

BRIEF REPORT

Growth Hormone Insensitivity Associated with a STAT5b Mutation

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THE SYNDROME OF GROWTH HORMONE INSENSITIVITY IS CHARACTERIZED by phenotypic features consistent with the presence of a growth hormone deficiency, but with normal-to-elevated circulating concentrations of growth hormone and resistance to exogenous growth hormone therapy.¹ Originally described by Laron and colleagues,² the syndrome, it has become increasingly apparent, involves considerable phenotypic heterogeneity, reflecting, at least in part, the complexity of the growth hormone signaling cascade. The majority of patients described to date have low serum concentrations of growth hormone-binding protein, the extracellular domain of the growth hormone receptor, as a result of mutations or deletions that affect the growth hormone-binding region of the receptor.³⁻⁵ A number of patients, however, have been described who have normal or even elevated serum concentrations of growth hormone-binding protein.^{6,7} These include patients with mutations in the growth hormone receptor that affect dimerization⁸ or the transmembrane and intracellular regions.⁹⁻¹²

Growth hormone insensitivity in patients who are positive for growth hormone-binding protein might also result from defects in the signaling of growth hormone receptor. Dimerization of the receptor activates Janus kinase 2 (JAK2), a receptor-associated kinase that phosphorylates both itself and the growth hormone receptor. The phosphorylated residues act as docking sites for a variety of molecules, which undergo subsequent phosphorylation by JAK2, resulting in the activation of the signal transducers and activators of transcription (STAT), phosphatidylinositol-3 kinase, and mitogen-activated protein kinase pathways. Collectively, these pathways mediate the growth-promoting and metabolic actions of growth hormone.

Although several patients with a phenotype of growth hormone insensitivity and normal growth hormone receptor (GHR) genes have been described, no specific molecular defects downstream of the receptor have been identified.¹³⁻¹⁶ We now describe a patient with the clinical and biochemical characteristics of growth hormone insensitivity, a normal GHR gene, and a homozygous missense mutation in the gene for STAT5b.

CASE REPORT

A 1400-g baby girl was born at 33 weeks of gestation to consanguineous Argentine parents. Paternal and maternal heights were 173.6 and 155.8 cm, respectively. There was no family history of growth failure, and the girl's younger sisters were of normal stature. At birth, she required care in a neonatal unit because of respiratory difficulties. Poor weight gain and growth failure were noted during the first three years of life, and evaluations for failure to thrive and malabsorption revealed no abnormalities. The girl was first referred to an endocrine center at seven years of age, when her height was 97 cm and her weight was 14 kg, both far below the 5th percentiles. She also had respiratory difficulties,

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N Engl J Med 2003;349:1139-47.

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with increased oxygen requirements; an eventual lung biopsy was interpreted as indicating lymphoid interstitial pneumonia. Studies were negative for human immunodeficiency virus, cytomegalovirus, and Epstein-Barr virus. The patient was treated with corticosteroids, with partial improvement, but she continued to have multiple episodes of bronchial obstruction. At the age of eight years, she presented with severe hemorrhagic varicella and subsequently had several episodes of herpes zoster. A progressive worsening of her pulmonary function resulted in a second lung biopsy at the age of 10 years (diagnosis, lymphoid interstitial pneumonia), and *Pneumocystis carinii* was isolated from the tissue.

A 12-month trial of growth hormone therapy resulted in no improvement in growth rate (Fig. 1A). At the age of 16.5 years, her height was 117.8 cm (7.5 SD below the mean for age), with normal body proportions and delayed secondary sex characteristics (Tanner stage III pubertal development). She had a prominent forehead, a saddle nose, and a high-pitched voice. In an insulin-arginine growth hormone stimulation test, the serum growth hormone concentration was 9.4 ng per milliliter after an overnight fast, with a peak concentration of 53.8 ng per milliliter after stimulation with 0.05 U of insulin per kilogram of body weight (normal value, >10), and the serum growth hormone-binding protein concentration was 1236 pmol per liter (normal range, 431 to 1892). Serum concentrations of insulin-like growth factor I (IGF-I), insulin-like growth factor-binding protein 3 (IGFBP-3), and the acid-labile subunit were all markedly low, with poor responses to seven consecutive daily injections of growth hormone (0.05 mg per kilogram of body weight per day) (Fig. 1B). All studies were approved by the institutional review board at Oregon Health and Science University, and written informed consent was obtained from the patient and her parents.

