An in-frame insertion in exon 3 and a nonsense mutation in exon 2 of the insulin receptor gene associated with severe insulin resistance in a patient with Rabson-Mendenhall syndrome

D. Müller-Wieland¹, E. R. van der Vorm², R. Streicher¹, W. Krone¹, E. Seemanova³, M. Dreyer⁴, H. W. Rüdiger⁵, S. R. Rosipal⁶, J. A. Maassen²

¹ Klinik II und Poliklinik für Innere Medizin der Universität zu Köln, Cologne, Germany

² Department of Medical Biochemistry, Sylvus Laboratories, Leiden, The Netherlands

³ Department of Clinical Genetics, Charles University Hospital, Praha-Motol, Czech Republic

⁴ Department of Diabetes and Metabolism, Krankenhaus Bethanien, Hamburg, Germany

⁵ Universitätsklinik für Innere Medizin IV, Abteilung für Arbeitsmedizin, Vienna, Austria

⁶ Department of Paediatrics, Hospital Poprad, Poprad, Slovak Republic

Summary. We have studied the structure and function of the insulin receptor in a patient (PK) with severe insulin resistance and Rabson-Mendenhall syndrome. Insulin binding to cultured fibroblasts from PK was almost not detectable and insulin-induced insulin receptor autophosphorylation and glucose uptake was abolished. The structure of the receptor gene was analysed by sequencing amplified products of the 22 exons with the flanking intron regions directly as well as after subcloning in pUCBM20 plasmids. Two mutant alleles of the insulin receptor gene were detected. One allele contains in-frame 12 additional base pairs in exon 3 coding for the amino acids Leu-His-Leu-Val located between Asp-261 and Leu-262 in the receptor's extracellular domain, being the first report of an insertion mutation of the insulin receptor gene. In the other allele Arg-86 in exon 2 is changed into a

stop codon. Therefore, PK is compound heterozygous at the insulin receptor locus. Direct cDNA sequencing indicates that both mutant alleles are expressed in the patient's fibroblasts. Studies of the parents' fibroblasts revealed that PK inherited the insertion mutation from the father and the nonsense mutation from the mother. Insulin binding to fibroblasts of the mother was reduced (63 % of control cells) and hormone binding to the father's cells shows a larger reduction (37 % of control cells), but less severe than the patient's cells (11 % of control). This investigation provides further evidence that the Rabson-Mendenhall syndrome is causally related to mutations in the insulin receptor gene.

Key words: Diabetes mellitus, insulin resistance, insulin receptor, Rabson-Mendenhall syndrome, insertion mutation.

Insulin resistance is the most common disorder of hormone action and a major cause of hyperglycaemia or reduced insulin-mediated glucose uptake in most patients with Type 2 (non-insulin-dependent) diabetes mellitus. It is primarily due to a defect of hormone action at the cellular level [1–5]. During the last few years major advances in biochemistry and molecular biology have led to the elucidation of some principle mechanisms of hormone action and their alterations in disease. Insulin affects multiple pathways regulating cellular metabolism and growth, but the first step in insulin action is the binding and activation of the insulin receptor [5–10]. The insulin receptor is a heterotetramer consisting of two extracellular α -subunits ($M = 135,000$) and two transmembrane β -subunits ($M = 95,000$). The α -subunit binds insulin and the intracellular domain of the β -subunit is an insulin-stimulated tyrosine-specific protein kinase [11]. Recently, several natural occurring mutations in the insulin receptor gene have been characterised in patients with insulin resistance and these characterisations have helped to elucidate the molecular mechanisms of insulin action [5, 12–17]. In this study we show a structural defect in the ex-

tracellular domain of the insulin receptor in an insulin-resistant patient with reduced cellular insulin binding due to two mutant alleles.

