

The New England Journal of Medicine

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Volume 333

OCTOBER 26, 1995

Number 17

MUTATIONS OF THE GROWTH HORMONE RECEPTOR IN CHILDREN WITH IDIOPATHIC SHORT STATURE

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Abstract Background. Short stature in children who are not deficient in growth hormone (GH) is probably caused by a variety of defects. Some children with idiopathic short stature have low serum concentrations of GH-binding protein, which is derived from the GH receptor. The possibility that low serum concentrations of GH-binding protein might indicate partial insensitivity to GH led us to investigate possible defects in the gene for the GH receptor in children with idiopathic short stature and low serum concentrations of GH-binding protein.

Methods. We studied 14 children with idiopathic short stature who were selected on the basis of normal GH secretion and low serum concentrations of GH-binding protein. Analysis of single-strand conformation polymorphisms and DNA sequencing were both used to identify mutations in the GH-receptor gene.

Results. Mutations in the region of the GH-receptor gene that codes for the extracellular domain of the receptor were found in 4 of the 14 children, but in none of 24 normal subjects. One of the four children with mutations was a compound heterozygote, with one mutation that reduced the affinity of the receptor for GH and a second mutation that may affect a function other than ligand binding. The remaining three children had single mutations in one allele of the gene. One mutation introduced a premature termination codon, and two caused substitutions of single amino acids in a structurally conserved domain of the receptor.

Conclusions. Some children with idiopathic short stature may have partial insensitivity to GH due to mutations in the GH-receptor gene. (N Engl J Med 1995;333:1093-8.)

THE cause of growth failure in the majority of short children who do not have a deficiency of growth hormone (GH) is unknown. These otherwise normal children with idiopathic short stature secrete normal amounts of GH in response to pharmacologic stimulation.¹ GH stimulates growth by binding to GH receptors, thereby stimulating the production of insulin-like growth factor I (IGF-I). Serum contains a GH-binding protein that is identical to the extracellular domain of the GH receptor. The importance of the GH receptor in modulating the growth-promoting action of GH is demonstrated by the abnormal growth of children with complete insensitivity to GH due to inactivating mutations in the gene for the GH receptor (Laron dwarfism).²

Children with idiopathic short stature may secrete GH normally and yet have a defect in the ability of target cells to respond to GH. Such a defect could occur

in either the GH receptor or the intracellular mediators of GH signaling. Serum concentrations of GH-binding protein³ are low in many children with idiopathic short stature,^{4,5} suggesting that such children may have abnormalities in the gene for the GH receptor. This possibility is supported by the observation that children with lower serum concentrations of GH-binding protein have lower serum IGF-I concentrations and higher mean 12-hour serum GH concentrations (suggesting a defect in the functioning of IGF-I as a negative regulator of GH secretion) than children with idiopathic short stature who have normal serum concentrations of this protein.⁶ Although most children with idiopathic short stature respond to treatment with recombinant GH with increases in their growth rates,⁷ their responses are more limited than those of children with GH deficiency who are treated similarly. This mild form of insensitivity to GH could be caused by less disruptive mutations in the gene for the GH receptor than are found in children with complete GH insensitivity (those with Laron dwarfism).²

We have proposed^{4,6} that the reduced serum concentrations of GH-binding protein in children with idiopathic short stature may serve as a marker for partial insensitivity to GH and may indicate mutations in the GH-receptor gene. To test this hypothesis, we analyzed

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Supported by Genentech, Inc. Dr. Carlsson is a consultant for Genentech.

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the coding region of that gene for mutations in a subgroup of 14 children with idiopathic short stature and low serum concentrations of GH-binding protein. We detected mutations in four of the children that would be expected to alter the structure of the extracellular domain.

