Splice Site Mutations Are a Common Cause of X-Linked Chronic Granulomatous Disease

By Martin de Boer, Ben G.J.M. Bolscher, Mary C. Dinauer, Stuart H. Orkin, C.I. Edward Smith, Anders Åhlin, Ron S. Weening, and Dirk Roos

Chronic granulomatous disease (CGD) is characterized by the absence of a respiratory burst in activated phagocytes. Defects in at least four different genes lead to CGD. Patients with the X-linked form of CGD have mutations in the gene for the β-subunit of cytochrome b558 (gp91-phox). We studied the molecular defect in four patients with X-linked CGD. In a fifth family, we studied the mother of a patient with X-linked CGD who had died before our investigations. Gp91-phox messenger RNA (mRNA) was reverse transcribed into cDNA and the coding region was amplified by polymerase chain reaction into three fragments. Sequence analysis showed the absence of the exon 7, 5, 3, and 2 sequences in patients 1, 2, 3, and 4, respectively. In carrier 5, we found both normal cDNA and cDNA that lacked 57 3'-nucleotides of exon 6. We analyzed the splice sites of the flanking introns of the missing exons. In patients 1, 2, and 3, we found single nucleotide substitutions within the first five positions of the downstream 5' donor splice sites. In patient 4, a similar substitution was found at position −1 of the 3' acceptor splice site of intron 1. In carrier 5, no mutation was found in the exon 6-intron 6 boundary sequence. Instead, a single substitution was observed in exon 6 (C→A at nucleotide 633) that created a new donor splice site. Apparently, mRNA splicing occurs preferentially at this newly created splice site. We conclude that the absence of the exon sequences in the gp91-phox mRNA of these patients is due to splicing errors. Of 30 European X-linked CGD patients studied by us so far, five appear to be caused by mutations that affect correct mRNA splicing. Thus, such mutations appear to be a common cause of X-linked CGD.

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Table 1. Primers Used for DNA Amplification

<table>
<thead>
<tr>
<th>Family</th>
<th>Exon</th>
<th>Primer Sequences on Flanking Introns</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>5'-tacctggaACTATTACTAAAGATCTCGG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(sense)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-taggaactCTATGTTTCTTCTAGACA-CAGG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(antisense)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5'-tacctggaACTAGCACCTCTCT-GAATTG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(sense)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-gaattacaCTGACAAAAGACTCTAAAG-TAAACCTGCGGAGT-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(antisense)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>5'-CCTCTAGCTAAAGCCATCGG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(sense)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-TTGATGAGCGTTTGGAAATATGAG-GAACTTAG-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(antisense)</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>5'-tacctggaTCCAGCTTTGTTGGGAATC-TAC-3'</td>
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<td>(sense)</td>
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<tr>
<td></td>
<td></td>
<td>5'-gaattacaCTGACCGCAAATATTG-CATG-3'</td>
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<tr>
<td>5</td>
<td>1</td>
<td>5'-tacctggaCCTTTGGCAGCCTACCTG-GAGTGTCTATCCCATCAGG-3'</td>
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<td></td>
<td>5'-GGACATGAATCTCTCTCATCAGG-3'</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>

The lower case letters indicate the BanHI and HindIII restriction sites for sense and antisense primers, respectively.

RESULTS

Heme absorbance spectroscopy and Western blot analysis of the cytochrome b558 subunits gp91-phox and p22-phox with monoclonal antibodies showed complete absence of cytochrome b558 in the neutrophils of the four X-linked CGD patients studied. In addition, there was no residual NADPH oxidase activity detectable after stimulation of patients' neutrophils with phorbol myristate acetate (PMA), serum-treated zymosan, or both. Analysis of NADPH oxidase activity in the cell-free system showed that the defect was localized exclusively to the particulate fraction of the patients' neutrophils.

We analyzed the mRNA of the four patients' monocytes for the cytochrome b558 subunits. Both gp91-phox mRNA and p22-phox mRNA of patients 1 through 4 were present on a Northern blot. The amount and mobility of these mRNAs were similar to normal (not shown).

