

## BRIEF REPORT

## Deficiency of the Circulating Insulin-like Growth Factor System Associated with Inactivation of the Acid-Labile Subunit Gene

Horacio M. Domené, M.S., Sonia V. Bengolea, M.D., Alicia S. Martínez, M.D., M. Gabriela Ropelato, M.S., Patricia Pennisi, Ph.D., Paula Scaglia, M.S., Juan J. Heinrich, M.D., Ph.D., and Héctor G. Jasper, M.D.

From the Endocrinology Research Center, Division of Endocrinology, Ricardo Gutiérrez Children's Hospital (H.M.D., A.S.M., M.G.R., P.P., P.S., J.J.H., H.G.J.); and the Division of Pediatrics, J.A. Fernández Hospital (S.V.B.) — both in Buenos Aires, Argentina. Address reprint requests to Mr. Domené at the Endocrinology Research Center (CEDIE), Division of Endocrinology, Ricardo Gutiérrez Children's Hospital, Gallo 1330, 1425 Buenos Aires, Argentina, or at hdomene@cedie.org.ar.

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THE GROWTH-PROMOTING ACTIONS OF GROWTH HORMONE WERE originally hypothesized to be mediated through a circulating liver-generated sulfation factor that later came to be known as insulin-like growth factor I (IGF-I).<sup>1</sup> This growth factor is produced in almost every tissue in the body.<sup>2</sup> In the cartilage growth plate, growth hormone–induced IGF-I<sup>3</sup> acts locally through autocrine–paracrine mechanisms.<sup>4</sup>

Some 80 to 85 percent of IGF-I circulates as a 150-kD ternary complex that includes the ligand itself, IGF-binding protein 3, and an acid-labile subunit.<sup>5</sup> The acid-labile subunit is a glycoprotein found almost exclusively in the circulation and produced in the liver under growth hormone stimulation.<sup>6,7</sup> This subunit stabilizes the IGF–IGF-binding protein 3 complex, reduces the passage of IGF-I to the extravascular compartment, and extends its half-life.<sup>8</sup> Recently, the role of circulating IGF-I in growth has been challenged by the finding that specific disruption of the hepatic *igf1* gene in mice, the main source of circulating IGF-I,<sup>9,10</sup> or the inactivation of the gene encoding the acid-labile subunit protein (*igfals*) in mice<sup>11</sup> has a minor effect on growth, despite causing a profound reduction in the serum IGF-I level.

In this report, we describe a 17-year-old boy who had a delayed onset of puberty, slow pubertal progress, and yet minimal slowing of his linear growth in association with an inactivation of the *IGFALS* gene.

## CASE REPORT

No data regarding gestation and the perinatal period were available, because the boy had been adopted at one week of age, at which time his length and weight were recorded as 47 cm and 2500 g, respectively. His childhood medical history was unremarkable. Psychomotor and neurologic development was normal. At 14.6 years of age, he was referred for evaluation of growth and pubertal delay, at which time his height was 145.2 cm (2.05 SD below the mean),<sup>12</sup> near the third percentile by Argentinean standards (2.37 SD below the mean<sup>13</sup> according to a Tanner growth chart). His weight was 35.9 kg (2.34 SD below the mean), his head circumference 55.2 cm (1.78 SD below the mean),<sup>13</sup> and his body-mass index (the weight in kilograms divided by the square of the height in meters) 17, with Tanner stage 1 for both sexual development and pubic hair; both testes were 3 ml in volume.<sup>14</sup> The clinical examination showed no abnormalities, except for mild micrognathia and truncal obesity. The bone age was 12.5 years.<sup>15</sup>

Routine laboratory analyses ruled out hematologic, liver, and renal diseases. Magnetic resonance imaging of the brain was normal. The growth hormone responses to provocative tests with the use of arginine and clonidine were normal, but there was a marked reduction of both IGF-I (31 ng per milliliter [4.0 nmol per liter]; 5.3 SD below the mean for his chronologic age) and IGF-binding protein 3 (0.22 μg per milliliter [7.7 nmol per

liter]; 9.7 SD below the mean).<sup>16</sup> After informed consent had been obtained, the patient was admitted to Ricardo Gutiérrez Children's Hospital in Buenos Aires, Argentina, for further study.

