

表題 インスリン受容体キナーゼ活性に必要な分子構造に関する研究
"Study on the structural requirement for kinase activity of insulin receptor"

論文の区分 博士課程

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Table of contents

Abstract.....	1
1. Introduction.....	3
2. Case presentation.....	9
2.1 Objective.....	9
2.2 Subject and methods.....	9
2.3 Results.....	12
3. Experiment on the R ¹⁰⁰⁰ E ¹⁰⁰¹ →Q mutation.....	19
3.1 Objective.....	19
3.2 Research design and methods.....	19
3.3 Results.....	25
4. Discussion.....	31
4. Conclusion.....	40
5. Acknowledgements.....	41
6. References.....	42

Abstract

In this study, the insulin receptor gene was analyzed in a patient with severe insulin resistance syndrome and her two children. The mutation found was previously reported as kinase defective due to deletion of a leucine residue in the first β strand of the kinase domain of the receptor. Inspired by this mutation, we hypothesized that the exact length of this β strand would be essential to the kinase activity. To verify this hypothesis, we made the site-directed mutation, which deletes a residue next to the leucine⁹⁹⁹.

1) A non-obese Japanese woman presented at 6 weeks of gestation in her first pregnancy with features of severe insulin resistance: fasting plasma glucose of 272 mg/dl, HbA1c level of 95 mmol/mol (10.8%), and fasting serum insulin of 68.8 μ U/ml. Neither anti-insulin nor insulin-receptor antibodies were detected. Direct sequencing analysis detected an in-frame heterozygous deletion mutation (Δ Leu⁹⁹⁹) in the β -subunit of the insulin receptor gene. Despite large daily doses (up to 480 units per day), the patient's postprandial plasma glucose level exceeded 200 mg/dl. In the patient's second pregnancy, the addition of metformin at a dose of 2250 mg per day achieved tighter glycemic control, with lower doses of total insulin (up to 174 units/day). Both newborns, who were found to carry the same mutation, were small for gestational age and developed transient hypoglycemia after birth.

2) An in-frame deletion mutant of three nucleotides that replaces Arg¹⁰⁰⁰ and Glu¹⁰⁰¹ with Gln (R¹⁰⁰⁰E¹⁰⁰¹→Q) was constructed by site-directed mutagenesis and expressed in Chinese hamster ovary (CHO-K1) cells. The insulin binding and expression level of mature insulin receptors were similar between the mutant and wild-type insulin receptors when expressed in CHO-K1 cells. The insulin-induced autophosphorylation of the R¹⁰⁰⁰E¹⁰⁰¹→Q insulin receptor was markedly decreased compared with wild type. Additionally, the level of Akt kinase phosphorylation in cells expressing the mutant receptor was impaired to nearly half that of cells expressing the wild type.

Deletion mutation R¹⁰⁰⁰E¹⁰⁰¹→Q caused significant damage to insulin-mediated signal transduction. The exact length of the first β strand in the kinase domain may be essential to the optimal positioning of the kinase structure.

1. Introduction

In recent decades, the prevalence of diabetes in pregnancy has been increasing (1-3). Hyperglycemia during pregnancy is associated with adverse perinatal complications. Pregnant women with diabetes are at an increased risk for developing weight gain, miscarriage and preeclampsia. The infants born to them are at higher risk for developing congenital abnormalities, macrosomia, perinatal hypoglycemia and subsequent long-term risk of diabetes and obesity (4). As these adverse outcomes are strongly correlated with maternal hyperglycemia (5), the main therapeutic goal in diabetic pregnancies is to maintain normoglycemia. Despite the vigorous attempts to achieve better glycemic control (6-12), it remains notoriously difficult to have successful pregnancy outcomes.

Insulin resistance is a precipitating factor for developing diabetes, particularly in pregnancies. Obesity and advanced age are two major causes of a common form of diabetes in pregnancies. Polycystic ovary syndrome (PCOS) is also an important underlying condition. A rare, more severe form of insulin resistance is defined as insulin resistance syndrome, which is further classified into three categories: types A, B and C. Type A insulin resistance syndrome is a synonym for haploinsufficiency of insulin receptor (INSR). Very severe forms with congenital anomalies, leprechaunism and Rabson-Mendenhall syndrome are caused by bi-allelic mutations in INSR.

The INSR is a heterotetrameric transmembrane glycoprotein composed of 2 α and 2 β subunits linked together by disulfide bonds (13). Human INSR maps to the short arm of chromosome 19, occupies more than 150 kilobase pairs of DNA and contains 22 exons (14). The α subunit is encoded by exons 1 through 11, while the β subunit is encoded by exons 12-22, including a juxtamembrane domain, tyrosine kinase domain, and carboxyterminal domain. The INSR is synthesized as a single polypeptide that includes the signal peptide, α subunit and β subunit. After deletion of the signal peptide, the proreceptor is glycosylated and forms a dimer. The dimerized proreceptor is processed to α and β subunits in the endoplasmic reticulum (ER) to form a mature $\alpha_2\beta_2$ heterotetramer linked by disulfide bonds (15). The mature INSR is finally transferred to the plasma membrane, where it binds insulin. When the INSR gene is mutated, especially in the α subunit, proreceptors may be trapped in the cytoplasm and cannot contribute to insulin binding. When kinase-negative mutant INSR (mINSR) coexists with the normal INSR (nINSR), three forms of receptors are made: nINSR-nINSR, nINSR-mINSR and mINSR-mINSR. It is also known that INSR can make a hybrid receptor with insulin-like growth factor-I receptor (IGF-IR). When tyrosine kinase is activated by insulin binding, the cross phosphorylation of INSR occurs between each half receptor. However, nINSR-mINSR and mINSR-mINSR cannot phosphorylate properly, and receptor activation is

impaired. Kinase-negative INSR can interfere with the activity of IGF-I through hybrid formation with IGF-IR (16).

Since the first description of an INSR mutation (17,18), nearly 132 causative mutations have been described (19). To our knowledge, however, only two cases of pregnant women with type A insulin resistance syndrome have been reported (20,21). Neither of them developed diabetes. Therefore, it remains unknown whether pregnancies in diabetic patients with type A insulin resistance syndrome have successful outcomes for both mother and fetus. It is also not known how to manage diabetes during the pregnancies.

Here, we report a case of pregnancy with severe insulin resistance in a diabetic patient with type A insulin resistance syndrome resulting from a deletion of leucine⁹⁹⁹ (ΔLeu^{999}) in INSR, which was previously reported in a non-diabetic woman with insulin resistance with occasional hypoglycemia (22). The INSR with ΔLeu^{999} is defective in tyrosine kinase activity, thereby compromising insulin signaling. Interestingly, this leucine residue is a part of a dileucine motif that mediates the intracellular sorting of the INSR (23). According to Haft et al.(23), a mutant INSR where the two leucine residues are both replaced with alanine has intact kinase activity. Therefore, the deletion of Leu^{999} abolishes the kinase activity of INSR, while mutation to alanine has no effect on its activity. To address these questions, we

constructed INSR with the R¹⁰⁰⁰E¹⁰⁰¹→Q mutation, which is adjacent to the dileucine motif and leaves it intact.

The INSR has two alternative splicing forms, INSR-A and INSR-B, dependent on 12 amino acids derived from exon 11 being absent or present, respectively. The amino acid number by Awata et al. (22) was INSR-B, while Haft et al. (23) used INSR-A; therefore, the same leucine was numbered as 987. Here we used the numbering of INSR-B, and the mutation ΔLeu^{999} found in our patient is located in the first β strand of the kinase domain of the INSR, which is necessary for insulin action (24) (Figure 1, A). The dileucine motif is also shown (Figure 1, B). The INSR has two tyrosine motifs and four dileucine sequences in the cytoplasmic tail of the β subunit. Insulin binding to the extracellular α subunit leads to the activation and subsequent phosphorylation of the receptor. In the absence of ligand, the INSR preferentially localizes on the cytoskeleton-rich microvilli of the cell surface. After ligand binding, the ligand-INSR complex leaves the microvilli and then moves in the plane of the plasma membrane (25,26) and redistributes to the nonvillous region, where it segregates into clathrin-coated pits. This internalization process requires two types of motifs within the juxtamembrane domain. The first type, tyrosine-based motifs GPLY (962-965) and NPEY (969-972), require activation of the receptor for association with the clathrin-coated pits (27,28), whereas the other type,

dileucine-containing sequence EKITLL (residues 994-999), does not need the tyrosine kinase activation of the receptor for internalization (29). These three motifs are also thought to be sorting signals for endocytosis.