METHODS

Serum concentrations of IGF-I, IGFBP-3, and acid-labile subunit (Diagnostic Systems Laboratories),¹⁷ growth hormone-binding protein (Esoterix Endocrinology), and growth hormone (Nichols Institute Diagnostics) were measured in the patient and control subjects. Primary fibroblast cultures were established from skin-biopsy specimens obtained from the patient and a healthy 30-year-old woman in compliance with ethics guidelines. Additional normal dermal fibroblasts from a 31-year-old woman

Figure 1 (facing page). Growth Curve of the Patient between the Ages of 4 and 16 Years (Panel A); Response of Insulin-Like Growth Factor I (IGF-I), Insulin-Like Growth Factor-Binding Protein 3 (IGFBP-3), and Acid-Labile Subunit to Stimulation with Growth Hormone (Panel B); and the Transcriptional Regulation of IGF-I (Panel C) and IGFBP-3 (Panel D) by Growth Hormone.

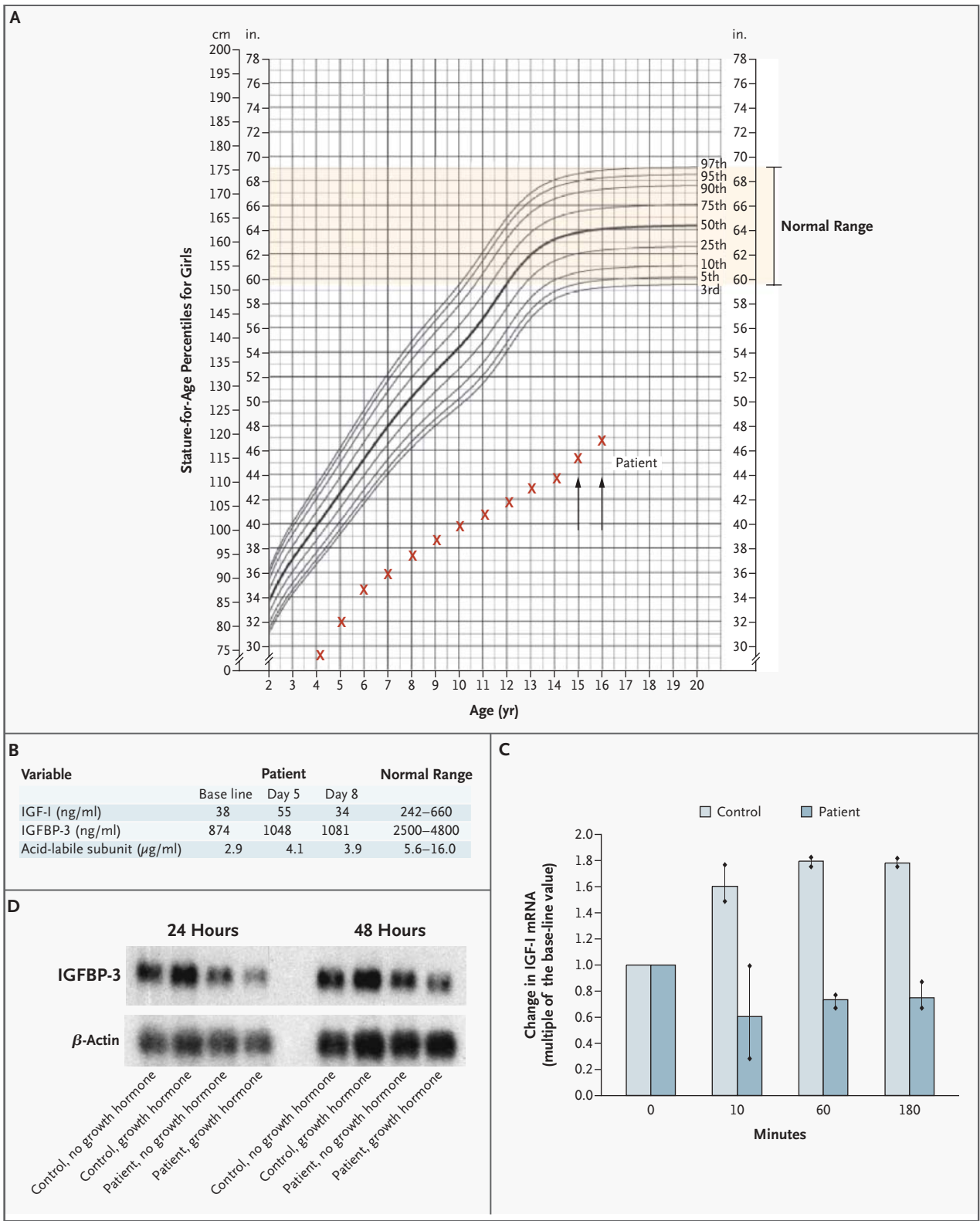
The arrows in Panel A indicate the 12-month period when the patient received growth hormone therapy. Panel B shows the response of IGF-I to the administration of human growth hormone when the patient was 16.5 years old (0.05 mg per kilogram of body weight per day for seven days). Laboratory studies performed at the age of 16.5 years also revealed a 46,XX karyotype and a bone age of 13.5 years. In Panel C, primary fibroblasts were incubated with growth hormone (500 ng per milliliter) for 0, 10, 60, and 180 minutes. Total RNA was collected, and the response of IGF-I messenger RNA (mRNA) to growth hormone was analyzed with the use of real-time quantitative polymerase chain reaction. Results are normalized to those for 18S and are expressed as the relative difference as compared with untreated cells. Data are the means (\pm SD) from two independent experiments. In Panel D, primary fibroblasts were treated with growth hormone (500 ng per milliliter) for 24 or 48 hours, and total RNA was collected. The results of Northern blotting for IGFBP-3 are shown; β -actin was used as a control.

