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Reduced mRNA and a Nonsense Mutation in the Insulin-Receptor Gene Produce Heritable Severe Insulin Resistance

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Summary

Leprechaunism is an autosomal recessive syndrome of severe insulin resistance and is characterized by intrauterine growth restriction, acanthosis nigricans, hirsutism, and loss of glucose homeostasis. Here we report a new female patient of Hispanic and Afro-American descent whose fibroblasts and lymphoblasts had markedly impaired insulin binding (less than 10% of that in controls). Insulin binding to lymphoblasts established from both unrelated parents was partially impaired. Insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF) binding to the patient's fibroblasts were within the normal range. Insulin stimulation of receptor autophosphorylation and kinase activity was markedly reduced in the patient's fibroblasts. The patient's fibroblasts had both a reduced number of immunoreactive insulin receptor (6% of those in controls) and concomitantly reduced amounts of insulin-receptor mRNA, suggesting that both mutations inherited by the patient reduced insulin-receptor mRNA. Sequencing of the insulin-receptor gene and cDNA indicated that the patient was heterozygous for a paternally derived mutation at bp 1333, converting Arg_{372} to a STOP codon. This nonsense mutation was observed in the insulin-receptor gene, but not in cDNA, indicating reduced amounts of mRNA for the allele containing this mutation. The coding sequence of the maternally inherited insulin-receptor allele was normal. Both the marked reduction in insulin-receptor mRNA in the compound heterozygous fibroblasts of the proband and the partially reduced insulin binding in maternal cells suggest that the maternally derived mutation is located in an insulin-receptor gene sequence that controls cellular mRNA content.

Introduction

The human insulin receptor is a heterotetramer composed of two extracellular α subunits which bind insulin and two β subunits which span the plasma membrane and have an intracellular tyrosine kinase domain (Ebina et al. 1985; Ullrich et al. 1985). Insulin binding to the α subunit of the receptor stimulates β subunit autophosphorylation and kinase activity. A single gene located on chromosome 19 codes for both the α and β subunits of the receptor (Seino et al. 1989). Mutations in this gene have been identified in patients with insulin-resistant syndromes, such as leprechaunism, Rabson-Mendenhall syndrome, and type A diabetes (Elsas et al. 1989; Kadowaki et al. 1990*a*). Leprechaunism is the most severe of these syndromes. It is inherited as an autosomal recessive trait and is characterized by intrauterine growth restriction, loss of glucose homeostasis, hyperinsulinemia, and characteristic physical features (Elsas et al. 1985*a*). Specific loss of insulin binding is a genetic discriminant for this syndrome in cells cultured from patients and first-degree relatives (Elsas et al. 1985*b*; Endo et al. 1987; Longo et al. 1989). Defective insulin binding is responsible for reduced insulin-stimulated receptor autophosphorylation and kinase activity in fibroblasts cultured from most patients with this syndrome (Endo et al. 1987; Maassen et al. 1988; Reddy et al. 1988). A significant exception is one unique patient whose fibroblasts had reduced insulin binding but constitutive

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activation of insulin-receptor autophosphorylation and kinase activity (Longo et al. 1990). Further studies of these natural mutations in the insulin-receptor gene promise to clarify the mechanism by which insulin accomplishes transmembrane signaling.

Three of the mutations characterized in patients with insulin resistance insert a premature STOP codon and reduce insulin-receptor mRNA levels (Ojamaa et al. 1988; Muller-Wieland et al. 1989; Kadowaki et al. 1990*a*, 1990*b*). In one of these patients, patient Minn, and in two other patients with type A insulin resistance, no mutations were found within the 22 exons of the insulin-receptor gene, and a second mutation was postulated but not demonstrated in the promoter region of the insulin-receptor gene (Kadowaki et al. 1990*b*; Imano et al. 1991). The promoter region and the 5′ region of the first intron of the insulin-receptor gene contain putative regulatory sequences, but their physiological importance is at present not known (Araki et al. 1987, 1991; Mamula et al. 1988; Seino et al. 1989; Tewari et al. 1989; McKeon et al. 1990; McKeon and Pham 1991).

Here we describe a new patient, Mount Sinai (Norton et al. 1990), with leprechaunism. Her cultured cells had absent insulin binding and immunoreactive insulin receptors. Insulin-receptor mRNA was also markedly reduced, indicating that both mutations impaired mRNA production and/or stability. Sequencing of the insulin-receptor gene identified a paternally inherited C-to-T transition at bp 1333, converting Arg_{372} to a STOP codon (R372X). The maternally inherited allele had no mutations within the protein-coding region, suggesting that this second mutation was localized in an insulin-receptor gene region involved in the control of gene expression.

