Severe Resistance to Insulin and Insulin-Like Growth Factor-I in Cells From a Patient With Leprechaunism as a Result of Two Mutations in the Tyrosine Kinase Domain of the Insulin Receptor

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We studied the biological properties of insulin receptors (IRs) and insulin-like growth factor-I (IGF-I) receptors in cultured fibroblasts from a patient with leprechaunism (leprechaun Par-1). Patient cells displayed normal insulin binding capacity and affinity. Basal in vivo autophosphorylation and in vitro exogenous kinase activity of patient IRs were elevated twofold to threefold compared with control receptors, and insulin had no further effect on these processes. Moreover, patient IRs were unable to promote the stimulation of metabolic and mitogenic pathways. IR substrate-1 (IRS-1) and mitogen-activated protein (MAP) kinase tyrosine phosphorylation and glycogen and DNA synthesis were not increased in the basal state in patient fibroblasts and were also insensitive to the stimulatory effect of insulin. As for IGF-I, although binding and receptor kinase activity were normal, the ability to stimulate glycogen and DNA synthesis was altered in patient cells. Two mutant alleles of the IR gene were detected by denaturing gradient gel electrophoresis (DGGE) and direct sequencing. The maternal allele contained a point mutation in exon 18 encoding the tryptophan-for-arginine substitution at position 1092, and the paternal allele had a point mutation in exon 20 substituting lysine for glutamic acid at codon 1179. Thereby, leprechaun Par-1 was a compound heterozygote for two missense mutations located in the IR β-subunit. The present investigation provides the first evidence that leprechaunism can be causally related to structural alterations in the tyrosine kinase domain of the IR. These alterations result in severe impairment of insulin and IGF-I action.

THE INSULIN RECEPTOR (IR) is a heterotetrameric protein composed of two extracellular α-subunits and two transmembrane β-subunits assembled in a disulfide-linked $\alpha_2\beta_2$-structure.[1,2] Each αβ-half-receptor is derived from one gene product that is processed into α- and β-subunits during holoreceptor formation. Binding of insulin to the α-subunit results in tyrosine autophosphorylation of the β-subunit and subsequent activation of this β-subunit as a tyrosine kinase toward intracellular substrates.[3]

One of the major proteins phosphorylated by the IR is the IR substrate-1 (IRS-1) that acts as a multisite docking protein via the interaction of its phosphotyrosines with *src*-homology 2 domains of several target proteins.[4] Binding of these proteins to IRS-1 is thought to represent a point of divergence for insulin's signal to elicit pleiotropic effects. Thus, mediation of insulin-stimulated glucose transport, glycogen synthesis, and other metabolic processes engages the IRS-1 with the p85 subunit of the phosphatidylinositol 3-kinase.[3,4] Otherwise, various evidence suggests that insulin-dependent mitogenesis involves phosphorylation of IRS-1 and/or Shc, another cytoplasmic IR substrate.[4,5] Binding of these substrates to the growth factor receptor–bound protein-2 could lead to activation of the $p21^{ras}$-mitogen–activated protein (MAP) kinase pathway.[5]

Inasmuch as the first step in insulin action is binding and activation of the IR, the IR gene has been the focus of much attention in studies of the genetic causes of insulin resistance. Indeed, many mutations in this gene have been found over the past few years in patients with a variety of syndromes of severe insulin resistance such as leprechaunism, type A insulin resistance, and Rabson-Mendenhall syndrome.[6,7] Identification of these mutations has helped to elucidate the IR structure-function relationship.

Leprechaunism is a rare autosomal recessive disorder characterized by intrauterine and postnatal growth restriction, hyperinsulinemia, abnormal glucose homeostasis, and short life expectancy.[8] Most of the affected patients described so far were homozygotes or compound heterozygotes for mutations in the IR α-subunit. These mutations impair insulin signaling by decreasing IR expression on the cell surface and/or IR affinity for insulin.[9-17] We[18] and others[14,19] have reported that some α-subunit alterations may also lead to a constitutive activation of IR kinase activity.

In the present study, we describe the biochemical and molecular properties of the IR from a new patient with leprechaunism (leprechaun Par-1). Insulin-like growth factor-I (IGF-I) signaling was also investigated in cultured fibroblasts from the patient.

SUBJECT AND METHODS

Subject

The patient Par-1 was a white male with leprechaunism. He was the third pregnancy of healthy nonconsanguineous parents. The first pregnancy was interrupted by a provoked abortion, and the second one produced a healthy female baby. In week 22 of the third pregnancy, growth retardation was noted, as well as interventricular communication. Because of progressive intrauterine growth retardation, a cesarean section was performed in the 35th week. Birth weight was only 930 g, length 36 cm, and head circumference 27 cm (all < the 3rd percentile). Leprechaunism was diagnosed by the presence of characteristic elfin facies, hirsutism, acanthosis

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nigricans, generalized lipoatrophy, and muscular hypotrophy. In addition, the newborn period was complicated by hyperglycemia (glucose level, 12.2 mmol/L), elevated levels of serum insulin ($>2,500$ pmol/L), and total resistance to exogenous insulin ($\leq 2,000$ U/kg/d). The patient had a normal karyotype. He died at 3 months of age from infectious syndrome.

