Genotype–phenotype correlation in inherited severe insulin resistance

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The insulin receptor is a ligand-activated tyrosine kinase. Mutations in the corresponding gene cause the rare inherited insulin-resistant disorders leprechaunism and Rabson–Mendenhall syndrome. Patients with the most severe syndrome, leprechaunism, have growth restriction, altered glucose homeostasis and early death (usually before 1 year of age). Rabson–Mendenhall syndrome is less severe, with survival up to 5–15 years of age. These disorders are transmitted as autosomal recessive traits. Here we report six new patients and correlate mutations in the insulin receptor gene with survival. Patients with leprechaunism were homozygous or compound heterozygous for mutations in the extracellular domain of the insulin receptor and their cells had markedly impaired insulin binding (<10% of controls). Mutations in their insulin receptor gene inserted premature stop codons (E124X, R372X, G650X, E665X and C682X), resulting in decreased levels of mature mRNA, or affected the extracellular domain of the receptor (R86P, A92V, D821, I898T and R899W). Three patients with Rabson–Mendenhall syndrome had at least one missense mutation in the intracellular domain of the insulin receptor (P970T, I1116T, R1131W and R1174W). Expression studies in CHO cells indicated that the R86P, A92V, D821, I898T, R899W and R1131W mutations markedly impaired insulin binding (<5% of control), while the P970T, I1116T and R1174W mutant receptors retained significant insulin-binding activity. These results indicate that mutations in the insulin receptor retaining residual insulin-binding correlate with prolonged survival in our series of patients with extreme insulin resistance.

INTRODUCTION

The human insulin receptor is a heterotetramer composed of two extracellular α subunits that bind insulin and two β subunits that span the plasma membrane and have an intracellular tyrosine kinase domain (1,2). 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 (3). Mutations in this gene cause the insulin-resistant syndromes leprechaunism, Rabson–Mendenhall syndrome and type A insulin resistance (4,5). Leprechaunism (OMIM 246200), the most severe of these syndromes, is characterized by intrauterine growth restriction, loss of glucose homeostasis, hyperinsulinemia, and dysmorphic features, with prominent eyes, thick lips, upturned nostrils, low-set posteriorly rotated ears, thick skin with lack of subcutaneous fat, distended abdomen, and enlarged genitalia in the male and cystic ovaries in the female (6–8). Cells from most patients with leprechaunism have absent insulin binding, although recent exceptions were reported (9,10). Patients with the slightly less severe Rabson–M endenhall syndrome (OMIM 262190) have different dysmorphic features, with premature or dysplastic teeth and gingival hyperplasia (11). In addition, they present pineal hyperplasia. In both cases, children have initially postprandial hyperglycemia and fasting hypoglycemia. The paradoxical fasting hypoglycemia is caused by inappropriately elevated insulin levels at the time of fasting, due to the excessive production of insulin by the pancreas of these patients, coupled to the prolonged half-life of the hormone for the inability of peripheral tissues to bind and remove circulating insulin (12). Patients with Rabson–M enden hall syndrome survive beyond 1 year of age and, with time, develop constant hyperglycemia followed by diabetic ketoacidosis and death. This is caused by

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a progressive decline of insulin levels, which become insufficient to prevent glucose synthesis in the liver and prevent release of fatty acid by adipocytes (12).

A number of different mutations in the insulin receptor gene have been reported in patients with leprechaunism and Rabson-Mendenhall syndrome (5). Although both syndromes are inherited as autosomal recessive traits, a clear correlation between genotype and phenotype has not yet been established (5). The difficulty in establishing genotype-phenotype correlation is due in part to the rarity of these syndromes and to the fact that individual centers have not analyzed a large number of patients. In addition, several of the mutations reported have not been analyzed in vitro in the patient’s cells or in transfected cells to determine their effect on insulin binding or signaling.

Here we report several new mutations in the insulin receptor gene of patients with leprechaunism and Rabson-Mendenhall syndrome. The mutant receptors were analyzed in the patients’ cells and expressed in mammalian cells to define the residual insulin-binding and signaling properties of the mutant insulin receptor. In our patient population, mutations markedly impairing insulin binding resulted in the most severe phenotype with early demise, while mutations leaving residual insulin-binding activity were associated with longer survival.

RESULTS

Insulin binding to cultured cells

Table 1 reports insulin binding to fibroblasts obtained from patients with leprechaunism and patient ATL-2 with Rabson-Mendenhall syndrome. Insulin binding was reduced to less than 10% of controls in fibroblasts derived from all patients with leprechaunism, while cells from patient ATL-2 with Rabson-Mendenhall syndrome retained about 18% of normal binding.