were purchased from BioWhittaker. Further details concerning cell culture are provided in Supplementary Appendix 1 (available with the full text of this article at <http://www.nejm.org>). Details of Western immunoblotting, genomic, and complementary DNA (cDNA) analysis, real-time quantitative polymerase chain reaction (PCR) for IGF-I transcripts, and Northern analysis for IGFBP-3 are also provided in Supplementary Appendix 1.

RESULTS

ANALYSIS OF THE GROWTH HORMONE RECEPTOR GENE AND PROTEIN

Sequencing of genomic DNA from the patient's lymphocytes did not show any mutations in the GHR gene. Sequencing of GHR cDNA amplified from fibroblasts by reverse-transcriptase PCR (RT-PCR) confirmed that the GHR coding region was normal. Corroborating the genetic data, normal growth hormone receptor was readily detected in fibroblast-cell lysates by immunoblot analysis (data not shown). These findings were consistent with the patient's normal serum growth hormone-binding protein concentration and suggested that the defect occurs downstream from GHR.



EXPRESSION OF IGF-I AND IGFBP-3 MESSENGER RNA

Growth hormone–induced regulation of the expression of IGF-I and IGFBP-3 was assessed in primary fibroblasts. IGF-I messenger RNA (mRNA) was monitored by real-time quantitative PCR 0, 10, 60, and 180 minutes after the addition of growth hormone (Fig. 1C). Primers for IGF-I were as follows: 5'TGCCCGGCTAATTTTTTGG3' (forward) and 5'CATGCCTGTAATCCCAGCAA3' (reverse) (further details are available in Supplementary Appendix 1). Transcriptional regulation of IGF-I mRNA was reproducibly induced in two independent cultures of normal fibroblasts; elevated mRNA concentrations were evident within 10 minutes after the administration of growth hormone, peaked at 60 minutes (with an 80 percent increase), and were sustained for 180 minutes. In contrast, the fibroblasts from the patient showed no increase in IGF-I transcription in response to growth hormone; there was even a reduction in transcription of 30 to 40 percent 60 minutes after treatment.

To assess the expression of IGFBP-3, total RNA was collected at 24 and 48 hours from untreated cells and from cells that had been incubated with growth hormone. Northern blotting analysis demonstrated that the level of expression of IGFBP-3 tripled in normal primary fibroblasts within 24 hours after growth hormone stimulation, whereas there was no such increase in the patient's cells (Fig. 1D). These observations reflected the markedly reduced serum concentrations of IGF-I and IGFBP-3, as well as the minimal increase in these proteins after the administration of growth hormone in vivo (Fig. 1B).