Patient, Material, and Methods

Patient

The proband (leprechaun Mount Sinai) was a black female, the second child of healthy unrelated parents of Hispanic and Afro-American descent. After a reportedly uncomplicated pregnancy, a caesarean section was performed as an emergency at 35 wk gestation, because of breech presentation and cardiac deceleration. The proband weighed 1,870 g, which was small for gestational age (below the fifth percentile). Apgar scores were at 1 at 1 min, 4 at 5 min, and 7 at 10 min. The neonatal period was marked by a meconium plug, rectal prolapse, and repeated, transient hypoglycemic episodes.

At 5 mo of age the patient was referred to the Mount Sinai Medical Center. She was small and had dysmorphic features, including prominent eyes with shallow orbits, wide and upturned nostrils, thick lips, low-set and abnormally folded ears, prominent female genitalia, a reducible rectal prolapse, marked hirsutism, breast hyperplasia, loose and pachidermatous skin, decreased adipose tissue, acanthosis nigricans, and abdominal distention. Magnetic resonance imaging of the head showed a diffusely thinned corpus callosum, sulcal prominence, and an enlarged cisterna magna (Norton et al. 1990). Pelvic ultrasound showed a retroverted uterus with bilaterally enlarged ovaries containing multiple cysts. Blood chemistry indicated an insulin level greater than 1,132 μU/ml and a glucose concentration of 83% mg/100 ml. The patient died of unknown cause at age 7 mo.

Material

Sera, growth media, and trypsin solutions were from Sigma. Na[^{125}I], and *gamma*-[^{32}P]ATP were from New England Nuclear; [^{32}P]dCTP was from Amersham, and insulin-like growth factor-I (IGF-I, receptor grade) and epidermal growth factor (EGF) were from Imcera. Porcine sodium insulin was from Calbiochem. Wheat-germ agglutinin (WGA) was from E-Y. Products for molecular biology were from Boehringer Mannheim. Sigma was the source of other chemicals.

Cell Culture

Fibroblast cultures were established from skin biopsies of both patient Mount Sinai at age 5 mo and matched controls. Additional control fibroblast cultures (GM 003348 and GM 005756) and fibroblasts from patient Minn (GM 005241) were obtained from the Coriell Institute for Medical Research (Camden, NJ). Cells were grown in Dulbecco-Vogt (DV) medium containing 15% FBS.

Lymphocytes from patient Mount Sinai at age 5 mo and from her parents were transformed by the Epstein-Barr virus. Control lymphoblasts were from the Coriell Institute for Medical Research and from controls established at the Emory Clinical Research Facility. Lymphoblasts were grown in RPMI-1640 medium supplemented with 15% FBS.

Ligand Binding

Ligand binding to cultured fibroblasts was performed at 20°C (for insulin and IGF-I) or 4°C (for EGF) in Earle's balanced salt solution (EBSS) buffered with tris(hydroxymethyl)-aminomethane (26 mM,

pH 7.4), according to a method described elsewhere (Longo et al. 1989). Insulin binding to lymphoblasts was measured at 16°C (Gavin et al. 1973). Ligand binding was normalized to cell proteins, measured by a modified Lowry procedure (Wang and Smith 1975), corrected for nonspecific binding (measured in the presence of 1–10 μg of cold ligand/ml) and expressed as femtomoles of ligand bound per milligram of cell protein.

Kinetics of ligand binding were analyzed by nonlinear regression according to a two-receptor model in the case of insulin and according to a single, noncooperative receptor in the case of IGF-I and EGF (Longo et al. 1989). In the text, nonlinear parameters are expressed as means ± SD.

Immunoassay of Insulin Receptors

Human fibroblasts were grown to confluence in 150-cm^2 flasks, were washed three times with ice-cold saline, were solubilized in 1% Triton X-100, and were centrifuged for 30 min at 4°C at 16,000 *g*. The supernatant was used to displace ^{125}I-radiolabeled insulin receptor from polyclonal anti-insulin receptor antibodies (Pezzino et al. 1989).

Insulin-Receptor Phosphorylation and Kinase Activity

Insulin-receptor autophosphorylation and kinase activity were measured in WGA-purified insulin receptors (25 μg of protein/fibroblast strain) according to a method described elsewhere (Endo et al. 1987; Longo et al. 1990). For autophosphorylation, insulin receptors were immunoprecipitated using a polyclonal rabbit anti-insulin receptor IgG that recognizes specifically the insulin receptor and does not precipitate significant amounts of IGF-I receptors (Endo et al. 1987).

RNA Analysis

Cellular RNA was extracted with guanidinium thiocyanate (Chomczynski and Sacchi 1987) and was fractionated on an oligo(dT) cellulose column (Sambrook et al. 1989). Poly(A)$^+$ RNA was separated by formaldehyde/agarose-gel electrophoresis, was transferred to nylon, and hybridized in high-stringency conditions to a [^{32}P]dCTP-cDNA extending from the *Eco*RI site at bp 1013 to the 3′ of the insulin receptor cDNA (Ebina et al. 1985; Ullrich et al. 1985). After autoradiography, blots were stripped and hybridized to the actin cDNA. Autoradiograms were analyzed by laser densitometry in two-dimensional mode. The area of the insulin-receptor mRNA was normalized to the actin signal before comparison.

