Abstract Chronic granulomatous disease (CGD) is a rare inherited immunodeficiency disease that leads to severe recurrent infections. CGD is caused by defects in the phagocyte NADPH oxidase, a multiprotein enzyme that reduces oxygen to superoxide, a precursor of microbicidal oxidants. Less than 6% of CGD patients have an autosomal recessive form of the disease caused by mutations in NCF-2. This gene encodes p67-phox, a cytosolic oxidase subunit that associates with membrane-bound flavocytochrome b₅₅₈ and regulates electron transfer. We studied six patients from five families with p67-phox deficiency and identified seven different mutant alleles. Patients from three of the kindreds were homozygous for their respective mutation, although the parents of only one family were known to be related. Five of the mutations have not previously been identified: (1) a missense mutation (383C→T) in exon 5, (2) a nonsense mutation (196C→T) in exon 3, (3) a missense mutation (230G→A) in exon 3, (4) a nonsense mutation (298C→T) in exon 4, and (5) a dinucleotide deletion (835–836 AC) from exon 9. Phagocytes from each of the patients analyzed failed to generate a measurable respiratory burst and had no detectable p67-phox protein. Our results further demonstrate that there is great heterogeneity among the mutations in p67-phox-deficient CGD patients, with no evidence for mutational hot-spots or a founder effect. Our data also support the hypothesis that the stability of p67-phox is particularly sensitive to missense mutations that cause amino acid substitutions within its N-terminal domain. In contrast, mutations predicting single amino acid changes elsewhere in the protein generally represent benign polymorphisms.

Introduction

Chronic granulomatous disease (CGD) is an inherited disorder of the innate immune system characterized by the absence or, more rarely, very low levels of superoxide production by the phagocyte NADPH oxidase. Superoxide and its toxic derivatives (e.g., hydrogen peroxide, hypochlorite) are necessary for killing many invading microorganisms, and consequently, CGD patients suffer from recurrent, sometimes fatal, bacterial and fungal infections and are usually diagnosed in infancy or early childhood (Roos and Curnutte 1999).

When activated by opsonized microorganisms or a variety of soluble stimuli, the phagocyte NADPH oxidase catalyzes the reduction of oxygen to form superoxide, which is released into the phagocytic vacuole or into the extracellular space. The enzyme is composed of at least five unique protein components. Two of them, gp91-phox and p22-phox, are integral membrane proteins and together form flavocytochrome b₅₅₈, the electron-transporting center of the oxidase. Three other proteins, p40-phox, p47-phox, and p67-phox, are found in the cytosol of resting phagocytes where they exist in a multiprotein complex, probably associated with the sub-membranous cytoskeleton. Activation of the NADPH oxidase system also requires the GTP-binding protein Rac (Rac1 or Rac2) and involves the association of these soluble components with the flavocytochrome (reviewed in Robinson and Badwey 1995; De Leo and Quinn 1996).
The incidence of CGD is approximately 1 in 250,000 individuals. The most common form of the disease, X91 CGD, is X-linked, accounts for ~65% of all cases, and is caused by mutations in the CYBB gene encoding gp91phox (Roos et al. 1996; Rae et al. 1998). The remaining cases are inherited in an autosomal recessive manner and are caused by defects in the genes for p22-phox, p47phox, or p67-phox (referred to as A22, A47, and A67 CGD, respectively). A67 CGD is the rarest form of the disease, accounting for less than 6% of all cases. To date, only twelve mutations have been described in the NCF-2 gene (locus 1q25) that encodes p67-phox, and all but one result in the total absence of protein product (i.e., A67° CGD; the superscript ° indicates the absence of the protein; de Boer et al. 1994; Ahlin et al. 1995; Tanugi-Cholley et al. 1995; Nuno et al. 1995; Leusen et al. 1996; Aoshima et al. 1996; Bonizzato et al. 1997; Patiño et al. 1999). NCF-2 spans approximately 40 kb and contains 16 exons (Kenney et al. 1993).

The primary structure of p67-phox reveals two SH3 domains, a single SH3-binding proline-rich region and four tetratricopeptide (34 amino acid) repeats (TPRs; Leto et al. 1990; Leto 1996). Tandem arrays of TPRs form scaffolds that mediate protein–protein interactions and the formation of multiprotein complexes, and are found in a wide variety of proteins (Chen et al. 1996). The presence in p67-phox of these three types of motif is consistent with the involvement of the protein in a complex with p40-phox and p47-phox. The precise roles of p67-phox and the other soluble phox proteins in initiating or maintaining NADPH oxidase activity are not known. It is clear that p67-phox is absolutely required for superoxide generation both in intact cells and in cell-free activation systems. In contrast, NADPH oxidase activation can be achieved in a cell-free system in the absence of p47-phox, provided that the concentrations of p67-phox and Rac are high (Freeman and Lambeth 1996; Koshkin et al. 1996; Cross et al. 1999). Many recent studies indicate that, whereas p47-phox acts primarily as a docking protein that promotes interactions between flavocytochrome b558 and the cytosolic components, p67-phox is more directly involved in regulating electron flow, possibly in conjunction with activated (GTP-bound) Rac, with which it is able to interact (reviewed in De Leo and Quinn 1996; Heyworth et al. 1999). There is also some evidence, albeit controversial, that p67-phox contains a catalytically essential binding site for NADPH (Smith et al. 1996).