GHR SIGNAL-TRANSDUCTION PATTERNS

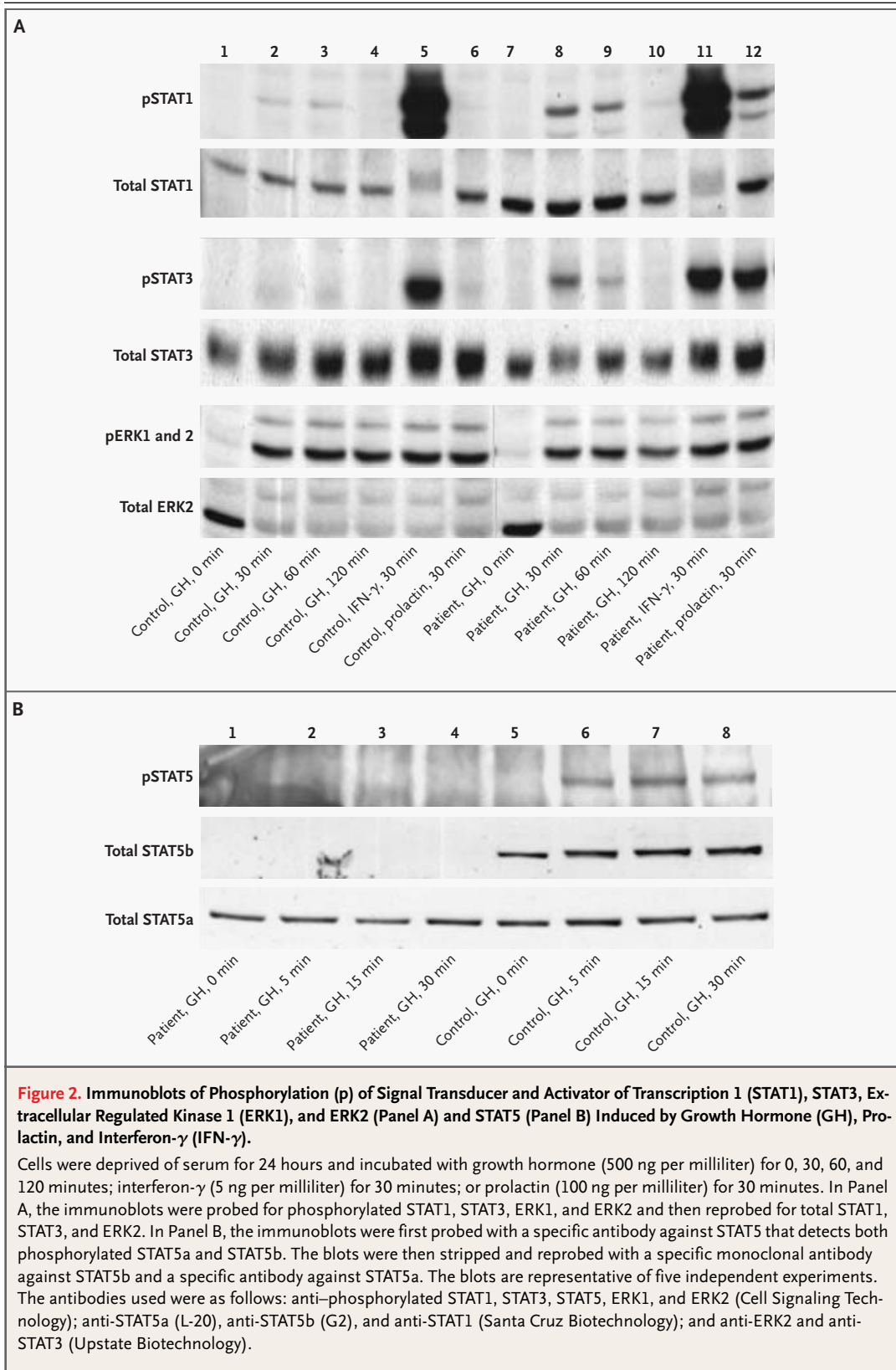
Signal transduction involving the growth hormone receptor occurs by means of at least three well-established pathways: the signaling module involving mitogen-activated protein kinase and extracellular regulated kinase 1 and 2 (ERK1 and 2), STAT, and the phosphatidylinositol-3 kinase–protein kinase B signaling module. A review of these pathways indicated that signal transduction from the growth hormone receptor to ERK1 and 2 was normal in normal fibroblasts and in primary fibroblasts from the patient, with a rapid onset of phosphorylation (within 5 minutes) (data not shown), which peaked within 30 minutes (Fig. 2A). Growth hormone–induced signaling through the STAT pathways (STAT1, STAT3, STAT5a, and STAT5b) was, however, aber-