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## METHODS

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### ENDOCRINE STUDIES

Serum levels of thyroxine, free thyroxine, triiodothyronine, thyrotropin, and prolactin were determined by means of electrochemoluminescence (Elecys, Roche), levels of follicle-stimulating hormone and luteinizing hormone by means of an immunofluorometric assay (Wallac Oy), and levels of growth hormone by means of an immunoradiometric assay (Biodata). Levels of cortisol and insulin were measured by means of a radioimmunoassay (Diagnostic Products Corporation), levels of testosterone and free testosterone by means of a radioimmunoassay, levels of IGF-binding protein 3 and sex hormone-binding globulin by means of an immunoradiometric assay, levels of the acid-labile subunit by means of an enzyme-linked immunosorbent assay (ELISA) (all from Diagnostic Systems Laboratories),<sup>17</sup> levels of IGF-I by means of a radioimmunoassay,<sup>16</sup> levels of growth hormone-binding protein by means of a time-resolved fluoroimmunoassay,<sup>18</sup> and levels of glycosylated hemoglobin by means of a turbidimetric inhibition immunoassay (Roche).

Nocturnal 12-hour profiles of growth hormone, luteinizing hormone, and follicle-stimulating hormone were determined in blood samples drawn every 20 minutes. Data were analyzed with the use of a cluster-analysis algorithm.<sup>19</sup>

### WESTERN LIGAND BLOTTING AND WESTERN IMMUNOBLOTTING

Serum IGF-binding proteins were evaluated by means of Western ligand blotting.<sup>20</sup> For Western immunoblotting, we used antibodies against the amino terminal of the acid-labile subunit (hALS<sub>1-34</sub>) and the carboxy terminal of the acid-labile subunit (hALS<sub>551-578</sub>, Diagnostic Systems Laboratories).<sup>21</sup> An antibody against human transferrin (Dako) was used as a control for protein loading and transfer.

### SIZE-EXCLUSION CHROMATOGRAPHY

Samples were chromatographed on a HiPrep 16/60 Sephacryl S-200HR column. Serum samples (in volumes of 100  $\mu$ l) were incubated overnight at 22°C with  $3.5 \times 10^6$  counts per minute of <sup>125</sup>I-labeled IGF-I, and were then cross-linked with the addition of disuccinimidyl suberate (Sigma Aldrich), as re-

ported previously.<sup>22</sup> Five hundred microliters was loaded into the column and 1-ml fractions were collected.

### MOLECULAR STUDIES

Genomic DNA was isolated from peripheral leukocytes by means of phenol-chloroform extraction. Exons 1 and 2 and contiguous intronic sequences, corresponding to the IGFALS gene (GenBank accession number AF192554), were amplified by polymerase chain reaction (PCR) and sequenced with the use of <sup>33</sup>P end-labeled dideoxy nucleotides, internal primers, and a thermostable DNA polymerase (ThermoSequenase, Amersham Biosciences). The sequences of oligonucleotide primers for PCR amplification were designed with the use of the Primer Detective Program<sup>23</sup> and the published sequence of the human IGFALS gene.<sup>24,25</sup> To determine the prevalence of the 1338delG, E35fsX120 mutation of the IGFALS gene in the Argentinean population, we studied 100 controls by means of single-strand conformation polymorphism analysis.<sup>26</sup>

Two highly polymorphic microsatellite markers located near the IGFALS locus (D16S521 and D16S3024) were studied. PCR was performed with the use of <sup>32</sup>P-labeled deoxyadenosine triphosphate, and reaction products were analyzed on 6 percent polyacrylamide gels. Twenty-three unrelated controls were genotyped in order to derive control allele frequencies.