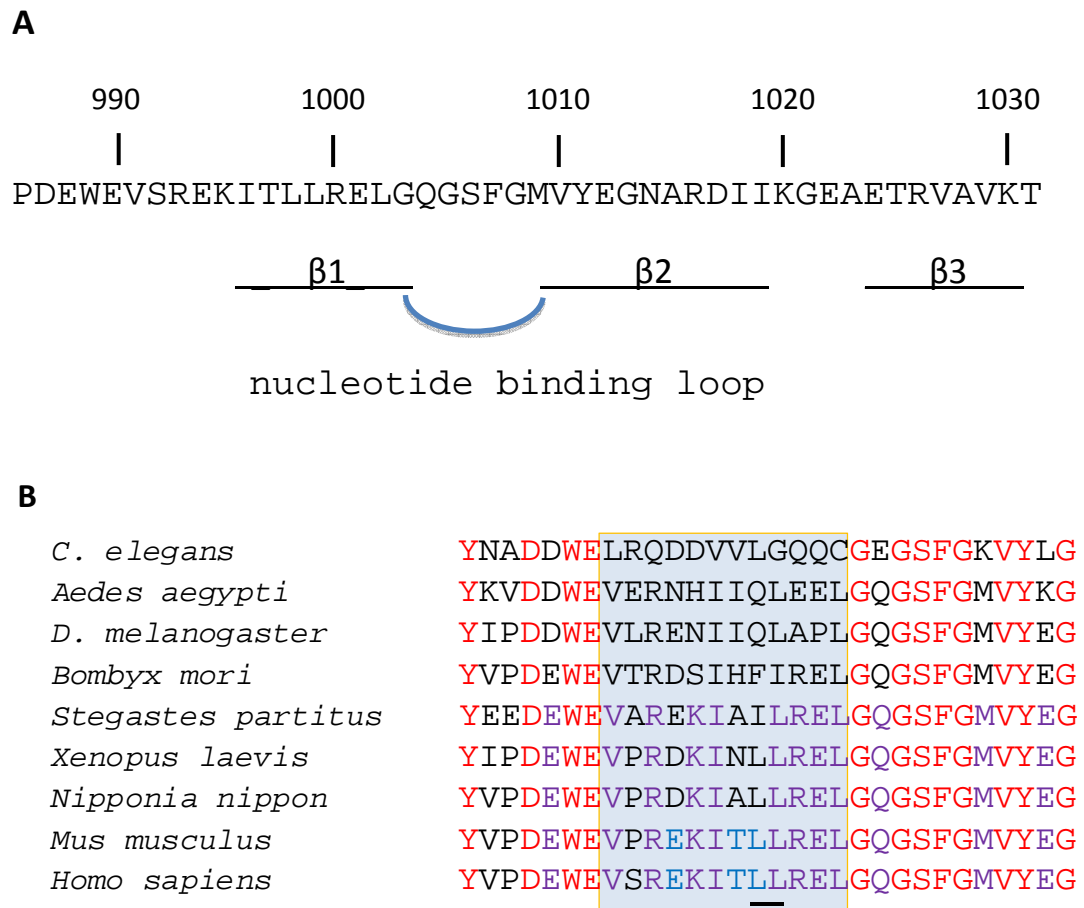


Figure 1. Mutation found in the patient and the sequences flanking this mutation (A) The deleted leucine residue was located in the first beta strand of the kinase domain. Leu⁹⁹⁹ is highlighted in red. (B) Sequence alignment of INSR (amino acids 984-1013) from different species: *C. elegans*, earth worm; *A. aegypti* to *B. mori*, insect; *S. partitus*, fish; *X. laevis*, amphibian; *N. Nippon*, bird; *M. musculus*, mouse; *Homo sapiens*. Residues with fixed length are highlighted. Dileucine motif is underlined.

A comparison of the amino acid sequences among species from earthworm to human revealed diverse amino acid changes in this region; however, the number of amino acids between the conserved flanking residues is exactly the same (Figure 1, B).

According to the represented crystal structure of the kinase domain of the INSR (30,31), the ΔLeu^{999} is located in the first β strand of the tyrosine kinase domain. Although this first β strand is far from the A-loop that moves dramatically after activation, the ATP-binding motif is very close; therefore, shortening by only 1 residue may change the optimal positioning of the kinase structure. We questioned whether the low kinase activity of ΔLeu^{999} is derived from the distortion of the kinase domain by the deletion of one residue or the disruption of the dileucine motif.

Next, to determine the effect of the deletion of 1 amino acid in the first β strand, we made a deletion mutation of Arg^{1000} with concomitant substitution of Glu^{1001} to Gln ($\text{R}^{1000}\text{E}^{1001}\rightarrow\text{Q}$), which is right next to Leu^{999} , and examined its influence on INSR functions. This mutation was planned because it introduces a new PvuII site that is convenient for construction. Although it is not a simple deletion of one residue, the residues corresponding to $\text{R}^{1000}\text{E}^{1001}$ in *C. elegans* are QQ. Therefore, we assumed that replacing to Q will not affect the kinase activity.

2. Case presentation

2.1. Objective

We aimed to explore the underlying molecular mechanism of severe insulin resistance in a patient whose pregnancies were successfully managed with massive insulin treatment. Our goal is therefore to contribute to the therapeutic approach in the safe management of pregnant women with insulin receptoropathy because to date, there are no definitive guidelines for an efficient treatment of such patients or reported experiences.

2.2. Subject and methods

Patient

The proband, a 31-year-old woman, was referred for the observation of high blood glucose, which was first noticed at 6 weeks of gestation in her first pregnancy. She was born to nonconsanguineous Japanese parents with a weight of 2,130 g. The patient's childhood was uneventful with no precocious puberty and normal menstruation cycles. At presentation, the patient was non-obese (BMI: 24.4 kg/m²).

Acanthosis nigricans, a skin symptom unique to the insulin resistance syndrome, was observed in the neck and axillary areas. Hyperandrogenism, a symptom of polycystic ovary syndrome that causes insulin resistance, was not present. Her blood

pressure was 121/80 mmHg and remained stable thereafter. The patient's parents had type 2 diabetes mellitus and had been treated with oral hypoglycemic agents for several years at the time of investigation. The patient's younger and only brother was normal with no symptoms of diabetes.

Written informed consent was obtained from the patient and her family members for this study, which was conducted with the approval of the ethics committee of the Jichi Medical University (Ethical board ID number: 13-57).

Genomic DNA isolation

Genomic DNA was isolated using the QIAamp DNA Blood Midi kit (Qiagen, Tokyo, Japan) from peripheral blood samples of the patient, the patient's parents and her children.

Amplification of genomic DNA

All 22 exons and intron-exon boundaries of INSR were individually amplified by polymerase chain reaction (PCR). The specific primer sets were used as described by Seino *et al.* (14), and the annealing temperature for each primer was determined by gradient PCR. A total of 200 ng of genomic DNA was amplified by PCR under the following conditions: denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, annealing at 50 - 65°C (varied for each primer) for 2 min, extension at

72°C for 3 min, and post-extension at 72°C for 15 min. PCR was performed using Taq DNA polymerase (Invitrogen, Tokyo, Japan), except for exon 1, which was amplified using Tks Gflex DNA polymerase (Takara Bio, Tokyo, Japan) according to the manufacturer's recommended PCR conditions. Amplified PCR products were electrophoresed on an agarose gel with ethidium bromide, and the desired DNA band was cut out under a UV light.