Sequence of the Insulin-Receptor Gene and cDNA

Genomic DNA was extracted from cells of patient Mount Sinai and her parents by the "salting out" procedure (Miller et al. 1988). DNA was amplified by PCR using primers flanking the exons of the insulin-receptor gene (Seino et al. 1990). cDNA synthesized from fibroblast and lymphoblast RNA was amplified by PCR using internal primers (table 1). Amplified double-stranded DNA was purified by low-melting-temperature agarose-gel electrophoresis and was sequenced directly by adding either one of the two end primers or an internal primer (table 1) labeled with [^{32}P]-ATP by the T4 polynucleotide kinase (Sambrook et al. 1989), *Taq* polymerase, and the four termination mixes, containing 250 μM of the nonterminating dNTP, 25 μM of the terminating dNTP, and either 50 μM ddGTP, 350 μM ddATP, 600 μM ddTTP, or 160 μM ddCTP. The reaction was separated on a 6%–8% acrylamide sequencing gel which was then dried and autoradiographed.

Results and Discussion

Insulin, IGF-I, and EGF Binding to Fibroblasts and Lymphoblasts

Figure 1 depicts a Scatchard analysis of insulin binding to lymphoblasts obtained from family Mount Sinai and a control. At the lowest insulin concentration (0.8 ng/ml), insulin binding to the patient's lymphoblasts was reduced to 2.7% of that in controls. Lymphoblasts established from both unrelated parents had partially defective insulin binding which was reduced to 31% and 42% of that in controls in the mother's and the father's cells, respectively. Curvilinear plots for insulin binding were observed in lymphoblasts obtained from the control and from both parents. Data were fitted to a two-receptor model to estimate the number of receptors and the dissociation constant (K_D) (Longo et al. 1989). Insulin binding to the high-affinity site is depicted in figure 1 as a straight line. Control lymphoblasts (fig. 1*A*) bound 141 ± 34 fmol of insulin/mg of cell protein to the high-affinity binding site (about 8,500 sites/cell), with a K_D of 0.19 ± 0.08 nM. Lymphoblasts from the proband's mother (fig. 1*C*) had a 76% reduction in the number of high-affinity sites but had normal affinity (K_D) = 0.12 ± 0.05 nM). Cells from the father (fig. 1*D*) had a 63%

Table 1

Oligonucleotides for Amplification and Sequencing of Insulin-Receptor cDNA

A. Primer Pairs for Amplification of Insulin-Receptor cDNA		
Upstream (+)	Fragment Size (bp)	Downstream (−)
5′-CCAGGACCTGCACCACAAA (996–1015)	631	5′-TTTCACAGGATGCCTTGTCC (1627–1608)
5′-CCACTATAACCCCAAACTCT (1503–1522)	1,107	5′-AGCCTGCAGCTCGATGCG (2610–2593)
5′-ATCTCCGGCTTGCGACACTTCA (2563–2584) ...	1,036	5′-TTCTTGGCGTTCAGGTAG (3599–3572)
5′-ATGGAGCTGATGGCTCACGG (3444–3463)	966	5′-GAACGATCTCTGGAACTCCACT (4410–4390)

B. Internal Primers for Sequencing (in addition to end primers)	
Upstream (+)	Downstream (−)
5′-TATGGGGCCAAGAGTGACATCATTT (1954–1977)	5′-ACCTTCTCAAAAGGCCTGTG (2540–2521)
	5′-ATCACTGGCACTGAGATACT (3150–3131)
	5′-AACTCAATCCGCTCTCGGAGAC (3350–3331)
5′-ATGAAACGGATTACTACCGG (3692–3712)	5′-GTGCGAGGAACGGTCCAG (4141–4124)

NOTE.—Nucleotide numbering is that of Ebina et al. (1985).

reduction of the number of high-affinity insulin-binding sites and had no significant change in the K_D value (0.12 ± 0.06 nM). The almost complete absence of insulin binding to the patient's lymphoblasts (fig. 1*B*) prevented kinetic analysis. Both the partial impairment of insulin binding to lymphoblasts of both parents and their different ethnic background suggested that their offspring was a compound heterozygote for two different mutations in the insulin-receptor gene.