Table 2 reports insulin binding to lymphoblasts obtained from patients with Rabson-Mendenhall syndrome and patient Mt Sinai with leprechaunism. Cells from patients with Rabson-Mendenhall syndrome retained significant residual insulin binding, which was 18–27% of the normal average. By contrast, insulin binding to lymphoblasts of patient Mt Sinai with leprechaunism was negligible.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Birth-weight (kg)</th>
<th>Gestational age (weeks)</th>
<th>Survival age</th>
<th>Specific binding of insulin (fmol/mg cell protein)</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATL-1</td>
<td>M</td>
<td>1.9</td>
<td>40</td>
<td>6 months</td>
<td>0.07 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>R86P/R86P</td>
</tr>
<tr>
<td>ATL-2</td>
<td>M</td>
<td>1.6</td>
<td>36</td>
<td>7 years</td>
<td>0.27 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>R1131W/R1116T</td>
</tr>
<tr>
<td>FL-1</td>
<td>F</td>
<td>1.9</td>
<td>37</td>
<td>26 months</td>
<td>0.06 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A92V/I898T</td>
</tr>
<tr>
<td>GE</td>
<td>F</td>
<td>2.0</td>
<td>40</td>
<td>7 weeks</td>
<td>0.07 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>665X/C682X</td>
</tr>
<tr>
<td>Mt Sinai</td>
<td>F</td>
<td>1.9</td>
<td>35</td>
<td>7 months</td>
<td>0.05 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>R372X/low RNA</td>
</tr>
<tr>
<td>NZ</td>
<td>M</td>
<td>1.6</td>
<td>40</td>
<td>24 months</td>
<td>0.02 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ΔN281/859-867 del</td>
</tr>
<tr>
<td>NY-1</td>
<td>F</td>
<td>1.9</td>
<td>35</td>
<td>4 months</td>
<td>0.01 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>E124X/E124X</td>
</tr>
<tr>
<td>VA</td>
<td>M</td>
<td>1.7</td>
<td>38</td>
<td>3 months</td>
<td>0.01 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>del2136-2139G650X/R399W</td>
</tr>
</tbody>
</table>

*Fibroblasts were cultured and evaluated for equilibrium binding of [125I]insulin (1 ng/ml) for 2 h at 20 °C. Non-specific binding, measured in the presence of 5 μg/ml of cold ligand, was subtracted from each point. Data are means ± SE of triplicates.

<sup>a</sup>Normal range (n = 18) 1.01–2.20; average 1.52 ± 0.15.

<sup>b</sup>P < 0.01 versus normal range, using analysis of variance.

Mutations in the insulin receptor gene

We sequenced each one of the 22 exons of the insulin receptor gene using PCR and primers in the flanking introns. PCR products were sequenced directly without subcloning and the mutations identified confirmed, when possible, by restriction analysis. Mutations are summarized in Table 3.

Patient FL-1 with leprechaunism was a compound heterozygote for a single-base-pair change (356C > T) converting the codon for Ala92 to that for Val (A92V) and a 2774T > C transversion converting the codon for Ile898 to that for Thr (I898T). The A92V mutation removed a Cfo I site from exon 2, while the I898T mutation added a novel Hinfl site in exon 14. Both mutations were confirmed by restriction analysis.

Patient GE was a compound heterozygote for two single-nucleotide insertions in exon 10. The paternal mutation inserted an extra G in position 2187 of the insulin receptor cDNA, converting the codon for Thr657 into that for Asp and causing a frameshift of the downstream sequence. This resulted in the premature insertion of a stop codon in position 665 (665X). The maternal mutation inserted an A in position 2263 of the insulin receptor cDNA, converting the codon for Cys682 to a stop codon (C682X). The paternal insertion (2187insG) created a novel BsmFI site in exon 10 of the insulin receptor gene, while the maternal insertion (2263insA) ablated one of the two M wol sites in exon 10 of the insulin receptor gene. Both mutations were confirmed by restriction analysis in the patient and both parents.

Patient NY-1 was homozygous for a single-base-pair change in exon 2 of the insulin receptor (451G > T), converting the codon for Glu124 (GAG) to that for stop codon (TAG) (E124X). This mutation ablated a BanII restriction site in exon 2 of the insulin receptor gene.

Patient VA with leprechaunism was a compound heterozygote for a four base-pair deletion in exon 9 removing bp 2136–2139 of the insulin receptor cDNA. The 4bp deletion caused a frameshift that inserted a premature stop codon at codon 650 of the insulin receptor cDNA (650X). This deletion created a novel Pvu II restriction site. The second mutation was a C-to-T transition in exon 14 converting the codon for Arg989 to that for Trp (R999W). This mutation ablated one of two M spl cut sites in exon 14 of the insulin receptor gene.

Table 1. Insulin binding to fibroblasts of patients with inherited severe insulin resistance
Equilibrium binding of $[^{125}I]$Insulin (1 ng/ml) to lymphoblasts was measured for 2 h at 16°C as in fibroblasts, except that the assay medium contained EDTA. Non-specific binding, measured in the presence of 5 μg/ml of cold ligand, was subtracted from each point. Data are means ± SE of triplicates.