Subjects, materials and methods

Subject

Patient PK was a Caucasian male with Rabson-Mendenhall syndrome. He was born at term after an uncomplicated pregnancy. He showed growth retardation (birth weight 2170 g), mental deficiency, acanthosis nigricans, lipoatrophy and muscular hypotrophy in the extremities, dry and thickened skin, hypertrichosis, enlarged ears and hands as well as external genitalia, and typical coarse facial features. The abdomen appeared distended, but there was no gross hepatosplenomegaly. He had four neonatal teeth and advanced dentition having supranumerary permanent teeth. Insulin resistance was apparent by moderate hyperglycaemia (blood glucose levels between 11–21 mmol/l) associated with elevated plasma insulin levels (fasting plasma insulin levels 200–300 mU/l). PK was injected with about 70 IU insulin per day, which failed to restore normoglycaemia, to prevent ketonaemia as well as ketonuria. The patient died in 1988 at the age of 10 years during a varicella infection. On autopsy the

pineal gland was not examined. His parents were not consanguineous. Both parents had atypical oral glucose tolerance tests as well as postprandial hyperglycaemia and the father later developed Type 2 diabetes.

Materials

Taq polymerase and thermocycler were from Perkin Elmer/Cetus (Norwalk, Conn., USA). Deoxynucleotides and Klenow enzyme were from Boehringer Mannheim (Mannheim, Germany). Other enzymes were from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). ^{35}S - and ^{32}P -labelled nucleotides as well as A14-mono [^{125}I]iodo insulin and [^{14}C]2-deoxyglucose were from Amersham

(Amersham, Bucks., UK). A polyclonal antibody against the insulin receptor has been described previously [18]. Monoclonal insulin receptor (αIR) and insulin-like growth factor I (IGF-I) receptor ($\alpha\text{IGF-I}$) antibodies were obtained from Amersham and Sanbyo (Uden, Netherlands), respectively. Monocomponent insulin was obtained from Sigma (St. Louis, Mo., USA). The protein markers were obtained using a high molecular weight kit from Bio-Rad (Hercules, Calif., USA). The 1 kilobase (kb) DNA ladder marker was obtained from Gibco (Gaithersburg, Md., USA), pUCBM20 was used as vector and DH5 α as bacteria. All other chemicals were purchased from Sigma, Pharmacia or Boehringer Mannheim.

Cell culture

Fibroblast cultures were initiated from skin biopsies according to standard techniques. Cells were grown in Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal calf serum and penicillin, neomycin, and streptomycin, at 37°C, 5% CO_2 , and 95% humidity. Cells were grown overnight in serum-free medium before experiments using insulin.

Insulin binding

Fibroblasts of the proband and his parents were grown to confluence in six-well dishes. Tracer amounts of ^{125}I -insulin were added and cells were further incubated with or without increasing concentrations of unlabelled insulin at 4°C for 16 h. After washing and solubilisation the specifically bound radioactivity was determined as described previously [19].

Uptake of 2-deoxyglucose

Fibroblasts were grown in 6-cm dishes until they had just reached confluency. Twenty-four hours before assaying for 2-deoxyglucose uptake, cells were washed five times with phosphate-buffered saline. Then 1.5 ml 50 mmol/l Hepes, pH 7.4, 120 mmol/l NaCl, 1.3 mmol/l MgSO_4 , 1.85 mmol/l CaCl_2 , 4.8 mmol/l KCl, containing insulin at various concentrations (indicated in the figures), was added. Cells were incubated for 1 h at 37°C. Subsequently 2-deoxyglucose (1- ^{14}C), sp. act. 4 Ci/mol) was added at a concentration of 7.5 $\mu\text{mol/l}$ and incubation was continued for 10 min at 37°C. Uptake of glucose was terminated by addition of 5 ml ice-cold phosphate-buffered saline and cells were lysed in 1 ml 0.1 mol/l NaOH. [^{14}C]-content was determined by liquid scintillation counting [20].

Autophosphorylation of purified receptor preparations

Preparation of partially purified insulin receptors by chromatography on wheat germ agglutinin sepharose and insulin-stimulated autophosphorylation was performed as previously described [20]. Insulin was added to 10 μl glycoprotein fraction (1 mg protein/ml) and incubation was continued for 1 h at 22°C. Phosphorylation was initiated by addition of 6 mmol/l MnCl_2 and ^{32}P -ATP (25 $\mu\text{mol/l}$; 200 Ci/mmol). Incubation was terminated after 6 min by addition of 1 ml 10% trichloroacetic acid. The protein pellet was washed with 1 ml ethanol and dissolved in 50 μl 50 mmol/l NaHCO_3 /0.1% SDS for immune precipitation as described before [18].