The two parents were phenotypically normal. The father presented with marked glycosuria and impaired oral glucose tolerance (fasting plasma glucose, 6.2 mmol/L; 30-minute plasma glucose, 11.1 mmol/L; 2-hour plasma glucose, 8.1 mmol/L). An investigation of glucose tolerance was not performed in the mother, since she did not develop glycosuria during the whole pregnancy. Her fertility was normal (four pregnancies in 5 years). Ultrasound examination did not reveal the presence of cystic ovaries. The parents were not further examined. They gave informed consent for the following study, which was conducted with the approval of an institutional review board.

Cell Culture

Primary cultures of fibroblasts were established from skin biopsies following standard procedures.[20] Cells were subcultured in 75-cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mmol/L glutamine, 10 mmol/L HEPES, 10% fetal calf serum (FCS), and antibiotics as previously described.[20] For the assays described below, cells were seeded in 6- or 12-well dishes or in 60-cm^2 plates.

Insulin and IGF-I Binding

[^{125}I]insulin (9 pmol/L, (3-[^{125}I]iodotyrosyl^{A14})insulin, 2,000 Ci/mmol; Amersham, Les Ulis, France) and [^{125}I]IGF-I (17 pmol/L, (3-[^{125}I]iodotyrosyl)IGF-I, 2,000 Ci/mmol; Amersham) binding to fibroblast monolayers (5×10^5 cells/well) was performed for 5 hours at 15°C in the presence of unlabeled insulin or IGF-I (0 to 1,000 nmol/L) as previously described.[20] The results are expressed as femtomoles of [^{125}I]insulin or [^{125}I]IGF-I bound per milligram of protein. [^{125}I]insulin (8.33 pmol/L) or [^{125}I]IGF-I (17 pmol/L) binding to wheat germ agglutinin (WGA)-purified receptors was performed for 16 hours at 4°C as previously described.[21]

In Vivo Phosphorylation Studies

Confluent fibroblasts (10^6 cells/plate) were incubated for 16 hours in serum-free DMEM, treated or not treated with 100 nmol/L insulin for 5 minutes at 37°C, quickly washed, frozen with liquid nitrogen, and lysed in 50 mmol/L HEPES (pH 7.4), 1% sodium dodecyl sulfate (SDS), 5 mmol/L EDTA, 100 mmol/L sodium fluoride, 10 mmol/L ATP, 10 mmol/L sodium orthovanadate, 4 mmol/L sodium pyrophosphate, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1,200 U/mL aprotinin, and 100 mmol/L dithiothreitol. Cell lysates were clarified by centrifugation, and supernatants (100 μg protein) were boiled for 5 minutes, separated on 7.5% SDS-PAGE, and transferred to a nylon membrane (Hybond-ECL Western; Amersham). Tyrosine phosphorylation of IR, IRS-1, and MAP kinase was assessed by incubating the sheet with an antiphosphotyrosine antibody (monoclonal IgG2b$_\kappa$, dilution 1:1,000; Upstate Biotechnology, Lake Placid, NY). Antibodies bound to the membrane were revealed by chemiluminescence (ECL Western blotting kit; Amersham). Intensity of the bands was determined by densitometric scanning and expressed as arbitrary units (AUs). The proteins were identified by reprobing the membrane with polyclonal antibodies directed against the IR (83-14; generous gift from K. Siddle), IRS-1 (Upstate Biotechnology), and p42^{MAPK} (Santa Cruz Biotechnology, Santa Cruz, CA).

In Vitro Insulin and IGF-I Receptor Exogenous Kinase Activity

WGA-purified receptors (5 μg) were incubated for 45 minutes at 22°C with 50 mmol/L HEPES buffer (pH 7.4) containing 0.1% Triton X-100 and insulin or IGF-I (0 to 100 nmol/L). Phosphorylation was initiated by adding 15 mmol/L $MgCl_2$, 2 mmol/L $MnCl_2$, and 50 μmol/L [γ-^{32}P]ATP (10 Ci/mmol; Amersham). The synthetic polypeptide poly(Glu,Tyr) (10 mg/mL) was then added for 10 minutes. The reaction was blocked by adding 10 mmol/L EDTA, 100 mmol/L sodium fluorure, 20 mmol/L sodium pyrophosphate, and 250 μg/mL bovine serum albumin. Aliquots were spotted onto Whatman P81 paper squares (Whatman, Clifton, NJ) that were extensively washed and air-dried. A correction was made for nonspecific absorption of [γ-^{32}P]ATP to the filters. The results are expressed as picomoles of [γ-^{32}P]ATP incorporated per milligram of substrate per microgram of protein.