Reduced insulin receptor mRNA in fibroblasts of patients with leprechaunism (Fig. 3) in fibroblasts of patients with leprechaunism. Increasing amounts of normal RNA generated increasing signals for both actin and the insulin receptor by RNase protection assay (Fig. 1A). However, when the insulin receptor signal was normalized for the actin signal, no significant variations were observed over a 4-fold concentration range (Fig. 1C), confirming the linearity of the assay. When RNA from patients’ cells was analyzed, the levels of insulin receptor mRNA in cells from patient GE was reduced to less than 10% of normal, as in cells from patient Mt Sinai, previously shown to have reduced insulin receptor mRNA levels (6). By northern blot analysis, the insulin receptor mRNA produces multiple bands, ranging in size from 5 to 10 kb (6,13,14). Control fibroblasts had two prominent bands of 7.5 and 9.5 kb (Fig. 2). Although bands of size similar to those seen in controls were visible in cells from patient NY-1, the levels of insulin receptor mRNA were reduced to 15% or less of those measured in control cells in fibroblasts from patient NY-1 and patient GE (Fig. 2).

In the case of patient VA (Fig. 3), expression of the allele containing the deletion and the premature insertion of a stop codon (del 2136-2139, Fig. 3A) was compared against expression of the other allele containing the missense mutation (R899W; Fig. 3B). The PCR-amplified insulin receptor cDNA from normal controls had two bands of 262 and 325 bp (Fig. 3C) after digestion with MspI. By contrast, insulin receptor cDNA from patient VA was missing one MspI site abolished by the R899W mutation, and had bands of 325 and 286 bp. The lack of the 262 bp band in the insulin receptor cDNA of patient VA indicated that the allele containing the

The 22 exons of the insulin receptor were amplified using flanking primers and sequenced (6). Mutations identified were confirmed by sequencing in both directions and on additional PCR products. When available, parents were also sequenced. The table reports the changes observed from the published sequence. Nucleotides in the insulin receptor cDNA are numbered from the initiating methionine. Historically, the amino acid position is numbered from the histidine, after removal of the 27-amino-acid signal peptide (1,2). When indicated, restriction sites added (+) or removed (−) by the mutation in the relative exon are indicated.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype</th>
<th>Mutation</th>
<th>Restriction enzyme</th>
<th>Mutation</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE</td>
<td>Leprechaunism</td>
<td>2050insG, 665X</td>
<td>BsmFI, exon 10</td>
<td>C682X</td>
<td>MwoI exon 10</td>
</tr>
<tr>
<td>NY-1</td>
<td>Leprechaunism</td>
<td>451G &gt; T, E124X</td>
<td>BanII, exon 2</td>
<td>C682X</td>
<td>BanII exon 2</td>
</tr>
<tr>
<td>VA</td>
<td>Leprechaunism</td>
<td>1998-2001delTGAG650X</td>
<td>PvuII, exon 9</td>
<td>C682X</td>
<td>MspI exon 14</td>
</tr>
<tr>
<td>CIN</td>
<td>Rabson-M endenhall</td>
<td>2989C &gt; A, P970T</td>
<td>—, exon 16</td>
<td>R899W</td>
<td>MspI exon 14</td>
</tr>
<tr>
<td>CG-1</td>
<td>Rabson-M endenhall</td>
<td>3601C &gt; T, R1174W</td>
<td>Rsal, exon 20</td>
<td>C682X</td>
<td>MspI exon 19</td>
</tr>
<tr>
<td>FL-1</td>
<td>Leprechaunism</td>
<td>356C &gt; T, A92V</td>
<td>CfoI, exon 2</td>
<td>C682X</td>
<td>Hinfl exon 14</td>
</tr>
</tbody>
</table>

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deletion of nucleotides 2136–2139 produced undetectable levels of stable mRNA.

Since some premature termination codons are associated with abnormal splicing, we checked for the presence of abnormal transcripts by PCR using cDNA primers located in the exons flanking the nonsense mutations identified. After RT-PCR and 40 cycles of amplification, bands of the predicted size were identified in control RNA, but not in RNA from patients GE and NY-1 (not shown).

Functional expression of mutations in the insulin receptors in Chinese hamster ovary (CHO) cells

To confirm their causative role, the mutations identified in the insulin receptor gene of patients with insulin resistance were recreated in the insulin receptor cDNA and stably expressed in CHO cells. Insulin binding to cells expressing normal and mutant receptors is reported in Table 4. Insulin binding to cells expressing the R86P, ΔN281, I898T, R899W and R1131W...
mutations increased only minimally above the levels measured in untransfected CHO cells and to less than 10% of the increase observed in cells expressing the normal insulin receptor cDNA. By contrast, cells expressing the P970T, I1116T and R1174W mutations increased their insulin binding significantly: up to 63% of the level measured in cells expressing the normal insulin receptor. Therefore, the major mechanism by which the R86P, A92V, DN281, I898T, R899W and R1131W impair insulin action is by impairing insulin binding.