Direct sequencing analysis of INSR gene

The resulting PCR products were purified using the QIAquick Gel extraction kit (Qiagen, Tokyo, Japan), followed by sequencing using the same forward and reverse primers used for PCR. The sequencing reaction was carried out using Big-Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan) with the following conditions: 96°C for 1 min, 25 cycles at 96°C for 30 sec, 50°C for 15 sec, and at 60°C for 4 min. The products were then purified using a sodium acetate-ethanol method and denatured at 100°C for 2 min. After cooling on ice for 5 min, the products were electrophoresed on an ABI Prism 310 Genetic analyzer (Applied Biosystems, USA).

Confirmation of mutation

The PCR product of exon 17 was subcloned into pTAC2 vector using a TA PCR cloning

kit (Biodynamics, Tokyo, Japan). Plasmids containing the PCR product were sequenced using M13 forward and reverse primers (Invitrogen, Tokyo, Japan) to confirm the mutation.

2.3. Results

Clinical and laboratory examination on admission

Elevated fasting plasma glucose (272 mg/dl) was noticed during the medical check-up of our patient during pregnancy for the first time in her life. The patient was asymptomatic, and she had had no chance to check her plasma glucose before. A 75 g oral glucose tolerance test showed marked fasting and post-load hyperglycemia (Table 1). The fasting serum insulin and HbA1c levels were also high (reference range: < 16 μ U/ml and 4.6-6.2%, respectively). The fasting serum C-peptide concentration was 2.1 ng/ml, and the molar ratio of insulin to C-peptide was low (0.68). Other hormone levels were within their reference ranges except for a moderately elevated level of testosterone (0.74 ng/ml; reference range 0.11-0.47 ng/ml). The patient's serum biochemical profile, including her lipid profile, was unremarkable. Polycystic ovary syndrome, which is known to cause insulin resistance, was ruled out by a computed tomography scan performed after delivery. Antibodies against insulin and INSR were undetectable. However, after insulin

administration, the anti-insulin antibody was detected at 749 nU/ml (reference range: <125 nU/ml).

Table 1. Clinical characteristics of the patient on admission

Age	31	
Height (cm)	150.7	
Weight (kg)	55.4	
BMI (kg/m ²)	24.4	
HbA1c (%)	10.8 (95 mmol/mol)	
75 g OGTT (HOMA-IR: 35.2)		
Time (min)	0	120
PG (mg/dl)	216	392
IRI (μU/ml)	66	501.1
C-peptide (ng/ml)	2.1	8.8
Urinary C-peptide (μg/day)	318.3	
Insulin treatment	Before	After
Insulin antibody	Negative	749
Insulin receptor antibody	Negative	
Testosterone (ng/ml)	0.74	

BMI, body mass index; OGTT, oral glucose tolerance test; HOMA-IR, homeostasis assessment of insulin resistance; PG, plasma glucose; IRI, immunoreactive insulin.

To determine whether the insulin resistance was familial, the blood of the patient's parents was taken. For the convenience of the parents, this was performed in a non-fasting condition. The patient's father showed severe insulin resistance (casual insulin 259 μU/ml, casual plasma glucose 252 mg/dl; 13.9 mmol/l), while the

patient's mother was not insulin resistant (casual insulin 16.5 μ U/ml, random plasma glucose 142 mg/dl; 7.8 mmol/l).

Genetic studies

The 22 coding exons and flanking introns of the INSR from the peripheral blood DNA of the patient, her parents and children were analyzed for potential mutations. A deletion of three bp (CTT) in one allele of the INSR was identified in the patient and resulted in the loss of leucine⁹⁹⁹ in exon 17. The same heterozygous mutation was also detected in the patient's father and both the first and second child (Figure 2). The sequence of the patient's mother was normal.

Clinical course of pregnancy and treatment

Due to severe hyperglycemia and severe insulin resistance, the patient was admitted to a hospital for treatment. The patient began insulin therapy consisting of 18 units/day of insulin aspart, a rapid-acting human insulin analogue, and 4 units/day of human insulin isophane along with caloric restriction (1600 kcal/day) (Figure 3). The insulin doses were progressively increased, but the patient showed severe insulin resistance, and the control of the postprandial plasma glucose was extremely difficult even with 300 units/day total insulin dose.

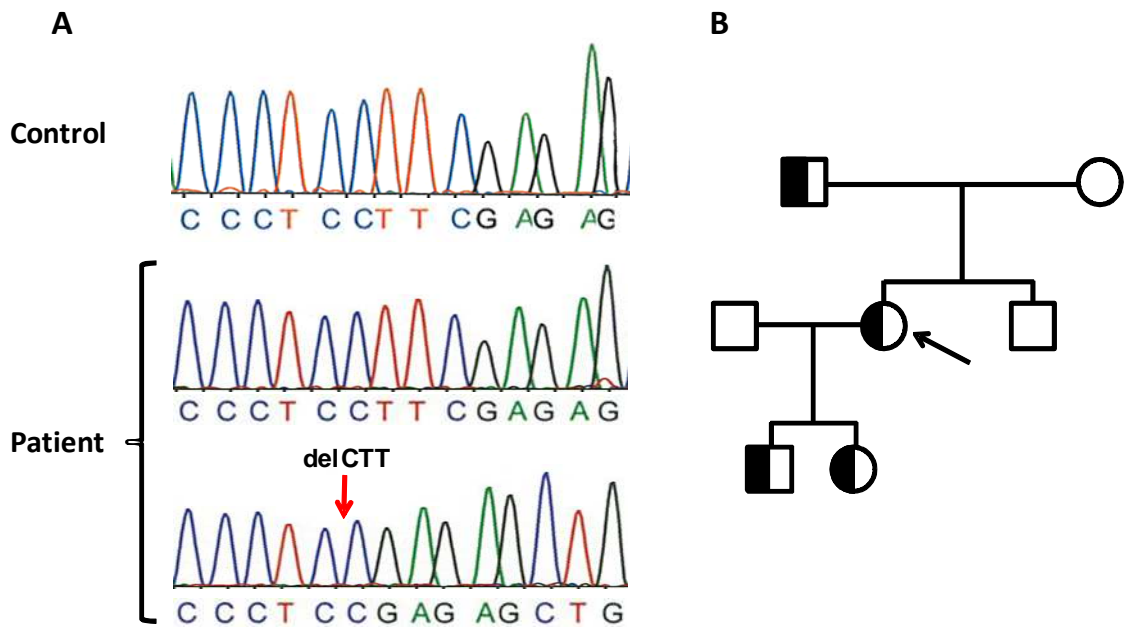


Figure 2. Nucleotide sequences of the region around ΔLeu^{999} in the INSR gene in a control subject and the patient. (a) Sequencing results of subcloned PCR product from exon 17. The red arrow indicates the heterozygous deletion of nucleotides at 2995_2997 in exon 17 (c.2995_2997delCTT) in the mutant allele of the patient (middle), according to the traditional nomenclature numbering the initial nucleotide coding the first amino acid of the α chain as +1. The wild-type allele was also found (bottom), indicating that the patient was a heterozygote for the mutation. (b) A family pedigree representing carriers of the heterozygous ΔLeu^{999} mutation. The patient's brother was not genotyped. The arrow denotes the proband; black denotes the mutant allele.

After half a month of treatment, when the insulin doses reached 380 units/day (insulin aspart 300 units/day and insulin isophane 80 units/day) at 17 weeks of gestation, the patient's blood glucose concentration was slightly decreased (average fasting and postprandial levels for the week were 78 and 206 mg/dl, respectively). The HbA1c concentration was lowered to 64 mmol/mol (8%). During the third trimester, because the patient's blood glucose began to increase, the insulin dose

was also gradually increased, reaching 480 units/day at 35 weeks' gestation. The patient gained only 1.6 kg of weight during the pregnancy. At 37 weeks' gestation, she vaginally delivered a boy with a weight of 2,224 g (<10th percentile) without any complications. Apgar scores at 1, 5 and 10 min were normal. The baby had no abnormality and showed only transient tachypnea. His blood glucose was 106 mg/dl just after birth, gradually fell to 20 mg/dl 3 h later despite treatment with intravenous dextrose, and rose to 83 mg/dl after 10 h.