METHODS

Study Subjects

We studied 14 children with idiopathic short stature, all but one of whom were selected from the Genentech National Cooperative Growth Study (Patient 1 was identified separately). To be included in that study, children had to be evaluated to determine the hormonal basis of their short stature and had to be willing to be followed thereafter. In the present study, standard-deviation (SD) scores for height and serum concentrations of GH-binding protein and IGF-I were calculated by subtracting the mean reference values for those variables in normal subjects from the values in the study subjects and dividing the difference by the standard deviation in the normal subjects. Children of both sexes were selected who had SD scores for height below -2.5 (except Patient 1),⁸ SD scores for the serum concentration of GH-binding protein (as measured by a ligand-mediated immunofunctional assay³ or, in the case of Patient 1, by charcoal separation⁹) below -2 , SD scores for the serum IGF-I concentration (as determined by the Nichols Institute, San Juan Capistrano, Calif.) below 0, maximal stimulated serum GH concentrations (as measured by various assays after stimulation by clonidine, insulin, glucagon, levodopa, or arginine) above $10 \mu\text{g}$ per liter, and no systemic illness.

The relatives of two patients (Patients 2 and 4) were studied to confirm the heritability of the mutations. Twenty-four normal adults served as controls. The statistical significance of differences between populations was calculated by Fisher's exact test.

Sample Preparation and Amplification

Lymphocytes were isolated from peripheral blood with either cell-separation tubes (LeucoPrep, Becton Dickinson, Franklin Lake, N.J.) or lymphocyte-separation medium (LSM, Organon Teknika, Durham, N.C.) and were transformed by Epstein-Barr virus (EBV).¹⁰ DNA was isolated both from EBV-transformed lymphocytes and directly from fresh lymphocytes (to control for EBV-induced mutations; QIAamp Blood Kit, Qiagen, Chatsworth, Calif.). Genomic fragments of the GH-receptor gene specific for exons 2 through 9 and their flanking splice sites were amplified by the polymerase chain reaction (PCR) with intronic primers. The coding portion of exon 10 was amplified in three overlapping fragments. The PCR products were electrophoresed in 2 percent agarose gels to test for contamination and verify fragment size.

Total RNA (5 to $10 \mu\text{g}$) was prepared from the EBV-transformed lymphocytes by the acid-phenol method¹¹ and was reverse-transcribed (RT kit, Perkin-Elmer, Foster City, Calif.) with random primers (Promega, Madison, Wis.). Complementary DNA (cDNA) from the GH receptor was amplified in a nested PCR (cycle conditions and primer sequences for both genomic PCR and reverse-transcriptase PCR are available on request).

Analysis of Single-Strand Conformation Polymorphisms

In the analysis of single-strand conformation polymorphisms of the products from each PCR reaction, 2 to $4 \mu\text{l}$ of the reaction mixture was mixed with an equal volume of loading buffer, denatured at 100°C for two minutes, and placed on ice. The samples were electrophoresed at room temperature in $0.5\times$ MDE gels (AT Biochem, Malvern, Pa.) with either 1 or 10 percent glycerol, according to the instructions of the manufacturer, after which the gels were dried and processed for autoradiography.

DNA Sequencing

Mutations detected as aberrant bands by analysis of single-strand conformation polymorphisms were confirmed by sequencing the PCR

products by the dye-terminator method and by sequencing 12 subclones by the dye-primer method with an ABI373 sequencer or an Ampli-Cycle kit (Applied Biosystems Division, Perkin-Elmer) and ^{32}P -labeled α -deoxyadenosine triphosphate (Dupont-New England Nuclear, Wilmington, Del.). Errors caused by *Taq* polymerase were avoided by sequencing the PCR products from different reactions and by sequencing 12 or more subclones of each PCR fragment.

