion of the p22-*phox* coding sequence is shown, along with the encoded amino acid residues, numbered according to their position in the protein sequence (21). As compared to the wild-type allele, a C → A substitution was identified in the patient described here.

Table 1. Flow cytometric determination of anti-p22 binding to intact and saponin-permeabilized neutrophils

	Mean fluorescence intensity (arbitrary units)			
	Normal neutrophils		Cytochrome *b*-negative neutrophils	
Antibody	Intact	Saponin	Intact	Saponin
Rabbit IgG	22 ± 3	37 ± 22	19	25
Anti-p22	161 ± 16	544 ± 53	129	96
+ peptide	—	72	—	—

Freshly isolated neutrophils were fixed without or with saponin and incubated with nonimmune rabbit IgG, affinity-purified anti-p22 alone (antibody p22-153), or antibody p22-153 in the presence of excess cognate peptide. For quantitation of antibody binding, cells were washed, incubated with a fluorescein-conjugated anti-rabbit antibody, and analyzed by flow cytometry. The results are expressed in mean fluorescence intensity units. Cytochrome *b*-negative neutrophils were obtained from a male with X-linked CGD whose neutrophils are devoid of cytochrome *b* by immunoblot and spectroscopy. Results shown are the mean ± SD of three separate experiments, with the exception of the competition study using excess cognate peptide, which was performed only once.

The Pro-156 → Gln substitution identified in this case of CGD occurs in the proline-rich, hydrophilic C-terminal portion of the p22-*phox* polypeptide. Flow cytometric studies suggested that this domain resides at the cytoplasmic face of the membrane. Previous reports examining the binding of two p22-*phox*-specific monoclonal antibodies by flow cytometry have suggested that p22-*phox* is a transmembrane protein (26, 34). However, in these studies, the epitopes to which the antibodies were directed were unknown. The cytoplasmic C-terminal domain of p22-*phox* could be involved in the interaction of the cytochrome with other oxidase components, and we speculate that the Pro-156 → Gln mutation may interfere with oxidase function by this route. In this regard, it is interesting that in two analogous cases of X-linked CGD with normal levels of a nonfunctional cytochrome *b*, the gp91-*phox* mutations involved the C-terminal portion of the polypeptide (5, 35), which also appears to be a cytoplasmic domain (36). In contrast, two other missense mutations in p22-*phox* associated with cytochrome-negative CGD occur at the junction of or within a relatively hydrophobic region (Arg-90 → Gln, Ser-118 → Arg) (9). These substitutions are associated with the absence of the encoded polypeptide, which presumably is either intrinsically unstable or is unable to form a heterodimer with the gp91-*phox* cytochrome subunit. Determining the mechanism by which the p22-*phox* Pro-156 → Gln mutation disables oxidase function may provide insights into the functional interactions of the cytoplasmic domain of cytochrome *b* with cytosolic constituents.

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