To evaluate whether the P970T, I1116T and R1174W mutations impaired insulin signaling, a dose–response curve for insulin stimulation of glucose transport was obtained. Glucose transport was half-maximally stimulated by insulin at 10.2 ± 2.7 pM in cells expressing the normal insulin receptor cDNA, as compared with 320 ± 15 pM in untransfected CHO cells. Cells expressing the P970T, I1116T and R1174W mutations had dose–response curves for insulin stimulation of glucose transport comparable to those measured in untransfected CHO cells (half-maximal stimulation of glucose transport was observed at 290–450 pM of insulin), indicating that the transfected receptors were not functional in signaling glucose transport (not shown).

**Table 4.** Insulin binding to CHO cells expressing normal and mutant insulin receptors

<table>
<thead>
<tr>
<th>Cells</th>
<th>Specific insulin binding (fmol/mg cell protein)</th>
<th>Percentage of wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental CHO cells</td>
<td>0.25 ± 0.02</td>
<td>0</td>
</tr>
<tr>
<td>Wild-Type IR</td>
<td>8.78 ± 0.11ab</td>
<td>100</td>
</tr>
<tr>
<td>R86P</td>
<td>0.25 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td>A92V</td>
<td>0.32 ± 0.04</td>
<td>1</td>
</tr>
<tr>
<td>AN281</td>
<td>0.27 ± 0.04</td>
<td>0</td>
</tr>
<tr>
<td>I898T</td>
<td>0.23 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td>R899W</td>
<td>0.58 ± 0.04ab</td>
<td>4</td>
</tr>
<tr>
<td>P970T</td>
<td>5.63 ± 0.31ab</td>
<td>63</td>
</tr>
<tr>
<td>I1116T</td>
<td>2.95 ± 0.31ab</td>
<td>32</td>
</tr>
<tr>
<td>R1131W</td>
<td>0.27 ± 0.03</td>
<td>0</td>
</tr>
<tr>
<td>R1174W</td>
<td>3.62 ± 0.33ab</td>
<td>40</td>
</tr>
</tbody>
</table>

Mutations in the insulin receptor gene were introduced by site-directed mutagenesis in the insulin receptor cDNA and stably transfected into CHO cells. Insulin (25 pM) binding was measured for 3 h at 20°C. Data are means ± SE of triplicates. Percentage of wild type is calculated after subtracting insulin binding by untransfected cells.

*a P < 0.01 versus parental CHO cells.

**Genotype-phenotype correlation**

Of the patients shown in Tables 1 and 2, those with a clinical diagnosis of leprechaunism died before 3 years of age, with an average survival of 10.2 ± 9.5 months. Patients clinically diagnosed with Rabson–Mendenhall syndrome survived at least 9 ± 1.4 years. The mutations identified in patients with leprechaunism all markedly impaired insulin binding to less than 5% of normal both in the patients’ cells (Tables 1 and 2) and when expressed in CHO cells (Table 4). By contrast, at least one of the mutations identified in patients with longer
survival left residual insulin binding in the patients’ cells (Tables 1 and 2) and when expressed in CHO cells (Table 4).

DISCUSSION

In this paper, we characterize several patients with inherited, severe insulin resistant syndromes and different survival, ranging from a few weeks to several years (Table 1). Two major phenotypes are recognized in these patients: leprechaunism and Rabson-Mendenhall syndrome. Both disorders are characterized by peculiar dysmorphic features and usually different outcomes, with patients with Rabson-Mendenhall syndrome surviving longer than patients with leprechaunism. Both syndromes are inherited as autosomal recessive traits. Although the clinical severity of patients with these severe syndromes varies among different families, the phenotype varies only slightly among multiple affected siblings within the same family, suggesting that genotype is the major predictor of phenotype (14–18). This differs from milder insulin-resistance syndromes, such as type A insulin resistance, in which other genes in addition to the insulin receptor, or environmental factors affect the severity of the phenotype (5).

Cells from all our patients with extreme insulin resistance had defective insulin binding (Tables 1 and 2). This defect was complete in cells from patients with leprechaunism and incomplete (with 18–27% residual binding) in fibroblasts or lymphoblasts from patients with Rabson-Mendenhall syndrome.