Assay of GH Binding

Recombinant extracellular domains of the receptor that contained the mutations were engineered by oligonucleotide-mediated site-directed mutagenesis, expression in *Escherichia coli*, and purification as described elsewhere.¹²⁻¹⁴ Affinity for GH was determined by competitive displacement of GH from the mutant receptors with radioiodinated GH.¹⁵ Dissociation constants were calculated by Scatchard analysis. A monoclonal antibody (Mab5) against the GH receptor was used to precipitate the GH-receptor-GH complex. This antibody prevents homodimerization of the receptor, allowing the dissociation constant for the initial 1:1 interaction to be determined independently of the effects of dimerization.^{12,16}

RESULTS

The clinical characteristics of the 14 children with idiopathic short stature are shown in Table 1. PCR fragments of individual exons of the GH-receptor gene that migrated with altered mobility were detected in 4 of these children (Patients 1, 2, 4, and 7) (Fig. 1), but in the 24 normal subjects only known polymorphisms in the DNA were found (in exon 6, guanosine or adenosine at base pair 558; and in exon 10, cytosine or adenosine at base pair 1630)^{17,18} (data not shown). Thus, there was a higher frequency of mutations of the GH-receptor gene in the children with idiopathic short stature who had low concentrations of GH-binding protein than in the normal subjects ($P = 0.014$). Each of the genomic PCR fragments thought to carry a mutation was sequenced in order to characterize the alteration that caused the aberrant band (data not shown). The GH-receptor messenger RNA (mRNA) of Patients 1 through 9 was also analyzed by reverse-transcriptase PCR; all the fragments were of the predicted sizes, and thus splicing alterations were ruled out (data not shown).

In the DNA from Patient 1, one of the genomic PCR fragments of exon 7 was aberrant (Fig. 1A) on analysis of single-strand conformation polymorphisms. DNA sequencing showed that a mutation at base pair 686 caused an arginine to be replaced with a histidine at amino acid 211 in the mature protein (Arg211His). No other abnormalities affecting the extracellular domain of the GH receptor were detected by sequencing PCR products from the exons encoding the extracellular domain. The likelihood that this patient carried a second mutation was therefore low. Patient 1 was found to be responsive to GH on the basis of an increase in the serum IGF-I concentration that occurred after the administration of GH (serum IGF-I, $56 \mu\text{g}$ per liter at base line and $179 \mu\text{g}$ per liter after four days of treatment with 0.1 mg of GH per kilogram of body weight per day) and an increase in the growth rate from less than 2 to 6.5 cm per year with GH therapy (0.05 mg per kilogram per day) (Table 1).

DNA from Patient 2 showed an aberrant band in one

genomic PCR product from exon 5 on analysis of single-strand conformation polymorphisms (Fig. 1B). DNA sequencing identified a mutation at position 418 in the cDNA that introduced a stop codon in place of cysteine at amino acid 122 (Cys122Stop). This mutant allele was most likely a null mutation, with no functional protein being produced. No other mutation was detected in this patient. Analysis of DNA from both the mother and the father of Patient 2 indicated that he had inherited the mutation from his mother (Fig. 2A). During the first year of treatment with 0.3 mg of GH per kilogram per week, his growth rate increased from 4.1 to 5.7 cm per year (Table 1), indicating a moderate response to exogenous GH. He had a puberty-associated growth spurt of 10.3 cm per year during the second year of treatment.

Patient 4 had abnormal bands on analysis of single-strand conformation polymorphisms in exons 4 and 6 (Fig. 1C and 1D) and reverse-transcriptase PCR fragments covering these regions. This child was a

Table 1. Clinical Characteristics of the 14 Children with Idiopathic Short Stature.

