JCI The Journal of Clinical Investigation

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J Clin Invest. 1989;84(6):2012-2016. https://doi.org/10.1172/JCI114393.

Research Article

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A Missense Mutation in the Neutrophil Cytochrome *b* Heavy Chain in Cytochrome-positive X-linked Chronic Granulomatous Disease

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Abstract

A membrane-bound cytochrome *b*, a heterodimer formed by a 91-kD glycoprotein and a 22-kD polypeptide, is a critical component of the phagocyte NADPH-oxidase responsible for the generation of superoxide anion. Mutations in the gene for the 91-kD chain of this cytochrome result in the X-linked form of chronic granulomatous disease (CGD), in which phagocytes are unable to produce superoxide. Typically, there is a marked deficiency of the 91-kD subunit and the cytochrome spectrum is absent (X^- CGD). In a variant form of CGD with X-linked inheritance, affected males have a normal visible absorbance spectrum of cytochrome *b*, yet fail to generate superxoxide (X^+ CGD). The size and abundance of the mRNA for the 91-kD subunit and its encoded protein were examined and appeared normal. To search for a putative mutation in the coding sequence of the 91-kD subunit gene, the corresponding RNA from an affected X^+ male was amplified by the polymerase chain reaction and sequenced. A single nucleotide change, a C → A transversion, was identified that predicts a nonconservative Pro → His substitution at residue 415 of the encoded protein. Hybridization of amplified genomic DNA with allele-specific oligonucleotide probes demonstrated the mutation to be specific to affected X^+ males and the carrier state. These results strengthen the concept that all X-linked CGD relates to mutations affecting the expression or structure of the 91-kD cytochrome *b* subunit. The mechanism by which the Pro 415 → His mutation renders the oxidase nonfunctional is unknown, but may involve an impaired interaction with other components of the oxidase.

Introduction

An unusual *b*-type cytochrome is an essential component of the phagocyte NADPH-oxidase, a membrane-bound enzyme system that generates large quantities of superoxide upon activation by a variety of particulate and soluble stimuli (1). The superoxide radical is converted into other potent microbicidal oxidants used to kill ingested microorganisms. Current evidence indicates that the oxidase is formed by multiple components that include a membrane-associated cytochrome *b* as well as several cytosolic proteins required for activity of the otherwise dormant oxidase (2–6). The cytochrome, a heterodimer of a 91-kD glycoprotein (heavy chain) and 22-kD polypeptide (light chain) (7, 8), has a very low midpoint potential (−245 mV) and is postulated to function as the terminal redox carrier in the transfer of electrons from NADPH to oxygen (1).

In the inherited disorder chronic granulomatous disease (CGD),[1] phagocytic cells are unable to generate superoxide (1, 9). Affected individuals develop severe and recurrent infections due to the lack of this important host antimicrobial pathway. CGD is genetically heterogenous, reflecting lesions in different components of the phagocyte oxidase (4–6, 10–12). The X-linked recessive form, which includes the majority of cases, results from mutations in the gene encoding the 91-kD subunit of the cytochrome *b* heterodimer (2, 3, 12). This accounts for the consistent absence of the cytochrome *b* heme spectrum previously noted in virtually all cases of X-linked CGD (13). Both the 91- and 22-kD chains are lacking in X-linked CGD despite the genetic deficiency of only the heavy chain, which suggests that the intracellular stability of the 22-kD species requires expression of the larger subunit (2, 7, 8).

The underlying defects in the gene for the 91-kD heavy chain that result in X-linked CGD appear to be heterogenous, but are largely unknown. Partial deletions of the gene, which is located at Xp21 (14), are rare (12). Complete gene deletions are associated with more complex phenotypes that include Duchenne muscular dystrophy, retinitis pigmentosa, and the McLeod phenotype (12, 14, 15). In the majority of typical cases of X-CGD, heavy chain gene structure is grossly normal by conventional Southern blot analysis (12). Levels of the mRNA for the heavy chain are typically, although not always, markedly deficient, presumably due to mutations that adversely affect the expression or stability of the mRNA (12, 16).

Rare variant forms of CGD with X-linked inheritance have been reported in which residual cytochrome *b* spectral activity is measurable (17–19). This subset of patients has been referred to as X^+ CGD, in contrast to those with the classic

This work was published in abstract form (1989. *Clin. Res.* 37:544a).

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Received for publication 27 July 1989 and in revised form 24 August 1989.