rant in the patient's cells. Induction of STAT1 phosphorylation by growth hormone was poor in normal primary skin fibroblasts, as described previously,¹³ and in the fibroblasts from the two control subjects (Fig. 2A). The patient's fibroblasts, on the contrary, demonstrated growth hormone–induced phosphorylation of STAT1 as early as 15 minutes after the administration of growth hormone, which peaked between 30 and 60 minutes and returned to base line by 120 minutes. The degree of phosphorylation of STAT1 in the patient's fibroblasts was 8 to 10 times as great as in the control cells (Fig. 2A). Interestingly, the total STAT1 concentration appeared to be at least twice as high in the patient's cells as in the control cells. As with STAT1, growth hormone–activated phosphorylation of STAT3 was also significantly enhanced in the patient's cells, with a peak increase by a factor of approximately 8 (Fig. 2A).

The enhanced activation of STAT1 and STAT3 in the patient's cells was also observed when the cells were treated with prolactin, a luteotrophic hormone whose actions, like those of growth hormone, are mediated by class I cytokine receptors. In contrast, when the cells were incubated with interferon- γ , a potent STAT1 activator whose actions are mediated by class II cytokine receptors, the activation of STAT1 and STAT3 was robust, and more important, it was the same as that in the normal cells (Fig. 2A).

IDENTIFICATION OF A MISSENSE MUTATION IN THE SH2 DOMAIN OF STAT5b

Although the signal transduction is mediated in part through STAT1 and STAT3, rodent knockout models (STAT5a^{-/-}, STAT5b^{-/-}, and STAT5a^{-/-}b^{-/-}) have demonstrated the requirement for STAT5b in the generation of IGF-I in response to treatment with growth hormone and for normal postnatal growth.¹⁸⁻²⁰ In normal fibroblasts, both STAT5a and STAT5b were detectable under basal conditions. Activation of STAT5 was evident but transient, with peak activity observed between 5 and 15 minutes after the administration of growth hormone (Fig. 2B) and a return to basal concentrations by 60 minutes. In the patient's cells, however (Fig. 2B), immunoblotting with antibodies specific to either STAT5a or STAT5b clearly demonstrated that STAT5b was only poorly detectable, whereas STAT5a was normally expressed. Furthermore, on treatment with growth hormone, no phosphorylation of STAT5a or STAT5b was detectable. These results suggested either that STAT5b was absent in the patient or that the epitope recognized by the specific (monoclon-



al) antibody was affected by a mutation and raised questions concerning the ability of this protein to be activated.

The 2.4-kb *STAT5b* coding region was amplified by RT-PCR from the patient's cells and from two control cell lines. A comparison of the DNA sequences indicated that the patient was homozygous for a single nucleotide change within the *src* homology (SH2) domain of *STAT5b* (Fig. 3A). The mutation was confirmed at the genomic level (data not shown). The mutation resulted in an amino acid substitution of proline (cct) for alanine (gct) at position 630 (A630P) (Fig. 3B). The patient's parents, as expected, were heterozygous for the mutation (Fig. 3B). Sequencing of the relevant *STAT5b* exon (exon 15) from 100 samples of normal genomic DNA (Coriell) revealed neither polymorphisms nor heterozygosity at this site.

The change in the DNA sequence introduced a *StyI* restriction site, which, as shown in Figure 3C, could be used to differentiate the mutated SH2 sequence from that of the wild type. Both the patient's mother and father carried the wild-type as well as the mutant products (Fig. 3C), corroborating the sequencing data. Furthermore, immunoblotting of lymphoblast-cell extracts from both parents showed the presence of wild-type *STAT5b* in concentrations equivalent to those of controls (data not shown).