The possibility of the deletion of one allele at the IGFALS locus was investigated by means of hot-stop PCR.<sup>27</sup> Briefly, 694-bp segments of DNA corresponding to exon 2 of the IGFALS gene were amplified from different amounts of genomic DNA (100, 300, and 600 ng of DNA per tube) from both our patient and a normal control, under standard PCR conditions. In the last PCR step, a <sup>32</sup>P end-labeled reverse primer was added. The PCR products were analyzed by means of a nondenaturing 10 percent polyacrylamide gel and autoradiography. The densitometric product (area  $\times$  intensity) in arbitrary units was obtained with the use of Scion Image software.

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## RESULTS

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The patient's thyroid function was normal, as were the prolactin and cortisol levels. Gonadal evaluation showed gonadotropin levels both at base line and after stimulation with gonadotropin-releasing hormone that were appropriate for a boy in early puberty, with testosterone values that matched the patient's Tanner stage (Table 1).

The study of the growth hormone–IGF system revealed normal stimulated growth-hormone values (Table 1), with marked reductions in the levels of both IGF-I and IGF-binding protein 3, which remained unchanged after stimulation with growth hormone (Table 2). Increased spontaneous nocturnal secretion of growth hormone was observed (Table 1), and suppression of growth hormone after the

oral intake of glucose was absent (Table 2). Remarkably, the acid-labile subunit was undetectable in the serum before and after growth-hormone stimulation (Table 2).

The sequencing of exons 1 and 2 of the IGFALS gene revealed a deletion of one of five consecutive guanines at positions 1334 through 1338. This frame-shift point mutation resulted in the substitution of a lysine for a glutamic acid at codon 35 and the appearance of an early stop codon at position 120 of the precursor form of the acid-labile subunit (1338delG, E35fsX120) (Fig. 1A).

In the absence of parental DNA, it is difficult to determine how the mutation arose. This mutation does not appear to be common in the Argentinean population, since it was not found in any of the 100 healthy controls. A germ-line chromosomal nondisjunction event is unlikely, because the patient was heterozygous for two microsatellites in the 16p13.3 region, near the locus of the IGFALS gene. Of the normal Argentinean controls, 60.9 percent were found to be heterozygous for D16S521, and 78.3 percent were heterozygous for D16S3024 — rates that are similar to those that were previously reported (71.0 percent and 86.0 percent, respectively<sup>33,34</sup>). Even when heterozygosity for both markers makes consanguinity an unlikely explanation, it is still possible that a crossover event has occurred, accounting for the heterozygosity despite the presence of possible consanguinity. Hot-stop PCR analysis of genomic DNA templates of a DNA fragment from exon 2 of the IGFALS gene revealed similar intensity in our patient and in a normal control (mean [±SD] ratio of the intensity in our patient to the intensity in the control, 0.92±0.10), suggesting that the patient most likely has both alleles at the IGFALS locus and is consequently homozygous for the mutation.

The presence of acid-labile subunit protein was assessed by means of Western immunoblotting. As depicted in Figure 1B, we could not demonstrate the presence of any protein band in the 84-to-86-kD region with the use of antibodies against the acid-labile subunit. In the Western ligand blotting assay (Fig. 1C), the patient with the acid-labile-subunit deficiency had a reduction in the 40-to-43-kD doublet, corresponding to IGF-binding protein 3, with no increase after treatment with recombinant human growth hormone. Size-exclusion chromatography showed that no ternary complexes were formed in this patient (Fig. 2). The formation of ternary complexes increases in a patient with a deficiency of growth hormone after therapy with recombinant human growth hormone, whereas there were

**Table 1. Laboratory Studies.\***

Study	Value	Normal Range
Nocturnal 12-hr gonadotropin profile		
Serum luteinizing hormone		
Level (mIU/ml)	2.9±2.7	4.9±0.4
Amplitude (mIU/ml)	2.6±2.2	3.5±0.5
Frequency of peaks (per hr)	0.75	0.57±0.05
Serum follicle-stimulating hormone		
Level (mIU/ml)	2.1±0.5	5.3±0.6
Amplitude (mIU/ml)	0.6±0.4	
Frequency of peaks (per hr)	0.33	
Base-line values		
Serum testosterone level (ng/dl)	87	20–240
Serum free testosterone level (pmol/liter)	13	≤20
Serum sex hormone–binding protein level (nmol/liter)	30	15–95
After the administration of human chorionic gonadotropin		
Serum testosterone level (ng/dl)	760	
Serum free testosterone level (pmol/ml)	134	
Serum sex hormone–binding protein level (nmol/liter)	14	
Serum growth hormone level (ng/ml)		
Base-line level	4.5	
Peak	31.0	>10.0
Nocturnal 12-hr growth hormone profile		
Level (ng/ml)	18.9±12.6	4.5±1.4
Amplitude (ng/ml)	16.3±11.0	8.5±4.3
Frequency of peaks (per hr)	0.42	0.33±0.08
Serum growth hormone–binding protein level (nM)	1.62	1.10–5.50