J. Clin. Invest.

0021-9738/89/12/2012/05 $2.00
Volume 84, December 1989, 2012–2016

1. *Abbreviations used in this paper:* ASO, allele-specific oligonucleotide; CGD, chronic granulomatous disease; PCR, polymerase chain reaction.

cytochrome-negative phenotype, designated as X^- CGD. X^+ CGD might result either from mutations at specific amino acid residues critical for the function of the 91-kD subunit or from defects in other, as yet undescribed, proteins encoded on the X chromosome. Here we present the molecular genetic analysis of one family with X^+ CGD, in which evidence for the former possibility has been obtained. This family is of particular interest as affected males generate virtually no superoxide, yet have normal levels of cytochrome *b* as measured by visible absorbance difference spectroscopy (19). The size and abundance of the 91- and 22-kD cytochrome subunits appeared normal in Western blots of neutrophil extracts. To search for a possible mutation in the coding sequence of the heavy chain gene, the polymerase chain reaction (PCR) was used to amplify the corresponding mRNA derived from an affected male. Sequence analysis of the amplified cDNA revealed a missense mutation that predicts a nonconservative amino acid substitution in the protein product.

Methods

Materials. Avian myeloblastosis virus reverse transcriptase was obtained from Molecular Genetic Resources, Tampa, FL, and RNAsin from Promega Biotec, Madison, WI. Taq DNA polymerase was from Perkin-Elmer Corp., Norwalk, CT/Cetus Corp., Emeryville, CA. Other enzymes were purchased from New England Biolabs, Beverly, MA. M13 vectors were from Pharmacia Fine Chemicals, Piscataway, NJ, and labeled (^{32}P; ^{35}S) deoxynucleotides were from Amersham Corp., Arlington Heights, IL. Synthetic oligonucleotides were prepared on a DNA synthesizer (model 340B; Applied Biosystems, Inc., Foster City, CA). All other reagents used were of analytical grade.

Western blot analysis. Western blots of neutrophil extracts were performed as described (2). Antiserum for the 91-kD heavy chain was prepared against a B-galactosidase fusion protein containing nearly the complete coding sequence of the heavy chain (2). Antiserum for the 22-kD light chain was prepared as previously described (2) against a peptide (SNPPPRPPAEAR) derived from the COOH-terminal region of this protein.

Preparation of RNA and DNA. Mononuclear cells were obtained from peripheral blood by Ficoll-Paque sedimentation and RNA prepared by guanidine-HCl precipitation (12). Genomic DNA was isolated from peripheral blood cell nuclei (20).

Northern blot analysis. RNA samples were electrophoresed in 1% agarose formaldehyde gels, transferred to nitrocellulose, and hybridized with a $\approx$ 1,400 base pair Pst I-Eco RI fragment of the heavy chain cDNA labeled by random priming as described (12).

DNA amplification. First strand cDNA synthesis was performed in 50 μl containing 50 mM Tris pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 500 μM of each dNTP, 10 mM dithiothreitol, 1,000 U/ml RNAsin, 40 μg/ml oligo(dT)12–18, 1 μg total cellular RNA, and 100 U of AMV reverse transcriptase. The mixture was incubated at 42°C for 60 min, then inactivated by heating to 67°C for 5 min. Overlapping cDNA fragments that span the $\approx$ 1.7-kb open reading frame of the 91-kD heavy chain transcript were then amplified by the polymerase chain reaction using three pairs of synthetic oligonucleotide primers derived from the heavy chain cDNA sequence (oligonucleotides 1, 2; 3, 4; 5, 6 in Table I). For each pair of primers, 10 μl of the first strand cDNA reaction was amplified in 100 μl as described (21). Certain portions of coding sequence in the heavy chain gene (see below) were amplified directly from genomic DNA (21) using oligonucleotide primers derived from flanking intron sequence (oligonucleotides 7, 8; 9, 10 in Table I). Oligonucleotides were synthesized with restriction enzyme sites at their 5′ ends (Hind III or Bam HI) to facilitate cloning.

Analysis of amplified target sequences. DNA fragments amplified from the coding region of 91-kD heavy chain cDNA and from genomic DNA were cloned into M13 derivatives and sequenced by the dideoxynucleotide chain termination method (22). Individual clones were pooled to prepare single-stranded template for sequencing to avoid potential artifacts due to nucleotide misincorporation in any single clone. Allele-specific oligonucleotide (ASO) probes (oligonucleotides 11, 12 in Table I) for the mutation identified in the X^+ CGD family and for the wild type sequence were used for direct screening of the corresponding exon (amplified from genomic DNA) in a slot blot format. The amplified DNA fragment was denatured with NaOH, immobilized on nitrocellulose, and hybridized with ^{32}P end-labeled ASO (23). Blots were washed in 6× standard saline citrate (0.9 M NaCl, 0.09 M sodium citrate, pH 7.5) at room temperature and then at 59°C for 10 min.