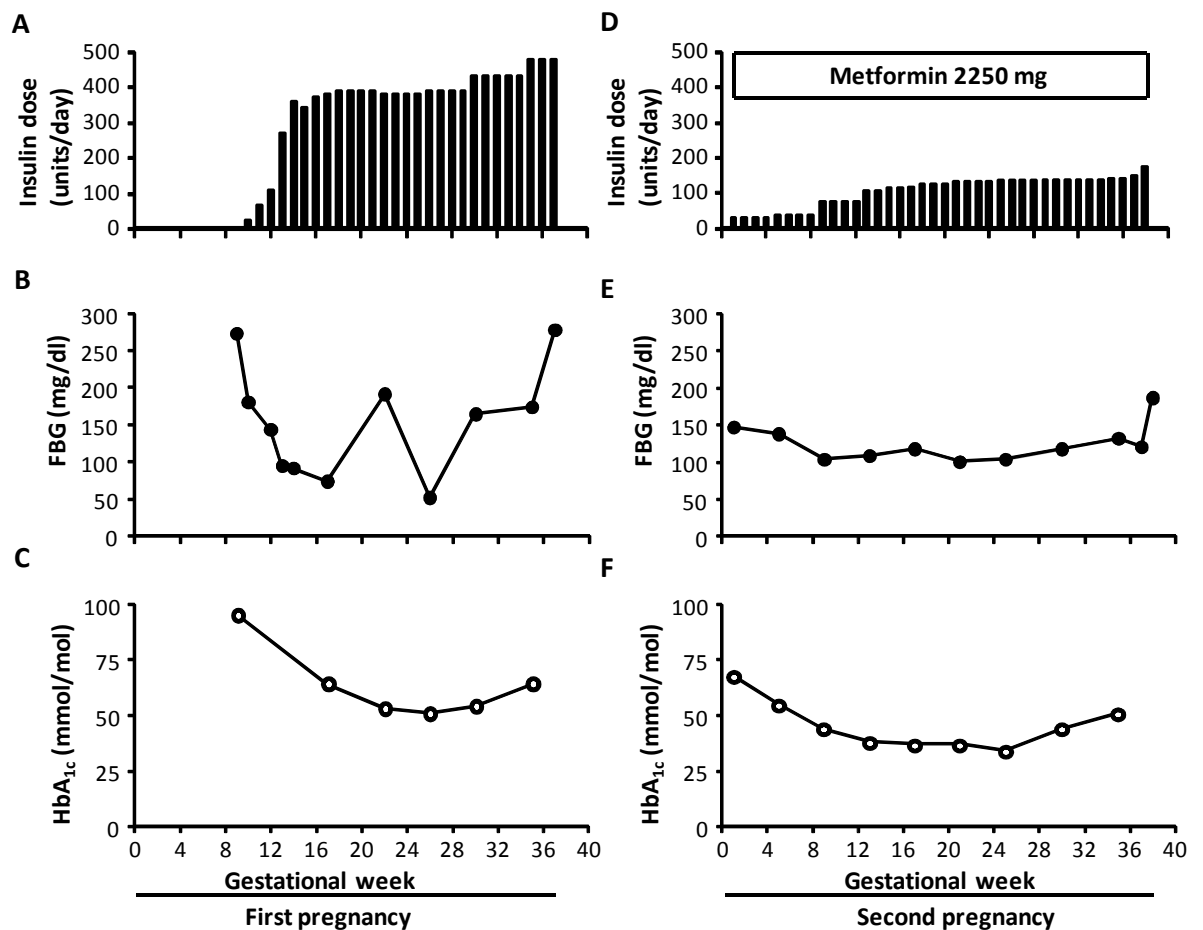


Figure 3. Fasting blood glucose and HbA1c measurements in response to insulin and metformin treatment. (a-c) First pregnancy and (d-f) second pregnancy.

After delivery, the insulin administration was ceased because of lactation, but the diet therapy (1400 kCal/day) alone was not sufficient for blood glucose control. The insulin treatment was therefore resumed with the same insulin analogues with total dose of 200 units/day (aspart 120 units/day and isophane 80 units/day). After fifteen months of treatment with the same dose of insulin, the HbA1c level was increased to 89 mmol/mol (10.3%). Two years after delivery, because of poor HbA1c control (98 mmol/mol (11.1%)), 1500 mg/day of metformin was added to the insulin treatment. The insulin and metformin doses were unchanged until the second admission, when the patient's HbA1c concentration was decreased to 68 mmol/mol (8.4%).

Almost two and a half years after her first delivery, the patient was hospitalized for glycemic control. The metformin dose was increased to 2250 mg/day, and her daily energy intake was decreased to 1200 kcal/day. Soon after discharge, the patient became pregnant again. Throughout the second pregnancy, metformin 2250 mg/day was administered along with an insulin regimen and caloric restriction (1600 kcal/day).

Insulin lispro was substituted for insulin aspart, with a starting dose of 36 and final dose of 150 units/day, and the insulin isophane dose ranged from 4 to 24 units/day. The patient's blood glucose was managed carefully with an average

HbA1c level achieved of 45 mmol/mol (6.3%). The patient gained only 1.3 kg of weight during the pregnancy. At 38 weeks of gestation, the patient successfully gave birth to a girl with a weight of 2,532 g (< 25th percentile). The baby had no abnormality, and the Apgar scores were normal. Just after delivery, the baby's blood glucose was 23 mg/dl but rose to 71 mg/dl within 3 h, after intravenous dextrose administration.

After the second delivery, metformin was discontinued. The HbA1c levels were 95 mmol/mol (10.8%), with total insulin doses of 114 units/day. Despite the relatively poor glycemic control before and after the two pregnancies, the only complication found at 1 year after the second delivery was simple diabetic retinopathy.

3. Experiment on the R¹⁰⁰⁰E¹⁰⁰¹→Q mutation

3.1. Objective

The deletion of Leu⁹⁹⁹ resulted in the loss of kinase activity (22). However, mutation of this same leucine to alanine had no effect on kinase activity (23). Thus, we hypothesized that leucine at this position is not essential to the kinase activity, but rather, the exact length of this region is required. To verify this hypothesis, a deletion of the one amino acid adjacent to Leu⁹⁹⁹ was analyzed.

3.2 Research design and methods

Materials

Chinese hamster ovary-K1 (CHO-K1) cells were cultured in F-12 Ham's medium (Sigma, Tokyo, Japan) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown at 37°C under an atmosphere of 95% humidified air and 5% CO₂. Restriction enzymes were either from Takara Bio, Toyobo, or New England Biolabs. AmpliTaq Gold was from Life technologies. The BigDye Terminator v3.1 Cycle Sequencing kit was from Applied Biosystems. Other reagents are either from Sigma-Aldrich Japan, Wako or Nacalai tesque unless otherwise indicated.

Site-directed mutagenesis and construction of expression vectors

A mutant human INSR (hINSR) was constructed by creating three fragments. First, the 5' terminal fragment of the hINSR cDNA was digested from pSV2-hINSR with HindIII and BamHI sites and subcloned into a pcDNA6/myc-HisA expression vector resulting in pcDNA6-hINSR1/myc-HisA (Figure 4; green line). The primers used for mutagenesis or verification of sequences in the subcloned vectors are described in Table 2.