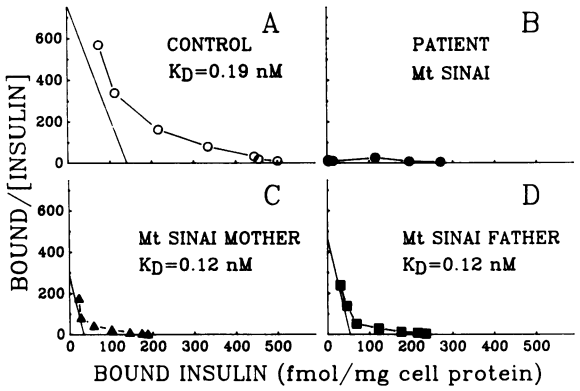

Figure 1 Scatchard plot of insulin binding to human lymphoblasts. Epstein-Barr virus–transformed lymphoblasts (1×10^6 cells/tube) from a control (*A*), patient Mount Sinai (*B*), her mother (*C*), and her father (*D*) were washed and incubated for 3 h at 16°C in EBSS, pH 7.4, containing 4% BSA and 0.5 mM bacitracin in the presence of 0.8–300 ng of ^{125}I-insulin/ml. Cells were then centrifuged and washed three times with ice-cold 0.1 M $MgCl_2$. Bound insulin was normalized to cell proteins and corrected for nonspecific binding (5 μg of cold insulin/ml). Points are means of triplicate observations. Nonlinear regression analysis of data by using a two-receptor model yielded the high-affinity component depicted as a straight line in panels *A*, *C*, and *D*, whose K_D is also reported in the same panels.

Figure 2*A* shows a Scatchard plot of insulin binding to fibroblasts obtained from patient Mount Sinai and from an age-matched control. At 0.6 ng/ml, [^{125}I]-insulin binding to the proband's fibroblasts was reduced to 4.1% of that in control cells. Analysis of the data according to a two-receptor model indicated that control fibroblasts bound 6.98 fmol of insulin/mg of cell protein (2,100 sites/cell) to the high-affinity site, with a K_D of 0.8 nM. Insulin binding to fibroblasts of patient Mount Sinai was markedly reduced and did not allow kinetic analysis.

Fibroblasts from some previously reported patients with leprechaunism also had reduced binding of IGF-I or EGF (Van Obberghen-Shilling et al. 1981; Reddy and Kahn 1989). IGF-I (1 ng/ml) binding to patient Mount Sinai's fibroblasts (3.9 ± 1.2 fmol/mg of protein) was within the normal range (range 2.2–6.3, mean 4.1 ± 1.9 [n = 18] fmol/mg cell protein). EGF (0.5 ng/ml) binding was also in the normal range (range 2.9–8.1, mean 5.3 ± 1.8 [n = 18] fmol/mg cell protein) in fibroblasts cultured from patient Mount Sinai (4.2 ± 0.4 fmol/mg cell protein), whereas it was reduced to 26% of that in controls in fibroblasts cultured from patient Minn with lepre-

