A Mutation in the Insulin Receptor That Impairs Proreceptor Processing but Not Insulin Binding*

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Here we report the identification of a new mutation in the α-chain of the insulin receptor, changing Trp142 into Ser using DNA from consanguineous parents who gave birth to a child with leprechaunism. The mutant receptor was expressed stably in CHO and transiently in COS-1 cells. It was found that the Ser412 mutant is not cleaved into α- and β-subunits and remains as a 210-kDa proreceptor at an intracellular site. This property of the mutant receptor is in line with the observed decreased insulin binding to the parental fibroblasts. Cross-linking experiments show that the Ser412 proreceptor is able to bind insulin with an affinity comparable to that of the wild-type α-chain. Despite its capacity to bind insulin, the mutant receptor is not autophosphorylated.

We postulate that the patient was homozygous for the Trp142—Ser mutation and that the mutation was responsible for the leprechaun phenotype. This is the first description of a transport-defective receptor with the mutation outside the tetrabasic processing site and a functional insulin binding domain. The ability of the Ser412 mutant to bind insulin in cross-linking experiments suggests that the impaired transport of the proreceptor to the cell surface is the primary cause for the binding defect to intact cells.

Recently, we and others investigated the effect of a number of different mutations in the α-chain of the IR on receptor properties by transfection experiments. The examined mutations came from patients with leprechaunism or the Rabson-Mendenhall syndrome. The mutations were alike in that they resulted in insulin receptor precursors that were deficient in receptor processing. The transport to the cell surface of these proreceptors is inhibited (2–6). The transport and processing defect of these mutants resulted from an altered conformation of the precursor receptor, as judged from studies with monoclonal antibodies recognizing conformational epitopes and a loss of insulin binding to the proreceptor.

This paper describes a new mutation in the α-chain of the IR, which is expected to be responsible for leprechaunism. As there was no material available from the deceased patient, material from both consanguineous parents was used for the experiments. The effect of the mutation on the properties of the IR was examined after overexpression of the mutant receptor in CHO and COS-1 cells.

EXPERIMENTAL PROCEDURES

Materials—Taq polymerase and thermocycler were from Perkin-Elmer. Deoxyribonucleotides, lactoperoxidase, and glucose oxidase were from Boehringer Mannheim. Other enzymes were from Pharmacia LKB Biotechnology Inc. (35)S Methionine, 32P-labeled nucleotides, Na32P, and A14 mon(125)Iioo-insulin from Amersham Corp. Bovine serum albumin, radioimmunoassay grade, and Sequenase version 2.0 were from U. S. Biochemical Corp. Dsaccinimidyl suberate (DSS) was from Pierce Chemical Co. Monocomponent human insulin was obtained from Novo, Bagsvaerd, Denmark. Immune precipitates were analyzed on 8% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) using as markers myosine (220 kDa), β-galactosidase (126 kDa), phosphorylase (97 kDa), bovine serum albumin (68 kDa), and ovalbumin (45 kDa). The protein markers were obtained by using a high molecular weight kit from Bio-Rad. Dried SDS-PAGE gels were exposed to Kodak XAR films using intensifying screens. In case of 35 labeling, the gels were impregnated with Amplify (Amersham).

Subject—Patient KG was a female born from a consanguous marriage. The parents originated from Turkey. After an uncomplicated pregnancy, she was born at term with typical clinical symptoms of leprechaunism: extreme dystrophic appearance, low ear implant, hypertrichosis, genital hypertrophy, and lipodystrophy. From day 3, the proband suffered from general infections, and at the age of 24 days, she died from septic shock. Autopsy showed hepatomegaly and marked hypertrophy of the islets of Langhans. No material from the patient was available. Skin biopsies and blood were obtained from the parents and used for the experiments.

Insulin Binding and DNA Sequencing—Determination of high affinity insulin binding sites on cultured fibroblasts (7) and DNA sequencing (8) has been described previously.