Table 2. Primers synthesized for mutagenesis

Primer name	Primer sequence
Primer 1	Sense 5'CTGGATCCAATCTCAGTGTC3'
Primer 2	Anti-sense 5'CCCCAGCTGAAGGAGGGTGAT3'
Primer 3	Sense 5'CTTCAGCTGGGGCAGGGCTCC3'
Primer 4	Anti-sense 5'CCTCTAGAGGAAGGATTGGACCGAGGCAAGG3'
Primer 5	Sense 5'CAGTGTTGTGATTGGAAG'

Underlines in primers 1, 2/3, and 4 indicate BamHI, PvuII and XbaI sites, respectively.

The second fragment was amplified using pSG5-hINSR as a template with primers 1 and 2 containing BamHI and PvuII sites, respectively (Table 2). The third fragment was amplified with primers 3 and 4 containing PvuII and XbaI sites, respectively (Table 2). The PvuII site of primers 2 and 3 contained a mutation, which introduces the deletion of GAG. Each of these two PCR products was subcloned into pTAC2

vectors using a TA cloning kit (Life technologies, Carlsbad, CA, USA), and the sequences were confirmed by direct sequencing analysis. Forward and reverse M13 primers and primer 5 were used for sequencing (Table 2). Confirmed fragments were digested with BamHI and PvuII or PvuII and XbaI, respectively, and subcloned into a BamHI and XbaI-digested subcloning vector by three-fragment ligation (Figure 4).

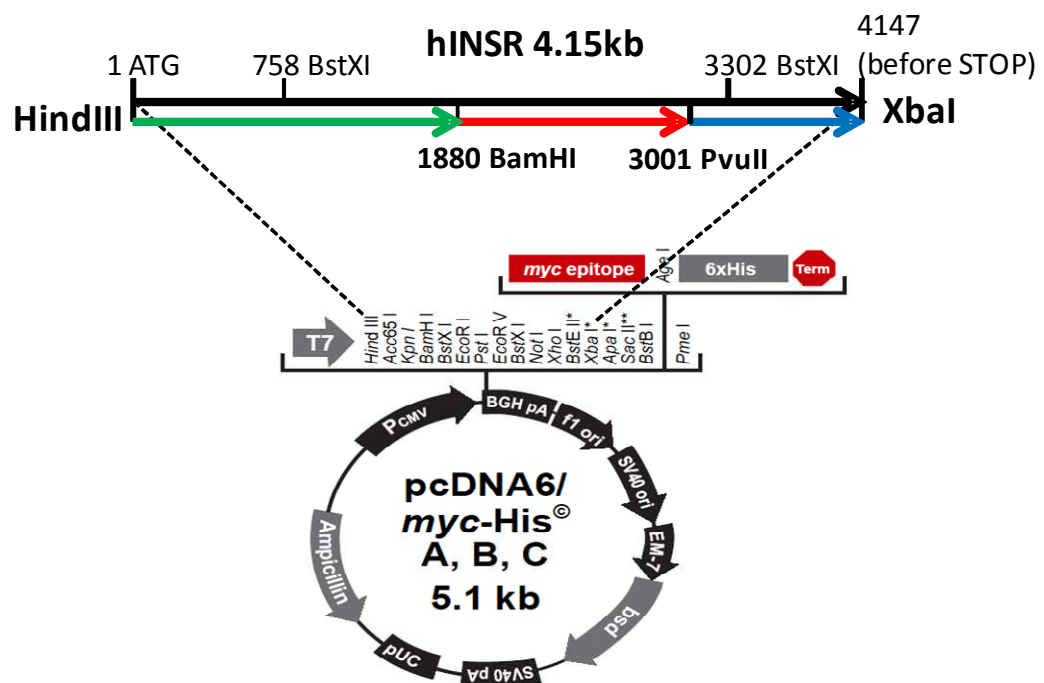


Figure 4. Construction of the expression vectors containing coding sequence of wild-type and mutant INSRs.

The resulting mutant fragment ($R^{1000}E^{1001} \rightarrow Q$) was digested with BamHI and XbaI and ligated with pcDNA6-hINSR1/myc-HisA digested with the same enzymes to obtain pcDNA6-hINSRmut/myc-HisA. To construct the wild-type hINSR, the BstXI

fragment (758 to 3302) containing the mutated site was replaced with the original hINSR to obtain pcDNA6-hINSR /myc-HisA. Sequences containing mutant and wild-type hINSR were confirmed by direct sequencing analysis using sequencing primer 5 (Table 2).

Transient transfection of cells and selection of stable transformants

Subconfluent CHO-K1 cells in 6-cm dishes were transfected with 5.5 µg of the expression vectors containing wild-type and mutant INSRs using Lipofectamine (Life technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. After 48 h, the cells were replated onto 10-cm dishes at a lower density, and the transfected cells were selected using 10 µg/ml of the antibiotic blasticidin S hydrochloride (MP Biomedicals LCC, Tokyo, Japan). After selection, independent colonies were picked up, grown in 96-well plates, and screened for a high level of expression of the INSRs by insulin binding.

Screening of stable clones by the ¹²⁵I-insulin binding assay

Subconfluent cells grown in 96-well plates were expanded to 12-well plates in duplicate, and one plate of each pair plates was subsequently used for insulin binding. Confluent cells were washed twice with ice-cold PBS and then incubated with 50 pM of ¹²⁵I- insulin (Perkin Elmer, Japan) in 500 µl binding buffer (100 mM

HEPES (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 10 mM glucose and 1% BSA) for 4 h at 4°C under constant shaking. After three washes, cells were solubilized with 0.1 N NaOH. Cell-bound radioactivity was determined in a γ -counter (Hitachi-Aloka Medical, Tokyo Japan). Nonspecific binding was determined in the presence of 1 μ M unlabeled insulin. Clones expressing wild-type and mutant INSRs were recovered from the remaining replica plates and used for further experiments.

Insulin binding assay

Stable clones grown in 12-well plates were washed twice with ice-cold PBS and then incubated with ¹²⁵I-labeled insulin (10, 20, 50 100, 200 pM) for 4 h at 4°C in 300 μ l binding buffer. Insulin binding to its receptor was measured as described in Alzahrani et al. (32). After four washes, the cells were solubilized with 0.1 N NaOH. Cell-bound radioactivity was assayed using a γ -counter. The protein concentration of each sample was determined by the bicinchoninic acid (BCA) protein assay and adjusted for calculation. Non-specific binding in the presence of 1 μ M unlabeled insulin was subtracted as background. The dissociation constant (K_d) and the total binding capacity (B_{max}) were calculated using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Western blot analysis

Stable transformants with wild-type and mutant ($R^{1000}E^{1001} \rightarrow Q$) INSR were stimulated with different concentrations of human insulin (1, 10, 100 nM) for 5 min at 37°C after 16 h serum starvation. Cells were washed with ice-cold PBS and solubilized in RIPA buffer (Cell signaling, Tokyo, Japan) in the presence of phosphate inhibitors. The protein concentration was determined by the Bradford method using bovine serum albumin as a standard and adjusted. 10-20 µg of cell lysates were resolved by 8% SDS—polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride (PVDF) membrane (Merk Millipore, Billerica, MA, USA) and analyzed by western blotting using the mouse monoclonal anti-myc tag antibody clone 4A6 for total INSR expression (Merk Millipore, Billerica, MA, USA) and anti-phosphotyrosine antibody (4G10) to detect tyrosine-phosphorylated receptor (Millipore, Japan). Antibodies against Akt, phospho-Akt (Ser473) and GAPDH (14C10, rabbit monoclonal antibody as a control for protein loading) were purchased from Cell Signaling Technology (Cell Signaling Technology, Japan). Blotting the total cell lysate with anti-phosphotyrosine antibody (4G10) (Merk Millipore, Billerica, MA, USA) gave high background. To overcome this problem, His-tagged INSR protein was purified using His60 Ni Superflour Resin (Takara Bio, Otsu Japan) and stimulated *in vitro* with 100 nM insulin, 2.5 mM $MnCl_2$ and 1 mM ATP for 10 min. After incubation,

1/4 volume of 4X sample buffer was added to the reaction mixture, and it was boiled and used for western blotting. Membranes incubated with the first antibody were washed and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody depending on the first antibody. After washing, the membranes were visualized with ECL plus western blot detection reagents (GE healthcare, Tokyo, Japan). The image acquisition and quantification of band intensity were performed with ImageQuant LAS4000 (GE healthcare, Tokyo, Japan).