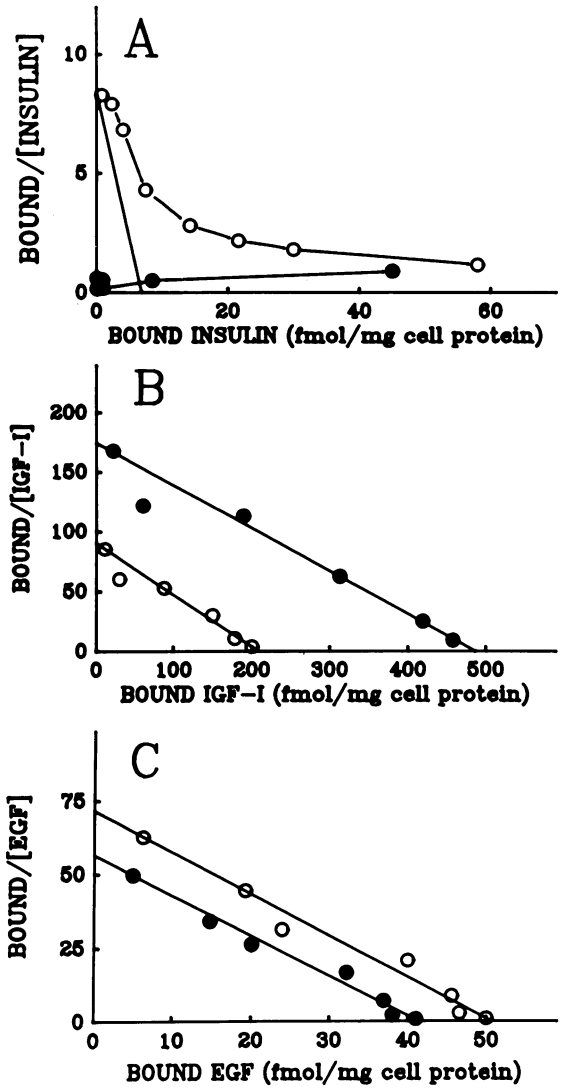

Figure 2 Scatchard plot of insulin (*A*), IGF-I (*B*), and EGF (*C*) binding to cultured fibroblasts. Confluent cultures of fibroblasts were washed and incubated for 2 h in EBSS supplemented with 4% BSA and 0.5 mM bacitracin at 20°C. Cells were then incubated for 2 h in the same buffer containing 0.6 ng of ^{125}I-Insulin/ml at 20°C (*A*), 1 ng of ^{125}I-IGF-I/ml at 20°C (*B*), or 0.6 ng of ^{125}I-EGF/ml at 4°C (*C*) and 1–300 ng of cold ligand/ml. Cells were then washed three times with ice-cold $MgCl_2$ 0.1 M and solubilized in 0.4 ml of 1 N NaOH containing 0.1% sodium deoxycholate. The cell extract was transferred to plastic tubes, counted for radioactivity, and analyzed for protein content. Nonspecific binding, measured in the presence of 1–10 μg of cold ligand/ml, was subtracted before plotting. Each point is the mean of triplicates. SDs were within 10% of the experimental value. Data were then evaluated by nonlinear regression analysis according to either a two-receptor model, for insulin binding, or a single-receptor model, for IGF-I and EGF binding. In *A*, the straight line represents high-affinity insulin binding. In *B* and *C*, the lines represent the best fit of the data, according to a single-receptor model. O—O = Control; ●—● = patient Mount Sinai.

chaunism (1.4 ± 0.2 fmol/mg cell protein), as observed by others (Reddy and Kahn 1989). Scatchard plots of IGF-I (fig. 2*B*) and EGF (fig. 2*C*) binding were linear, indicating that these growth factors bound to a single class of noncooperative receptors on the plasma membrane of human fibroblasts. The K_D for IGF-I binding to fibroblasts from patient Mount Sinai was 2.8 ± 0.6 nM, and it was 2.3 ± 0.8 nM in the matched control. The number of IGF-I receptors, estimated from the maximal binding of IGF-I, was also in the normal range in fibroblasts from patient Mount Sinai (486 fmol/ng of cell protein, compared with a control range of 208–535 fmol/mg of cell protein). Fibroblasts from patient Mount Sinai bound 41 fmol of EGF/mg of cell protein (normal range 35–121 fmol/mg cell protein), with a K_D of 0.73 ± 0.10 nM, compared with a K_D of 0.71 ± 0.17 nM in control fibroblasts. Normal EGF and IGF-I binding to fibroblasts from patient Mount Sinai indicated that neither reduced EGF nor IGF-I binding, observed in other patients with the same syndrome (Van Obberghen-Shilling et al. 1981; Reddy and Kahn 1989), are an obligate effect of impaired insulin receptors in fibroblasts cultured from patients with leprechaunism.

Insulin-Receptor Protein and mRNA in Human Fibroblasts

The number of insulin receptors on fibroblasts cultured from patient Mount Sinai was determined by displacement of radiolabeled human insulin receptor from a rabbit polyclonal antibody (Pezzino et al. 1989). Fibroblasts from patient Mount Sinai had 0.61 ng of immunoreactive receptors/mg of protein, compared with a normal mean ± SD of 9.5 ± 2.8 ng/mg of protein. Thus, fibroblasts from patient Mount Sinai had about 6% of control immunoreactive insulin receptors, consistent with the observed reduction in insulin binding.

We next evaluated the amount of poly(A)$^+$ RNA specific for the insulin receptor in fibroblasts cultured from patient Mount Sinai (fig. 3). The insulin-receptor mRNA produces multiple bands of size 5–10 kb (Ullrich et al. 1985; Ojamaa et al. 1988; Longo et al. 1989; Muller-Wieland et al. 1989). The variation in size is due to multiple 3′ ends, presumably reflecting alternative polyadenylation (Tewari et al. 1989). Insulin-receptor mRNA from control cells had prominent bands at 5.2 and 7.5 kb. Fibroblasts from patient Mount Sinai had reduced insulin-receptor mRNA levels, which were 5%–15% of control values when quantitated by laser densitometry in two separate experiments. This reduction in steady-state mRNA