PATIENT No.	SEX/AGE* (YR)	HEIGHT	SERUM GH-BINDING PROTEIN	SERUM IGF-I	MAXIMAL SERUM GH†	GROWTH RATE		RESPONSIVE TO GH‡
						BEFORE TREATMENT	DURING 1ST YR OF GH THERAPY	
			SD score§	µg/liter	centimeters per year			
1	M/3.0	-5.1	Not detectable¶	-0.7	42.0	<2.0	2.2, 6.5	Yes
2	M/11.6	-3.2	Not detectable¶	-1.2	18.8	4.1	5.7	Yes
3	M/7.8	-3.3	-2.7	-4.5	12.5	3.1	7.1	Yes
4	M/8.7	-2.9	-2.8	-4.2	20.7	5.0	6.0	No
5	M/7.8	-3.4	-2.6	-3.7	48.9	2.9	Not treated**	—
6	F/14.6	-5.5	-2.8	-8.2	18.0	2.7	7.8	Yes
7	F/3.5	-3.2	-2.6	-2.3	19.2	7.1	Not treated	—
8	M/9.3	-2.5	-2.8	-6.1	18.7	2.1	7.2	Yes
9	M/10.0	-3.2	-3.0	-2.3	20.8	1.2	7.3	Yes
10	M/7.9	-3.2	-2.8	-3.0	15.6	4.4	8.4	Yes
11	M/9.8	-1.6	-2.3	-2.0	19.3	4.2	9.1	Yes
12	F/7.9	-3.1	-2.5	-3.5	21.6	6.3	9.0	Yes
13	M/12.8	-3.4	-2.3	-4.1	16.3	1.5	8.4	Yes
14	M/8.1	-3.5	-2.0	-3.2	11.7	3.9	8.3	Yes

*At the time of enrollment in this study.

†Denotes the maximal serum GH concentration in response to various stimuli, including clonidine, insulin, glucagon, levodopa, and arginine.

‡Responsiveness to GH was defined as an increase in the serum IGF-I concentration or in the growth rate during the first year of treatment with GH.

§SD scores were calculated by the following formula: (value in study patient) - (reference mean value)/(reference SD). The reference populations used were those of Hamill et al.⁸ for height, Carlsson et al.⁴ for serum GH-binding protein, and a group of patients studied at the Nichols Institute (San Juan Capistrano, Calif.) for serum IGF-I.

¶The serum concentration of GH-binding protein was less than 2 percent of the ¹²⁵I-labeled human GH bound in 50 µl of serum in Patient 1, and less than 31.2 pmol per liter of serum in Patient 2.

||At doses of GH of 0.03 and 0.05 mg per kilogram per day, respectively.

**This patient was not compliant with GH therapy.

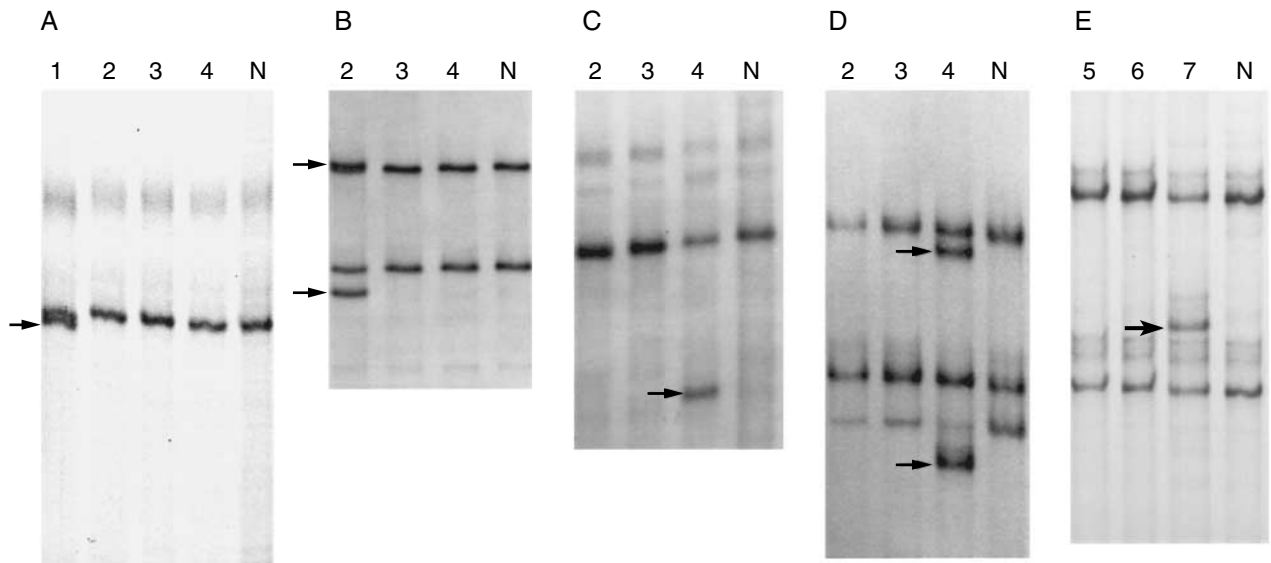


Figure 1. Analysis of Single-Strand Conformation Polymorphisms in PCR Fragments of Genomic DNA from Four Children with Idiopathic Short Stature Who Had Mutations in Their GH-Receptor Genes.

