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Research Article

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Recurrent Nonsense Mutations in the Growth Hormone Receptor from Patients with Laron Dwarfism

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Abstract

In addition to its classical effects on growth, growth hormone (GH) has been shown to have a number of other actions, all of which are initiated by an interaction with specific high affinity receptors present in a variety of tissues. Purification of a rabbit liver protein via its ability to bind GH has allowed the isolation of a cDNA encoding a putative human growth hormone receptor that belongs to a new class of transmembrane receptors. We have previously shown that this putative growth hormone receptor gene is genetically linked to Laron dwarfism, a rare autosomal recessive syndrome caused by target resistance to GH. Nevertheless, the inability to express the corresponding full-length coding sequence and the lack of a test for growth-promoting function have hampered a direct confirmation of its role in growth. We have now identified three nonsense mutations within this growth hormone receptor gene, lying at positions corresponding to the amino terminal extremity and causing a truncation of the molecule, thereby deleting a large portion of both the GH binding domain and the full transmembrane and intracellular domains. Three independent patients with Laron dwarfism born of consanguineous parents were homozygous for these defects. Two defects were identical and consisted of a CG to TG transition. Not only do these results confirm the growth-promoting activity of this receptor but they also suggest that CpG doublets may represent hot spots for mutations in the growth hormone receptor gene that are responsible for hereditary dwarfism. (*J. Clin. Invest.* 1991. 87:1098–1102.) **Key words:** human growth hormone receptor • stop mutation • Laron dwarfism

Introduction

Growth hormone (GH)¹ exhibits a variety of biological effects on somatic growth, development, metabolism (1), and the im-

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1. *Abbreviations used in this paper:* GH, growth hormone; GH-BP, GH-binding protein; GH-R, GH-receptor; LTD, Laron-type dwarfism; PCR, polymerase chain reaction.

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mune system (2). While these effects are well understood, little is known of the mechanisms by which GH elicits the intra- and intercellular events leading to physiological responses. Nevertheless, it is widely assumed that the multiple actions of GH are initiated by the binding of the hormone to specific receptors in the plasma membrane of target cells, followed by a series of postreceptor events. Specific high affinity GH binding sites, presumed to be receptor proteins, have been extensively described (3). More recently, a specific binding protein has been purified from rabbit liver (4) and its sequence used to obtain cDNA clones encoding both rabbit and human putative GH receptors (GH-R) (5). On the basis of these nucleotide sequences, putative GH-R cDNAs of various species (6–8) have been cloned. These cDNAs code for proteins containing a single membrane-spanning domain; they share sequence identity with prolactin receptors isolated in a variety of species (9–12) and certain homologies with a new cytokine receptor family (13).

Although when expressed in cultured cells clones of the putative rabbit GH-R and a truncated human GH-R give rise to specific binding (5), the growth-promoting activity of these receptors has not been demonstrated, mainly because sequence analysis provides no clues as to the signaling pathway from which an *in vitro* functional test (related to receptor activation following GH binding) could be derived. An alternative approach is to seek mutations of this receptor in selected patients with dwarfism. In a first step toward this goal, we have recently shown (14), using linkage analysis, that patients with Laron-type dwarfism (LTD) (15), a rare autosomal recessive GH-resistance syndrome (16), are good candidates for abnormalities within this gene. However, the inability to express a full-length cDNA in eucaryotic cells has excluded any possibility of testing the functional importance of the molecular variants already described (14, 17).

To circumvent these difficulties, we have designed a strategy based on the identification in LTD patients of unambiguous molecular defects, i.e., stop mutations, in the coding sequence of this gene.

Methods

Patients. We examined six unrelated patients with clinical and biological features of Laron dwarfism, i.e., severe growth retardation with very low serum levels of insulin-like growth factor I, despite increased levels of GH. All the patients were born from consanguineous unions; four of them were of Mediterranean extraction (families A, B, C, and D); the last two patients were of Northern European descent (families E and

F). Plasma GH-binding proteins were evaluated as previously described (14).