Construction of Mutant Expression Vectors—Recombinant polymerase chain reaction was used to create the Ser412 mutant receptor from a cDNA clone encoding the wild-type human insulin receptor (WT-HIR). With two specific oligonucleotide sets, two cDNA fragments (A and B) were amplified, both containing the mutation. Fragment A was amplified with primers 1057 and 1377 (AAGGTGTGCGACCTCTCTAGA; TTT-
GCTCCACGTGAGAGCTGCTTTA). Fragment B with primers 1352 and 1661 (TAAAGGCACCTCTCGGACTGGACAAA; GGGCTCTCTTGTTAGAACAGC). Primers 1352 and 1377 are complementary 26-mers with the mutated nucleotide at position 1364 (indicated by bold italics).

Fragment A (1057–1377) and fragment B (1352–1661) were purified by low melting agarose gel electrophoresis. They were isolated and precipitated together at 97 °C. Renaturation yielded the original fragments, and also the heteroduplexes A-B. In this mixture the heteroduplex was selectively reamplified with primers 1057 and 1661, resulting vectors were transfected into (2–600 bacteria. Colonies expressing the Ser412 mutant vector (Ser412-HIR) were identified by colony hybridization with a 32P-labeled mutant oligonucleotide. By DNA sequencing, the correct sequence of the Ser412-HIR vector was reconfirmed.

Transient Expression of the Insulin Receptor (+ Exon 11) in COS-1 Cells—COS-1 cells were used for transient expression of the WT-HIR and Ser412-HIR Sr-α vector (the vector containing the WT-HIR+ exon 11 was kindly provided by Dr. R. A. Roth). DNA (4 μg) in 4 μl of 0.1 × TAE (10 mM Tris-acetate, 1 mM Na2EDTA) was mixed with 186 μl of H2O, 50 μl of 1.5× CaCl2, and 250 μl of 2 × transfection buffer (78 mM NaCl, 13.0 mM Hepes, 0.38 mM Na2HPO4, pH 7.0).

The COS-1 cells were grown to 40–60% confluence, and the DNA mixture was added dropwise in 5 ml of Dulbecco’s modified Eagle’s medium. After 18 h at 37 °C in a 2–4% CO2 atmosphere, the precipitation was completed. The medium was then changed to standard Dulbecco’s modified Eagle’s medium, and the cells were cultured for another 56 h at 5% CO2.

Stable Expression of the Insulin Receptor (−Exon 11) in CHO Cells—The WT-HIR (−exon 11) vector was transfected in CHO cells using the calcium phosphate method, as described before (3). Single colonies resistant to phosphate method, as described before (3). Single colonies resistant to 5 μg/ml G-418 were picked and brought to high expression by amplification on 400 μm methotrexate.

(F)S/Methionine Labeling of Cells, Enzymatic Deglycosidation, Western Blot, and Cell Surface Iodination—Labeling, enzymatic deglycosidation, Western blot, and cell surface iodination were as described previously (2, 3).

Cross-linking of 125I-Insulin to the Insulin Receptor—CHO cells expressing WT and mutant receptors were grown in 6-cm dishes to confluence. Cells were washed with phosphate-buffered saline and lysed in 200 μl of ice-cold lysis buffer (50 mM Hepes, pH 7.4, 0.1 mM EDTA, 1% Triton X-100). Nuclei were removed by centrifugation, and 25 μl (150 μg of protein) of total cell lysate was incubated with 2.5 μl of 125I-insulin (0.25 μCi, 2000 Ci/mmol) overnight at 4 °C. DSS (final concentration 200 μM) was added, and after 20 min on ice the reaction was terminated by 1 μl of 2× TCA (1% in pH 7.5). Sample buffer was added, and the mixture was analyzed by SDS-PAGE and autoradiography.

The affinity of the (pro)receptor was determined at four concentrations of 125I-insulin: 0.5, 1.0, 5.0, and 10.0 × 10−8 μM (specific activity 2000 Ci/mmol).