A normal subject (N) is also included in each panel. Panel A shows exon 7 in Patients 1, 2, 3, and 4; the extra band in the product from Patient 1 (arrow) indicates the presence of a mutation. Panel B shows exon 5 in Patients 2, 3, and 4, with aberrant bands (arrows) in the sample from Patient 2. Panel C shows exon 4, and Panel D exon 6, in Patients 2, 3, and 4; aberrant bands (arrows) are seen in the two samples from Patient 4. Panel E shows exon 7 in Patients 5, 6, and 7, with an aberrant band (arrow) in the sample from Patient 7. All the samples were electrophoresed in a 0.5× MDE gel, with either 10 percent (Panels A, B, and C) or 1 percent (Panels D and E) glycerol.

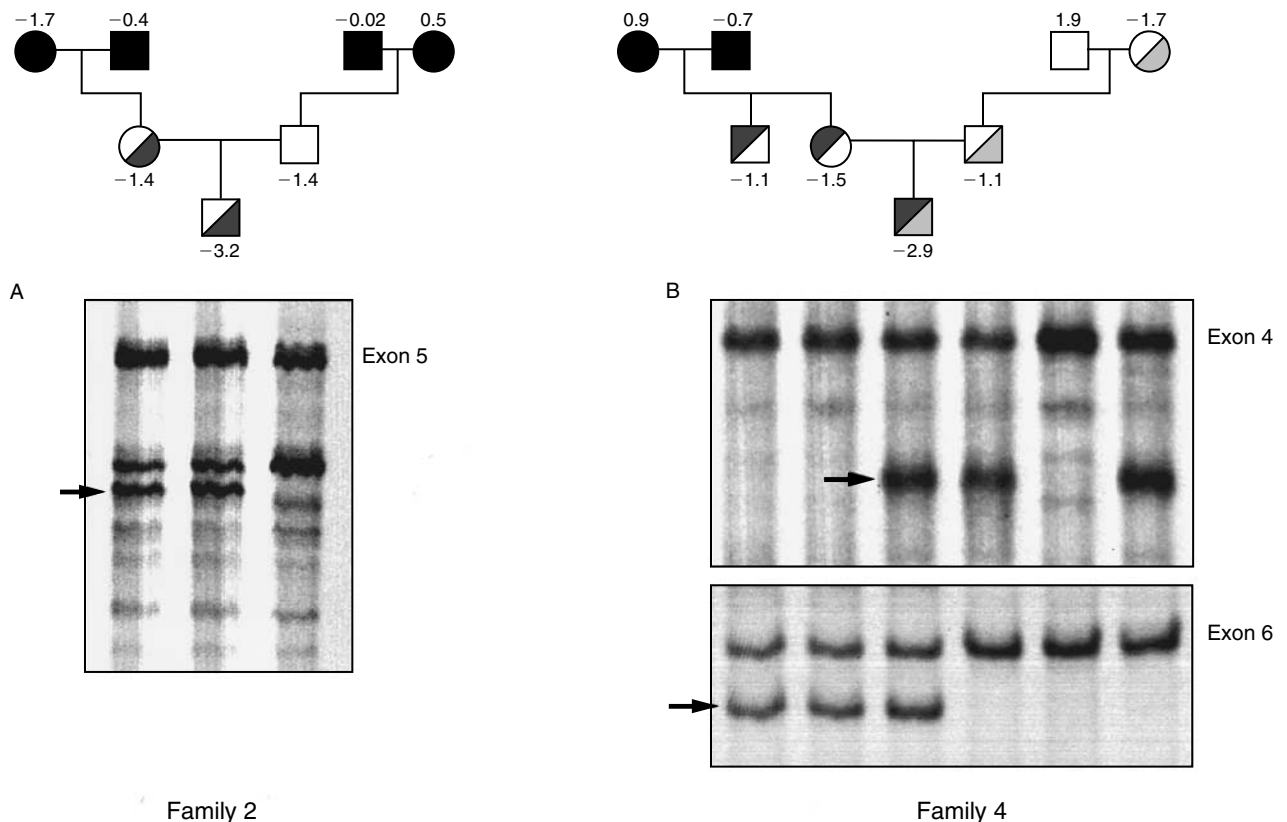


Figure 2. Pedigrees of Two Families in Which Mutations of the Gene for the GH Receptor Were Detected in One Child.

The pedigree for Family 2 (Panel A) is shown above the analysis of single-strand conformation polymorphisms in the genomic PCR products from exon 5 (box). Gray symbols denote alleles for the mutation in exon 5, open symbols wild-type alleles, solid symbols alleles of unknown identity in family members who could not be tested, square symbols male family members, and circles female family members. The SD scores for height of all three generations are shown adjacent to the corresponding symbols. The arrow indicates the mutant base. The pedigree for Family 4 (Panel B) is shown above the analyses of exons 4 and 6 in that family. Light gray symbols denote the mutation in exon 4, and dark gray symbols the mutation in exon 6; the other symbols are as in Panel A.

compound heterozygote, with both a mutation in exon 4 that introduced a lysine in place of a glutamic acid at position 44 (Glu44Lys) and a mutation in exon 6 that introduced a cysteine in place of an arginine at position 161 (Arg161Cys) (Table 2). The two mutations were found in different subclones spanning exons 4 through 6 (data not shown); thus, one mutation was found in each allele. Genetic analysis of the patient's family members (Fig. 2B) indicated that the mutation in exon 4 was inherited from the paternal side of the family and the mutation in exon 6 from the maternal side. Patient 4 did not have severe growth failure, but his growth rate increased slightly — from 5.0 cm per year before treatment to 6.0 cm per year — during six months of treatment with GH (0.35 mg per kilogram per week).