STABILITY AND ACTIVITY OF MUTANT *STAT5b*

To evaluate the stability and phosphorylation of the mutant *STAT5b* (A630P) further, we used a COS-7-cell expression system and interferon- γ , which can activate both *STAT5a* and *STAT5b* (data not shown). In COS-7 cells, treatment with interferon- γ results in the activation of endogenous *STAT5* (Fig. 3D, lanes 7 and 8). Transient transfection with the vector pcDNA3.1 resulted in the same pattern of phosphorylation (Fig. 3D, lanes 1 and 4). When wild-type *STAT5b* was overexpressed, it was constitutively activated but was still clearly responsive to interferon- γ -induced activation (Fig. 3D, lanes 3 and 6). In contrast, overexpression of mutant *STAT5b* (A630P) showed a pattern of *STAT5* phosphorylation that was similar to that of untransfected, or vector-transfected, cells (Fig. 3D, lanes 2 and 5). This was not due to a lack of expression of mutant *STAT5b*, since the overexpressed protein was readily detected, albeit less well recognized by the antibody than the wild-type protein (Fig. 3D, lanes 2 and 5). These results demonstrate that the mutant *STAT5b* can be stably expressed but cannot be

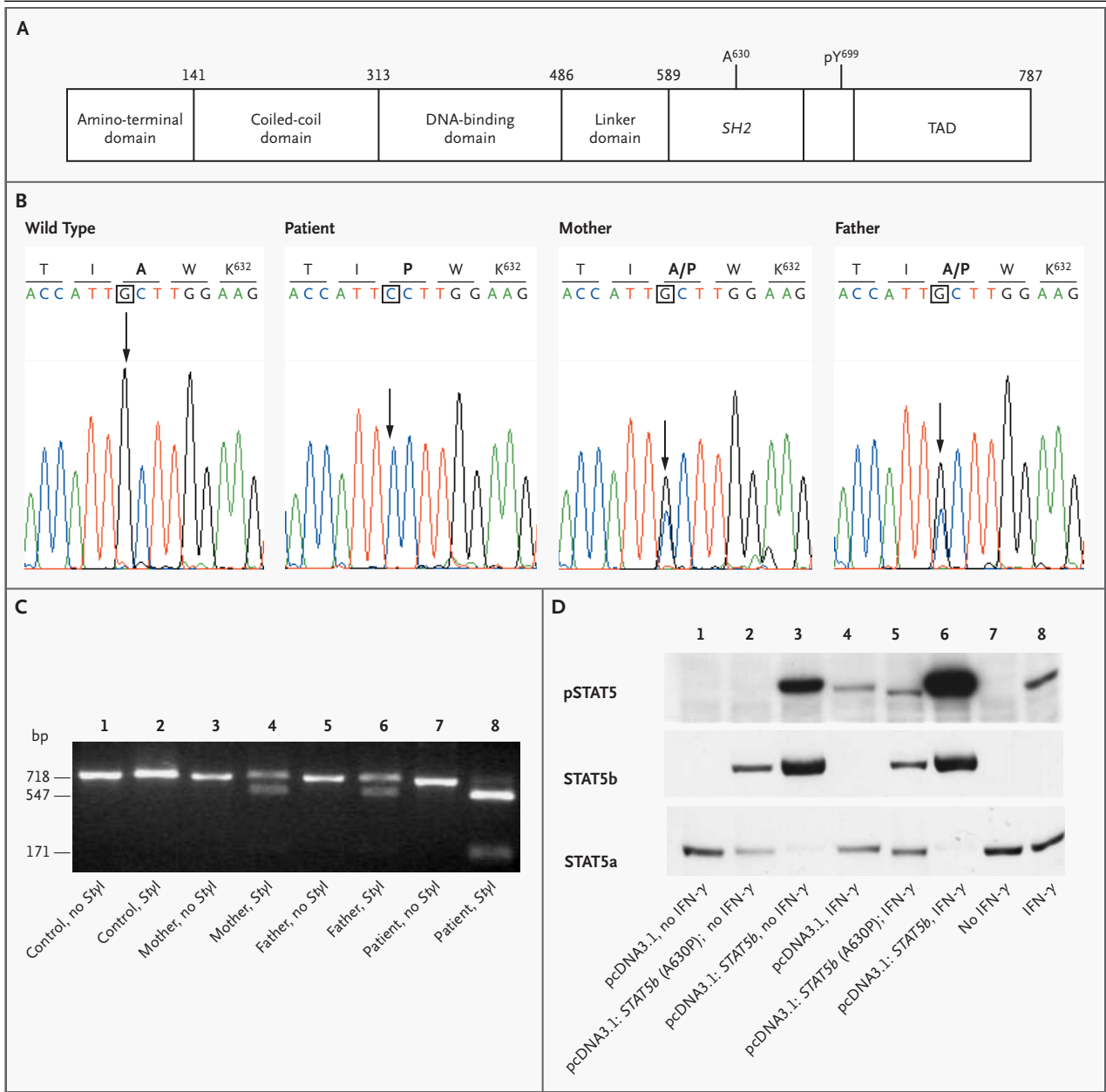
Figure 3 (facing page). Domains of the Wild-Type *STAT5b* Protein (Panel A), Mutation in the *STAT5b* Gene from the Patient (Panel B), Differentiation of the Wild-Type Sequence from That of the Mutated Sequence in the *src*-Homology-2 (SH2) Domain (Panel C), and Stability and Activity of the Mutant *STAT5b* (Panel D).