Polymerase chain reaction

Genomic DNA (1 μg) was subjected to 30 cycles of polymerase chain reaction (PCR) amplification [21] using the primer sets shown below. Each reaction was performed in a volume of 100 μl containing

Fig. 1. Insulin receptor binding of fibroblasts of 15 healthy donors (shaded area) and patient PK. All experiments were performed in triplicate

Fig. 2. Autophosphorylation of partially purified receptors for insulin and insulin-like growth factor I (IGF-I) of a control subject and patient PK. Insulin (IR) and IGF-I receptors (IGF-IR) were partially purified from fibroblasts by chromatography on wheat germ agglutinin sepharose. Ligand stimulated autophosphorylation was performed as described in Subjects and methods. Receptor preparations were incubated without (lanes a and d) or with either insulin (lanes b and e) or IGF-I (lanes c and f). After incubation the IR and IGF-IR were immunoprecipitated with a monoclonal antibody against the IR (lanes a, b, d, and e) or the IGF-IR (lanes c and f) and analysed by SDS-PAGE and autoradiography. β indicates the β -subunit of the IR and the IGF-IR

50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 10 $\mu g/ml$ gelatin; 50 $\mu mol/l$ each of dATP, dGTP, dCTP, and dTTP; 0.15 μg each of oligonucleotide primer and 2.5 units of Taq DNA polymerase. After initial denaturation at 94°C for 5 min, samples were subjected to 30 cycles of amplification with an automated DNA thermal cycler; annealing at 50–60°C for 2 min, extension at 72°C for 2 min, and denaturation at 94°C for 1 min. When cDNA was amplified, total cellular RNA was extracted [22] and incubated for 30 min at 37°C with reverse transcriptase in the absence of the respective 5' oligonucleotide [23].

DNA sequencing

Nucleotide sequences of the insulin receptor gene were obtained directly and after subcloning. DNA (10 ng) was directly sequenced according to Casanova et al. [24] using the United Biochemical Sequence kit version 2.0. PCR-amplified DNA fragments of each exon were subcloned into pUCBM20 plasmids [25] for sequence analysis by the dideoxy chain determination method [26].

Oligonucleotides

Intron primers sets according to Seino et al. [27] were used for PCR amplification. For analysis of exon 3 an additional set of primers (5'-CGACCATCTGTAAAGTCACAC-3'/5'-GAAGCTGAAGTTACACAGC-3') and a specific oligo for the mutant allele (5'-TTCTGCCAGGACCTACAC-3') were used.

Results

Insulin binding and insulin action. Insulin binding to cultured fibroblasts of PK and 15 separate control subjects is shown in Figure 1. Insulin binding was markedly decreased and almost undetectable in the patient's cells. To determine insulin receptor autophosphorylation, the glycoprotein fraction was isolated and incubated with insulin or IGF-I in the presence of ^{32}P -ATP as indicated in Figure 2. Immunoprecipitations were performed using monoclonal antibodies either to the receptor for insulin or for IGF-I and the precipitates were analysed by SDS-PAGE. In agreement with reduced cellular insulin binding, the hormone did not induce autophosphorylation of the insulin receptor β -subunit. There was no alteration of the insulin receptor phosphorylation in the basal state. Similar results were obtained using a polyclonal antibody to the insulin receptor (data not shown). By contrast, incubation of cells with IGF-I led to a marked increase in the autophosphorylation of the IGF-I receptor (Fig. 2), but not of the insulin receptor (data not shown). Correspondingly, binding of IGF-I to cultured fibroblasts in tracer concentrations was not altered (data not shown). Insulin-stimulated 2-deoxyglucose uptake in the patient's fibroblasts is shown in Figure 3. In contrast to control cells, insulin-induced uptake of 2-deoxyglucose was greatly diminished in the patient's cells.