Like Patients 1 and 2, Patient 7 had an alteration in a single allele (Fig. 1E). A mutation at base pair 726 introduced an aspartic acid in place of a glutamic acid at position 224 (Glu224Asp). Neither analysis of single-strand conformation polymorphisms nor direct sequencing of the region of the gene that coded for the extracellular domain identified a second mutation in this patient. Patient 7 was not treated with GH.

We then investigated the effects of these mutations on the ability of the receptor to bind GH. Residue

Glu44 is in direct contact with the GH molecule¹⁹ (Fig. 3), and it is known that the replacement of Glu44 with alanine reduces GH binding.¹² The introduction of a lysine at position 44 reduced binding by a factor of 330 as compared with the binding of the wild-type receptor (Table 2). By contrast, residue Arg161 is not directly involved in the interaction between GH and its receptor¹⁹; its mutation to cysteine reduced binding by a factor of 2.1 (Table 2). Residue Arg211 is on the surface of the receptor away from the region that binds GH.¹⁹ The mutant Arg211His protein had an affinity for GH similar to that of the wild-type receptor (Table 2). However, the level of expression of the mutant receptor was much lower (by a factor of 10,000) than that of the wild-type receptor. We were unable to express the Arg211Gly mutation found in some children with Laron dwarfism²⁰ in our system (data not shown). The conservative Glu224Asp substitution was expressed at normal levels and resembled the Arg211His mutant protein in that its affinity for GH was nearly normal (Table 2).

DISCUSSION

Our finding of mutations in the gene for the GH receptor provides support for the suggestion that some

children with idiopathic short stature and low serum concentrations of GH-binding protein have partial insensitivity to GH.⁶ Single-strand conformation analysis can detect approximately 80 percent of known mutations in experimental systems^{21,22}; therefore, the children we studied may have additional mutations that were missed. In addition, there could be defects in the noncoding regions of the gene for the GH receptor or in other genes encoding products that influence growth.