\* Plus–minus values are means ±SD. Hormone levels were measured at base line and after adequate stimuli. Growth hormone was measured after a 30-minute intravenous infusion of arginine (0.5 g per kilogram of body weight) and after the oral administration of clonidine (100 µg per square meter of body-surface area); testosterone, free testosterone, and sex hormone–binding globulin were measured seven days after the administration of human chorionic gonadotropin (2500 IU on days 1 and 4). To convert values for testosterone to nanomoles per liter, multiply by 0.347; to convert values for free testosterone to picomoles per liter, multiply by 3.47. The normal ranges given for the base-line levels are those for boys at Tanner stage 2. Normal ranges for the serum luteinizing hormone level and the serum follicle-stimulating hormone level are from Goji and Tanizake<sup>28</sup>; the normal range for the peak serum growth hormone level is from Martínez et al.<sup>16</sup>; and the normal range for the nocturnal 12-hour growth hormone level is from Ropelato et al.<sup>29</sup>

**Table 2. Responses of the Insulin-like Growth Factor (IGF) System and Carbohydrate Metabolism to Treatment with Recombinant Human Growth Hormone (rhGH).\***

Variable	Base Line	With rhGH Treatment				
		5 Days	7 Days	1 Mo	3 Mo	6 Mo
<b>IGF-I</b>						
Level (ng/ml)	31	39	20	20	29	23
No. of SD below mean	5.3	4.7	6.3	6.3	5.4	6.0
<b>IGFBP-3</b>						
Level ( $\mu$ g/ml)	0.22	0.21	0.28	0.27	0.41	0.33
No. of SD below mean	9.7	9.9	8.9	9.0	7.6	8.3
<b>ALS level (<math>\mu</math>g/ml)</b>						
	ND	ND	ND	ND	ND	—
<b>Glucose-tolerance test</b>						
<b>Insulin level (<math>\mu</math>IU/ml)</b>						
Base line	32				28	36
Peak	260				237	308
120 Min	44				187	191
<b>Glucose level (mg/dl)</b>						
Base line	100				93	82
Peak	145				155	156
120 Min	85				126	92
<b>Growth hormone level (ng/ml)</b>						
Base line	1.9				4.3	6.3
60 Min	11.1				32.0	3.2
<b>Ratio of fasting glucose level to insulin level</b>						
	3.12				3.31	2.27
<b>Insulin-sensitivity index<sup>†</sup></b>						
	1.53				1.57	1.25
<b>Glycosylated hemoglobin (%)</b>						
	5.7				5.6	5.3

\* Serum insulin-like growth factor I (IGF-I), IGF-binding protein 3 (IGFBP-3), and the acid-labile subunit (ALS) were measured before (base line) and after five days of stimulation with rhGH (0.03 mg per kilogram of body weight per day), as well as after seven days and one, three, and six months of treatment with rhGH (0.17 mg per kilogram per week). Serum glucose, insulin, and growth hormone were measured after the oral administration of 75 g of glucose in glucose-tolerance tests performed before rhGH treatment and after three and six months of treatment. Normal values for the ratio of the fasting glucose level (in milligrams per deciliter) to the insulin level (in micro-international units per milliliter) are greater than 4.5.<sup>30</sup> Glycosylated hemoglobin was measured in blood samples drawn before treatment and after three and six months of rhGH treatment; the normal range is 4.8 to 6.0 percent. To convert values for IGF-I to nanomoles per liter, multiply by 0.13; to convert values for IGFBP-3 to nanomoles per liter, multiply by 35; to convert values for ALS to nanomoles per liter, multiply by 15.8; to convert values for glucose to millimoles per liter, multiply by 0.0555; to convert values for insulin to picomoles per liter, multiply by 7.175. ND denotes nondetectable.