Glycogen Synthesis

Confluent fibroblasts (5×10^5 cells/well) were deprived of 10% FCS for 16 hours and then incubated for 2 hours with insulin or IGF-I (0 to 100 nmol/L) and 2 μCi/mL D-[U-^{14}C]glucose (310 mCi/mmol; Amersham). Glycogen was extracted as described previously.[22] The results are presented as the percent over the basal value, which is expressed as picomoles of D-[U-^{14}C]glucose incorporated into glycogen per milligram of protein per hour.

DNA Synthesis

Subconfluent fibroblasts (10^5 cells/well) were maintained for 48 hours in serum-free DMEM to obtain quiescent and synchronized cells. The medium was replaced by 1 mL fresh DMEM containing 0.3% FCS and increasing concentrations of insulin or IGF-I (0 to 100 nmol/L). After a 17-hour incubation at 37°C, 0.5 μCi [methyl-^{3}H]thymidine (25 Ci/mmol; Amersham) was added for 4 hours. The cells were washed three times with ice-cold phosphate-buffered saline and treated with 5% trichloroacetic acid at 4°C for 30 minutes. The cells were then rinsed with 80% ethanol, lysed in 0.5N NaOH, and counted for radioactivity. The results are presented as the percent over the basal value, which is expressed as disintegrations per minute per milligram of protein.

Denaturing Gradient Gel Electrophoresis Analysis

Genomic DNA was isolated from cultured fibroblasts and used as a template for polymerase chain reaction (PCR). IR exons 2 to 22 and their flanking introns were amplified by PCR and analyzed by denaturing gradient gel electrophoresis (DGGE) under conditions previously described in detail.[23] The nucleotide sequence of PCR products showing an altered migration pattern on denaturing gels was determined with the Sequenase T7 DNA polymerase version 2.0 sequencing kit (U.S. Biochemical, Cleveland, OH). Amino acids are numbered according to Ebina et al.[1]

Statistical Analysis

Results are presented as the mean ± SEM for the indicated number of experiments. Differences between mean values were evaluated by Student's *t* test.

RESULTS

Insulin Binding to Cultured Fibroblasts

Maximal binding of a tracer concentration of [^{125}I]insulin was similar in patient and control fibroblasts (0.22 ± 0.01 and 0.29 ± 0.05 fmol [^{125}I]insulin bound/mg protein, respec-

tively; Fig 1). The apparent insulin binding affinity was not significantly modified in patient cells compared with control cells (ED_{50}, 0.9 and 0.4 nmol/L, respectively). Scatchard analysis of the data showed that the total insulin binding capacity did not appreciably differ between the two cell types (data not shown).

In Vivo IR Autophosphorylation

Autophosphorylation of the IR was evaluated in vivo by Western blot analysis using immunodetection with an antiphosphotyrosine antibody. Insulin 100 nmol/L increased the IR β-subunit phosphorylation 3.8-fold in control cells (Table 1). In Par-1 cells, there was a marked increase in basal IR autophosphorylation 330% of the basal value obtained in control cells). This was associated with poor hormone stimulation of IR autophosphorylation (1.3-fold stimulation).

In Vitro IR Exogenous Kinase Activity

Using poly(Glu,Tyr) as an exogenous substrate, the basal kinase activity of WGA extracts obtained from patient fibroblasts was elevated by approximately twofold compared with control WGA preparations (36.8 ± 6.1 and 19.7 ± 1.7 pmol ^{32}P/mg substrate/μg protein, respectively, $P < .05$, n = 4; Fig. 2). In addition, patient IRs showed a marked reduction in insulin-stimulated kinase activity compared with control IRs, whatever the concentration of insulin. At 1 nmol/L, a concentration at which insulin does not activate the IGF-I receptor, the hormone stimulated IR kinase activity by 70% and 23% over basal in control and patient WGA preparations, respectively.