Sequence analysis and expression studies in CHO cells confirmed that the mutations in the insulin receptor gene identified in these patients affected insulin binding (Tables 3 and 4). Patients whose cells failed to bind insulin were homozygous or compound heterozygous for mutations abolishing insulin binding, either for the premature insertion of a stop codon or for a structural alteration in the insulin receptor preventing insulin binding. Two patients (GE and NY-1) were homozygous or compound heterozygotes for null insulin receptor alleles. All the nonsense mutations identified in this study (E124X, 650X, 665X and 682X) and in one of our previous patients (R372X) (6) were associated with greatly reduced insulin receptor mRNA levels (Figs 1–3). Other mutations in the insulin receptor gene resulting in premature stop codons also reduce insulin receptor mRNA levels (13,16). However, this is not always the case. Among patients homozygous for nonsense mutations in the insulin receptor gene, cells from patient Qatar-1 (855X) had normal mRNA levels (19). A second patient (Richmond) homozygous for the R786X nonsense mutation had absent PCR amplification of insulin receptor cDNA (20), while RNA was not evaluated in the third patient (Cam-1) homozygous for the K121X nonsense mutation (21). Thus, reduced mRNA levels are not necessarily associated with nonsense mutations in the insulin receptor gene. Figure 4 reports nonsense mutations in the insulin receptor whose effect on mRNA levels was determined either by measurement of mRNA levels or absence of the relevant cDNA. Some nonsense mutations were the result of single nucleotide changes, and others the result of frameshifts or small deletions or insertions (Table 5). Natural mutations reported in the insulin receptor gene introduce 10 UGA (opal), 5 UAG (amber), and 1 UAA (ochra) premature termination codons. There was no clear association between specific types of stop codons and effect on mRNA levels, even though the single ochra codon, similar to the natural termination codon of the insulin receptor gene, was associated with normal mRNA levels. Reduced insulin receptor mRNA or
cDNA was associated with E124X (this paper), W133X (22), R372X (6), 650X (this paper), 665X (this paper), C682X (this paper), R786X (20), 801X (23), R897X (13), R1000X (22) and 1118X (23) mutations. By contrast the presence of normal levels of insulin receptor mRNA or cDNA was reported with the 34X (24), R86X (25), Q672X (26) and 855X (19) mutations. Importantly, multiple nonsense mutations within exons 2 (R86X, E124X and W133X) and 10 (665X, Q672X and C682X) had a discordant effect on mRNA levels, with two mutations (R86X and Q672X) not reducing transcript levels (25,26).

The reduction of mRNA levels with premature termination codons occurs through nonsense-mediated RNA decay (27,28) and is not expected to result in protein formation. Termination codons located more than 55 nucleotides upstream of the 3’-most exon–exon junction usually mediate nonsense-mediated mRNA decay (27,28). The recognition of abnormally terminated mRNA is probably mediated by ribonucleoproteins that, at time of splicing, bind close to the exon–exon junction and flag to the mRNA surveillance machinery messages prematurely terminated (29). The additional nonsense mutations identified in this study, when added to those already known in the insulin receptor gene, indicate that the mechanism seems more complex and that there are other factors, in addition to the location of the premature stop codon, that are important determinants of RNA stability. The study of the effect on RNA stability of these and other natural nonsense mutations can shed light on the nature of these factors.

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The missense mutations identified in our patients with leprechaunism and shorter survival affected the extracellular portion of the insulin receptor and abolished or markedly reduced insulin binding (Fig. 5). The A92V mutation identified

![Diagram](image-url)

**Table 5. Effect of nonsense mutations in the insulin receptor gene on mRNA levels**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation</th>
<th>Nonsense codon</th>
<th>mRNA levels</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK</td>
<td>22-31del 34X</td>
<td>UGA (opal)</td>
<td>Normal</td>
<td>24</td>
</tr>
<tr>
<td>PK</td>
<td>337C &gt; T R86X</td>
<td>UGA (opal)</td>
<td>Normal</td>
<td>25</td>
</tr>
<tr>
<td>Camp</td>
<td>442A &gt; T K121X</td>
<td>UAG (amber)</td>
<td>Reduced</td>
<td>21</td>
</tr>
<tr>
<td>NY-1</td>
<td>451G &gt; T E124X</td>
<td>UAG (amber)</td>
<td>Reduced</td>
<td>22</td>
</tr>
<tr>
<td>A-1</td>
<td>479G &gt; A W133X</td>
<td>UAG (amber)</td>
<td>Reduced</td>
<td>6</td>
</tr>
<tr>
<td>Mt Sinai</td>
<td>1195C &gt; T R372X</td>
<td>UGA (opal)</td>
<td>Reduced</td>
<td>22</td>
</tr>
<tr>
<td>VA</td>
<td>1998-2001del 650X</td>
<td>UGA (opal)</td>
<td>Reduced</td>
<td>6</td>
</tr>
<tr>
<td>GE</td>
<td>2050insG 665X</td>
<td>UGA (opal)</td>
<td>Reduced</td>
<td>6</td>
</tr>
<tr>
<td>Ark-1</td>
<td>2095C &gt; T Q672X</td>
<td>UAG (amber)</td>
<td>Normal</td>
<td>26</td>
</tr>
<tr>
<td>GE</td>
<td>2125insA C662X</td>
<td>UAG (amber)</td>
<td>Reduced</td>
<td>6</td>
</tr>
<tr>
<td>Richmond</td>
<td>2437C &gt; T R786X</td>
<td>UGA (opal)</td>
<td>Reduced</td>
<td>20</td>
</tr>
<tr>
<td>RM-E</td>
<td>2480-2487del 801X</td>
<td>UGA (opal)</td>
<td>Reduced</td>
<td>23</td>
</tr>
<tr>
<td>Qatar</td>
<td>2630-2642del 2630-</td>
<td>UAA (ochra)</td>
<td>Normal</td>
<td>19</td>
</tr>
<tr>
<td>Mt Sinai</td>
<td>2642insACTCA 855X</td>
<td>UGA (opal)</td>
<td>Reduced</td>
<td>13</td>
</tr>
<tr>
<td>RM-1</td>
<td>3079C &gt; T R1000X</td>
<td>UGA (opal)</td>
<td>Reduced</td>
<td>22</td>
</tr>
<tr>
<td>Osaka-S</td>
<td>3408del 1118X</td>
<td>UGA (opal)</td>
<td>Reduced</td>
<td>23</td>
</tr>
</tbody>
</table>

