Original Article

Critical role of the length of the first β strand in insulin receptor kinase activity

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Abstract

Background
Mutations in the tyrosine kinase domain of the insulin receptor gene cause a monogenic syndrome of insulin resistance in humans. The first β strand in this kinase domain is close to the nucleotide binding loop, and its length is conserved through several species. To test whether the exact length of this region is essential for the kinase activity of the insulin receptor, we constructed a one-amino-acid deletion mutant in the first β strand of the tyrosine kinase domain.
Methods
Deletion of Arg^{1000} with concomitant substitution of Glu^{1001} to Gln（$R^{1000}E^{1001}$→ Q）was generated by site-directed mutagenesis. Chinese hamster ovary cells were transfected with mutant or wild-type human insulin receptor cDNA, and stable clones with similar binding activity were screened by insulin binding assay and used for further experiments. Receptor expression, kinase activity and downstream insulin signaling were examined by western blot analysis.
Results
Mature insulin receptor expression was comparable between the wild-type and mutant cells. The mutant insulin receptor showed markedly defective tyrosine kinase activity. Akt kinase phosphorylation was severely reduced, indicating that downstream insulin signaling was also impaired by the mutant receptor.
Conclusion
This study suggests that the deletion of one residue in the first β strand results in a distortion of the optimal positioning of the kinase structure, thereby compromising the kinase activity of the insulin receptor.
（Keywords：tyrosine kinase, insulin receptor, mutation, insulin resistance）

I. Introduction

Binding of insulin to the insulin receptor leads to the autophosphorylation of several tyrosine residues and subsequent activation of the tyrosine kinase, which is a prerequisite for the metabolic actions of insulin in humans[1)]. Mutations in the kinase domain are known to cause insulin resistance and diabetes mellitus[2-5)]. We recently found a family with a 3 base pair deletion in exon 17 of the insulin receptor（INSR）gene that resulted in deletion of Leu^{999}（ΔLeu^{999}）[6)]. The proband was heterozygous for this mutation, had severe insulin resistance and developed overt diabetes mellitus. The same mutation has been previously reported to cause severely impaired kinase acitivity[7)]. Interestingly, the same leucine residue is a part of a dileucine motif that mediates intracellular sorting of the insulin receptor[8)]. According to Haft et al.[8)], a mutant INSR where the two leucine residues are both replaced with alanine has intact kinase activity. Therefore, deletion of Leu^{999} abolishes the

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kinase activity of INSR while mutation to alanine has no effect on it.

The insulin receptor is synthesized as a single polypeptide with a signal peptide and α and β subunits ligated together. After deletion of the signal peptide, the proreceptor is processed to α and β subunits, which are ligated with a disulfide bond and finally form an α2β2 heterotetramer. Purified insulin receptor is known to be activated *in vitro* with insulin. The extracellular α subunit contains an insulin binding region and the β subunit has transmembrane domain and an intracellular tyrosine kinase domain. Traditionally, the amino acid numbering of the insulin receptor does not include the 27 amino acid signal peptide. The INSR has two alternative splicing forms dependent on the absence or presence of the 12 amino acids encoded by exon 11[9]. Here we use a numbering system including exon 11. The insulin receptor internalization process requires two types of motifs within the INSR β subunit, namely tyrosine based [10, 11] and dileucine containing sequences[12].

It was our question whether the low kinase activity of Δ Leu^{999} derives from distortion of the kinase domain or from problems in the maturation and recycling process. The crystal structure of the kinase domain of the insulin receptor has revealed that Leu^{999} is included in the first β (β1) strand in the kinase domain[13, 14]. This β1 strand is very close to the ATP-binding motif (Figure 1), therefore shortening it by only one residue may disrupt the tertiary structure of the INSR, thereby compromising its tyrosine kinase activity. Comparison of amino acid sequences among species revealed that although the leucine residue is not conserved, the number of amino acids in the interval between the conserved motifs is the same from *C. elegans* to human (Table 1 A). It is also possible that Δ Leu^{999} decreases tyrosine kinase activity by impairing interactions with other proteins through the dileucine motif. To address these questions, we made a mutant replacing Arg^{1000} and Glu^{1001} with a single Gln (R^{1000}E^{1001}→ Q), which is adjacent to the dileucine motif. We have used JPred 4 prediction server[15] and recorded prediction by JPred program for several one amino acid deletions around this region. The results are shown in Table 1 B. The predicted secondary structures were primarily beta sheets although the length were different and only in the case of deletion of I^{996} resulted in change to helix.