Southern analysis. DNA samples prepared from peripheral blood leukocytes were digested with the restriction enzymes Eco RI, Hind III, Eco RV, Bam HI, and Pst I, and run on a 1% agarose gel. After transfer to a nitrocellulose membrane, the filters were hybridized to a GH-R cDNA probe synthesized with primers P1 (5'-GCTCTGTGATCA-GAGGCGAA-3') and P2 (5'-GGTTTAAACATTGTTTTGGC-3') (Fig. 1 a) designed to amplify the full-length coding sequence by means of the polymerase chain reaction (PCR) (18), as previously described (14). These PCR primers are based on published cDNA sequence data (5).

Amplification of genomic DNA by the polymerase chain reaction. Exons 4 and 7 were amplified by the polymerase chain reaction catalyzed by Taq DNA polymerase using the patients' genomic DNA as described previously (18) and primers homologous to flanking intronic sequences (17): P3 (5'-AGGATCACATATGACTCACC-3') and P4 (5'-AGGAAAATCAGAAAGGCATG-3'), and P5 (5'-TAGTGTT-CATTGGCATTGAG-3') and P6 (5'-ACAAAAGCCAGTTAGC-TAC-3'), respectively (Fig. 1 b).

Direct sequence analysis of PCR products. Using an asymmetric ratio of primers (50 pmol/1 pmol) in the polymerase chain reaction, we amplified exons 4 and 7 (with two sets of primers: P3-P4 and P5-P6, respectively) from 1 µg of leukocyte DNA from each individual (Fig. 1 b). The single-strand DNA was used directly as a template for DNA sequencing as described (19). The sequencing primer was the same as the 3' primer used for the polymerase chain reaction. An intronic segment (located between exons 9 and 10) that has been shown to contain polymorphic sites (14) was amplified and directly sequenced as previously described (14).

Numbering systems for amino acids, nucleotides, exons, and frameworks. The amino acids and nucleotides in the coding sequence of the GH-R are numbered according to Leung et al. (5). Exons are numbered according to Godowsky et al. (17). The association of nucleotide sequence polymorphisms within the GH-R gene defines six gene frameworks that are numbered according to Amselem et al. (14).

Results

Determination of nucleotide sequences of exons 4 and 7. After ruling out a major deletion or rearrangement at the GH-R locus by Southern analysis, we sought single point mutations at this locus. One mechanism for generation of mutations involves spontaneous deamination of 5-methylcytosine to thymine (20), resulting in C to T transitions if deamination originally occurs on the coding strand, and G to A transitions if

deamination originally occurs on the noncoding strand. Since the modified base 5-methylcytosine occurs primarily at CpG dinucleotides in vertebrates (21), we hypothesized that some mutations in LTD would be at such sites. We screened the 2-kb full-coding region of the putative GH-R cDNA for the presence of CpG doublets. Among the 17 CpG dinucleotides detected (see Fig. 1 a), we selected those that belong to a CGA (arginine) codon. These codons are important because a CGA to TGA transition creates a premature termination codon, a defect with known deleterious functional consequences. Only two CpG doublets lying in exons 4 and 7 (nucleotides 181-182 and 703-704, respectively) were found to occur in a CGA codon (arginines 43 and 217, respectively) (Fig. 1 a).

Using the sequences of the introns flanking exon 4 and 7 as a guide, we selected two pairs of oligonucleotide primers that allowed the full protein-coding portion of these exons and flanking splice donor and acceptor sites to be amplified. Thereafter, direct genomic sequencing (19) was used to determine the nucleotide sequences of the amplified genomic DNA (Fig. 2). This method has several advantages, including the ability to determine the sequences of both alleles of the GH-R gene simultaneously. At positions where the two alleles differ, two bands will be visible in the sequencing ladder; each of the two bands will show approximately half the normal intensity.