The gp91-phox mRNAs of the four patients and carrier 5 were amplified in three overlapping fragments as described. Figure 1 shows the analysis of the PCR fragments on an agarose gel. The PCR fragment I is apparently smaller in patients 2, 3, and 4. In patient 1 and carrier 5, apparently smaller fragments II are observed. In addition, in carrier 5, a normal-sized fragment is present, in agreement with the carrier state. All five individuals gave normal-sized fragments III (not shown). The additional smaller sized fragment I in lane b was found in three separate RNA samples from patient 1, and was not due to intron-exon mutations. Therefore, the origin of this smaller-sized fragment remains obscure.

Sequence analyses of all the PCR fragments were performed. As shown in Fig 2, the sequences of exons 7 and 5 were absent in patients 1 and 2, respectively. In patient 3, the sequence of exon 5 was absent from the cDNA, and in patient 4, the sequence of exon 2 was absent from the cDNA. In carrier 5, two coinciding sequence ladders are observed for fragment II (Fig 2, 5): panel one is identical to

Fig 1. Size analysis of the PCR-amplified fragments of gp91-phox cDNA. Ten percent of the PCR amplified DNA products were run on a NuSieve 2:1 agarose gel and stained with 0.05% (wt/vol) ethidium bromide. Lane g, molecular weight markers (1,500 to 200 bp, with 100 bp difference); lanes a through f, PCR fragments 1 (6 to 633); lanes h through m, PCR fragments II (530 to 1,210). Lanes a and h are a control and lanes b through f and i through m of patients 1 through 5, respectively.
SPLICE SITE MUTATIONS IN X-LINKED CGD

Fig 2. Sequence analyses of amplified gp91-phox cDNA. Sequence ladders of the mutated regions of the four patients and the mother of patient 5 are as indicated. The control sequences are shown to the left. The horizontal lines point to the splice junctions where exons 7, 5, 3, and 2 are skipped in patients 1, 2, 3, and 4, respectively. In carrier 5, only part of exon 6 is skipped. Splice sites between exons 6 and 7 fall within codon 225, and between exons 7 and 8 between codons 268 and 269. Splice sites between exons 4 and 5 fall within codon 113, and between exons 5 and 6 between codons 161 and 162. Between exons 2 and 3 the splice site falls between codons 47 and 48, and between exons 3 and 4 between codons 84 and 85. Splice sites between exons 1 and 2 fall between codons 15 and 16, and between exons 2 and 3 between codons 47 and 48. The newly created splice site on exon 6 (carrier 5) lies within codon 208. Codon numbering is according to Orkin. The (-) strand has been sequenced.

The control sequence, while in the other sequence the 3' end of exon 6 (nucleotide 630 through 686) is missing. These results are consistent with the apparently smaller PCR fragments shown in Fig 1.

To investigate whether the absence of the exon sequences in the patients was due to splicing errors, we amplified the flanking intron sequences from genomic DNA by PCR (see Table 1 for primer sequences). In all five individuals, PCR products of the expected sizes were obtained, indicating that the absence of the exons in the gp91-phox mRNAs was not due to a gene deletion of these sequences. When the donor-splice regions of the missing exons were sequenced, single-base substitutions were observed in the consensus donor splice sequences of introns 7 (t → a at position +2 in patient 1), 5 (a → t at position +3 in patient 2), and 3 (g → a at position +5 in patient 3) (Fig 3). In patient 4, a single-base substitution was observed in the consensus acceptor splice sequence of intron 1 (g → a at position −1). No other mutations were identified. Therefore, we conclude that the absence of exons 7, 5, 3, and 2 in the gp91-phox mRNA of patients 1, 2, 3, and 4, respectively, is due to mRNA splicing defects resulting in exon skipping.

Fig 3. Sequence analysis of PCR-amplified genomic DNA containing the mutated sites. (1) Sequence of exon 7-intron 7 boundary; (2) sequence of exon 5-intron 5 boundary; (3) sequence of exon 3-intron 3 boundary; (4) sequence of exon 2-intron 1 boundary; (5) sequence of the newly created splice site on exon 6. The sequence of each patient is as indicated in the figure, with the control sequence at the left. Arrows point to the mutations. The (-) strand has been sequenced.
Fig 4. Mutations in the gp91-phox gene causing X910 CGD. Short stretches are shown of the nucleotide sequences and predicted amino acid sequences that contain the mutations. Upper case letters indicate coding sequences, and lower case letters indicate intron sequences. Exon sequences in genomic DNA are shown within boxes. Arrowheads in the cDNA sequences show the splice sites. Arrows indicate the mutations in the patients. The amino acid numbering on top of the sequences is according to Orkin.27 In contrast to the published gene structure,29 we found a CCAG sequence at the acceptor site of intron 6.
Figure 4 summarizes the results of the mutation analyses of these four patients.