The dissociation rate of the 125I-insulin-insulin receptor complex was examined by incubation with 1.0 × 10−8 M nonradioactive insulin. At intervals of 5 min, samples were taken and subjected to cross-linking. Analysis of receptor labeling was as described above.

Autophosphorylation of Insulin Receptors—CHO cells were grown to confluence in 6-cm dishes, washed in phosphate-buffered saline, and lysed in 200 μl of ice-cold lysis buffer (50 μg of protein) lysates were used, and autophosphorylation was performed with and without 50 μM insulin. In case of autophosphorylation on immune precipitates, the cells were grown to confluence and lysed in 300 μl of ice-cold lysis buffer. Nuclei were removed by centrifugation. The supernatant was diluted to 15 μl). Incubation was for 6 min, after which total protein was precipitated by adding 1 ml of 10% trichloroacetic acid. The mixture was centrifuged, and the pellet was washed with 70% ethanol. The protein pellet was dissolved in SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

Insulin Binding to Fibroblasts—In most cases, fibroblasts from leprechaun patients show a loss of high affinity insulin binding sites (8). In relatives of the patients, who were heterozygous for mutations in the IR, we observed an approximately 70% loss of these sites. As no cells were available from the deceased proband KG, we examined fibroblasts from the parents of KG (641 and 642) for the number of high affinity insulin binding sites. In the binding experiment, we included fibroblasts from an individual (RD 330) who is heterozygous for a Pro320 mutation in the IR. Homozygosity for the Pro320 mutation results in leprechaunism and total loss of high affinity insulin binding to fibroblasts. This mutant is transport-defective when expressed in CHO cells (3). As control we used fibroblasts from healthy individuals. Insulin binding experiments with fibroblasts from RD 330, 641, and 642 showed comparable binding values which were approximately 30% of the values seen in fibroblasts from 10 controls (Fig. 1). This observation suggests that in the deceased leprechaun KG, IR mutations were involved that are linked to a loss of high affinity insulin binding sites.

Sequence Analysis—To determine the presence of mutations in the IR, DNA was isolated from parental blood. The coding region of the IR gene was amplified, and the polymerase chain reaction product was sequenced. We detected a non-polymorphic mutation in exon 6 changing a tryptophan (TGG) into a serine (TGC) at position 412 (Fig. 2). Both parents are heterozygous for this Ser412 mutation.

Insulin Binding to Cells Expressing the Ser412 IR—To examine the effect on the properties of the IR of the conversion of tryptophan to serine at position 412, we introduced this mutation in IR cDNA by recombinant polymerase chain reaction. Subsequently, CHO cells were transfected stably with an SV40 driven vector containing the sequence for WT-HIR and Ser412-HIR, respectively. This vector contains a dihydrofolate reductase gene, which enables amplification with methotrexate. Overexpression was obtained after selection with G-418 in the presence of increasing concentrations of methotrexate.

Insulin binding studies showed in WT-HIR CHO cells a marked increase of binding compared to untransfected CHO cells. The number of high affinity sites in WT-HIR CHO cells ranges between 2 × 106 and 1 × 106/cell in various individual clones. In the parental CHO cells and the Ser412-HIR CHO cells,
the number is 5 x 10^7/cell.

Similar results were seen in transient experiments in COS-1 cells, using the Sr-α-derived expression vector (not shown). From these studies we conclude that expression of the Ser412 mutant does not result in the appearance of high affinity insulin binding sites on the cell surface.

**[35S]Methionine Labeling on Transfected CHO Cells**—Parental CHO cells and cells expressing WT and Ser412 mutant IRs were labeled with [35S]methionine for 8 h. Lysates of the cells were immune precipitated with a polyclonal antibody against the IR. Analysis of the immune precipitates on SDS-PAGE showed comparable expression of the proreceptor in case of the Ser412-HIR CHO cells and the WT-HIR CHO cells. However, no bands with a migration corresponding to the α- or β-chains were detected in the Ser412-HIR CHO cells (Fig. 3A), even after overexposure. The same result was obtained using a monoclonal antibody (RPN 538, Amersham) against the IR, which recognizes an epitope on the α-chain that is sensitive to conformational changes (Fig. 3B). These results suggest that the Ser412 mutant is not converted into α- and β-subunits.