We used this approach to identify mutations in the GH-R genes from six patients with Laron dwarfism. In all six patients, no plasma GH binding activity was detected (data not shown). Three patients, members of consanguineous pedigrees, were found to be homozygous for a nonsense mutation: (a) patients from families C (subject C3, Fig. 2 a) and D (data not shown) were homozygous for a mutation substituting the opal chain termination codon (TGA) for arginine (CGA) at codon 43 (R43X); (b) patient E3 was homozygous for a C to A transversion resulting in the conversion of a cysteine (TGC) into a premature termination signal (TGA) at codon 38 (C38X) (Fig. 2 b). In addition to those three patients, we also determined the nucleotide sequence of both alleles of the GH-R gene of their parents and siblings. As expected from autosomal recessive inheritance, we found the parents heterozygous for the mutant allele (Fig. 2 a: C1 and C2; Fig. 2 b: E1 and E2), whereas the healthy siblings were genotypically normal (C4, Fig. 2 a) or carriers (family D, data not shown). Using this targeted strategy for mutation detection, our analysis did not reveal any abnor-

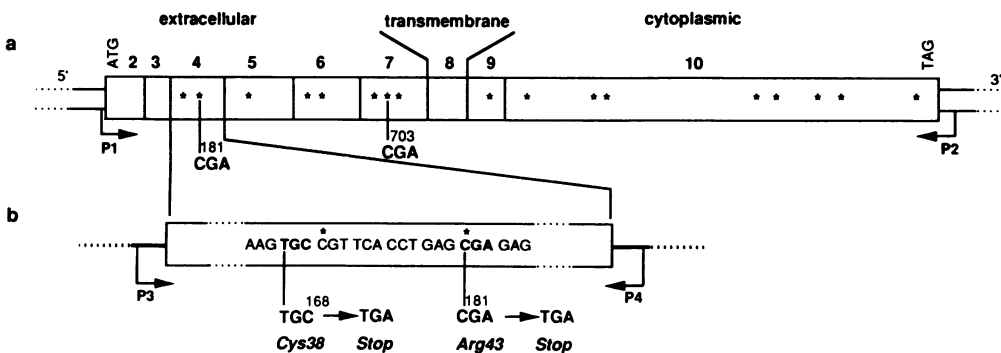


Figure 1. (a) Diagrammatic representation of the human growth hormone receptor cDNA. (b) Nucleotide and amino acid changes found within exon 4 in patients with Laron dwarfism. The open reading frame is indicated by an open box, the untranslated mRNA region by double lines, and introns by thick black lines. The numbers above the cDNA indicate the corresponding exons according

to Godowsky et al. (17). The arrows indicate locations and directions of PCR primers used in cyclic thermal DNA amplifications. The position of each CpG dinucleotide is marked by an asterisk within the coding sequence. Two CpG doublets (nucleotides 181-182, 703-704) lie in a CGA codon identified below the cDNA in exons 4 and 7, respectively. A partial sequence of exon 4 is represented within the open box at the bottom.