Nucleotides affected by the mutations are numbered from the starting methionine. Historically, the amino acid position is numbered from the histidine after removal of the 27-amino-acid signal peptide (1,2).
in patient FL-1 is located close to the R86P mutation and one of the major insulin binding sites of the receptor (7,30). The ΔN281 mutation, found in two unrelated patients with leprechaunism (8,31), was associated with absent insulin binding to the patient’s cells in one study (8), but near-normal insulin binding in another study (31). Insulin-binding studies in fibroblasts can be difficult to interpret given the low levels of expression of the insulin receptor gene. Expression studies were not conducted in either case, and our data in transfected CHO cells indicate that the ΔN281-mutant insulin receptor does not bind insulin (Table 3). The I898T and R899W mutations are located in the extracellular portion of the β subunit of the insulin receptor and close to the T910M mutation identified in another patient with leprechaunism (23). The T910M mutation was found to affect insulin binding by impairing receptor processing (23), and it is likely that the two novel mutations identified in this study reduce insulin binding via a similar mechanism.

In our patients with Rabson-Mendenhall syndrome and prolonged survival, at least one mutation was located in the intracellular portion of the receptor fsubunit. The R1131W mutation, identified in two unrelated patients with Rabson-Mendenhall syndrome (ATL-2 and CIN), abolished insulin binding when expressed in CHO cells despite its intracellular location. Three other mutations in intracellular residues (P970T, I1116T and R1174W) reduced insulin binding to about 50% of normal. These data are compatible with insulin-binding data in cells from these patients showing a reduction of insulin binding to about 25% of normal (Tables 1 and 2) (11,12). Therefore, even intracellular mutations in the insulin receptor can reduce insulin signaling by impairing, at least in part, insulin binding. The R1174W mutation was previously identified in a patient with leprechaunism (32), and was found not to impair insulin binding in transiently transfected CHO cells when binding was normalized to receptor number. However, the R1174W-mutant receptors were degraded more rapidly than normal receptors (32,33). This might result in reduced steady-state levels of insulin receptors on the plasma membrane, resulting in the decrease in insulin binding reported here in the patient’s cells and in transfected CHO cells.

The P970T, I1116T and R1174W substitutions prevented insulin stimulation of glucose transport, indicating their causative role in the insulin resistance observed in our patients. The mechanism by which this occurs has not yet been defined. These substitutions affect conserved domains of the insulin receptor and are predicted to impair either the kinase activity of the receptor or its interaction with intracellular substrates. Specifically, the P970T mutation affects the consensus sequence (969-NPEY-972) for the binding of insulin receptor substrate 1 (IRS-1) (34,35), and is the first natural mutation reported in such an important area of the insulin receptor. The I1116T substitution affects an α-helical region to which the catalytic loop of the insulin receptor kinase is attached (36). A hydrophobic amino acid is present in this position in a number of tyrosine kinases, including the epidermal growth factor, platelet-derived growth factor and fibroblast growth factor receptors (EGFR, PDGFR and FGFR), and the tyrosine kinases c-Src, c-Abl and cAPK (36). The R1174W substitution affects the activation loop of the tyrosine kinase domain of the insulin receptor (36). A positively charged amino acid in this position is conserved among several tyrosine kinases as well (36). Previous studies of this latter mutation have confirmed that the R1174W substitution abolishes insulin-stimulated receptor autophosphorylation and kinase activity (32).

Taken together, our data indicate that, within the limits of our patient population, the most severe phenotype with early demise was observed when both mutations completely abolished insulin binding and subsequent insulin action. By contrast, when one mutation retained residual insulin-binding activity, the survival was longer. While the majority of patients reported to date seem to fit within this classification, there are notable exceptions. There are patients with leprechaunism in whom the mutations affect the intracellular portion of the insulin receptor (9,10). However, in one case, the mutations identified were not expressed in CHO cells (9), and an effect on insulin binding cannot be excluded in view of our results with the R1131W mutation. In the second case, the patient was older than 1 year of age at the time of the study and an affected brother died at 7 years of age with the same syndrome (10), despite a phenotype of leprechaunism. The prolonged survival of patients with leprechaunism within this family indicates that residual insulin binding (and probably action) correlates with survival, rather than the specific phenotype of leprechaunism or Rabson-Mendenhall syndrome. Therefore, the two diseases should be considered as a continuous of a spectrum, in which the specific mutation and the degree of impairment of insulin action predicts survival, rather than the type of syndrome.

MATERIALS AND METHODS

Patients

Several new patients are reported in this manuscript. A brief description of each new patient will follow. Details on patients ATL-1 (7,14), ATL-2 (11,12), Mt Sinai (6) and NZ (8) can be found in previous papers.