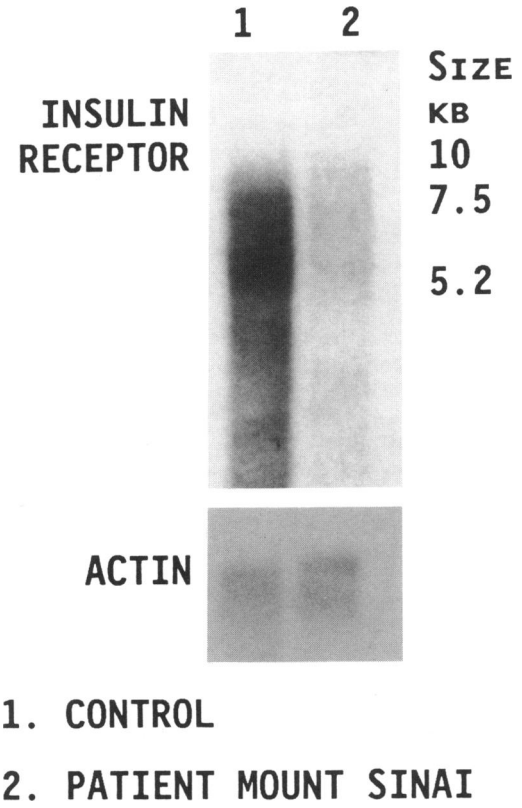

Figure 3 Northern blot of insulin-receptor mRNA from fibroblasts of patient Mount Sinai and a control. Five micrograms of poly(A)$^+$ RNA were separated by formaldehyde–agarose gel electrophoresis, were transferred to nylon, and were hybridized, in high-stringency conditions, to the insulin-receptor cDNA labeled with [^{32}P]-dCTP. The blot was then stripped and hybridized to the actin cDNA. Insulin-receptor mRNA quantitation by laser densitometry indicated that patient Mount Sinai's levels were reduced to 5%–15% of control values in two separate experiments.

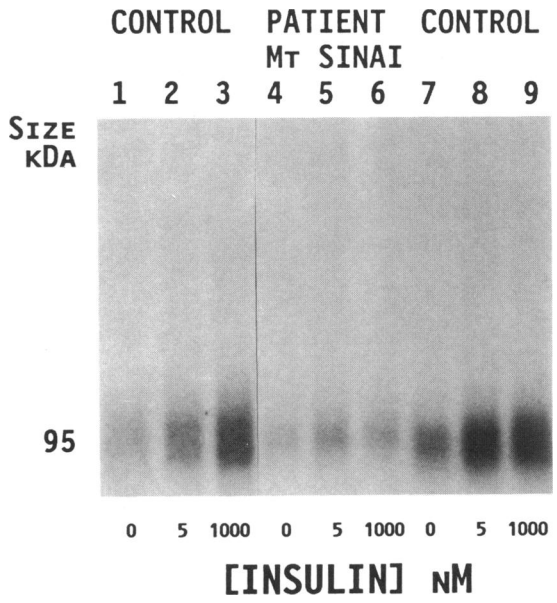

Figure 4 Autophosphorylation of insulin receptors from fibroblasts of controls and patient Mount Sinai with leprechaunism. Twenty-five micrograms of WGA-purified insulin receptors were incubated for 2 h at 22°C in the presence of the indicated concentrations of insulin and then for 15 min in the presence of [^{32}P]ATP (50 μM, 8 μCi/nmol). After immunoprecipitation with a polyclonal antibody (Endo et al. 1987), insulin receptors were separated by SDS-PAGE (5% acrylamide) in reducing conditions (100 mM DTT) and were visualized by autoradiography for 36 h at −70°C. The experiment was repeated three times with similar results. Lanes 1–3 and 7–9, Controls. Lanes 4–6, Patient Mount Sinai.

levels was consistent with reduced amounts of immunoreactive receptors. The marked decrease in insulin-receptor mRNA levels in the proband's cells suggested that both the paternal and maternal mutations had an additive effect in reducing insulin-receptor mRNA levels.

Insulin-Receptor Phosphorylation and Kinase Activity

Insulin-receptor autophosphorylation and kinase activity were determined in insulin receptors of human fibroblasts partially purified by WGA chromatography (fig. 4). Insulin stimulated autophosphorylation of the 95-kD β subunit of the receptor two- to threefold in receptors from two matched controls. Half-maximal stimulation was obtained with about 5 nM of insulin. Insulin stimulation of β-subunit phosphorylation in patient Mount Sinai's fibroblasts was reduced to about 8% of that in controls (mean of three separate experiments), when Cerenkov counting was used to quantify the 95-kD band (table 2).

The kinase activity toward an exogenous substrate of receptors purified from patient Mount Sinai was also decreased (table 2). Insulin stimulated the phosphorylation of poly (Glu:Tyr) about twofold in control receptors and only 27% above basal in receptors from patient Mount Sinai's fibroblasts, the absolute increase corresponding to 14% of that observed in control receptors.