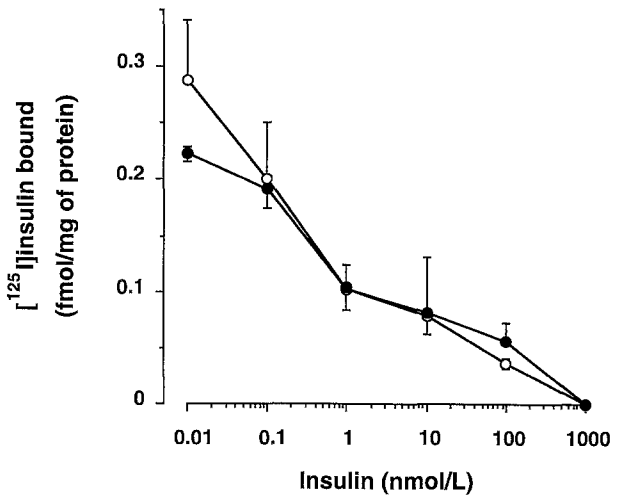

Fig 1. Insulin binding to control and patient fibroblasts. Confluent fibroblasts from the control (○) and patient Par-1 (●) were deprived of FCS for 16 hours and incubated for 5 hours at 15°C with [125I]insulin 9 pmol/L and graded concentrations of unlabeled insulin (0 to 1,000 nmol/L). Cells were then lysed in 0.5N NaOH, and cell-associated [125I]insulin was quantified. Data are the mean ± SEM of 6 experiments performed in triplicate.

Table 1. In Vivo IR Autophosphorylation

Fibroblasts	IR Autophosphorylation (AU)	
	Basal	Insulin
Control	5.5 ± 0.9	21.3 ± 4.6*
Par-1	18.2 ± 5.1*	23.5 ± 4.8

NOTE. Data are the mean ± SEM of 3 to 4 experiments.
*$P < .05$ *v* basal value of control cells.

In Vivo IRS-1 and MAP Kinase Phosphorylation

We next studied the relationship between IR tyrosine kinase activity and cytoplasmic signaling in intact cells. For this purpose, tyrosine phosphorylation of IRS-1 and MAP kinase was evaluated by Western blot analysis using an antiphosphotyrosine antibody. In the absence of insulin, basal levels of phosphorylation were similar in control and patient cells. With 100 nmol/L insulin, neither IRS-1 (275% ± 45% and 111% ± 6% of basal in control and Par-1 fibroblasts, respectively) nor MAP kinase (225% ± 23% and 124% ± 9% of basal in control and Par-1 fibroblasts, respectively) phosphorylation was increased in patient cells.

Glycogen and DNA Synthesis

To test the metabolic effect of insulin on glucose metabolism, we evaluated insulin stimulation of labeled glucose incorporation into glycogen. Since insulin poorly stimulates glucose transport in human fibroblasts,[20] this assay mainly reflects the effect of the hormone on glycogen synthesis.

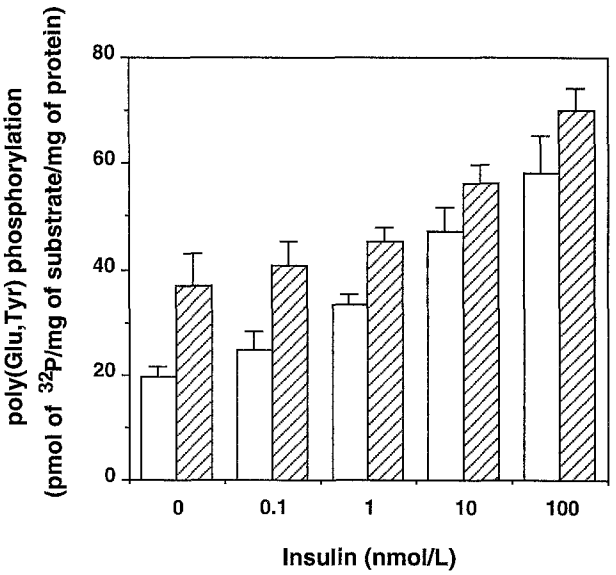

Fig 2. IR exogenous kinase activity in WGA extracts obtained from control and Par-1 fibroblasts. Equal amounts of WGA-purified IRs from control (□) and Par-1 (▨) fibroblasts were incubated with graded concentrations of insulin (0 to 100 nmol/L) for 45 minutes at 22°C followed by 10 minutes with 50 μmol/L [γ-32]ATP (10 μCi) and 10 mg/mL poly(Glu,Tyr). Aliquots of the incubation mixture were spotted onto Whatman P81 paper squares, which were extensively washed in phosphoric acid and counted for radioactivity. Data are the mean ± SEM of 4 experiments.

Basal levels of glycogen synthesis were similar in control and Par-1 cells (66 ± 10 and 72 ± 14 pmol glucose incorporated into glycogen/mg protein/h, respectively). Nevertheless, the stimulation of glycogen synthesis by insulin was severely altered in patient cells compared with control fibroblasts (Fig 3A). At 100 nmol/L, the effect of the hormone was 98% ± 13% and 47% ± 14% over basal in control and patient cells, respectively.

As for DNA synthesis, basal levels were similar in control and patient cells (371 ± 80 and 370 ± 89 dpm/mg protein × 10^3 in control and patient cells, respectively). The concentration-response curve for stimulation of DNA synthesis by insulin showed a decreased response in patient cells compared with control fibroblasts (Fig 3B). At 100 nmol/L, insulin stimulated this process by 85% ± 24% and 25% ± 16% over basal in control and patient cells, respectively. 10% FCS similarly increased DNA synthesis in control and patient fibroblasts (eightfold to ninefold stimulation).