Data analysis

The data are presented as the mean \pm standard deviation (SD) unless otherwise indicated. One-way analysis of variance (ANOVA) was performed to analyze the differences between groups. For comparison between two groups the t-test was used. P-values less than 0.05 were considered statistically significant.

3.3. Results

Structure of the constructed INSR.

One amino acid-shortening mutation was made in the first β strand of the INSR deletion of the three base pair GAG resulted in the loss of Arg at 1000 with a concomitant substitution of Glu at 1001 to Gln ($R^{1000}E^{1001} \rightarrow Q$) (Figure 5, A). This artificial deletion mutation is located next to the ΔLeu^{999} mutation found in our

Insulin binding

Following transfection and selection by blasticidin, the cells with high levels of insulin binding were identified by ^{125}I -insulin binding. Each stable clone expressing WT and mutant INSR that bound 6-8% of the total ^{125}I -insulin radioactivity was selected for the subsequent analyses. Clones expressing the mutant INSR that bound a similar amount of insulin were obtained at a comparable frequency to the clones expressing wild-type INSR. The affinities of the wild-type and mutant INSR to insulin were similar. The total binding capacities of the clones expressing wild-type and mutant INSR were also similar (Figure 6).

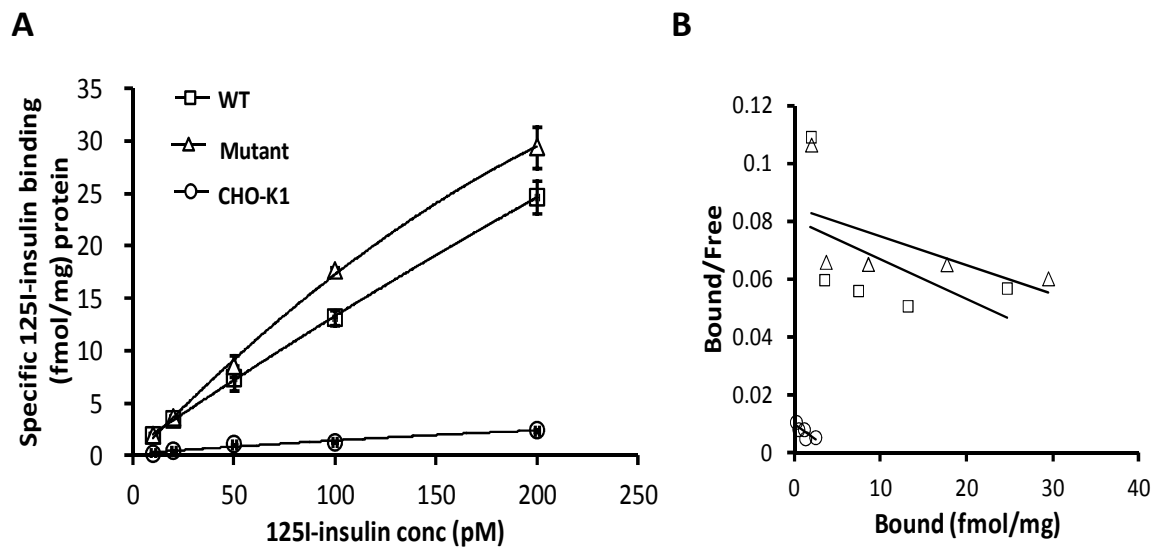


Figure 6. ^{125}I -insulin binding by CHO-K1 clones expressing wild-type and mutant $\text{R}^{1000}\text{E}^{1001}\rightarrow\text{Q}$ INSR cDNA. (A) Cells were incubated with different concentrations of ^{125}I -insulin in 300 μl binding buffer for 4 h at 4°C . After washing, cells were solubilized, and radioactivity was measured using a γ -counter. (B) The representative result of the Scatchard plot of the WT and mutant INSR is shown. B_{max} for WT and $\text{R}^{1000}\text{E}^{1001}\rightarrow\text{Q}$ INSR was 125.0 and 114.4, respectively. K_d values for the WT and mutant INSR were 819.4 and 573.3, respectively.

INSR expression in CHO-K1 cells

Stable clones encoding wild-type and mutant ($R^{1000}E^{1001} \rightarrow Q$) INSRs were stimulated with 100 nM of insulin, and cell lysates of the purified receptors were analyzed by western blotting. Expression levels of mature receptors were comparable in both wild-type and mutant CHO-K1 cells, but the expression level of proreceptors was elevated in mutant INSR (Figure 7, A).

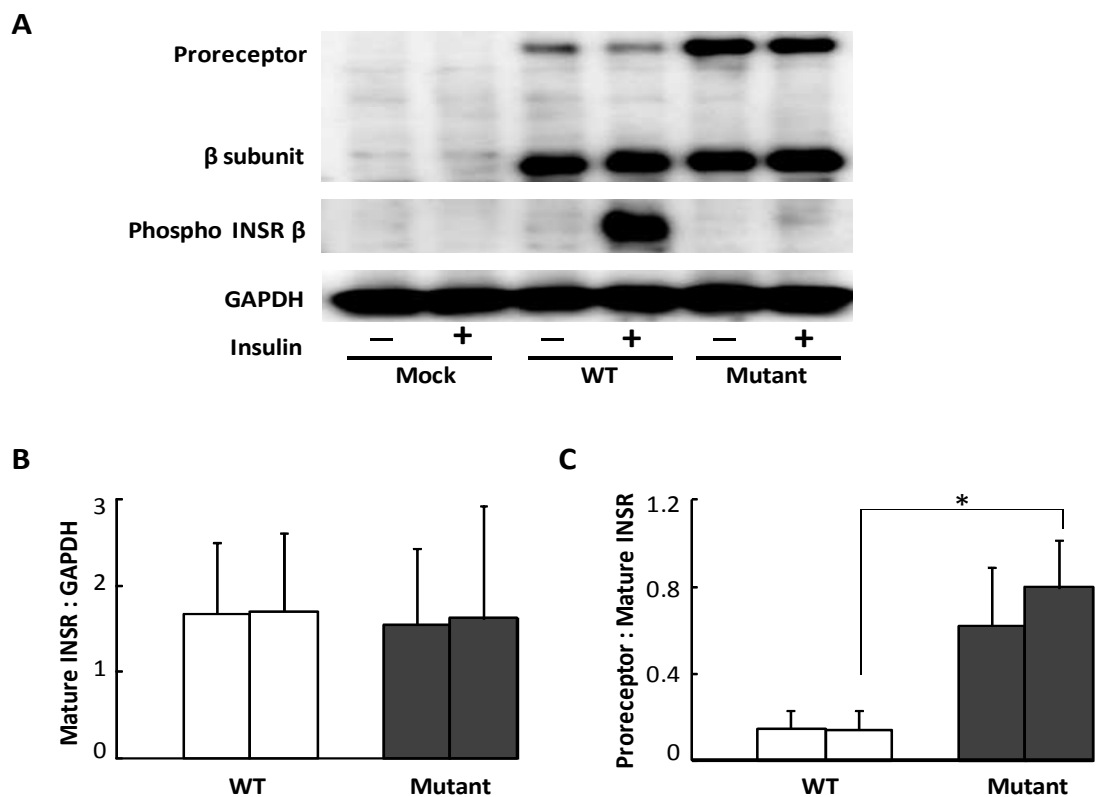


Figure 7. (A) Expression and insulin-induced autophosphorylation of wild-type (WT) and mutant ($R^{1000}E^{1001} \rightarrow Q$) INSRs in CHO-K1 cells. Cells were stimulated with or without insulin (100 nM) for 5 min at 37°C and analyzed by western blot analysis (n=3 per group). The levels of INSR proreceptor (210 kDa), mature β -subunit of INSR (97 kDa), and GAPDH (37 kDa) as a loading control were detected. (B) Quantified values for the levels of mature INSR (β -subunit) detected as a ratio to levels of GAPDH. (C) Quantified values for the levels of proreceptor detected as a ratio to levels of mature INSR. Representative data of three independent experiments. One-way ANOVA was performed, and (*) indicates the value $p < 0.05$.