Table 1 A. Comparison of amino acid sequences spanning Leu^{999}

Sequences of *Caenorhabditis elegans* (earth worm), *Aedes aegypti* (insect), *Drosophila melanogaster* (insect), *Bombyx mori* (insect), *Stegastes partitus* (fish), *Xenopus laevis* (amphibian), *Nipponia Nippon* (bird), *Mus musculus* (mammal) and *Homo sapiens* (mammal) are shown. Residues conserved from *C. elegans to H. sapiens* are in bold. Leu^{999} is underlined.

Species	Amino acid sequences
C. elegans	**Y**NA**D**D**WE**LRQDDVVLGQQC**G**E**GSFG**K**VY**L**G
Aedes aegypti	**Y**KV**D**D**WE**VERNHIIQ<u>L</u>EEL**G**Q**GSFG**M**VY**K**G**
D. melanogaster	**Y**IP**D**D**WE**VLRENIIQ<u>L</u>APL**G**Q**GSFG**M**VY**E**G**
Bombyx mori	**Y**VP**D**E**WE**VTRDSIHFIREL**G**Q**GSFG**M**VY**E**G**
S. partitus	**Y**EE**D**E**WE**VAREKIAI<u>L</u>REL**G**Q**GSFG**M**VY**E**G**
Xenopus laevis	**Y**IP**D**E**WE**VPRDKINL<u>L</u>REL**G**Q**GSFG**M**VY**E**G**
N. nippon	**Y**VP**D**E**WE**VPRDKIAL<u>L</u>REL**G**Q**GSFG**M**VY**E**G**
Mus musculus	**Y**VP**D**E**WE**VPREKITL<u>L</u>REL**G**Q**GSFG**M**VY**E**G**
Homo sapiens	**Y**VP**D**E**WE**VSREKITL<u>L</u>REL**G**Q**GSFG**M**VY**E**G**

Table 1 B. Prediction of the secondary structure of several sequences spanning Leu^{999} using JPred server.

Sequences with deletion of E^{1001} to I^{996} are shown. Predictions are indicated below and E indicates sheet and H indicates helix. Gaps are inserted for convenience of comparison.

Sequence	Secondary structure prediction
Human IR (984-1013)	YVPDEWEVSREKITLLRELGQGSFGMVYEG ----------EEEEE--------EEE--
R^{1000}E^{1001}>Q	YVPDEWEVSREKITLLQ LGQGSFGMVYEG ----------EEEE- -------EE---
deletion of E^{1001}	YVPDEWEVSREKITLLR LGQGSFGMVYEG -----EE-----EEEEE -------EE---
deletion of R^{1000}	YVPDEWEVSREKITLL ELGQGSFGMVYEG -----EE-----EEEE --------EE---
deletion of L$^{999/998}$	YVPDEWEVSREKITL RELGQGSFGMVYEG -----EE-----EEE E------EEEE---
deletion of T^{997}	YVPDEWEVSREKI LLRELGQGSFGMVYEG -----EEE----E EE---------EE---
deletion of I^{996}	YVPDEWEVSREK TLLRELGQGSFGMVYEG ---------- HHHHH------EEE---

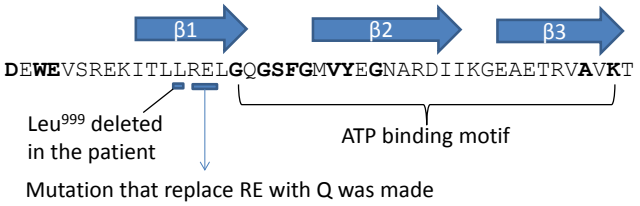

Figure 1. Location of the mutation in relation to secondary structure

Sequences flanking Leu999 are shown. Residues conserved from *C. elegans* to humans are indicated in bold. The positions of the first three β strands are indicated by arrows.