IGF-I Binding and Signaling

The binding of a tracer concentration of [^{125}I]IGF-I was similar in cultured fibroblasts from the control and Par-1 (10.9 ± 3.4 and 10.6 ± 2.6 fmol [^{125}I]IGF-I bound/mg protein, respectively). In addition, IGF-I binding affinity was not modified in Par-1 cells. IGF-I increased the phosphorylation of poly(Glu,Tyr) to a comparable extent in WGA extracts from control and Par-1 fibroblasts (51 and 71 pmol ^{32}P incorporated/mg substrate/μg protein at 100 nmol/L IGF-I, respectively), with a half-maximal response at 0.5 nmol/L. However, IGF-I stimulation of glycogen and DNA synthesis was severely altered in Par-1 cells compared with control cells (Fig 4). IGF-I 100 nmol/L stimulated glycogen synthesis by 2.4-fold (142% ± 22% over basal) and 1.4-fold (39% ± 7% over basal) in control and patient fibroblasts, respectively (Fig 4A). As for DNA synthesis, IGF-I 100 nmol/L failed to activate this process in Par-1 fibroblasts (82% ± 12% and 2% ± 5% over basal in control and patient fibroblasts, respectively; Fig 4B).

DGGE Analysis of the IR Gene

Molecular screening of the IR gene by DGGE showed that PCR-amplified exon 18 from the patient and his mother migrated in multiple bands on a denaturing gel, suggesting the presence of a nucleotide variation (Fig 5A). Direct sequencing of the DNA fragments identified a heterozygous point mutation substituting Trp (TGG) for Arg (CGG) at codon 1092. DGGE also evidenced a sequence modification in PCR-amplified exon 20 from the patient and his father (Fig 5B). Direct sequencing identified in these fragments a G-to-A substitution at codon 1179, changing Lys (AAG) for Glu (GAG). These mutations were not found in genomic DNA from 30 control individuals (data not shown).

DISCUSSION

To date, more than 50 different alterations have been identified in the IR gene, approximately 15 having been characterized in patients with leprechaunism.[6,7,9-19] In these patients, both alleles of the IR gene are generally mutated. Affected individuals are either homozygotes for the same mutation in the case of consanguineous kindreds, or compound heterozygotes if there is no inbreeding. Most of the mutations identified so far in patients with leprechaunism map in the IR α-subunit and result in defective biosynthesis,[13,15] abnormal transport of the IR to the plasma membrane,[10-12,16,17] altered insulin binding,[9,14] and/or deregulated kinase activity.[14,18,19]

In contrast, the Arg1092 → Trp and Glu1179 → Lys mutations identified in the IR gene from patient Par-1 both