<sup>†</sup> The insulin-sensitivity index is calculated as  $10,000 \div [\text{the fasting glucose level (in milligrams per deciliter)} \times \text{the fasting insulin level (in micro-international units per milliliter)} \times \text{the mean glucose level (in milligrams per deciliter)} \text{ during the oral glucose-tolerance test} \times \text{the mean insulin level (in micro-international units per milliliter)} \text{ during the oral glucose-tolerance test}]^{1/2}$ ; normal values are greater than 2.4.<sup>31,32</sup>

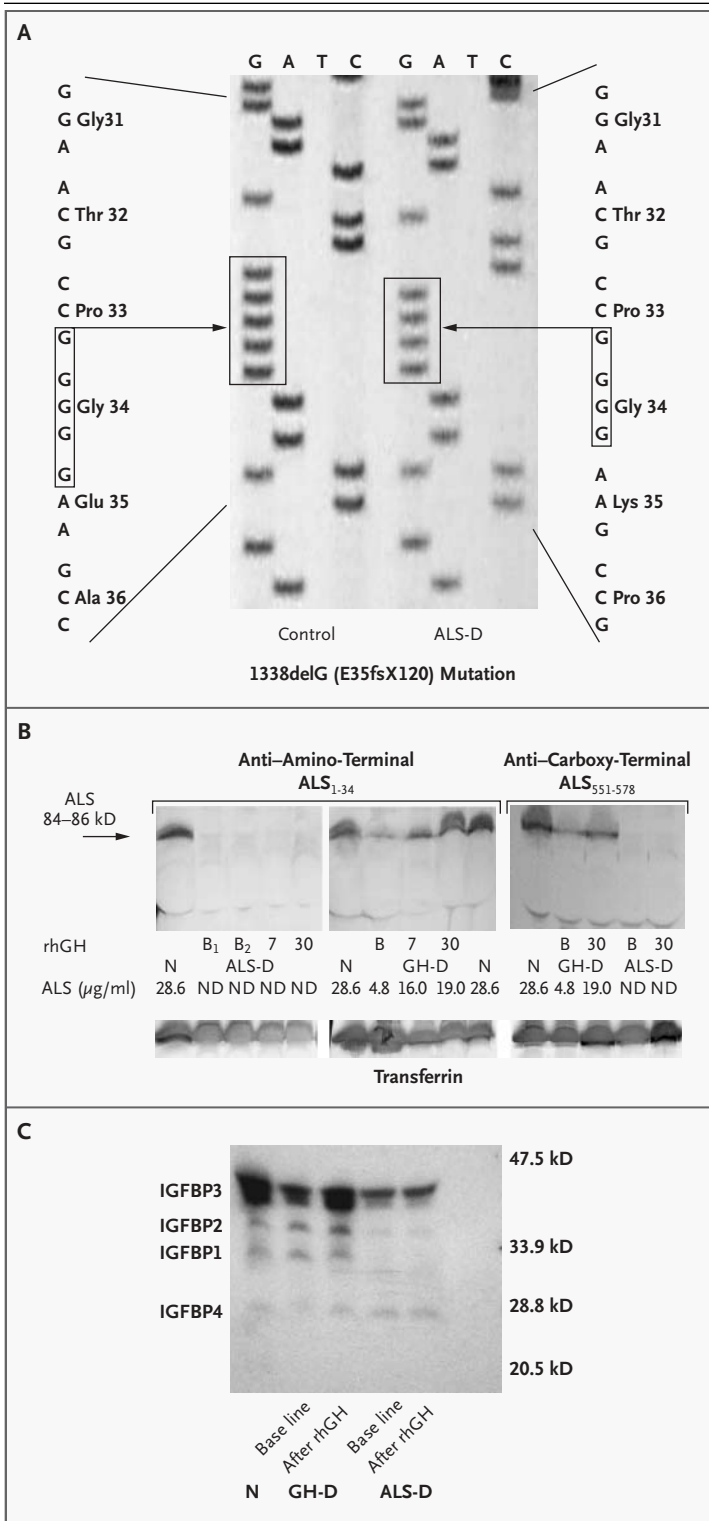
no such complexes in the patient with the acid-labile-subunit deficiency, even after such treatment.