In Panel A, TAD denotes transcription activation domain, A alanine, and pY the tyrosine at position 699 that is phosphorylated. Numbers indicate the amino acid positions. Panel B shows an electrophoretogram of the wild-type DNA sequence, the mutant sequence in the patient, and the sequences in her mother and father, both of whom were heterozygous for the mutation. Arrows indicate the relevant nucleotide that is changed in the patient's DNA. The predicted amino acid alteration is shown. As shown in Panel C, the substitution of proline for alanine at position 630 (A630P) introduces a novel *StyI* restriction site into the *STAT5b* gene. Polymerase-chain-reaction (PCR) primers were used to amplify the SH2 and transcription activation domains of *STAT5b* complementary DNA from lymphocytes from a control subject, the patient's mother and father, and the patient. *StyI*-restriction digests of the PCR products confirmed the patient's homozygosity and parental heterozygosity. In Panel D, mutant *STAT5b*, which is overexpressed in COS-7 cells, is not activated by interferon- γ (IFN- γ). Representative immunoblots are shown from four independent experiments. COS-7 cells were incubated alone (lanes 7 and 8) or transiently transfected with pcDNA3.1 (lanes 1 and 4), pcDNA3.1:*STAT5b* (A630P) (lanes 2 and 5), or pcDNA3.1:*STAT5b* (lanes 3 and 6). After 24 hours, cells were deprived of serum for 24 hours and then incubated alone or with interferon- γ (100 U per milliliter) or not treated for 60 minutes. Cell lysates were collected and immunoblotted to determine the phosphorylation (p) of *STAT5* induced by interferon- γ , as well as the total *STAT5b* and *STAT5a*, as described in the legend to Figure 2B. The apparent lack of detectable *STAT5a* in lanes 3 and 6 is due to the inefficient stripping of antibody against *STAT5b* from the blot. Fresh blots probed with antibody against *STAT5a* confirmed that *STAT5a* was unaffected by the overexpression of wild-type or mutant *STAT5b* (data not shown).

activated by either growth hormone (Fig. 2B) or interferon- γ (Fig. 3D).

DISCUSSION

We describe a patient with a homozygous mutation in the gene for *STAT5b*, resulting in IGF-I deficiency and growth hormone insensitivity. The patient had abnormal postnatal growth, facial dysmorphism, and markedly reduced serum concentrations of IGF-I, IGFBP-3, and acid-labile subunit. Serum concentrations of these three proteins remained abnormally low, despite seven days of treatment with



growth hormone, and her growth rate failed to increase in response to one year of growth hormone therapy. Her concentrations of growth hormone-binding protein were normal, as measured by a ligand-mediated binding assay in serum and by immunoblotting of fibroblast lysates, reflecting the fact that her GHR gene and protein were normal. Although there was no family history of growth retardation, the parents were first cousins, a relationship consistent with the occurrence of classic autosomal recessive transmission.