Patient GE with leprechaunism was a Caucasian female, the first child of healthy unrelated parents of European descent. Pregnancy was complicated by oligohydramnios and the patient was born at 40 weeks of gestation via Caesarean section. The proband weighed 1960 g (small for gestational age), her length was 44 cm and the head circumference was 34.5 cm. At birth, the infant was noted to have dysmorphic features, such as a small face with prominent eyes and thick lips, large, pointed ears, depressed nasal bridge, wide nostrils, absence of subcutaneous fat, wrinkled and loose skin, hirsutism, acanthosis nigricans, disproportionately large genitilia with bilateral inguinal hernia, and a protuberant abdomen. A systolic ejection murmur was also noted. This prompted further diagnostic evaluation, which revealed hypertrophic obstructive cardiomyopathy. Unstable blood sugars with severe hypoglycemia (down to 21 mg%) and elevated insulin levels (up to 800 μU/ml) were noted shortly after birth. A abdominal ultrasound revealed bilateral polycystic ovaries, with liver, spleen, kidneys, bladder and uterus of normal size and structure.

The progressive increase in abdominal distention prompted a laparotomy and surgical removal of the ovaries shortly after 7
weeks of age. The peritoneal cavity was almost completely occupied by large cystic ovaries, which were removed. Histologic examination was consistent with bilateral juvenile granulosa cell tumor of the ovaries. A liver biopsy was also performed during surgery for the cholestatic appearance of the liver. Liver histology was consistent with cytomegalovirus hepatitis. Two days after surgery at 55 days of age, the patient died of refractory heart failure. An autopsy was not performed. A detailed clinical report of this patient has been published (37).

Both parents of the proband were phenotypically normal. An oral glucose tolerance test performed in both parents was normal, but elevated insulin levels were measured before and during the test, consistent with mild asymptomatic insulin resistance.

Patient FL-1 with leprechaunism was a Caucasian female, the first child of healthy unrelated parents. Pregnancy and delivery were uncomplicated. The patient was born at 37 weeks of gestation via natural delivery. Birthweight was 1800 g (small for gestational age), length was 44.5 cm and head circumference was 30 cm. For the intrauterine growth restriction, she remained in the hospital for 10 days. Initial studies indicated presence of antibodies against cytomegalovirus. At about 1 month of age, she was readmitted to the hospital for fever (40°C) and irritability. Physical examination was significant for low weight (2.9 kg), decreased subcutaneous fat, generalized hirsutism, gingival hyperplasia, breast enlargement, heart murmur, distended abdomen, and prominence of clitoris and labia majora. Echocardiogram documented left ventricular murmur, distended abdomen, and prominence of clitoris and hirsutism, gingival hyperplasia, breast enlargement, heart murmur, distended abdomen, and prominence of clitoris and labia majora. Echocardiogram documented left ventricular hypertrophy and an atrial septal defect. Abdominal ultrasound demonstrated bilateral cystic ovaries. Routine laboratory studies indicated severe hyperglycemia (422 mg/dL). The child had poor weight gain and was noted to have dysmorphic features. The skin was thickened and coarse. Acanthosis nigricans was present in the neck, axilla and groin. Ears had thickened helices. The nose was upturned with a broad tip. The mouth was wide with thick lips. The palate was narrow and crowded. Cardiac and lung exam were normal. The liver edge was palpable 2 cm below the costal margin. Genitalia were normal for a male, with a large phallus. Uterus and ovaries were difficult to palpate in the pelvis. The family history was significant for consanguinity and distant relatives with diabetes mellitus. Glucose tolerance tests on the parents revealed normal tolerance but moderate hyperinsulinemia, with fasting hypoglycemia and postprandial hyperglycemia. Insulin levels were elevated (535, 1084 and 4394 μU/ml). After 2 months in the newborn nursery, he was discharged home with a weight of 2000 g. He was fed via gastric tube a diet rich in complex carbohydrates every 3 hours to prevent hypoglycemia. He had a progressive worsening of his respiratory status, and failed to thrive. At 3 months of age, he presented fever (38.5°C) and acute deterioration of his respiratory status. A blood count indicated leukocytosis with bandemia. He had normal blood pH and blood gases (on oxygen) with normal electrolytes. His average blood glucose was 106 ± 66 mg/dl (n = 10) with one value of 20 and one of 280 mg/dl. He died of heart failure shortly after admission. Both parents are phenotypically normal.