Although six months of treatment with recombinant human growth hormone (at a dose of 0.17 mg per kilogram of body weight per week) caused a reduction in the subscapular skin-fold thickness (from 0.72 SD above the mean to 0.01 SD above the mean),<sup>13</sup> there was no beneficial effect on either the velocity of growth<sup>35</sup> (which was 6.2 cm per year before treatment and 4.6 cm per year after treatment) or the serum levels of IGF-I, IGF-binding protein 3, and the acid-labile subunit (Table 2). An oral glucose test performed before and after treatment with recombinant human growth hormone showed nor-

mal glucose levels with high basal and exaggerated peak insulin responses, indicating insulin resistance. Blood glycosylated hemoglobin levels remained normal during treatment with recombinant human growth hormone (Table 2).

#### DISCUSSION

The patient we describe was found to have a complete absence of the acid-labile subunit of the circulating IGF ternary complex; this absence was associated with subtle growth retardation and delayed puberty. The association of markedly subnormal IGF-I and IGF-binding protein 3 with normal or el-



**Figure 1. Mutation in the *IGFALS* Gene (Panel A), Western Immunoblot of Acid-Labile Subunit (ALS) (Panel B), and Western Ligand Blot of Insulin-like Growth Factor (IGF)-Binding Proteins (Panel C).**

Sequencing of exon 2 encoding for the *IGFALS* gene (Panel A) shows a deletion of one of five consecutive guanines at bases 1334 through 1338 in the patient with the acid-labile-subunit deficiency (ALS-D). This frameshift mutation results in the substitution of lysine for glutamic acid at codon 35 and the appearance of an early stop codon at position 120 (1338delG, E35fsX120). Panel B shows that no 84-to-86-kD ALS protein band was detected in the patient with the ALS deficiency (ALS-D), with the use of antibody against amino-terminal ALS<sub>1-34</sub>, before or after treatment with recombinant human growth hormone (rhGH). In a patient with a growth hormone deficiency (GH-D), the ALS protein band progressively increased from base line after treatment with rhGH. A sample from a normal child (N) is also included. Nor was any 84-to-86-kD ALS protein band detected in the ALS-deficient patient with the use of an antibody against carboxy-terminal ALS<sub>551-578</sub>, whereas in the growth hormone-deficient patient, an increment was observed in the ALS protein band after treatment with rhGH. Serum ALS values, measured by means of an enzyme-linked immunosorbent assay, are also included. ND denotes nondetectable. Transferrin immunoblots were used as controls for the comparison of protein load and transfer. The Western ligand blot (Panel C) shows that both a patient with growth hormone deficiency (GH-D) and the ALS-deficient patient (ALS-D) had a reduction in the 40-to-43-kD doublet corresponding to IGF-binding protein 3, but whereas there was an increase in the growth hormone-deficient patient after 30 days of growth hormone treatment, there was no such increase in the ALS-deficient patient. A sample from a normal child (N) is also included. IGFBP denotes IGF-binding protein.

evated levels of growth hormone is found in patients with either an insensitivity to growth hormone<sup>36</sup> or biologically inactive growth hormone,<sup>37,38</sup> but in these conditions, the magnitude of the growth-factor deficiency usually correlates with the severity of the growth impairment.<sup>39</sup> Our patient's minimal growth impairment may be explained by normal local production of IGF-I, acting through autocrine-paracrine mechanisms,<sup>3,4</sup> or by normal or near-normal levels of circulating free IGF-I. Unfortunately, we could not determine the free IGF-I levels. However, normal free IGF-I levels were reported in mice with a liver-specific inactivation of the *igf1* gene,<sup>40</sup> despite profound reduction in the serum IGF-I level. It has been suggested that free IGF-I might be the regulator of growth-hormone secretion in humans<sup>41</sup>; therefore, the increased growth hor-