II. Materials and methods

A. Materials

Restriction enzymes were either from Takara Bio (Otsu, Japan), Toyobo (Osaka, Japan), or New England Biolabs Japan (Tokyo, Japan). pTAC2 vector was from Biodynamics (Tokyo, Japan). AmpliTaq Gold, TA cloning kit, the BigDye Terminator v3.1 Cycle Sequencing kit and Lipofectamine were from Life Technologies Japan (Tokyo, Japan). F-12 Ham's medium was from Sigma-Aldrich Japan (Tokyo, Japan). Blasticidin S hydrochloride was from MP Biomedicals LCC (Tokyo, Japan). ^{125}I- insulin was from PerkinElmer Japan (Yokohama, Japan). RIPA buffer was from Cell signaling (Tokyo, Japan). The γ-counter was from Hitachi-Aloka Medical, (Tokyo, Japan). Polyvinylidene fluoride (PVDF) membrane, anti-phosphotyrosine antibody (4G10) and monoclonal anti-myc tag antibody clone 4A6 were from Merk Millipore (Billerica, MA USA). Antibodies against Akt, phospho-Akt (Ser473) and GAPDH (14C10, rabbit monoclonal antibody) were from Cell Signaling Technology Japan (Tokyo, Japan). His60 Ni Superflour Resin was from Takara Bio (Otsu, Japan). ECL plus western blot detection reagents and Image Quant LAS4000 were from GE healthcare (Tokyo, Japan). Other reagents are either fromWako Pure Chemical Industries (Osaka, Japan) or Nacalai tesque (Kyoto, Japan) unless otherwise indicated.

B. Construction of wild-type (WT) and mutant human INSR (hINSR) expression vectors

A mutant human insulin receptor (hINSR) was constructed by creating three fragments. First, the 5' terminal fragment of hINSR cDNA was digested from pSV2-hINSR with HindIII and BamHI and subcloned into pcDNA6/myc-HisA resulting in pcDNA6-hINSR1/myc-HisA. The second fragment was amplified using pSG5-hINSR as template with primers 5'CTGGATCCAATCTCAGTGTC3' and 5'CCCCAGCTGAAGGAGGGTGAT3' containing BamHI and PvuII sites, respectively. The third fragment was amplified with primers 5'CTTCAGCTGGGGCAGGGCTCC3' and 5'CCTCTAGAGGAAGGATTGGACCGAGGCAAGG3' containing PvuII and XbaI sites, respectively. Each of these two PCR products was subcloned into a pTAC2 vector using a TA cloning kit and the sequences were confirmed by direct sequencing analysis. Forward and reverse M13 primers and a sequencing primer, 5'CAGTGTTGTGATTGGAAG', were used for sequencing. 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. The resulting mutant fragment ($R^{1000}E^{1001}$→ 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 wild-type hINSR, a BstXI fragment (758 to 3302) containing the mutated site was replaced with the original hINSR to obtain pcDNA6-hINSR /myc-HisA.

C. Selection of stable transformants and screening by ^{125}I-insulin binding assay

Chinese hamster ovary-K1 (CHO-K1) cells were maintained at 37℃ in F-12 Ham's medium supplemented with 10% fetal calf serum, 100U/ml penicillin and 100 μg/ml streptomycin. The mutant or WT plasmids were transfected into CHO-K1 cells using Lipofectamine. Stable clones carrying WT or mutant INSR vector were selected by blasticidin S hydrochloride and used for insulin binding assays. Clones were plated in 12-well plates in duplicate. When the cells were confluent, one of each pair plates was washed twice with ice-cold PBS and then incubated with 50 pM of ^{125}I- insulin in 500 μl binding buffer (100 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose and 1% BSA) for 4 h at 4℃ with constant agitation. After three washes, the cells were solubilized with 0.1 N NaOH. Cell bound radioactivity was determined in a γ-counter. Nonspecific binding was determined in the presence of 1 μM unlabeled insulin. Mutant and wild-type clones (each three clones) with comparable binding levels were recovered from the replica plate and used for further experiments.