Patient CIN with Rabson-Mendenhall syndrome was born after a full-term pregnancy complicated by preterm labor and oligohydramnios. He was the first child of a 21-year-old mother. The birthweight was 2138 g (small for gestational age). He was diagnosed with diabetes at 3 weeks of age and initiated on insulin therapy. This was stopped after verifying the lack of response and determination of high circulating levels of insulin (above 2000 μU/ml), indicative of insulin resistance. He has been managed with a sugar-free diet, glipzide, and glucophage, with glucose levels between 200 and 400 mg/dl. Other medical problems include repeated episodes of toxic synovitis of the left hip, and repeated ear infections requiring placement of ear tubes and tonsillectomy. He was noted to have early eruption of permanent teeth at 3 years of age. Developmentally, he is mildly delayed. He sat at 9 months and walked at 2 years of age. Speech development, however, was normal. On examination at 5 years of age his weight was 13.9 kg ( < 5th percentile, appropriate for a 32-month-old boy), height was 95.4 cm ( < 5th percentile, appropriate for a 34-month-old boy), head circumference was 47.5 cm ( < 5th percentile, appropriate for a 14-month-old boy). He appeared very tiny, with coarse facial features. The skin was thickened and coarse. A canthosis nigricans was present in the neck, axilla and groin. Ears had thickened helices. The nose was upturned with a broad tip. The mouth was wide with thick lips. The palate was narrow and high-arched. Eight permanent teeth were present, and were irregularly placed and crowded. Cardiac and lung exam were normal. The liver edge was palpable 2 cm below the costal margin. Genitalia were normal for a male, with a large phallus. Fingers were short and broad, with mild flexion contractures. Neurological examination was normal. The child developed ketoacidosis at 8 years of age.

The family history was significant for absence of consanguinity and distant relatives with diabetes mellitus. Glucose tolerance tests on the parents revealed normal tolerance but moderate hyperinsulinemia, with fasting insulin levels of 78.1 and 43.8 μU/ml in the mother and father, respectively.

Patient CG-1 is an 8-year-old Hispanic female with Rabson-Mendenhall syndrome. Only limited information is available on this patient. On physical examination, she had growth restriction, with height and weight below average, and mild developmental delay. She has dysmorphic features,
including a high arched, V-shaped palate with dysplastic teeth, hirsutism, acanthosis nigricans, breast enlargement and clitoromegaly. A random insulin level was 500 µU/ml, with a simultaneous glucose level of 85 mg/dl. The mother has a normal glucose tolerance test. No information is available about the father.

Materials

Sera, growth media and trypsin solutions were from Sigma (St Louis, MO). Radiochemicals, including \[^{125}\text{I} \]insulin, were from Amersham. Products for molecular biology were from Roche Molecular Biochemicals (Indianapolis, IN). Sigma was the source of other chemicals.

Cell culture

Fibroblast cultures were established from skin biopsies of patients and their parents for diagnostic purposes. Control fibroblast cultures (GM 003348 and GM 005756) were obtained from the Coriell Institute for Medical Research (Camden, NJ) or from the repository of the Division of Medical Genetics (n = 14). The research use of these cells was approved by the Emory University Institutional Review Board. Fibroblasts from patients ATL-1, ATL-2, Mt Sinai and N2 were obtained as previously described (6–8,11). Cells were grown in Dulbecco–Vogt (DV) medium containing 15% fetal bovine serum. Lymphoblasts were grown in RPMI medium containing 15% fetal bovine serum.

Insulin binding

Insulin binding to lymphoblasts and cultured fibroblasts was performed at 20°C in Earle’s balanced salt solution (EBSS) buffered with tris(hydroxymethyl)aminomethane (26 mM, pH 7.4), as previously described (14). Ligand binding was normalized to cell proteins, corrected for non-specific binding (measured in the presence of 5 µg/ml of cold ligand), and expressed as femtomoles of ligand bound per micromolars of cell protein (14).

DNA and RNA analysis

Genomic DNA was amplified by PCR using primers flanking the 22 exons of the insulin receptor gene (6). Amplified DNA was purified and sequenced as previously described (6). Sequencing results were confirmed by enzymatic digestion using the enzymes indicated in Table 3. Southern blot analysis to exclude gross deletions was performed using EcoRI and BamHI as restriction enzymes for genomic DNA and three different insulin receptor cDNA probes covering the whole coding region (39).

Cellular RNA was extracted with guanidinium thiocyanate and poly(A) + RNA was analyzed by northern blot analysis using a 3 kb fragment corresponding to the 3’ end of the insulin receptor cDNA as a probe (6,14). After autoradiography, the radioactivity in each band was measured by an InstantImager (Packard). The blot was then stripped and hybridized to the actin cDNA for normalization.

RNA protection assay was performed using the manufacturer’s protocol (Ambion) on total RNA using antisense-labeled RNA to actin and insulin receptor. Bands on the gel were quantified using the InstantImager (Packard) as above.

Mutagenesis of the insulin receptor cDNA

The mammalian expression vector containing the insulin receptor cDNA (40) was mutagenized by site-directed mutagenesis using the Quik Change system (Stratagene, La Jolla, CA), following the manufacturer’s instructions. The final clones were sequenced to confirm the presence of the mutation and the absence of PCR artifacts. The clones were transfected into CHO cells using lipofectamine. Cells were selected for 2 weeks in 0.8 mg/ml of G418 and then used for insulin binding (performed as described above) and insulin stimulation of glucose transport. Insulin stimulation of glucose transport in transfected CHO cells was measured and analyzed as previously described (30,40).

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