Table I. Sequence of Oligonucleotide Primers

Oligonucleotide	
1	5′ acttaggatcc TGCCACCATGGGGAACTGGGCTGTGAATGAG 3′ (6)
2*	5′ gaataagctt GTACCAAAAGACTTCAAAGTAAGACCTCCGGATG 3′ (633)
3	5′ tactggatcc TGTTGGCAGGCATCACTGGAGTTGTCATCACGC 3′ (530)
4*	5′ gaataagctt GAACACATCTTCACTGGCAGTGCCAAAGGGCCCATC 3′ (1210)
5	5′ tactggatcc AATGCTTGTGGCTGTGATAAGCAGGAGTTTCAA 3′ (1111)
6*	5′ tagtaagctt AGCATTATTGAGCATTTGGCAGCACAACCCACA 3′ (1784)
7	5′ tactggatcc ATGATTATTAGCCAATTTCTGATAAAAGAA 3′
8*	5′ tagtaagctt CGAGAAGTCAGAGAATTTATAACTTATCTCTTGTTGGT 3′
9	5′ tactggatcc TGAAGAGCAAGACATCTCTGTAACTA 3′
10*	5′ tagtaagctt ACTGCTCTAAGGCCCTCCGATAAAT 3′
11*	5′ GGATGCGAAGGGTGTGA 3′
12*	5′ GGATGCGAAGTGTGTGA 3′

Oligonucleotides 1–10 were used as primers for PCR amplification. Lowercase letters indicate noncomplementary bases that include Bam HI or Hind III sites that can facilitate DNA cloning. Oligonucleotides 1–6 were derived from the cDNA sequence for the 91-kD heavy chain [2, 12], and the number indicates the position within the cDNA sequence of the most 5′ nucleotide of the primer. Oligonucleotides 7, 8 and 9, 10 were derived from genomic sequence flanking exon 1 and exon 10, respectively (unpublished data). Oligonucleotide 11 and 12 are complementary to sequence in exon 10 for the wild type and X^+ alleles, respectively, and used for ASO hybridization. * Antisense strand.

Results

Neutrophils obtained from affected males in a family with an X^+-CGD allele display a normal cytochrome *b* spectrum yet fail to produce superoxide (19). This phenotype is compatible either with a mutation in the gene encoding the 91-kD heavy chain that results in a nonfunctional cytochrome or a defect at an unrelated locus on the X chromosome that encodes another, as yet undescribed, protein critical to the function of the phagocyte oxidase. The mRNA for 91-kD subunit in X^+ males in this family was grossly normal by Northern blot analysis (Fig. 1, lane *2*). The size and abundance of both the heavy and light chains of the cytochrome also appeared normal in a Western blot of X^+ neutrophil extracts (Fig. 2, lane *2*), in contrast to the classic cytochrome-negative form of X-linked CGD in which both chains are markedly deficient (Fig. 2, lane *3*).

The sequence of the protein coding region of the cytochrome *b* heavy chain transcript in affected males in this family was determined in its entirety from cDNA derived from peripheral blood mononuclear cell RNA. Three overlapping segments of the ≈ 1.7-kb open reading frame of the heavy chain transcript were obtained by PCR amplification using specific primers (see Table I). The initiator ATG lies only 11 nucleotides downstream from the cap site (2) and is encompassed within the most 5′ synthetic oligonucleotide primer (primer 1 in Table I). Therefore, to obtain the extreme 5′ sequence, it was necessary to amplify genomic DNA using flanking intron primers (primers 7, 8; Table I). Upon completion of this analysis, only a single nucleotide substitution, a C → A transversion at nucleotide 1,256 (Fig. 3), was evident. This mutation predicts a nonconservative substitution of proline to histidine at residue 415 of the 570 amino acid heavy chain.