Thus, in fibroblasts from proband Mount Sinai, autophosphorylation and kinase activity of insulin receptors were reduced in parallel with the reduced number of receptors. Since the reduction of receptor phosphorylation was not greater than the reduction in insulin binding, the kinase function of residual receptors was not affected by the mutations in this patient. In another patient with inherited severe insulin resistance (type A diabetes), a single mutation in the α subunit of the

Table 2

Effect of Insulin (1 μM) on Insulin-Receptor Phosphorylation and Kinase Activity

	β-Subunit Phosphorylation[a] (cpm)		Phosphate Incorporation[b] (pmol/μg protein)	
	Basal	Insulin	Basal	Insulin
Control A	19.1 ± 4.1	71.7 ± 4.7[c]	3.9 ± .1	7.7 ± 1.7[c]
Control B	35.6 ± 3.2	69.9 ± 4.8[c]	3.5 ± .1	9.4 ± .1[c]
Mount Sinai	18.3 ± 1.2	21.6 ± 6.6	2.6 ± 1.1	3.3 ± 1.8

[a] [^{32}P]Incorporation into the β subunit of the insulin receptor purified from human fibroblasts. Gel slices corresponding to bands on autoradiograms such as that shown in fig. 4 were quantified by Cerenkov counting. Each value represents the mean ± SD of three independent experiments.

[b] Kinase activity of insulin receptors purified from fibroblasts of controls and of patient Mount Sinai with leprechaunism. Three micrograms of WGA-purified insulin receptors were incubated for 90 min at 22°C in the absence or presence of insulin (1 μM). [^{32}P]ATP (60 μM, 0.7 μCi/nmol) and poly (Glu: Tyr, 4:1) at a final concentration of 2 mg/ml were then added to each tube, and the reaction was allowed to proceed for 30 min. The total reaction was spotted on Whatman paper, and unincorporated ATP was removed by several washings in 10% trichloroacetic acid. Each value is expressed as mean ± SE of three independent determinations.

[c] Different from paired basal at $P < .01$, with analysis of variance.

receptor impaired both receptor processing and kinase activity (Accili et al. 1991).

Sequence of the Insulin-Receptor cDNA and Gene of Patient Mount Sinai

Both cDNA, synthesized from fibroblast and lymphoblast mRNA, and genomic DNA were amplified using PCR and either cDNA primers (table 1) or primers in the flanking region of each exon (Seino et al. 1990). Amplified DNA was sequenced directly without subcloning, by using either one of the end primers or an internal primer labeled with [^{32}P]ATP and the T4 polynucleotide kinase. Patient Mount Sinai was heterozygous for a mutation in exon 5 of the insulin-receptor gene, converting Arg_{372} to a STOP codon (fig. 5). Both a C and a T nucleotide were present at bp 1333. The same mutation was present in paternal DNA but not in maternal DNA (fig. 5, *Lower panel*). This mutation has not been reported in other patients with inherited insulin resistance and was not present in five other controls and in three other patients with leprechaunism whom we have evaluated (Atl-1, Atl-2, and New Zealand). When we sequenced several preparations of insulin-receptor cDNA synthesized from different RNA isolates from fibroblasts and lymphoblasts of patient Mount Sinai, we failed to find this mutation. This suggested that transcripts of the paternal allele containing the premature STOP codon were present in reduced amounts as compared with transcripts of the maternal allele. Other patients with severe insulin-resistant syndromes have mutations that insert a premature STOP codon in the insulin-receptor gene. Most of these nonsense mutations are associated with decreased insulin-receptor mRNA (Kadowaki et al. 1990*a*, 1990*b*). Reduction of steady-state mRNA levels by the premature insertion of a STOP codon has been observed in other genes, such as those coding for β-globin (Atweh et al. 1988), β-hexosaminidase (α-chain) (Myerowitz and Costigan 1988), apolipoprotein CII (Fojo et al. 1988), and dihydrofolate reductase (Urlaub et al. 1989). The molecular mechanisms involved are still unclear (Urlaub et al. 1989).

Sequencing of the remaining insulin-receptor gene (exons 1–12 and their flanking regions, coding for the first 2,680 bp of the insulin-receptor cDNA) and cDNA (bp 1050–4350) revealed no variations from the published sequence (Ebina et al. 1985; Ullrich et al. 1985). However, both the marked reduction in insulin-receptor mRNA levels in the patient's fibroblasts (fig. 3) and the partial impairment of insulin binding to maternal cells (fig. 1) suggest the presence of a different mutation in the maternally inherited insulin-receptor allele, a mutation which also reduces insulin-receptor mRNA levels. The presence of two mutations inserting premature STOP codons in both alleles of the insulin receptor is probably incompatible with life (Kadowaki et al. 1990*a*). Thus, the maternally derived mutation in patient Mount Sinai must reduce mRNA by a different mechanism. Leprechaun Minn is heterozygous for a nonsense mutation