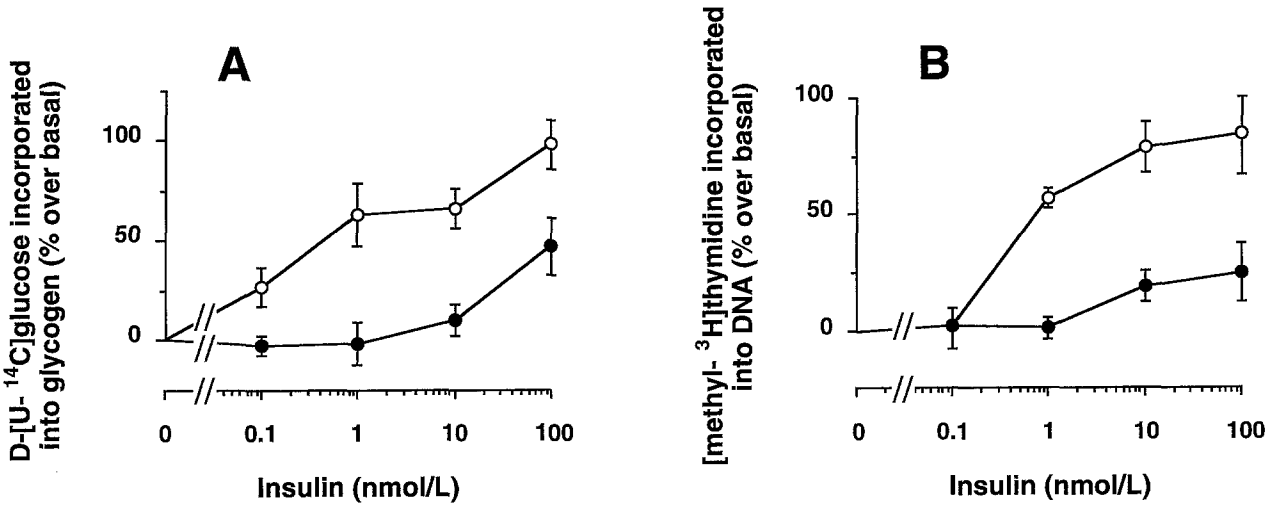

Fig 3. Insulin stimulation of glycogen (A) and DNA (B) synthesis in control and patient fibroblasts. Confluent fibroblasts from control (○) or patient Par-1 (●) were incubated for 16 hours (glycogen synthesis) or 48 hours (DNA synthesis) in serum-free DMEM. For glycogen synthesis, cells were incubated for 2 hours with insulin (0 to 100 nmol/L) and 2 μCi/mL D-[U-^{14}C]glucose. Glycogen was extracted as described previously.[22] For DNA synthesis, the medium was replaced by fresh DMEM containing 0.3% SVF and graded concentrations of insulin (0 to 100 nmol/L). After 17 hours at 37°C, 0.5 μCi/mL [methyl-^{3}H]thymidine was added for 4 hours. Labeled DNA was then evaluated. Data are the mean ± SEM of 4 experiments.

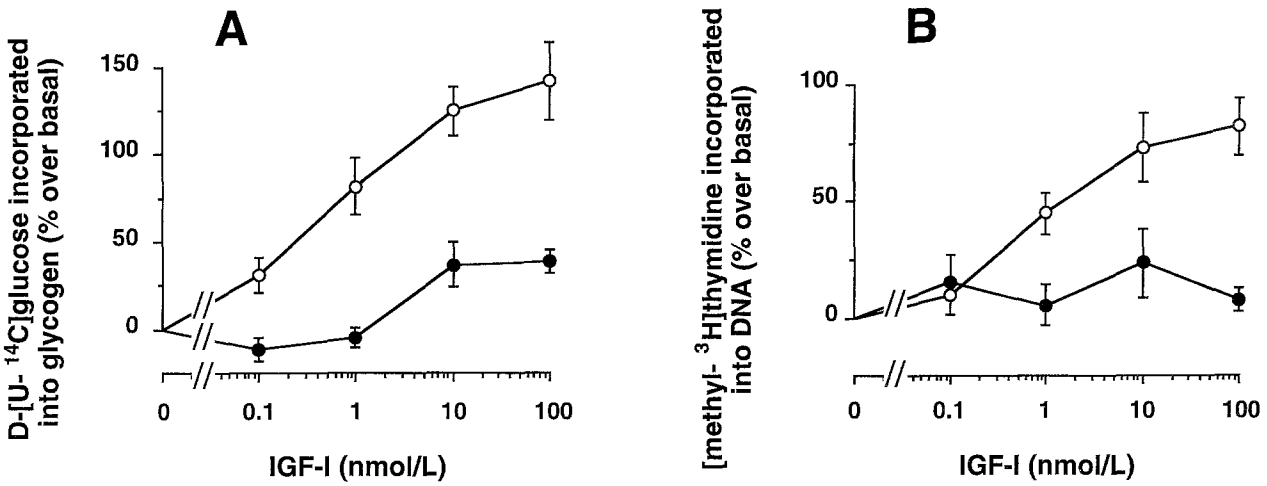

Fig 4. IGF-I stimulation of glycogen (A) and DNA (B) synthesis in control (○) and patient Par-1 (●) fibroblasts. Glycogen and DNA synthesis were evaluated as described for Fig 3, except that IGF-I was used as the stimulator. Data are the mean ± SEM of 4 experiments.

occur in the tyrosine kinase domain of the protein and map outside of the putative "catalytic loop" (amino acids 1131 to 1137). The two substitutions are not conservative in nature. Indeed, arginine is a large positively charged amino acid, whereas tryptophan is a nonpolar aromatic residue. Similarly, glutamic acid and lysine differ considerably with respect to charge and size. Several arginines (codon CGX, where X is any base) have been reported to be mutated throughout the IR gene[13,14,24-28] (and this study). This may result from the fact that the CpG sequence is a substrate for methylation and that the presence of 5-methyl cytosine predisposes DNA replication to errors.