Because some of the proreceptors are trapped in the maturation process and cannot contribute to insulin binding, cells expressing mutant INSR must produce more proreceptor to achieve comparative levels of insulin binding. As a result, an increased ratio of proreceptors to mature receptors was detected (Figure 7, B and C). Mock-treated CHO-K1 cells should express their intrinsic receptor at a low level because insulin signaling to Akt kinase is also mediated in the non-transfected cells (see below); however, intrinsic INSR is not detected by the anti-myc antibody blotting.

Signaling pathways

To evaluate whether the R¹⁰⁰⁰E¹⁰⁰¹→Q mutation affects INSR activity and downstream signaling, levels of insulin-induced phosphorylation were examined. The autophosphorylation of purified mutant INSR stimulated by 100 nM insulin in vitro was markedly reduced compared with that in wild-type cells (Figure 7, A). The autophosphorylation of intrinsic INSR in CHO-K1 cells could not be detected by blotting the total lysate with anti-phosphotyrosine antibody (data not shown). However, insulin stimulation induced clear phosphorylation of Akt kinase in CHO-K1 cells (Figure 8, A), suggesting that the low level of the intrinsic receptor is sufficient to mediate insulin signaling to Akt kinase.

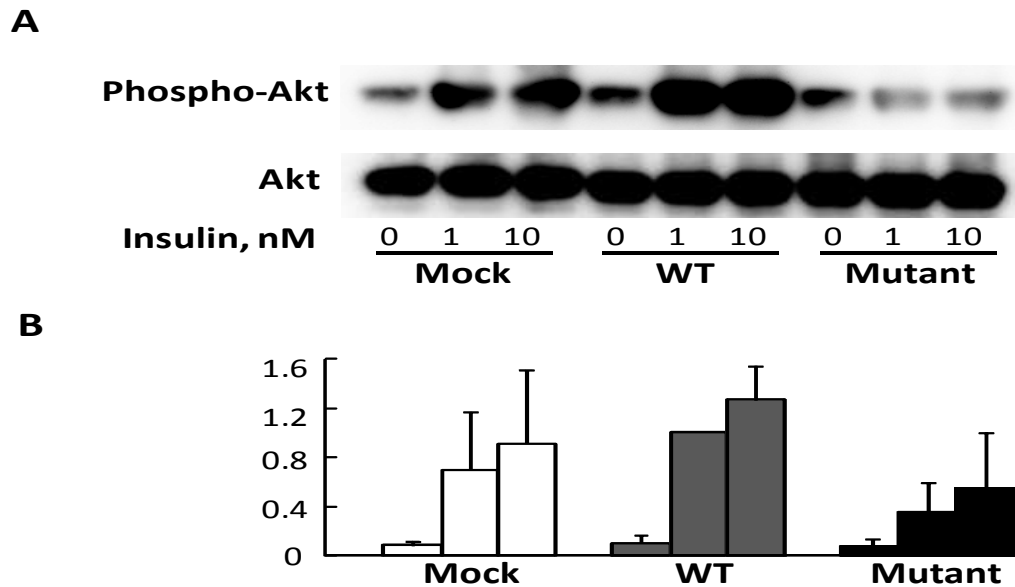


Figure 8. Insulin-stimulated phosphorylation of Akt kinase in cells expressing wild-type and mutant INSR (A) Analysis of levels of phosphorylated and unphosphorylated Akt. CHO-K1 cells transfected with wild-type (WT) and mutant ($R^{1000}E^{1001} \rightarrow Q$) INSRs were serum starved for 16 h and then stimulated with (1, 10 nM) or without insulin for 5 min at 37°C (n=3 per group). Representative gels from several independent experiments are shown. (B) Quantified values show the ratio of phosphorylated to unphosphorylated protein from three independent experiments. One-way ANOVA was performed to determine the statistical significance between groups.

Insulin-stimulated Akt phosphorylation was severely decreased in cells expressing the mutant receptor compared with those expressing the WT receptor or with CHO-K1 cells (Figure 8 A). These data suggest that the mutant INSR has not only lost its kinase activity but has also gained dominant-negative activity over the intrinsic INSR.

3.3. Discussion

As our patient was profoundly hyperglycemic early in her pregnancy, according to the diagnostic criteria of diabetes in pregnancy (33), she was considered to have overt diabetes in pregnancy. The patient did not have polycystic ovary syndrome, one of the common causes of severe insulin resistance. Type B insulin resistance syndrome was also excluded because of the absence of INSR antibodies in her plasma. As the patient showed a classical triad of type A insulin resistance syndrome: acanthosis nigricans in the neck and axillary areas, moderate hyperandrogenemia (testosterone 0.74 ng/ml) and severe insulin resistance (HOMA-IR: 35.2), we suspected the genetic defect in the INSR gene as the underlying molecular mechanism of her insulin resistance. Sequencing analysis revealed a heterozygous deletion mutation in exon 17 of the INSR gene (ΔLeu^{999}). The same mutation was previously reported in a hyperinsulinemic patient, who did not have diabetes, in the same area of Japan, suggesting that both patients shared the same ancestors, although we could not find any overlap in the pedigrees (22). We have not performed functional analysis of this mutation because according to Awata et al. (22), the ΔLeu^{999} mutation had significantly decreased tyrosine kinase activity of the INSR gene.

In insulin-resistant patients with INSR mutations, hyperglycemia is extremely

difficult to treat. Uncontrolled hyperglycemia during pregnancy associates with increased rates of perinatal complications and risk for the development of diabetes and obesity in offspring (34); therefore, we aimed to maintain near-normal glycemic control in our patient during her pregnancy and improve pregnancy outcomes. In the patient's first pregnancy, due to insulin resistance, her endogenous insulin was not able to decrease the blood glucose. Exogenous insulin was required, and insulin aspart and insulin isophane were used to control the blood glucose. Insulin aspart has been reported to control postprandial glucose efficiently in patients with gestational diabetes (9), and although the dose used in our patient was substantially higher (5.7-6.6 units/kg for gestational age \geq 26 weeks) than the dose used in that study (0.9 units/kg), the postprandial glycemic control in our patient was unfavorable, exceeding 200 mg/dl, and required supplementary insulin isophane (1.3-1.8 units/kg/day).

Before the patient's second pregnancy, metformin was added to the insulin regimen in an attempt to reduce insulin resistance and the total insulin dose. Metformin has been shown to be an effective and safe treatment option for gestational diabetes (6,35), although in almost half of the cases, supplementary insulin was needed to achieve the target level of glucose. Although large numbers of observational studies have reported no teratogenic effect with metformin use in

pregnancy, the fact that metformin crosses the placenta still raises concerns over its safety (36). The American College of Obstetrics and Gynecology and the UK National Institute of Health and Care Excellence (NICE) recommend that metformin can be used to treat women with gestational diabetes (36), whereas the American Diabetes Association (ADA) recommends that metformin should only be used in the context of clinical trials (37). In Japan, however, metformin use during pregnancy is recommended only if the benefit outweighs the risk.