Carriers of autosomal recessive CGD are difficult to detect as their phagocytes show normal staining in the nitroblue tetrazolium (NBT) slide test and frequently generate superoxide at rates within the normal range (Roos and Curnutte 1999). Identifying mutations in individuals with the A22 and A67 forms of CGD provides an unambiguous means of detecting carriers and the basis for performing pre-natal diagnosis in affected families. A47 CGD is more problematic because of the presence of one or more closely related pseudogenes (Görlich et al. 1997). In this study of six patients from five families with A67° CGD, we have found seven mutant alleles in the gene for p67-phox, five of which have not been described previously. In addition, we have found two novel polymorphisms and confirmed that a previously identified nucleotide change that alters 389His→Gln does not cause CGD but also represents a benign polymorphism. Two missense mutations described here support the hypothesis that changes in amino acids within the TPR motifs of p67-phox adversely effect the stability of the protein (Leto 1996; Ponting 1996).

**Materials and methods**

**CGD patients and families**

Blood samples were obtained from CGD patients and their family members with appropriate Institutional consent and were sent by the referring physicians to the investigators at The Scripps Research Institute.

Patient 1a is a 14-year-old male and patient 1b is his brother who died at the age of 10. Their family is native to Venezuela and includes one brother who has CGD (aged 13 months), two healthy sisters, and one healthy brother. A fifth male sibling died at the age of 4, possibly of CGD. The absence of superoxide generation from the patients’ neutrophils was established with a microtiter plate NBT assay (Virella et al. 1990). The absence of p67-phox and presence of p47-phox was established by immunoblotting with neutrophils from patient 1a. The paternal grandmother and maternal grandfather of patients 1a and 1b are first cousins.

Patient 2 is the 2-year-old son of unrelated Hispanic parents with no known history of CGD. His neutrophils had undetectable superoxide generation in response to phorbol myristate acetate (PMA), whereas neutrophils from the patient’s mother produced superoxide at a near normal rate. Protein immunoblotting revealed the absence of p67-phox from the patient’s neutrophils and normal levels of p47-phox, p22-phox and gp91-phox. Both the patient and his mother had normal levels of flavocytochrome b558 as measured spectrophotometrically.

Patient 3 is the 9-year-old daughter of unrelated parents of European origin, both of whom were normal in the dihydrorhodamine (DHR) flow cytometric test for neutrophil oxygen radical production. The original diagnosis of CGD in the patient was made by the absence of NBT reduction and confirmed by the absence (with FMLP) or extremely low level (with PMA) of neutrophil superoxide generation. The absence of p67-phox and presence of p47-phox and p22-phox was demonstrated by protein immunoblot.

Patient 4 is the 2-year-old daughter of unrelated parents originally from India. She presented with pneumonia at the age of one month and lymphadenitis caused by Serratia marcescens at three months. She was diagnosed as having CGD by a negative NBT test and by the absence of superoxide generation. Her parents’ phagocytes produce normal levels of superoxide. The patient also had normal levels of flavocytochrome b558 suggesting that her defect was most likely to be in the p47-phox or p67-phox gene, but freshly isolated neutrophils were not available for immunoblotting purposes. Analysis of her genomic DNA revealed a normal exon 2 sequence in NCF-1, the gene encoding p47-phox. As more than 90% of A47° CGD patients analyzed to date are homozygous for a GT deletion in this exon, this led us to believe she had the A67° form of the disease. This was confirmed by direct sequencing of her genomic DNA as described below.

Patient 5 is the 18-month-old son of unrelated Hispanic parents. He presented at the age of 6 weeks with multiple abscesses in his liver, spleen, and lungs and was diagnosed as having CGD by the absence of NBT reduction. As freshly isolated neutrophils were unavailable for immunoblotting, we used a process of elimination similar to that described for patient 4 to identify the molecular defect. The NBT test for both parents was within the normal range (~95% positive), suggesting that the mother was unlikely to be a carrier of X-linked CGD. In addition, single-strand conforma-