Interestingly, other missense mutations have been identified at codons 1092 and 1179 in pedigrees with leprechaunism[29,30] and type A insulin resistance.[30]

Comparison of the amino acid sequence of several

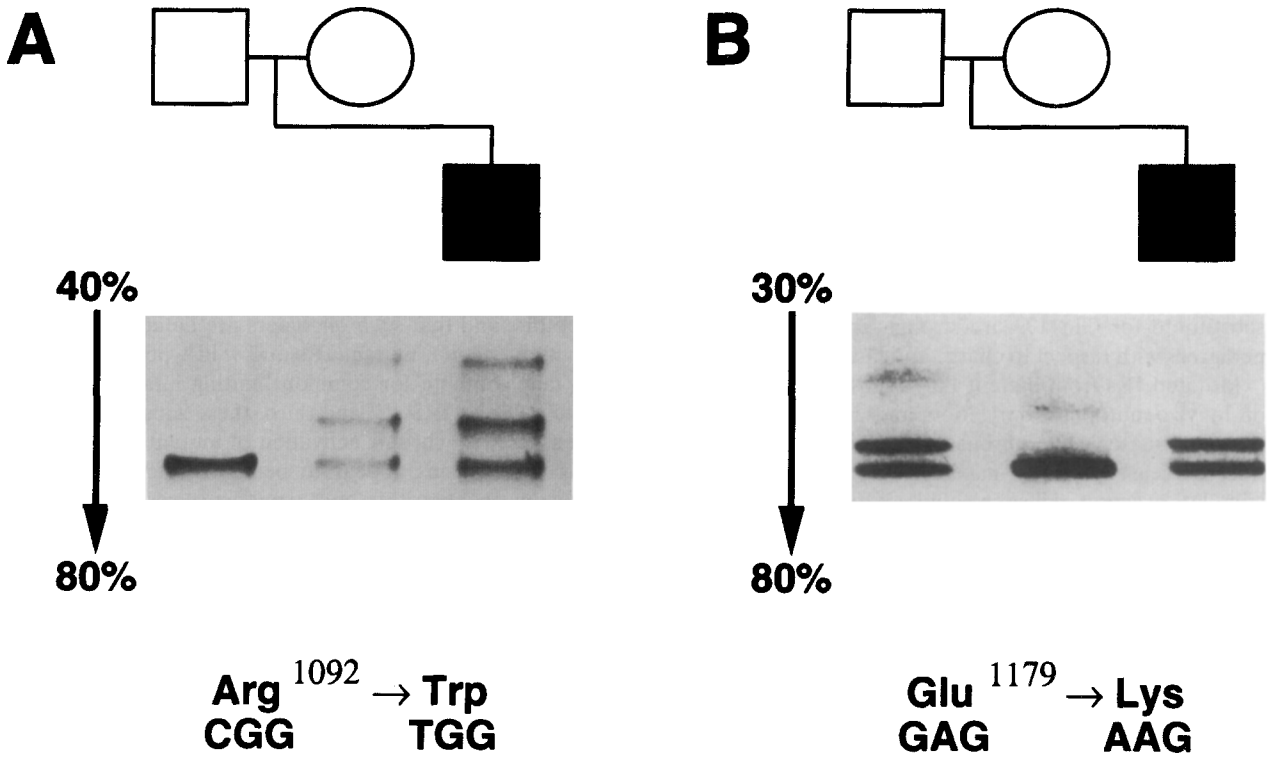

Fig 5. DGGE analysis of IR exons 18 (A) and 20 (B) from patient Par-1 and his parents. PCR-amplified exons 18 and 20 were analyzed on a 40% to 80% (6 hours at 160 V) and a 30% to 80% (3 hours at 160 V) denaturing gel, respectively. Gradients were oriented parallel to electric fields. Multiple bands observed with DNA fragments from patient Par-1 (exons 18 and 20), the mother (exon 18), and the father (exon 20) revealed the presence of heterozygous nucleotide variations. Direct sequencing showed that Trp1092 was changed for Arg in exon 18, and that Glu1179 was changed for Lys in exon 20.

tyrosine and serine-threonine kinases shows that Glu1179 is one of the IR residues that is strictly conserved in all protein kinases, whereas Arg1092 is not.[31] The recent determination of the crystal structure of the IR tyrosine kinase domain[32] revealed that the highly conserved Glu1179 residue does not play a direct role in catalysis, but is important for structure stabilization by establishing a salt bridge with Arg1253.

Several missense mutations have been characterized in the cytoplasmic domain of the IR from patients with type A insulin resistance.[26-30,33-39] Except for one case,[26] all affected individuals were simple heterozygotes and had less severe insulin resistance than patients with leprechaunism. In the current study, we describe the first patient with leprechaunism to have inherited different missense mutations in the kinase domain of each allele of the IR gene. Otherwise, it has been reported in a preliminary study that the Arg1092 → Gln mutation caused type A insulin resistance in a heterozygote state and leprechaunism in a homozygote state.[30] Taken together, these findings suggest that the presence of one or two IR alleles mutated in the tyrosine kinase domain may determine the clinical manifestations of type A insulin resistance and leprechaunism, respectively.