**Figure 2. Results of Cross-Linking Followed by Size-Exclusion Column Chromatography.**

Serum samples from a normal control (Panel A), a patient with a growth hormone deficiency (Panel B), and the patient with the acid-labile-subunit deficiency (Panel C) were analyzed as described in the Methods section. The regions corresponding to the ternary complexes are shown in the shaded boxes. Arrows indicate the positions of markers of the molecular weight. In Panel A, serum from a normal control shows the formation of ternary complexes. In Panel B, the formation of ternary complexes peaks after treatment with recombinant human growth hormone (rhGH) in a growth-hormone-deficient patient. In Panel C, even after rhGH treatment, there was no increase in the formation of ternary complexes in the patient with the acid-labile-subunit deficiency.

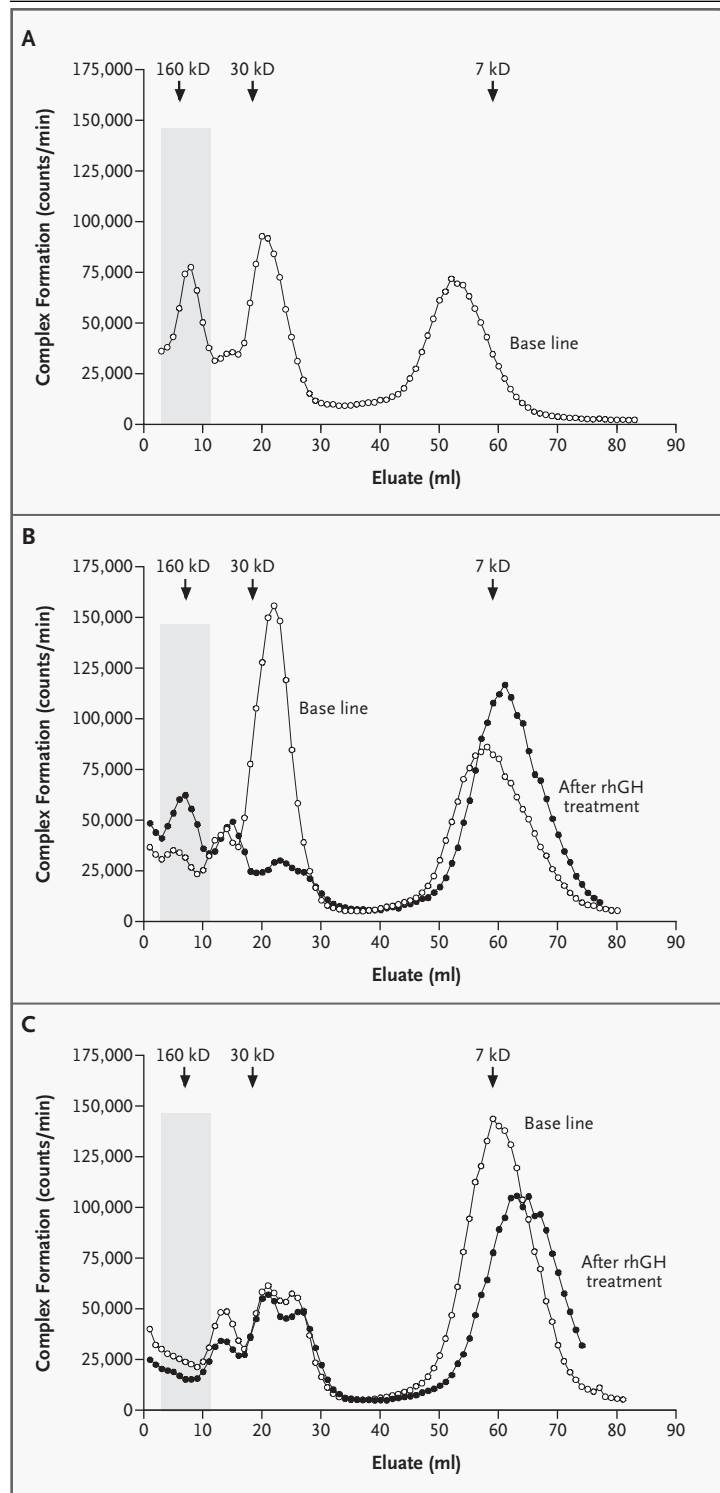
hormone secretion observed in our patient might be taken as indirect evidence of low levels of free IGF-I.