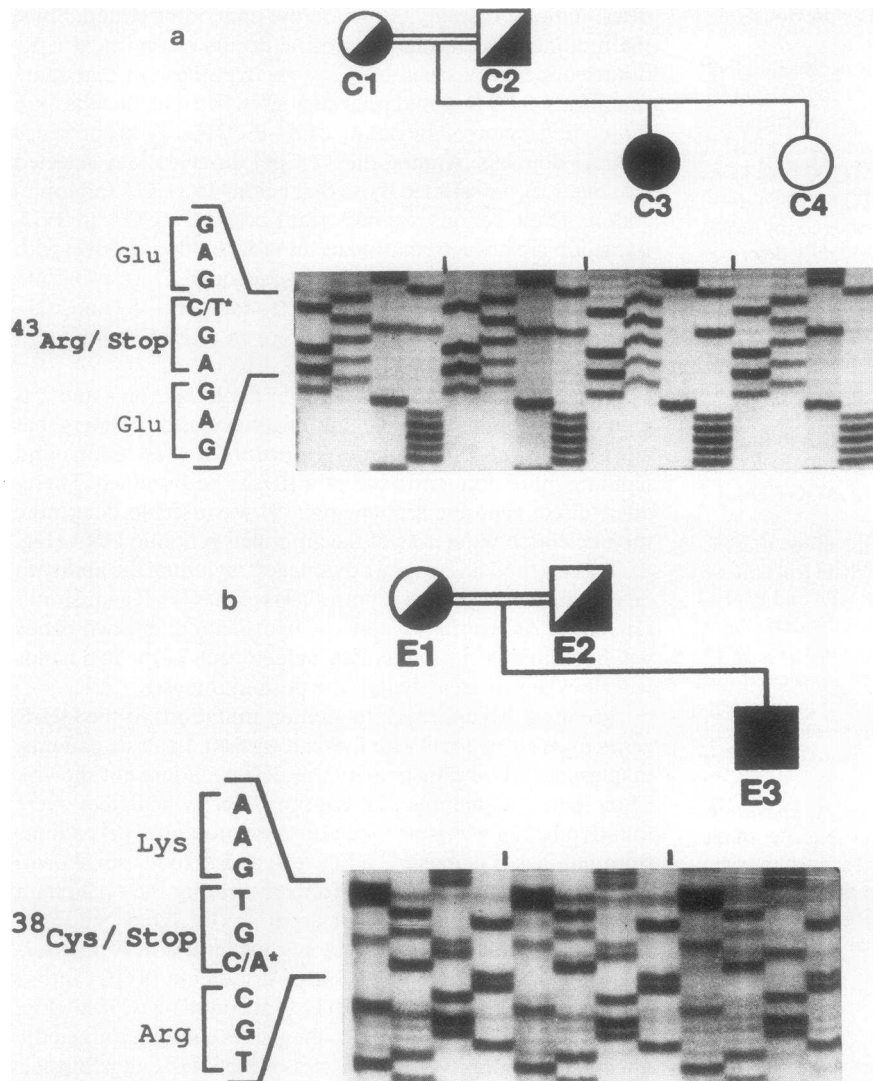


Figure 2. (a) Partial pedigree and corresponding GH-R nucleotide sequence analysis of two families with autosomal recessive Laron dwarfism. The solid symbols signify obvious clinical disease phenotype, and the partially filled symbols indicate heterozygote carrier status for the disease allele. The double bar between the parents indicates consanguineous unions. The order of nucleotides is A, G, C, T. Patient C3 is homozygous for a C-to-T transition within codon 43 generating a TGA codon (*stop*) in place of a CGA codon (*arginine*). The phenotypically normal parents (C1 and C2) bear the wild type and mutant (*asterisk*) alleles, whereas the sibling (C4) bears only the wild type allele. (b) Patient E3 is homozygous for a C to A transversion resulting in the conversion of a cysteine (TGC) into a premature termination signal (TGA) at codon 38. Similarly, the heterozygous parents (E1 and E2) display both the wild type and mutant (*asterisk*) alleles.

mality in exons 4 and 7 and their adjacent splice acceptor and donor sites in patients from families A, B, and F.

Nucleotide polymorphisms associated with the growth hormone receptor mutant alleles. We have previously described the presence of six polymorphic sites within an intervening sequence located in the part of the gene (flanked by exons 9 and 10) encoding the intracellular domain of the receptor. Their association has been shown to define six GH-R gene frameworks (14). The mutation R43X (families C and D) was found to be associated with two different frameworks (III and II, respectively), and mutation C38X with framework I (family E).

Discussion

In these investigations, we identified nonsense mutations in the growth hormone receptor gene in patients with Laron dwarfism, a rare autosomal recessive syndrome associated with GH resistance. We used a strategy based on the observation that

recurrent mutations occur frequently at CpG sites within a number of genes and give rise to genetic diseases (22). With this in mind, we sought to characterize unambiguous mutations, i.e., stop mutations, by analyzing the nucleotide sequence of each exon that contains a CGA codon (*arginine*) that is prone to generate a TGA stop codon. In the GH-R coding sequences, only two exons (4 and 7) were found to contain such a codon.