**Western Blot Analysis of COS-1 Cells**—The properties of the Ser412-HIR were also examined in COS-1 cells. The WT-HIR and the Ser412-HIR were expressed transiently using the SR-α-derived expression vector. Two days after transfection, the IR was visualized by Western blotting using a polyclonal antibody against a peptide epitope on the β-chain of the IR. In case of the WT-HIR expression, next to the proreceptor at 210 kDa the β-chain (95 kDa) was also present. Expression of the Ser412-HIR showed, as in CHO cells, only the 210-kDa band. In the untransfected COS-1 cells, no insulin receptor expression was seen (Fig. 4).

**Enzymatic Deglycosylation**—To determine whether the 210-kDa band actually represents the proreceptor, immune precipitates from the WT-HIR CHO and the Ser412-HIR CHO cells were subjected to enzymatic deglycosylation with endoglycosidase H and neuraminidase. After endoglycosidase H treatment, the 210-kDa band shifted to a 180-kDa band in both cell lines. Use of neuraminidase showed no shift of the 210-kDa bands (Fig. 5). This behavior is characteristic for the proreceptor of the IR, as it lacks sialic acid residues (9), and underscores that the 210-kDa band seen in the immune precipitates is indeed the proreceptor.

**Cell Surface Iodination in CHO Cells**—To investigate whether the mutant proreceptor is transported to the cell surface, the various CHO cell lines were subjected to cell surface radiiodination using the lactoperoxidase-catalyzed procedure. After cell lysis, immune precipitation, SDS-PAGE and autoradiography, cells expressing the WT receptor showed clearly the α-chain at 135 kDa and the β-chain at 95 kDa, whereas in the mutant receptor neither the IR chains nor the proreceptor could be detected. It should be noted that the nonimmune serum precipitates a labeled protein, migrating at a position of approximately 85 kDa (Fig. 6).

**Cross-linking of [125I]Insulin to the Receptor in Cell Lysates from CHO Cells**—As the proreceptor did not reach the cell surface, the binding defect to intact cells seems a secondary effect. To examine the ability of the mutant proreceptor to bind insulin, cell lysates of transfected CHO cells were prepared and used for insulin binding studies. It was found that whole cell lysates of CHO cells showed high binding values, which could not be competed with an excess of nonradioactive insulin. To
circumvent this problem of non-specific binding, an indirect assay was used to determine the amount of insulin bound to receptors in whole cell lysates. The method is based on covalent cross-linking of \(^{125}\)I-insulin to the IR. Cell lysates from CHO cells expressing WT-HIR and Ser\(^{412}\)-HIR were incubated overnight with \(1 \times 10^{-9}\) \(M^{125}\)I, followed by addition of the cross-linker DSS. Protein was analyzed by SDS-PAGE and autoradiography. In the WT-HIR CHO cells, the \(\alpha\)-subunit (135 kDa) and also (in small amounts) the proreceptor (210 kDa) were labeled. In the Ser\(^{412}\)-HIR CHO cells, only the proreceptor was seen. Radioactive insulin was competed away by addition of excess of nonradioactive insulin (Fig. 7A). Thus, the Ser\(^{412}\) mutant proreceptor can be cross-linked to insulin. The experiments were also done with various concentrations of insulin (0.5, 1, 5, and \(10^{-9}\) \(M\)) and labeled (pro)receptor was quantitated by densitometry. The yield of labeled (pro)receptor depends on the amount of IR-insulin complex formed and is an indication for the \(K_d\) of insulin binding. The observed dependence of cross-linking on the insulin concentrations is similar for the WT \(\alpha\)-subunit and the Ser\(^{412}\) proreceptor. From the concentration dependence, it is estimated that the half-maximal labeling in both situations is reached at 2–4 \(\text{nM}^{125}\)I-insulin (Fig. 7B). In addition, the dissociation rate of the IR-insulin complex was found to be similar in both situations. This was examined by incubating the cell lysates with \(^{125}\)I-insulin for 18 h, followed by addition of 1 \(\mu\)M nonradioactive insulin. Subsequently, at 5-min intervals, DSS was added and the labeling of the \(\alpha\)-subunit in case of the WT IR and the proreceptor in case of the Ser\(^{412}\) mutant was determined (Fig. 8A). Densitometry of the (pro)receptor bands is represented as percentage of the value measured at \(t = 0\). The decline is comparable in both cell lines (Fig. 8B). The \(t_{1/2}\) for dissociation is approximately 19 min.