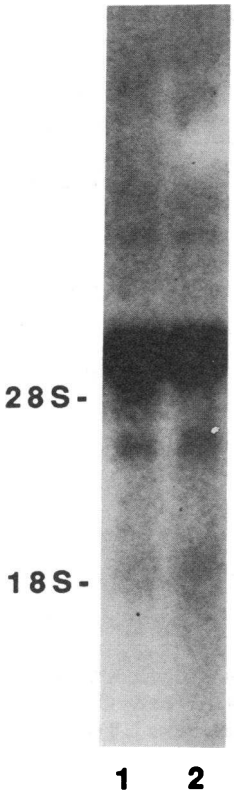

Figure 1. Northern blot analysis. Peripheral blood mononuclear cell RNA was fractionated on a denaturing agarose gel, transferred to nitrocellulose, and probed with a ^{32}P-labeled Pst I-Eco RI fragment derived from the cDNA for the cytochrome *b* 91-kD heavy chain. Lane *1*, normal control; lane *2*, X^+ CGD.

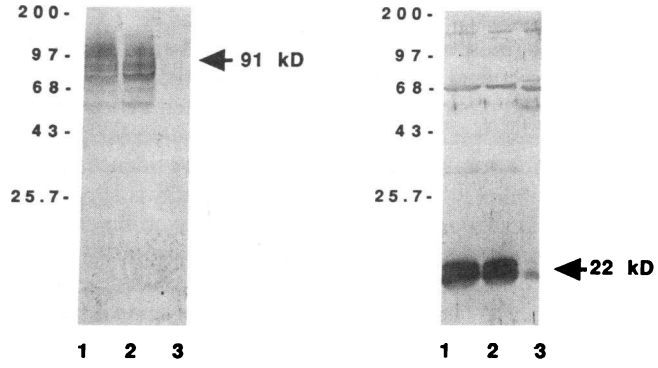

Figure 2. Western blot analysis. Neutrophil extracts were fractionated on a polyacrylamide gel, transferred to nitrocellulose, and probed with antisera directed against the cytochrome *b* 91-kD heavy chain (*left*) or 22-kD light chain (*right*). The arrows indicate the heavy and light chains. The 22-kD light chain frequently appears as a doublet after separation by denaturing gel electrophoresis (7). The numbers on the left of each blot denote the molecular weight standards (kD). Lane *1*, normal control; lane *2*, X^+ CGD; lane *3*, X^- CGD.

The C → A transversion might represent either a critical mutation in the 91-kD heavy chain or an irrelevant polymorphism. To address this issue, allele-specific oligonucleotide probes were used to examine the occurrence of the C → A mutation in genomic DNA samples from 14 unrelated normal controls and 10 unrelated patients with classical X^- CGD. Two brothers with the X^+ variant form of CGD studied here and their parents were also analyzed by this approach. The exon in which the identified mutation occurs was amplified from genomic DNA (using primers 9, 10; Table I) and analyzed by differential hybridization with each of two oligonucleotide probes, one for the wild type sequence (primer 11) and one for the mutation identified in the X^+ variant (primer 12). The C → A mutation was found only in the two X^+ brothers and their mother, an obligate carrier (lanes *1, 3, 4* in Fig. 4). The wild type allele was also present in their mother, consistent with the carrier state. The father carried the wild type allele (lane *2*, Fig. 4), as did all the normal controls (representative samples shown in lanes *5, 6*; Fig. 4), and patients with X^- CGD (not shown).

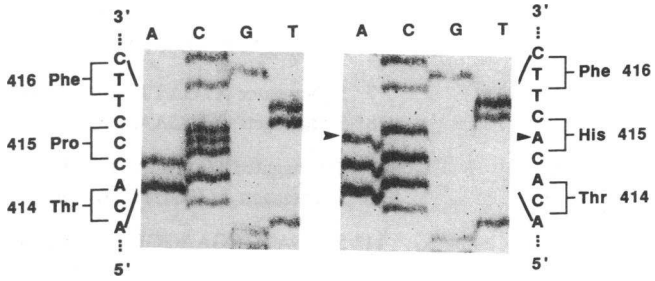

Figure 3. Sequence analysis of amplified 91-kD heavy chain cDNA. Sequence analysis was performed by the dideoxy chain termination method after cDNA fragments amplified by PCR were cloned into M13 derivatives. A normal control is shown on the left, and sequence obtained from the patient with X^+ CGD is shown on the right. The arrows indicate the mutation.