Structure of the insulin receptor gene in PK. To determine whether decreased insulin binding and insulin action in PK could be due to a structural alteration of the insulin receptor gene, the 22 exons with the flanking intron regions were amplified and analysed directly as well as after sub-

Fig. 3. Insulin-stimulated uptake of 2-deoxyglucose (2-DOG) from a control (O) subject and patient PK (X). Unstimulated uptake in control cells corresponds to 0.25 nmol 2-DOG/ 8×10^5 cells

cloning, sequencing at least four separate clones. Using both methods two different mutations were consistently found altering the primary amino acid sequence of the insulin receptor protein. One mutant allele showed a single nucleotide substitution changing codon CGA for Arg-86 into TGA in exon 2. This stop codon results in a truncation of the extracellular domain after Ser-85 (Fig. 4). The other mutation was found in exon 3 (Fig. 5). Amplified DNA-fragments of exon 3 were separated by a 6% PAGE. In contrast to a single amplified fragment of appropriate length in control individuals, two fragments were seen in PK. The size of one fragment corresponded to the single fragment observed in control subjects, the other fragment was slightly larger. The two fragments were isolated, amplified separately and sequenced directly as well as after subcloning. DNA sequence analysis revealed that the difference in length of the larger fragment was due to an additional 12 base pairs (bp) (5'-CTACACCTGGTT-3') inserting in-frame between nucleotide 1002 and 1003. Accordingly, amplification of this mutant allele using an insertion mutation specific primer revealed only a single fragment in PK and none in control subjects (data not shown). The 12 bp code for the amino acids Leu-His-Leu-Val between Asp-261 and Leu-262. Direct sequencing of PK's cDNA-fragment containing the nonsense mutation in exon 2 showed that both alleles are expressed in patient's fibroblasts (Fig. 6). Amplification by the PCR of reverse transcribed cellular RNA using primers for exon 3 confirmed the expression of both alleles (data not shown).

Inheritance of the mutant insulin receptor alleles. To study the inheritance of the two mutant insulin receptor alleles DNA was isolated from fibroblasts of the mother and father. The mother was found to be heterozygous for the nonsense mutation in exon 2 and the father heterozygous for the insertion mutation in exon 3 (data not shown). Therefore, PK is compound heterozygous, the stop codon being the maternal allele and the insertion mutation being

Fig. 5. Amplification and partial nucleotide sequence of exon 3 of the insulin receptor gene in patient PK. To the left genomic DNA fragments of control subjects (lane a and c) and the patient (lane b) corresponding to exon 3 were amplified by the polymerase chain reaction and analysed by a 6 % PAGE. The two fragments seen in the patient PK (lane b) were isolated and again amplified showing a single large fragment containing the insertion (lane f) and a small fragment being the wild type allele (lane g). The two separated fragments were directly sequenced as well as subcloned. To the right the partial nucleotide sequence of the mutant allele is shown containing an in-frame insertion mutation of 12 base pairs (5'-CTACACCTGGTT-3') in exon 3. DNA sequence of a subcloned fragment is shown. The insertion codes for the amino acids Leu-His-Leu-Val between Asp-261 and Leu-262

for gene [34–38], most of which appeared to be associated with decreased levels of mRNA [35–38]. However, similar to the nonsense mutation at codon 672 in the paternal allele of leprechaun Ark-1 [34], direct cDNA sequencing of our patient showed that the allele with the nonsense mutation at codon Arg-86 is expressed almost to the same degree as the other insulin receptor allele.

The other mutant insulin receptor allele of PK contains

in-frame insertion mutation of four amino acids between Asp-261 and Leu-262. This is the first insertion mutation identified in the insulin receptor gene. A search in the European Molecular Biology Laboratory gene bank using the sequence of the inner 10 of the 12 inserted bp revealed a full match to nucleotide sequences of transcription factor NF-1 [39] and human non-erythroid α -spectrin [40]. The four amino acids are mainly hydrophobic and are inserted between two cysteine residues (Cys-259 and Cys-266). Interestingly, this receptor sequence is part of a region which has no homology to the IGF-I receptor [41–43] and therefore might play an essential role in determining ligand specificity. Studies of ligand binding by chimeric insulin/IGF-I receptors indicate that the region between amino acid residues 230 and 285 in the insulin receptor might be part of the domain determining ligand specificity [33]. The structural alterations in this receptor region might affect insulin receptor binding of IGF-I. However, partially purified insulin receptors of PK were not autophosphorylated by incubation with IGF-I, indicating that IGF-I cannot activate the insulin receptor kinase in these cells. Several missense mutations have been characterized in the N-terminal α -subunit of the insulin receptor which are all associated with incomplete processing and defective transport [36, 44–48] to the plasma membrane. Cor-