In the second pregnancy of the patient, because insulin lispro has more evidence on safety during pregnancy than insulin aspart (38), we used insulin lispro. Combined therapy of metformin with insulin lispro and insulin isophane was able to reduce the total insulin dose to 174 units/day, which is less than half of the dose in the first pregnancy (480 units/day), supporting the hypothesis that metformin spares insulin by sensitizing insulin action through the suppression of hepatic glucose output or by increasing glucose uptake by peripheral tissues. The similar efficacy of the addition of metformin to insulin has been reported in patients with Type 2 diabetes mellitus (39,40), but not in pregnancy. We have not compared the hypoglycemic effects of insulin aspart and lispro in a head-to-head manner in our patient. Alternatively, the effect of these two insulin analogues without metformin can be observed during the periods after the first and second pregnancies when

metformin use was discontinued because of lactation. The insulin dose and glycemic control were similar during the lactation periods (each 15 months); therefore, we assume that the impact of lispro and aspart were similar for glycemic control in our patient. Weight gain during her pregnancies was minimal with caloric restriction (1.6 kg and 1.3 kg for the first and second pregnancy, respectively, with 32 kCal/kg ideal body weight/day), while 3.5 kg was gained even with stricter caloric restriction (24 kCal/kg ideal body weight/day) between pregnancies. It is also possible that more strict adherence to the caloric restriction resulted in better glycemic control in the second pregnancy judging from the smaller weight gain.

It is worth mentioning that both babies and their mother, who were carriers of the same heterozygous mutation of the INSR gene, were all small for gestational age. Intrauterine growth is regulated by various genetic and epigenetic factors (41). Intrauterine and postnatal growth retardations have been reported in patients with a bi-allelic defect of the INSR gene, such as that found in leprechaunism and Rabson-Mendenhall syndrome, while haploinsufficiency of INSR, type A insulin resistance syndrome, is not usually associated with intrauterine growth deficiency (42). It is possible that the ΔLeu^{999} mutation compromises insulin signaling more severely than most other mutations by a dominant negative effect, resulting in phenotypic similarity to patients with bi-allelic defects. It is also plausible that the

strict restriction of calorie intake in the mother compromised the intrauterine growth of the babies.

In summary, we experienced a successful outcome of pregnancies in a patient with type A insulin resistance syndrome caused by ΔLeu^{999} mutation in the INSR gene. Despite the large doses of insulin therapy, no abnormalities were observed in the babies except for transient hypoglycemia and low body weight. The additional use of metformin has significantly reduced the required doses of exogenous insulin and achieved glycemic control that is superior to insulin therapy alone. The combined therapy of insulin with metformin may be a practical tool for the management of severe insulin resistance associated with pregnancy.

Mutations in the INSR gene have been classified into five groups with regard to the interference with the major steps required for the normal function of the INSR: receptor biosynthesis, transport of receptors to cell surface, insulin binding, receptor tyrosine kinase activity and degradation of receptor (43). Binding of insulin to the INSR leads to the autophosphorylation of several tyrosine residues and the subsequent activation of the tyrosine kinase, which plays a necessary role in the metabolic actions of insulin in human. Most of the mutations in the tyrosine kinase domain of the INSR abolish its tyrosine kinase and thus cause insulin resistance. (22,44-48). In the present study, a one amino acid deletion mutation was made in

the $\beta 1$ strand (residues 996 to 1004) that lies just before the ATP binding motif. Two compound and one heterozygous deletion mutations have been reported in this $\beta 1$ strand (22,44,46). All carriers of these mutations show insulin resistance; however, the mechanisms underlying the insulin resistance caused by these mutations are different. Moritz et al. (46) reported type A insulin resistance with severely impaired insulin binding resulting from the skipping of exon 2 from the maternally inherited allele and a paternally inherited mutation of Ile to Phe at position 996; however, the physiological meaning of the latter alteration has not been fully described. Another case of type A insulin resistance was found to be caused by a compound heterozygous mutation (44). One allele encoded an Arg⁹⁹³Gln mutation, and the other had a nonsense mutation at Arg¹⁰⁰⁰. INSR purified from the patient's transformed lymphocytes had very low kinase activity, suggesting that the Arg⁹⁹³Gln mutation results in compromised kinase activity. These reports indicate that missense Ile⁹⁹⁶Phe and deletion Δ Leu⁹⁹⁹ mutations in the $\beta 1$ strand can result in compromised tyrosine kinase activity.

The deletion of one Leu⁹⁹⁹ residue results in a markedly defective tyrosine kinase activity (22). However, when the dileucine motif LL⁹⁹⁸⁻⁹⁹⁹ containing the same Leu residue is replaced with alanine, insulin binding and kinase activity are intact, but internalization of the receptor is reduced (23). It is now well established that this

dileucine motif has a dual role and participates in both receptor internalization and anchoring INSR on microvilli (49). Both of these processes occur independently of kinase activation. Anchoring on microvilli requires an additional dileucine-like motif II¹⁰¹⁸⁻¹⁰¹⁹. According to the crystal structure of hINSR's tyrosine kinase domain, these LL⁹⁹⁸⁻⁹⁹⁹ and II¹⁰¹⁸⁻¹⁰¹⁹ dileucine-like motifs are located within the β 1 and β 2 strands, respectively, and lie in close proximity. This may suggest that these residues interact with other proteins.

The R¹⁰⁰⁰E¹⁰⁰¹→Q mutation described in this study retains the dileucine, though the kinase activity was found to be severely impaired. These data may suggest that a leucine at this position is not required but that the length of the strand is critical.

A sequence comparison of species from *Caenorhabditis elegans* to *H. sapiens* reveals that a region of the first β strand contains diverse amino acid changes, including at residue Leu⁹⁹⁹, but the number of amino acids between the conserved flanking residues is always exactly the same. This result inspired us to analyze the effect of deleting other residues close by. For the convenience of construction, Arg¹⁰⁰⁰ and Glu¹⁰⁰¹ were mutated to a single Gln because this mutation makes an artificial PvuII site. The expression of mutant INSR was confirmed by insulin binding assay. However, proreceptor processing was delayed in the cells expressing the mutant receptor compared with those of wild type. This may result in a low

expression level of mature INSR on the cell membrane. However, low expression is unlikely to be the major cause of insulin resistance because we could select mutant clones that bind a similar level of insulin with comparable frequency as clones expressing wild type. To adjust the amount of the mature INSR on the cell surface, stable clones expressing the mutant and wild-type INSR were isolated by insulin binding, and clones that bind similar amounts of insulin were used for analyses. Our case described above also demonstrates that the same heterozygous mutation (ΔLeu^{999}) found in our patient and her father was the only cause of their severe insulin resistance, as no other reasonable mutation was identified, and it likely resulted in defective tyrosine kinase activity of the INSR. In the present study, the tyrosine kinase activity of the $\text{R}^{1000}\text{E}^{1001}\rightarrow\text{Q}$ mutant receptor was severely reduced as in the case of ΔLeu^{999} . To show that the mutant receptor had a dominant-negative effect over the intrinsic INSR of the CHO-K1 cells, blotting with anti-phospho-Akt antibody was performed. Unfortunately, with ANOVA, the difference in the pAkt level did not reach statistical significance. When the pAkt levels of cells expressing wild type and mutant INSRs stimulated by 1 nM insulin were compared by t-test, a significant reduction was shown in the cells expressing mutant INSR. There seems to be a discrepancy between the severe insulin resistance of the patient and the incomplete impairment of insulin signaling in the

CHO-K1 cells expressing mutant INSR. Chinese hamster INSR might not hybridize efficiently with human INSR, and hence, an obscure dominant-negative effect was shown. These data may show the importance of the exact length of this region, although we have not made a series of deletion mutants of the residues nearby. This region is in the first β strand, which supports the nucleotide binding loop as shown in Figure 1, A and Figure 5, B. Therefore, the deletion of one residue in this strand may result in distortion of the ATP positioning.

In summary, we suggest that deleting one amino acid in the first β strand can disrupt the tyrosine kinase activity of the INSR by changing the optimal positioning of the kinase structure.

4. Conclusion

The present work provides the following practical and theoretical contributions to the understanding of insulin resistance:

1. Severe insulin resistance caused by the kinase-negative mutation of INSR could be managed with intensive insulin therapy combined with metformin, even in a pregnant case.
2. The contribution of the INSR mutation to small body weight of the newborn is suggested, but confirmation by other cases is required.
3. The exact length of the first β strand of the kinase domain is suggested to be essential to the kinase activity of INSR.

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