The father of Par-1, who is heterozygous for the Glu1179 → Lys mutation, presents with impaired glucose tolerance, whereas the mother, who is heterozygous for the Arg1092 → Trp mutation, does not have any clinical signs suggestive of type A insulin resistance. This is presumably relevant to the variable phenotypic expression of heterozygous mutations in the IR kinase domain.[7,30,33-35]

Patient IRs displayed normal insulin binding characteristics. This is in accordance with the results obtained with most[26-28,34,35,38,39] but not all[30,33,36,37] β-subunit–mutated IRs. In contrast to the Lys1179 mutation described in the present report, the Asp1179 mutation reported by Imamura et al[30] was associated with a marked decrease of insulin binding on patient cells. This appeared to be due to an accelerated degradation of the IR. The discrepancy in these findings may be attributed to the fact that amino acids substituted for Glu1179 are strongly different in the two pedigrees with respect to charge and bulkiness.

Mutated IRs from patient Par-1 showed elevated levels of in vivo autophosphorylation and in vitro exogenous tyrosine kinase in the absence of insulin. These activities were increased to near-maximal levels, so that further stimulatory action of insulin could not be observed. Most of the naturally occurring mutations previously described in the cytoplasmic domain of the IR alter protein function by impairing insulin-stimulated but not insulin-independent IR kinase activity: Arg993 → Gln[26]; ΔLeu999[33]; Gly1008 → Val[38]; Ala1048 → Asp[35]; Arg1131 → Gln[28]; Ala1134 → Thr[34]; Ala1135 → Glu[37]; Met1153 → Ile[36]; Arg1174 → Gln[27]; Glu1179 → Asp[30]; Trp1193 → Leu[30]; and Trp1200 → Ser[39]. The unique β-subunit mutation described so far that activates IR exogenous kinase activity at the basal level is Arg1164 → Gln.[40]

IRS-1 plays an early role in transmission of the insulin signal beyond the plasma membrane.[3,4] In intact cells, the IR phosphorylates IRS-1 on multiple tyrosine residues, and this results in the activation of downstream signals. Tyrosine phosphorylation of IRS-1 in intact Par-1 fibroblasts did not correlate with IR kinase activity, since it did not show an elevated basal level. In addition, tyrosine phosphorylation of IRS-1 was not increased by insulin in these cells. One interpretation of these data is that Trp1092 and Lys1179 mutations modified the conformation of the IR β-subunit so that the substrate was sequestered but not phosphorylated by the tyrosine-phosphorylated receptor.[41] These data support the hypothesis that efficient phosphorylation of IRS-1 requires not only IR tyrosine phosphorylation, but also some structural integrity of the cytoplasmic β-subunit.[42] Otherwise, chronic activation of the receptor may lead to desensitization of some downstream steps in insulin signaling. This could result from serine/threonine phosphorylation of IRS-1, a process shown to preclude further phosphorylation of the substrate on tyrosine residues.[43]

Parallel results were obtained when the phosphorylation of MAP kinase was studied in patient fibroblasts, indicating that the mitogenic $p21^{ras}$ signaling pathway could not be activated in patient cells. Given that insulin-stimulated phosphorylation of IRS-1 and MAP kinase are important intermediaries for the regulation of metabolism and growth by insulin,[3,4] it was not surprising to observe desensitization of glycogen and DNA synthesis to insulin action in patient fibroblasts.

IGF-I stimulation of glycogen and DNA synthesis was also altered in fibroblasts from patient Par-1. Concomitant defects in the signaling of tyrosine kinase receptors other than IRs have already been reported in fibroblasts from patients with leprechaunism having mutations in the IR gene.[44-46] It is suggested that these associated defects may contribute to the severe growth retardation observed in affected individuals.[47] Several hypotheses have been advanced to account for the inhibition of IGF-I signaling by kinase-deficient IRs. First, it has been proposed that mutant IRs form hybrid oligomers with wild-type IGF-I receptors, and that such oligomers are defective in kinase activity.[48] Second, mutant IRs and wild-type IGF-I receptors may compete for common limiting intracellular substrates such as IRS-1,[41] and third, these substrates may be desensitized by chronic activation of mutant IRs. The two latter mechanisms appear to be more relevant in fibroblasts, given that this cell type expresses more IGF-I receptors than IRs, and that no major alteration of IGF-I receptor kinase activity has been detected in patient cells. As discussed earlier, it can be proposed that mutant IRs sequestered IRS-1 and/or stimulated its serine/threonine phosphorylation, thus inhibiting its tyrosine phosphorylation by kinase-competent IGF-I receptors.[41]

In conclusion, the present study describes two novel mutations in the IR β-subunit resulting in severe impairment of tyrosine kinase function and in alterations of insulin and IGF-I signaling. This is the first evidence that leprechaunism can be causally related to structural alterations in the tyrosine kinase domain of the IR. The present

findings reinforce the hypothesis that the phenotypic expression of the disease may result from concomitant defects in the signaling of several growth factors. Overexpression of each mutant IR in fibroblastic cell lines should help to evaluate the relative contribution of each mutation to insulin and IGF-I resistance.

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