D. Insulin binding assay

Stable clones grown in 12-well plates were washed twice with ice-cold PBS, then incubated with ^{125}I-labeled insulin (10, 20, 50 100, 200 pM) for 4 h at 4℃ in 300 μl binding buffer. Insulin binding to its receptor was measured as described previously[16]. After four washes, cells were solubilized with 0.1 N NaOH. Cell bound radioactivity was assayed using a γ-counter. Non-specific binding in the presence of 1 μM unlabeled insulin was subtracted as background.

E. Western blot analysis

Stable transformants with WT and mutant ($R^{1000}E^{1001}\rightarrow Q$) INSR were serum-starved for 16 h before stimulation with different concentrations of human insulin (1, 10, 100 nM) for 5 min at 37℃, followed by rinsing with ice-cold PBS. Cells were solubilized in RIPA buffer in the presence of phosphatase inhibitors. Protein concentration was determined by the Bradford method using bovine serum albumin as a standard and adjusted. Cell lysates were resolved by 8% SDS-polyacrylamide gel electrophoresis, then transferred to PVDF membrane and analyzed by western blotting using mouse monoclonal anti-myc tag antibody clone 4A6 for total INSR expression. Antibodies against Akt or phospho-Akt (Ser473) were used for detection of total or activated Akt kinase. Anti-GAPDH blotting was used as a control for protein loading. Blotting the total cell lysate with anti-phosphotyrosine antibody (4G10) gave high background. To overcome this problem, His-tagged INSR protein was purified using His60 Ni Superflour Resin 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. Image acquisition and quantification of band intensity were performed with the ImageQuant LAS4000.

F. Data analysis

Data are presented as the mean ±standard deviation (SD) unless indicated otherwise. One-way ANOVA was used for comparison among groups. P-values less than 0.05 were considered statistically significant. Post hoc analysis was done by Tukey method.

III. Results

A. Insulin binding by the $R^{1000}E^{1001}\rightarrow Q$ INSR

Following transfection and selection with blasticidin, the clones with high levels of insulin binding were screened using ^{125}I-insulin. Clones that bound over 8 percent of total ^{125}I-insulin radioactivity were selected for the subsequent analyses. Clones expressing the mutant INSR that bound a similar amount of insulin were obtained at a comparable frequency with the clones expressing wild-type INSR. The affinities of the wild-type and mutant INSR to insulin were similar (Figure 2 A). The Scatchard plot of the clones expressing wild-type and mutant INSR were also similar (Figure 2 B).