**Autophosphorylation of Mutant Receptors**—Cell lysates were also used to examine the ability of the Ser\(^{412}\) proreceptor to undergo insulin-stimulated autophosphorylation. For that, cell lysates from WT-HIR CHO cells and Ser\(^{412}\)-HIR CHO cells were incubated with or without 50 \(\text{nM}\) insulin in the presence of \([\text{\(^{32}\)P}]\)ATP. Total protein was precipitated and analyzed by SDS-PAGE.
with autoradiography. The relative amount of labeled (pro)receptor was determined by densi-
tometry. Wild-type CHO cells showed a strong signal at the position of the P-chain (95 kDa) after insulin stimulation, comparable to that seen when partially purified IRs were used. The Ser412-HIR CHO cells did not show any induction at the position of the P-chain. We also performed quantitation of the rate of dissociation.

PAGE and autoradiography (Fig. 9). The lysates from the WT-HIR CHO cells were analyzed by SDS-PAGE, followed by autoradiography. Lanes 1–5, WT-HIR CHO cells; lanes 6–10, Ser412 CHO cells. Lanes 1 and 6, initial situation; lanes 2–5 and 7–10 represent labeling after 5, 10, 15, and 20 min of incubation in the presence of excess nonradioactive insulin. Relative amount of labeled (pro)receptor was determined by densitometry. Wild-type (♦) and mutant (+) values were plotted against the time of incubation with excess of non-radioactive insulin.

**Discussion**

A number of IR mutations found in patients with leprechaunism or Rabson-Mendenhall syndrome interfere with transport of the IR to the cell surface (2, 3, 10). The patients are usually homozygous or compound heterozygous for the mutations. Cultured cells from these patients show, in general, a loss of high affinity insulin binding sites on the cell surface (5, 6). Individuals who are heterozygous for such a mutation, like the parents of these patients, show a decrease in high affinity insulin binding sites to approximately 30% of control fibroblasts. This is due to a partially dominant effect of the mutant receptor (2, 11).

We detected a new mutation (Ser for Trp at amino acid position 412) in the insulin receptor gene in DNA from both parents of a patient who exhibited phenotypical features of leprechaunism. As the parents are heterozygous, we postulate that the deceased child was homozygous for this serine (TCG) for tryptophan (TGG) substitution. We cannot confirm this assumption, because no material from the proband is available.

Fibroblasts from the parents show a marked reduction of the number of high affinity insulin binding sites. A similar situation is observed in fibroblasts from heterozygous relatives of leprechaun Geldermaesen (Pro289) and leprechaun H (Arg31). The Pro289 and the Arg31 IR mutants are impaired in receptor processing and transport (2, 3), the same defect could be expected in case of the Ser412 mutant. We examined the properties of the Ser412 mutant receptor, expressed in CHO and COS-1 cells. In CHO cells the insulin receptor expression vector lacked exon 11, whereas the vector used for COS-1 cells had exon 11. In both cases the synthesized Ser412-HIR appears as a proreceptor that is not transported to the cell surface. We observe that the Ser412 proreceptor (− exon 11) expressed in CHO cells has an affinity for insulin similar as the WT-HIR. In addition, it seems that the WT proreceptor (− exon 11), which is present in small amounts in the CHO cells expressing WT-HIR (cf. Fig. 7, lane 1), has a similar affinity for insulin as the WT-α-chain. This is inferred from the observation that the ratio of affinity-labeled WT-α and WT-proreceptor (Fig. 8A) is similar to the antisynthetic levels of processed and unprocessed receptor (Fig. 7). Thus, we observe no marked decrease in affinity of both WT and Ser412 proreceptors for insulin. Other groups have shown that proreceptors resulting from mutations in the tetrabasic cleavage site have lower affinities for insulin as processed α-chains (12, 13). However, it has been pointed out (13) that the affinity of the proreceptor for insulin depends on the cell type in which the proreceptor is expressed, which may explain the similar affinities of processed and unprocessed receptors for insulin that we observed. These authors also provide evidence that the presence of exon 11 in the proreceptor has no major effect on insulin binding.