In the female carrier of family 5, no mutations in the exon 5-intron 5 boundary sequence were observed. Instead, a single-base substitution was found within exon 6 (C → A at position 635) in addition to the normal sequence, consistent with the X-linked carrier state (Fig 3). No other mutations were identified. Because part of the mRNA of this individual lacked the 3’ part of exon 6, starting at nucleotide 631, it appears that this mutation has created a new donor splice site at nucleotide 630 to 635 (GTAAA). This is shown in Fig 4.

To confirm the mutations found, fragments containing the mutated sites were PCR amplified from genomic DNA isolated from all five individuals, some available family members, and 72 control alleles. For patients 1 to 4, these PCR products were hybridized with allele-specific oligonucleotides (data not shown). Only the PCR fragments amplified from the DNA of the four patients hybridized to the oligonucleotides containing the corresponding mutant sequence. Because the mutation in family 5 predicts the disappearance of a recognition site for the restriction enzyme Rsal, the PCR fragments (205 bp) amplified from the genomic DNA derived from the mother and sister of patient 5 and 36 unrelated female donors were digested with this enzyme. The PCR products of the 72 control alleles were completely digested by Rsal into two fragments of 112 and 93 bp, respectively, whereas only half of the PCR product of the alleles of the mother and sister of patient 5 were digested (not shown).

**DISCUSSION**

In the inherited disease CGD, phagocytes fail to produce superoxide upon stimulation due to a defect in the NADPH oxidoreductase. CGD is a very heterogeneous disorder, and defects in at least four different gene products can cause CGD. The most common form of CGD is the X-linked form, in which gp91-phox (the β-subunit of cytochrome b558) is affected.

The molecular defect has been characterized in a number of X-linked CGD patients. Interestingly, all patients were found to have a different genetic defect, ranging from gene deletions to point mutations in the coding region. In the four patients we describe in this report, deleted gp91-phox mRNAs were found. Sequence analysis of the gp91-phox mRNA showed the absence of exon 7, 5, 3, and 2 sequences, respectively. Analyses of the patients’ genomic DNA showed that these exons were not deleted in the gene. Point mutations in the consensus donor splice regions were identified in patients 1, 2, 3, and 3, which cause aberrant mRNA splicing with exon skipping. Because the abnormal splicing alters the reading frame in patients 1 and 2, these mutations predict the occurrence of frameshifts and premature termination of the synthesized protein in these patients at positions 230 and 133, respectively. The absence of exon 3 in the mRNA of patient 3 leads to an in-frame deletion and therefore to a shortened polypeptide. In patient 4, a point mutation was found at position −1 of the consensus acceptor splice region at the intron 1-exon 2 boundary, which apparently causes aberrant mRNA splicing with skipping of exon 2. This also leads to an in-frame deletion and therefore to a shortened polypeptide.

In the mother of patient 5, a point mutation was found in the coding region of exon 6. Apparently, this mutation has created a new donor splice site that is preferred over the original, nonmutated donor splice site of intron 6. The abnormal mRNA splicing results in the frameshift deletion of the 3’ 57 nucleotides of exon 6, and creates a premature stop codon at amino acid position 206. An alternative consequence of the C-633 → A substitution found in family 5 is the creation of a TAA stop codon at amino acid position 207 in a correctly spliced product, which predicts premature termination of protein synthesis. However, this transcript was not detected by DNA sequencing of cDNAs (Fig 2), which showed a truncated exon 6 sequence in apparently the same amount as the wild-type sequence (Fig 2). We presume, therefore, that patient 5 suffered from CGD due to alternative splicing of exon 6 in the gp91-phox mRNA.

Mutations in or around splice junctions are a well-recognized cause of inherited human disease, with β-thalassemia being the first and perhaps best studied example. Mutations around the 5’ splice site can lead to either alternative splicing with use of cryptic splice sites or exon skipping, as is reported here. Other reports of exon skipping due to donor splice site mutations include Ehlers-Danlos syndrome type IV and acatalasemia. Of 30 European X-linked CGD patients studied by us so far, 5 appear to be caused by mutations that affect correct mRNA splicing. Thus, such mutations are a common cause of X-linked CGD.

**REFERENCES**

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