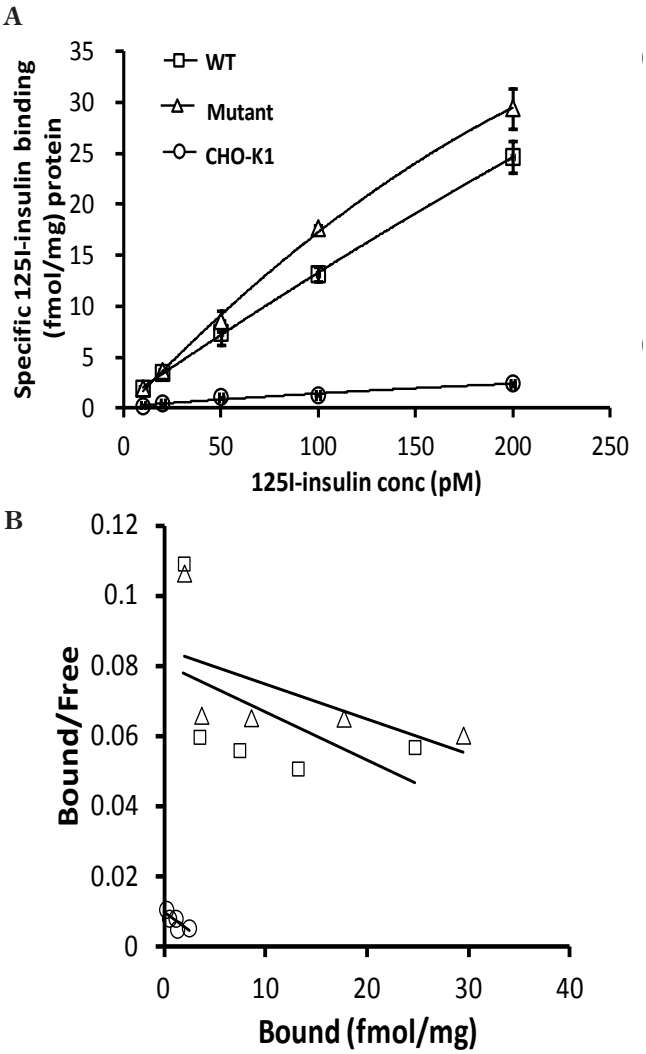

Figure 2. ^{125}I-insulin binding by CHO clones expressing WT and mutant INSR.

(A) Cells were incubated with different concentrations of ^{125}I-insulin in binding buffer for 4 h at 4℃. After washing, the 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. Regression lines are also indicated in the figure.

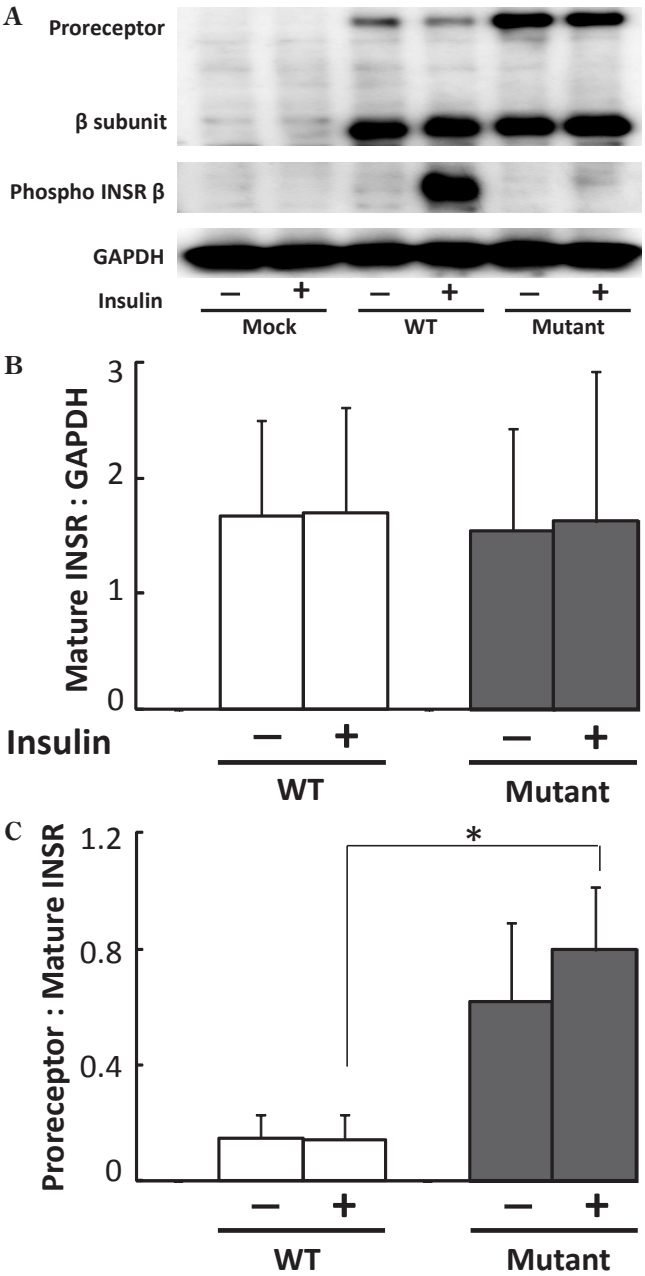

Figure 3. Expression of wild-type and mutant INSRs and β subunit phosphorylation.