When autophosphorylation is considered, we observe no insulin-stimulated autophosphorylation using the Ser412 proreceptor. Insulin proreceptors have been shown to undergo insulin-stimulated autophosphorylation, albeit with a lower efficiency as WT receptors (12, 13). Despite its ability to bind insulin, the Ser412 proreceptor does not show an increase in autophosphorylation when insulin is added, indicating that
Trp412 somehow is important to transduce the insulin binding signal to activation of the Tyr-kinase.

Enzymatic deglycosylation experiments, showing the lack of sialic acid, suggest that the proreceptor is already retained in the endoplasmatic reticulum or early Golgi. This observation implies that the folding of the mutant receptor is incorrect. Chaperone proteins can interact with these misfolded proreceptors, resulting in defective transport. Previously described mutations, which prevented receptor transport were likely to affect receptor folding, like the Arg251, Pro253, and Arg259 (1) mutants. These mutations occurred near cysteine residues involved in disulfide bond formation. The Ser412 IR is not located in a cysteine-rich domain, and a protein prediction program showed no major effect on the probability of α-helix or β-sheet formation. An additional indication that the mutation does not affect the folding of the entire α-chain of the receptor comes from immune precipitation experiments with the monoclonal antibody RPN 538. This is an antibody directed against a conformational epitope on the α-chain. RPN 538 recognizes the WT proreceptor but not the transport-defective mutant receptors Pro253 and Arg251. However, this monoclonal antibody does recognize the Ser412 mutant.

Direct insulin binding experiments with the Ser412-HIR in CHO cell lysates were inconclusive due to the high background binding in CHO cells. Indirect binding experiments by cross-linking proved that the proreceptor was still able to bind insulin with an affinity comparable to that of the wild-type IR. The suggested domains of the insulin receptor that determine the specificity are located at the amino acid region 1–68 (14–16), and the cysteine-rich domain between residue 205–310 (16–18) or a domain between residue 450–601 (19). The immunological and insulin binding data on the Ser412 proreceptor show that the overall folding of the α-chain is not affected by the mutation. The regions 1–68, 205–310, and, probably, 450–601 are important for correct insulin binding. Therefore, it seems that the properties of the receptor in the Ser412 mutant are only locally altered around position 412. Remarkably, this apparently minor change in structure is sufficient to inhibit intracellular IR transport completely. Although the conformation of the Ser412 mutant has not been altered grossly, insulin-stimulated autophosphorylation of the receptor is abolished. It has been reported recently by Collier et al. (20) that a loss of four N-linked glycosylation sites in the α-chain by changing Asn into Gln creates a transport-defective receptor. We have no evidence that, in the case of the Ser412 receptor mutant, the glycosylation pattern is grossly altered, suggesting that the conformational change introduced by the conversion of Trp into Ser is directly responsible for the transport defect.

In conclusion, we have found a new mutation in the IR changing a tryptophan into a serine at position 412 in exon 6 of the IR. This is the first mutation associated with impairment of IR function due to defective transport of the proreceptor to the cell surface, without directly interfering with its insulin binding capacity. The proband was most likely homozygous for this mutation and presumably this caused the leprechaun phenotype.

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