Studying genomic DNA from six unrelated patients with LTD, we first looked for a gene deletion by means of Southern blotting and hybridization with a full-length coding cDNA probe. Genomic DNA from control subjects and LTD patients displayed the same pattern, ruling out a major structural defect in the patients. We then focused our study on exons 4 and 7 to look for a stop mutation. Direct sequencing of the PCR-amplified exons allowed us to analyze these exons and their adjacent consensus sequences in a single step.

At one of the expected sites (*arginine* 43), we characterized a CGA to TGA transition (R43X) in two unrelated patients, born of consanguineous parents (families C and D), and homo-

zygous for this defect. In both families, the parents displayed both the normal and mutant alleles. These individuals were of Mediterranean extraction, and determination of their GH-R frameworks showed that this mutation was associated with two different chromosomal backgrounds (frameworks III and II, respectively).

A different stop mutation (C38X), TGC (cysteine 38) to TGA (stop), located in the same exon (nucleotide 168) was detected in a third patient (E3), but it did not involve a CpG dinucleotide (Figs. 1 *b* and 2 *b*). The patient, born of Northern European consanguineous parents, was homozygous for this defect that was associated with framework I.

Only two molecular variants have so far been described in LTD patients. One is a missense mutation (phenylalanine 96 to serine) (14), while the other is a partial and complex gene deletion (17). Due to difficulties in expressing the full-length cDNA in eucaryotic cells, it has not been possible to test the functional importance of the two variants in vitro. The partial deletion involves nonconsecutive exons (exons 3-5-6 from a gene that contains at least 10 exons). Several observations (5, 17) indicate that alternative splicing occurs during expression of the GH-R gene, but very little is known about the significance of this phenomenon, in particular about its possible role in the generation of receptor isoforms. Therefore, it cannot be excluded that a shorter mRNA could lead to the synthesis of a functional receptor. The nature and position of the mutations we have now identified preclude the expression of the putative functional GH-R containing 620 residues: codons 38 and 43 lie early in the amino terminus of the extracellular domain of the GH-R; a nonsense mutation at these positions results in a truncated protein and deletes a large portion of both the GH-binding domain and the full transmembrane and intracellular domains. Overall, these genetic defects identified in LTD patients confirm the role of the GH-R cloned by Leung et al. (5) in the transduction of the growth signal, and further illustrate the molecular heterogeneity of LTD.

It is interesting that sequence homology between the extracellular domain of the GH-R and a soluble plasma growth hormone-binding protein (GH-BP) has been observed (23, 24). In the light of various observations (5, 25, 26) suggesting that GH-R could give rise to GH-BP, we expected the nucleotide changes in codons 38 and 43 to result in the absence of GH binding activity of the plasma GH-BP; this was indeed the case. This result throws light on the relationship between GH-R and GH-BP. In theory, GH-BP could be synthesized from a second gene (distinct from the GH-R gene) or produced either by proteolytic cleavage or different processing of a precursor of the receptor mRNA. The identification of the molecular defects within the extracellular domain of the receptor in patients with an absence of detectable GH-BP binding activity allows us to rule out the first hypothesis. This conclusion is in agreement with the sequence data obtained for rat and mouse GH-Rs and GH-BPs suggesting that GH-BP in these species is likely to be an alternative spliced form of the receptor (6, 7).

Finally, two of the stop mutations described involve a CpG dinucleotide. CpG is the most common site of methylation in mammalian DNA, and deamination of 5-methylcytosine will lead to a C to T transition. Our results illustrate the power of this mutation detection strategy focused on CGA (arginine) codons of GH-R cDNA. Such codons are prone to generate

TGA stop mutations, as borne out by our findings. Since the same mutation (arginine 43 to stop) was found to be associated with two different alleles (frameworks II and III), we can conclude that it occurred independently. This description of a recurrent mutation in the growth hormone receptor responsible for a form of hereditary dwarfism supports the notion that CpG doublets are hot spots for mutations in this gene.

It is likely that mutations causing Laron dwarfism will encompass a wide range of genetic alterations. The delineation of the underlying genetic defect in other patients with Laron dwarfism will lead to a better understanding of the functional organization of GH-R and related receptors as well.

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