The described stop codon in exon 2 leads to a truncation of the receptor protein in the extracellular region after Ser-85 thereby deleting the entire receptor domains of hormone binding and protein kinase. Multiple nonsense mutations have been described in the insulin recep-

Fig. 6. Partial nucleotide sequence of the patient's insulin receptor cDNA obtained from fibroblasts. Total RNA of fibroblasts were isolated, reverse transcribed to cDNA and amplified by polymerase chain reaction (PCR) using primers for the region corresponding to exon 2. After amplification the PCR products were sequenced directly. Both alleles are expressed, since both DNA sequences the wild type (CGA) and the mutated one (TGA) are observed

Fig. 7. Binding of ^{125}I -insulin to fibroblasts from patient PK (▲), the mother (●), the father (■), and a control subject (○). Displacement curves are shown of bound radioactive insulin by increasing levels of non-radioactive insulin. Specific tracer binding of control cells represent 100% of binding. Cells were incubated with insulin at 4°C for 16 h. Means of duplicate determinations of one representative experiment are shown. A total of four separate experiments were performed

respondingly, preliminary observations using transient transfections of in vitro mutated insulin receptor cDNA in cos cells indicate that the described in-frame insertion of four amino acids in the extracellular domain of the insulin receptor impairs proper intracellular receptor processing and transport to the plasma membrane (E. van der Vorm, unpublished observations).

Direct cDNA sequencing of reversed transcribed and amplified RNA indicate that both mutant insulin receptor alleles are expressed in fibroblasts of PK. Since the stop codon allele will most likely not produce a functioning

protein the receptor oligomers of PK contain probably only insertion mutated α -subunits. The mother, who is heterozygous for the stop codon, exhibits a moderate reduction in insulin binding most likely caused by a decreased number of functional intact insulin receptors encoded by the wild-type allele. The father appears to be not only genetically but also biochemically heterozygous for the insertion mutation exhibiting significantly decreased insulin binding in fibroblasts, but not to the same degree as PK.

The patient did not exhibit the typical features of leprechaunism such as early death, extreme hyperinsulinaemia and fasting hypoglycaemia. When both the clinical phenotype and abnormalities in plasma glucose levels are considered, it appears to be more likely that PK suffered from Rabson-Mendenhall syndrome [49, 50], characterized by less severe insulin resistance, death near puberty, and abnormal dentition. The insulin receptor gene has been sequenced in two other patients with Rabson-Mendenhall syndrome. One patient [36] was also compound heterozygous. Similar to PK this patient RM-1 had a nonsense mutation at codon 1000 and a missense mutation in the amino-terminal extracellular receptor domain at codon 15. The missense mutation causes retardation of post-translational receptor processing and transport rate to the plasma membrane, as well as five-fold reduction in the affinity of the insulin receptor to insulin [45].

In the past different genetic syndromes of severe insulin resistance have been described on the basis of clinical features. Recently, molecular genetics of the insulin receptor show that different mutations in the insulin receptor gene cause different or similar alterations in the structure-function relationship of the receptor protein and thereby in insulin action. Increasing evidence indicates that the degree of functional disturbance in the cellular pathway of insulin action dictates the clinical picture of insulin-resistant patients. The most severe structural alterations caused by homozygous and compound heterozygous mutations are found in patients with leprechaunism and in Rabson-Mendenhall syndrome. However, it is interesting to note that PK had only a relatively mild degree of insulin resistance despite the severe cellular binding defect and the complete absence of insulin-stimulated receptor autophosphorylation. The residual stimulatory effect of insulin on glucose uptake seen in patient's fibroblasts is probably mediated by hormone binding to the IGF-I receptor. A similar situation is seen with fibroblasts of leprechaun G [44] and Winn-1 [36]. The reason why a similar functional loss of insulin receptor can result in leprechaunism or Rabson-Mendenhall syndrome is unknown and additional factors may contribute to the pathogenesis of the full leprechaun phenotype.

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Dr. D. Müller-Wieland
 Klinik II und Poliklinik für Innere Medizin
 der Universität zu Köln
 Joseph-Stelzmann-Str. 9
 D-50924 Köln
 Germany