Most reported cases of growth hormone insensitivity involve mutations or deletions of the GHR gene.^{2-5,8-12} Several cases of putative defects downstream of the GHR gene have now been reported in patients with phenotypes of growth hormone insensitivity.¹³⁻¹⁶ In two of these studies,^{15,16} growth hormone failed to induce tyrosine phosphorylation of the STAT proteins, although no specific molecular abnormalities were identified. Indeed, the *STAT5b* gene from each of the 14 children with idiopathic short stature described by Salerno et al.¹⁶ was se-

quenced and found to be normal.²¹ Rodent models of STAT knockouts, on the other hand, have indicated that of the STAT proteins, STAT5b is essential for growth hormone–induced activation of IGF-I and for normal postnatal growth.^{19,20} Interestingly, female mice with STAT5b knockouts were largely unaffected, whereas males, which are usually approximately 30 percent larger than female mice, resembled females in size. This loss of sexually dimorphic growth patterns in mice contrasts with the dramatic growth failure observed in our patient. Furthermore, STAT5a and STAT5b do not appear to be fully redundant in humans, since normal concentrations of STAT5a did not prevent the insensitivity to growth hormone in our patient.

The missense mutation in our patient is in exon 15 of the STAT5b gene, which encodes half of the SH2 domain. No polymorphism or heterozygosity was detected in this exon in control subjects. The SH2 domain of the STAT genes is highly conserved and is necessary for the docking of these latent proteins to ligand-activated phosphorylated receptors^{22,23} and for their subsequent dimerization.²⁴ The amino acid affected, alanine at position 630, is deep within the SH2 domain, in a β -sheet, β C, that is part of an antiparallel β -sheet structure essential for the binding of phosphate groups.²⁵ Prolines are incompatible with a functional β -sheet structure. A change from alanine to proline is therefore predicted to be disruptive, affecting the functionality of the SH2 domain, distorting the epitopes recognized by antibodies, and changing the overall stability of the protein. When the mutant STAT5b was overexpressed, it was detectable in COS-7 cells, suggesting that its expression can be stable. Epitope recognition and functionality, however, were clearly affected by the mutation, since the mutant protein was poorly recognized by commercially available antibodies against STAT5b. Most important, despite being stably expressed, the mutant protein could not be activated by either growth hormone or interferon- γ .

One consequence of the mutant STAT5b is the

failure to activate genes transcriptionally, as demonstrated by the dysregulation of IGF-I and IGFBP-3 both in vitro and in vivo. Aberrant regulation of other proteins is also to be expected, including the negative regulators of STAT signaling, which could potentially account for the enhanced phosphorylation of STAT1 and STAT3 that we observed. The increase in total STAT1 concentrations in our patient is consistent with that observed in the STAT5b^{-/-} rodent model.¹⁹

In addition to mediating the actions of growth hormone, STAT5b is also important for the actions of many other growth factors and cytokines (interleukins²⁶ and interferons²⁰). The signal transduction of prolactin was abnormal in our patient's fibroblasts, and in the COS-7–cell model, interferon- γ activated wild-type STAT5b but failed to activate the mutant STAT5b. STAT5b has an important role in the proliferation and differentiation of T cells,²⁶⁻²⁹ and a nonfunctional STAT5b is likely to decrease cell-mediated immunity, as has been demonstrated in STAT5b^{-/-} mice^{18-20,28} and as suggested by the results in our patient's cells when they were exposed to interferon- γ . Hence, we hypothesize that the immunologic problems in our patient are probably a direct manifestation of the mutated STAT5b. Further studies of immunodeficiencies in this and similar patients are clearly warranted.

In summary, the STAT5b mutation represents a clearly defined postreceptor molecular abnormality in the human growth hormone signaling cascade. It is likely that other defects in the JAK-STAT system will be identified in patients with various degrees of IGF deficiency and growth hormone insensitivity. The combined phenotype of growth hormone insensitivity and immunodeficiency is consistent with the presence of such signaling defects.

Supported by grants (CA58110 and DMD17-00-1-0042, to Dr. Rosenfeld) from the National Institutes of Health, and by a grant from Pharmacia.

We are indebted to Dr. Ellen Magenis (Department of Genetics, Oregon Health and Science University) for performing a skin biopsy to obtain the fibroblast cultures used in this study.

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