(A) Expression and insulin-induced autophosphorylation of wild-type (WT) and mutant ($R^{1000}E^{1001}$→ Q) insulin receptors in CHO-KI cells. Purified INSR extracted from cells expressing wild-type and mutant INSR were stimulated *in vitro* with or without insulin (100 nM) for 5 min at 37℃ and analyzed by western blot analysis. The levels of insulin receptor (INSR) proreceptor (210 kDa), mature β-subunit of INSR (97 kDa), and GAPDH (37 kDa) as a loading control were detected. Representative gels from several independent experiments are shown. (B) Quantified values for the levels of mature insulin receptor (β-subunit) detected as a ratio with levels of GAPDH (n=3 per group). (C) Quantified values for the levels of proreceptor detected as a ratio to levels of mature insulin receptor (n=3 per group). One-way ANOVA was performed, and (*) indicates the value $p<0.05$.

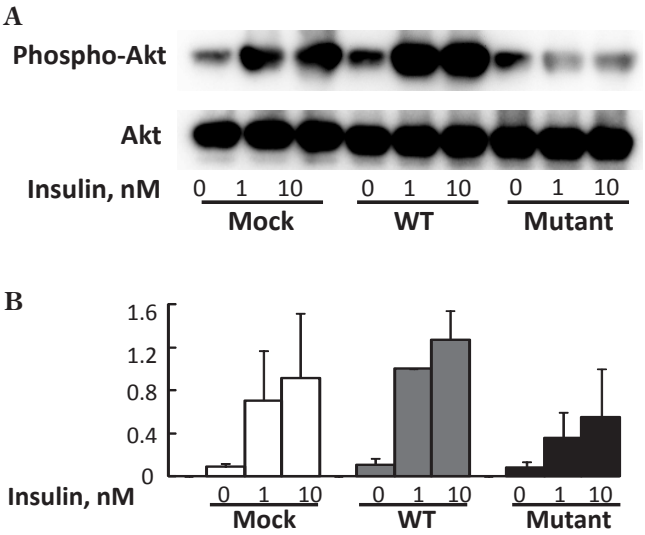

Figure 4. Insulin-stimulated phosphorylation of Akt kinase in cells expressing wild-type and mutant INSR

(A) Analysis of levels of phosphorylated and total Akt. CHO-KI cells transfected with wild-type (WT) or mutant ($R^{1000}E^{1001}$→ Q) INSR were serum starved for 16 h, then stimulated with (1, 10 nM) or without insulin for 5 min at 37℃ (n=3 per group). Representative gels from several independent experiments are shown. (B) Quantified values show the ratio of phosphorylated Akt to total Akt from three independent experiments. The difference among mock, WT and mutant in each insulin concentration did not reach statistical significance by one-way ANOVA.

B. INSR expression in CHO-K1 cells

Stable clones encoding wild-type and mutant ($R^{1000}E^{1001}$→ Q) INSRs were stimulated with 100 nM of insulin and total cell lysates were analyzed by western blotting. Expression levels of mature receptors were comparable in both WT and mutant CHO-K1 cells, but the expression level of proreceptors was elevated in mutant INSR (Figure 3 A). This is because the proreceptor is not expressed on the cell surface and hence does not contribute to insulin binding. As a result, an increased ratio of proreceptors to mature receptors was detected (Figure 3 B and C). Mock-treated CHO-K1 cells should express their intrinsic receptor at a low level because insulin signaling to Akt kinase is mediated also in the non-transfected cells (see below); however, intrinsic INSR is not detected by the anti-myc antibody blotting.