Three of the four children who had mutations in the GH-receptor gene (Patients 1, 2, and 4) were treated with GH, and had marginal growth in response to therapy (Table 1), suggesting that they may be partially insensitive to GH because of dysfunctional GH receptors. Patient 1 appeared to have a dose-dependent growth response to GH (Table 1). The poor response of Patient 4 probably reflects the effect of a mutation in each allele of the gene for the receptor, including one that reduces the affinity of the receptor for GH by a factor of 330. It is likely that there is a continuum of GH responsiveness ranging from complete insensitivity to GH, as occurs in children with Laron dwarfism, to idiopathic short stature in children with partial GH insensitivity (such as the children described here), to the normal phenotype.

In Families 2 and 4, the known carriers of mutations of the GH-receptor gene had heights below the mean. Patient 4 was a compound heterozygote for the Glu44Lys and Arg161Cys substitutions and was more severely affected than his heterozygous parents (Fig. 2B). Patient 2 (with a Cys122Stop mutation in one allele) was more severely affected than his mother, who carried the same genotype (Fig. 2A), suggesting that he had inherited an as yet undefined mutation from his father that affected the expression of the structurally normal GH-receptor allele or another step in growth regulation. The former possibility is suggested by the lack of detectable GH-binding protein in this patient's serum (Table 1).

The implication that heterozygous mutations in the GH-receptor gene can have mild phenotypic conse-

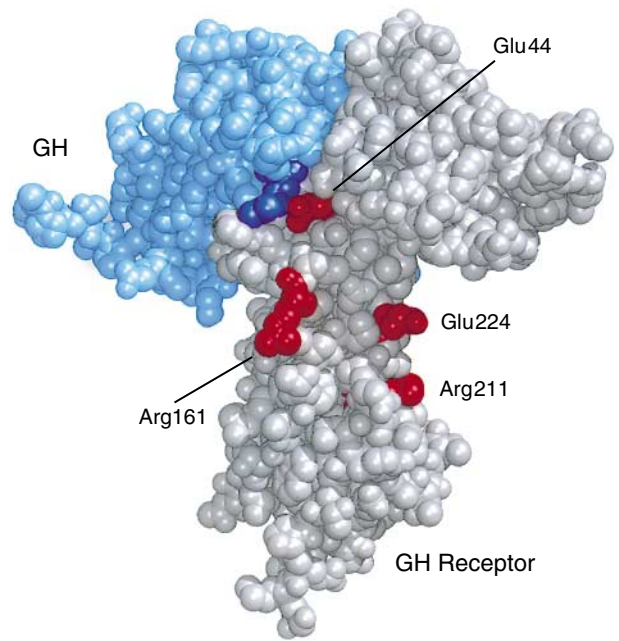


Figure 3. Structure of the GH-Receptor-GH Complex. The structure of human GH (blue) and serum GH-binding protein (gray) was derived from crystallographic coordinates¹⁹ (and de Vos AM, Utsch M: personal communication); the position of mutations associated with idiopathic short stature is shown in red. The GH-receptor residue Glu44 interacts with the Arg64 (dark blue) on the GH molecule (light blue).

quences has been suggested by studies of children with Laron dwarfism and their families. Heterozygotes for mutations in the GH-receptor gene (i.e., parents and siblings of children with Laron dwarfism) may have mild abnormalities of growth^{23,24} and low serum concentrations of GH-binding protein.^{25,26} Partial GH insensitivity that results in short stature may arise in carriers of heterozygous mutations of the GH-receptor gene under the influence of mutations in other genes or when the alterations confer a dominant phenotype.

The five mutations identified in our four patients

Table 2. Mutations in the Gene for the GH Receptor in Four Children with Idiopathic Short Stature.