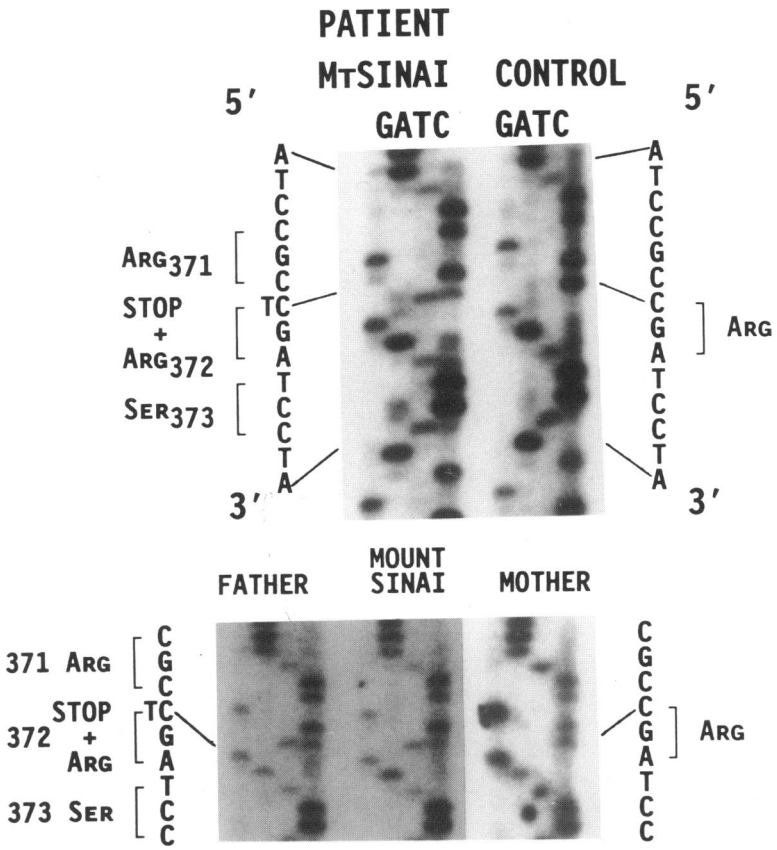

Figure 5 Partial sequence of the insulin-receptor gene in patient Mount Sinai. Genomic DNA was amplified using primers specific for exon 5 of the insulin-receptor gene (Seino et al. 1990). Both alleles were sequenced directly in both directions, without subcloning. The patient is heterozygous for a mutation at bp 1333, converting Arg_{372} to a STOP codon. The lower panel shows that the same mutation was present in paternal DNA but not in maternal DNA.

(R897X) that reduces insulin-receptor mRNA (Kadowaki et al. 1990*b*). An as yet unidentified second mutation also reduces both insulin-receptor mRNA (Ojamaa et al. 1988; Muller-Wieland et al. 1989) and protein levels (Elsas et al. 1989; Pezzino et al. 1989) to values comparable to those reported here in cells from patient Mount Sinai. In patient Minn, as well as in other patients with type A diabetes (Imano et al. 1991), the second mutation was not within the coding region of the insulin-receptor gene and was postulated to be in regulatory sequences of the insulin-receptor gene. A similar mutation probably affects the maternally inherited insulin-receptor allele of patient Mount Sinai. The insulin-receptor gene spans more than 120 kb on the short arm of chromosome 19 (Seino et al. 1989). The insulin-receptor gene promoter is like other housekeeping promoters in that it has no TATA or CAAT boxes, is extremely GC rich, and has multiple transcriptional initiation sites (Tewari et al. 1989; Araki et al. 1991). Among the published sequences of the promoter region of the insulin-receptor gene, there are several differences which are probably attributable to the high GC content of this region (see McKeon et al. 1990, table 1). The high GC content also impairs efficient amplification and sequencing of this region by PCR, and all of the studies published so far have used genomic subclones to investigate this region. These two technical problems prevent routine analysis of the insulin-receptor gene promoter in patients with extreme insulin resistance.

It has been proposed that clusters of binding sites for the transcription factor Sp1, located in the 5′ region of the gene, play a role in the regulation of insulin-receptor gene expression (Tewari et al. 1989; Araki et al. 1991). However, different laboratories have obtained conflicting results on the relative importance

that Sp1 binding sites have for promoter activity (Araki et al. 1987, 1991; Mamula et al. 1988; Seino et al. 1989; Tewari et al. 1989; McKeon et al. 1990). Other enhancer regions in the 5′ end and the first intron of the insulin-receptor gene contain binding sites for the CAAT/enhancer binding protein, which may be implicated in tissue-specific expression (McKeon and Pham 1991). It is not known what role these sequences have in overall regulation of gene expression in vivo. Multiple sites are expected to contribute to promoter activity in housekeeping genes (Barrera-Saldana et al. 1985), and it is unlikely that a single point mutation within any one site will explain the markedly decreased steady-state levels of insulin-receptor mRNA in fibroblasts of patient Mount Sinai (fig. 3) and in other insulin-resistant patients (Kadowaki et al. 1990*a*, 1990*b*; Imano et al. 1991). Elucidation of the sequences which are involved in the control of insulin-receptor gene expression—and their study in patients whose cells have reduced mRNA levels—may clarify the mechanisms by which point mutations may affect the activity of housekeeping promoters and thereby result in severe insulin resistance.

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