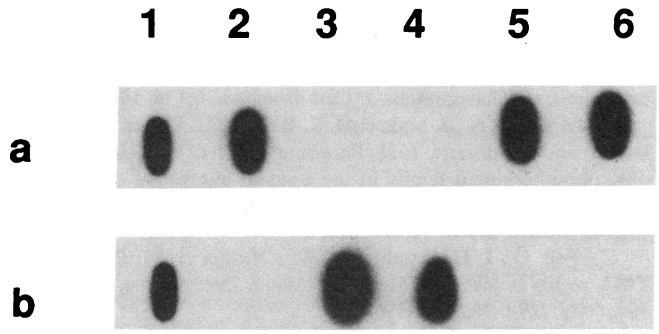

Figure 4. Allele-specific oligonucleotide analysis. The exon containing the C → A mutation detected in the X^+ CGD patient was amplified from genomic DNA by PCR, transferred to nitrocellulose, and hybridized to oligonucleotide probes specific to the wild type sequence (*a*) or X^+ CGD sequence (*b*). Lane *1*, mother of X^+ CGD brothers; lane *2*, father of X^+ CGD brothers; lanes *3* and *4*, X^+ CGD brothers; lanes *5* and *6*, normal controls.

Discussion

We have identified a missense mutation in the gene encoding the 91-kD heavy chain of the phagocyte cytochrome *b* heterodimer in a family with a rare cytochrome-positive variant form of X-linked CGD. Whereas classic X-linked CGD is characterized by mutations in the heavy chain gene that result in the absence of the encoded protein, this variant is distinguished by the presence of an apparently normal-sized heavy chain in neutrophils obtained from affected males. A search for the molecular basis of this disorder led to the identification of a single nucleotide alteration (C → A) in codon 415, which predicts a nonconservative substitution of a histidine residue for a proline in the encoded protein. As functional assays for cloned heavy chain cDNAs have not yet been developed, the effect of the His 415 replacement cannot be tested directly. Nonetheless, this substitution does not appear to represent an irrelevant polymorphism, since it is nonconservative and was not detected in classic X^- patients or in normal controls. These results strengthen the concept that all forms of X-linked CGD are due to mutations in the cytochrome *b* heavy chain locus at Xp21 that ultimately affect either the expression or primary structure of the 91-kD subunit. Evidence for additional X-linked loci responsible for CGD is lacking.

Although cDNAs for both protein chains of the cytochrome *b* heterodimer have been cloned (12, 24), key functional domains of these polypeptides have not been fully defined. These include the location of the heme prosthetic group, the site of interaction between the two subunits, and their topologic organization in the membrane compartment. The cytochrome is also likely to interact with other components of the phagocyte oxidase such as an NADPH-binding protein and several cytosolic proteins important for activation of the oxidase (4–6).

Our findings strongly suggest that a replacement of histidine for proline at residue 415 results in a nonfunctional phagocyte oxidase and chronic granulomatous disease and, as such, identify one critical amino acid residue in the cytochrome *b* heavy chain. The substitution of a positively charged residue for a proline might alter the folding properties of the peptide backbone. Nevertheless, several properties of the cytochrome *b* heterodimer are maintained. First, the association between the mutant 91-kD heavy chain and the 22-kD light chain is not grossly disrupted, as the two proteins copurify normally (Curnette, J. T., J. K. Hurst, C. A. Parkos, A. J. Jesaitis, and H. Rosen, manuscript in preparation). In addition, the microenvironment of the heme prosthetic group appears to be unaffected as the visible absorbance spectrum is normal (19). Furthermore, recent studies on partially purified mutant cytochrome have indicated that the Raman and electron paramagnetic resonance spectra are also normal, as is the midpoint potential (Curnutte, J. T., J. K. Hurst, C. A. Parkos, A. J. Jesaitis, and H. Rosen, manuscript in preparation). Since the redox properties of the mutant cytochrome appear to be intact, the basis of the profound functional effect on superoxide production may involve an abnormal interaction with other components of the oxidase. A 47-kD cytosolic protein that undergoes phosphorylation upon activation of the NADPH-oxidase (25, 26) has been reported to associate with the COOH terminus of the heavy chain (27). This association appears to be required for complete phosphorylation of the 47-kD species, as only partial phosphorylation occurs in classic cytochrome-negative X-linked CGD (25, 28). However, since phosphorylation was found to be normal in the X^+ CGD variant (19), the interaction between the 47-kD protein and mutant heavy chain may be preserved. It is likely that a more complete understanding of the structure of the cytochrome *b* heterodimer and the identity and function of associated proteins will be required to clarify the mechanism by which the Pro 415 substitution results in a nonfunctional cytochrome *b*.

Acknowledgments

Dr. Dinauer is the recipient of a Clinical Investigator Award (K08 HL-02253-01), Dr. Curnutte is an Established Investigator of the American Heart Association, and Dr. Orkin is an Investigator of the Howard Hughes Medical Institute. This work was supported in part by a grant from the National Institutes of Health (HD-18661).

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