GENE STUDIED	BASE CHANGE	AMINO ACID ALTERATION	DISSOCIATION CONSTANT*		FUNCTIONAL EFFECT
			IN STUDY SAMPLE	MUTANT: WILD-TYPE	
Wild-type receptor	—	—	0.3 ± 0.1†	—	—
Patient 1, exon 7	G → A at position 686	Arg211His‡	0.50 ± 0.02	1.4	Expression reduced by factor of 10,000§
Patient 2, exon 5	T → A at position 418	Cys122Stop	Not done		Truncated protein
Patient 4					
Exon 4	G → A at position 184	Glu44Lys	112 ± 19	330	Reduced affinity for GH
Exon 6	C → T at position 535	Arg161Cys‡	0.73 ± 0.15	2.1	Extra cysteine, potentially misfolded
Patient 7, exon 7	G → C at position 726	Glu224Asp	0.54 ± 0.07	1.6	Potentially aberrant subcellular localization

*Dissociation constants, used as a measure of affinity for GH, were determined by competitive displacement as described in the Methods section, with ¹²⁵I-labeled human GH used as a tracer and with a monoclonal antibody (Mab5) used to precipitate the GH-receptor-GH complexes. The reference value and those for the study samples are presented as means ± SE. Samples were analyzed in triplicate. The ratio of the dissociation constant for the mutant GH-receptor-GH complex divided by that for the wild-type receptor-GH complex is a measure of GH binding.

†According to Fuh et al.¹³

‡Some children with complete insensitivity to GH (Laron dwarfism) have been found to be homozygous for mutations of this amino acid.

§As compared with the wild-type receptor.

were confined to the extracellular domains of the receptors. The Glu44Lys substitution caused the affinity of the receptor for GH to decrease by a factor of 330, whereas each of the other mutations had a much smaller effect on ligand binding (Table 2). The Arg161Cys mutation probably has a major effect on receptor function, because homozygosity for this alteration is associated with complete insensitivity to GH. The poor expression in *E. coli* of the proteins coded by the Arg211His mutant gene and the Arg211Gly mutant gene²⁰ suggests that these proteins are not folded in a stable fashion. This observation also correlates with our failure to detect serum GH-binding protein in Patient 1 (Table 1), implying that the Arg211His mutation may function as a dominant negative phenotype or that the second allele in this patient is poorly expressed. Mutations of Glu224 do not alter GH binding or protein concentrations (Table 2), but they may not be localized in the cells in a normal fashion and therefore may not be accessible to ligand (GH).²⁷

We conclude that a subgroup of children with idiopathic short stature and clinical findings suggestive of partial insensitivity to GH have mutations in the gene for the GH receptor that may reduce the function of the receptor. Because the patients studied were selected partly on the basis of reduced serum concentrations of GH-binding protein, the identified mutations may affect ligand binding directly (as with Glu44Lys) or may potentially reduce the availability of cell-surface receptors (as with Arg161Cys, Arg211His, and Glu224Asp). Three of the children with mutated GH-receptor genes who were treated with exogenous GH had marginal growth in response to treatment, again suggesting partial insensitivity to GH. Other children with idiopathic short stature and similar clinical characteristics may have mutations of this gene that result in partial GH insensitivity.

We are indebted to Dr. Aida Metzberg for the EBV-producing cell line B95-8, to Mr. Peter Compton for statistical consultation, to Dr. A.M. de Vos for providing unpublished crystallographic coordinates, to Mr. Hal Mulvihill for collecting samples, to Dr. William Wood for technical advice, to the Genentech oligonucleotide-synthesis group, to Dr. Harvey Guyda for the determination of serum GH-binding protein in Patient 1, and to all who have contributed data to the National Cooperative Growth Study.

APPENDIX

In addition to the study authors, the Growth Hormone Insensitivity Study Group includes P. Blackett, S. Casella, S. Clark, D. Donaldson, J. Gonzalez, D. Jelley, R. Levy, M. MacGillivray, R. Mauseth, W. Moore, P. Saenger, J. Vandermeulen, and D. Wilson.

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