Although recent studies have shown that the test of IGF-I generation has a sensitivity of only about 77 percent,<sup>42</sup> the lack of response to this test of both IGF-I and IGF-binding protein 3 may suggest a diagnosis of growth hormone insensitivity<sup>43</sup> or, alternatively, instability of IGF-I and IGF-binding protein 3 in the circulation. The finding of undetectable levels of the acid-labile subunit steered the investigation to the *IGFALS* gene.

The complete deficiency of circulating acid-labile subunit appears to result from a frame-shift mutation 1338delG, E35fsX120 in the *IGFALS* gene, which encodes a truncated and probably inactive protein. If it is expressed, secreted, and stable in the circulation, this mutant acid-labile-subunit protein would retain only 7 amino-terminal amino acid residues corresponding to the acid-labile subunit after the cleavage of the 27-amino-acid signal peptide and would lack the domains required for binding to IGF-binding protein 3.<sup>44</sup>

Given the uniqueness of this case, we cannot prove that there is a relation between the acid-labile-subunit deficiency and the patient's clinical condition. However, we speculate that the lack of acid-labile-subunit protein might be involved in a subtle impairment of linear growth, a delay in the onset and slow progress of puberty, and a certain degree of insulin resistance.

In our patient, as in the mouse with a targeted inactivation of the *igfals* gene (the ALS-knockout mouse),<sup>11</sup> the absence of the acid-labile subunit causes a marked decrease in the serum levels of IGF-I and IGF-binding protein 3. The minimal



slowing of growth observed is in agreement with the 13 percent reduction in body weight found in the ALS-knockout mouse, indicating that the absence

of the acid-labile subunit leads to growth impairment, albeit mild, after birth.<sup>11</sup>

Pubertal delay could be related either to a reduction in the circulating IGF-I level or to the patient's biologic background. Pubertal retardation is a common finding in patients with a growth hormone deficiency<sup>45</sup> and has also been reported in a patient with a deletion of the IGF1 gene,<sup>46</sup> suggesting that circulating IGF-I has a role in pubertal development.

The insulin resistance may result from increased secretion of growth hormone. However, because IGF-I has been shown to facilitate the action of insulin,<sup>47</sup> a reduction in the circulating IGF-I level may contribute to insulin resistance. Insulin insensitivity has also been observed in mice with liver-specific deletions of the *igf1* gene.<sup>48,49</sup>

In contrast to the patient with a deletion of the IGF-1 gene,<sup>46</sup> who had retarded psychomotor development and hearing impairment, the patient with an acid-labile-subunit deficiency had normal neurologic development, suggesting that locally produced IGF-I, or a normal level of circulating free IGF-I, is sufficient for normal brain development. Although the reduction in skin-fold thickness during growth hormone treatment points to a sensitivity of adipose tissue to growth hormone, the absence of an acceleration in growth could be related to the failure of growth hormone to increase the level of circulating IGF-I. A deleterious effect of acid-labile-subunit deficiency at the growth-plate level could not be ruled out.

The acid-labile-subunit deficiency caused by the inactivation of the IGFALS gene is associated with a severe disruption in the growth hormone-IGF axis, underscoring the important physiologic role of this member of the IGF family.<sup>50</sup> These findings support the hypothesis that the circulating total IGF-I level might not be the major mediator of the growth-promoting actions of growth hormone. Perhaps, then, the main roles of circulating IGF-I might be the feedback control on growth hormone secretion and the regulation of carbohydrate metabolism through the facilitation of insulin action.<sup>51</sup> Further investigation may reveal whether the disorder in our patient represents a rare case of acid-labile-subunit deficiency caused by an uncommon molecular defect or whether mutations in the IGFALS gene might be involved in other cases of delayed growth and pubertal development in children.

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