C. Signaling pathways

The autophosphorylation of purified mutant INSR stimulated by 100 nM insulin *in vitro* was markedly reduced compared with that in WT cells (Figure 3 A). The autophosphorylation of intrinsic INSR or tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) 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 4), suggesting that the low level of the intrinsic receptor is sufficient to mediate insulin signaling to Akt kinase. 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 despite the amount of total Akt kinase was similar (Figure 4). Similar results were observed in two out of three independent experiments. Unfortunately, data from three independent experiments did not reach statistical significance by ANOVA. 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.

D. Discussion

Most of the mutations in the kinase domain of the INSR abolish its tyrosine kinase activity and thus cause insulin resistance. In the present study, a deletion mutant was made in the β1 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 β1 strand[7, 17, 18]. All carriers of these mutations show insulin resistance ; however, the mechanisms underlying the insulin resistance caused by these mutations are different. Moritz et al.[17] reported type A insulin resistance with severely impaired insulin binding resulting from 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[18]. One allele encoded an Arg^{993} Gln mutation and the other had a nonsense mutation at Arg^{1000}. INSR purified from the patient's transformed lymphocytes had very low kinase activity, and suggesting that the Arg^{993} Gln mutation results in compromised kinase activity. These reports indicate that missense Ile^{996} Phe and deletion ΔLeu^{999} mutations in the β1 strand can result in compromised tyrosine kinase activity. When the dileucine motif $LL^{998\text{-}999}$ is replaced with alanine, insulin binding and kinase activity are intact, but internalization of the receptor is reduced[8]. It is now well established that this dileucine motif has a dual role with participation in receptor internalization and anchoring INSR on microvilli[19]. Both these processes occur independently of kinase activation. Anchoring on microvilli requires an additional dileucine-like motif $II^{1018\text{-}1019}$. According to the crystal structure of hINSR's tyrosine kinase domain, these $LL^{998\text{-}999}$ and $II^{1018\text{-}1019}$ dileucine-like motifs are located within the β1 and β2 strands, respectively, and lie in close proximity. This may suggest that these residues interact with other proteins. The deletion of one Leu^{999} residue results in markedly defective tyrosine kinase activity[7]. Interestingly, the replacement of dileucine to alanine has no effect on kinase activity[8]. The $R^{1000}E^{1001} \rightarrow Q$ mutation described in this study retains the dileucine, though the kinase activity was found to be severely impaired. These data may suggest that leucine at this position is not required, but the length of the strand is critical.

Sequence comparison of species from *C. elegance* to *H. sapiens* reveals that a region of the first β strand contains diverse amino acid changes including residue Leu^{999}, but the number of amino acids between the conserved flanking residues is always exactly the same. This inspired us to analyze the effect of deletion of other residues close by. For the convenience of construction, Arg^{1000} and Glu^{1001} were mutated to single Gln because this mutation makes an artificial PvuII site. Expression of the mutant insulin receptor was confirmed by an insulin binding assay. However, proreceptor processing was delayed in cells expressing the mutant receptor compared with those expressing 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. 3 stable clones have been established for both wild-type and mutant cells and two most abundantly expressing clones were used for further analysis. To adjust the amount of the mature insulin receptor on the cell surface, stable clones binding similar amounts of insulin were used for the analysis. In the present study, the tyrosine kinase activity of the $R^{1000}E^{1001} \rightarrow Q$ mutant receptor was severely reduced as in the case of ΔLeu^{999}. We tried to detect the tyrosine phosphorylation of IRS-1 which is an upstream mediator of insulin signaling by western blotting. Unfortunately, expression level of intrinsic IRS-1 in CHO-K1 cells did not allow us to detect its phosphorylation by blotting total cell lysate with anti-phosphotyrosine antibody. Alternatively, we used blotting with an anti-phospho-Akt antibody because Akt phosphorylation is a downstream event and the signal is amplified. Blotting with anti-phospho-Akt antibody could reveal that the mutant receptor had reduced Akt phosphorylation as compared with CHO-K1 cells. However, the reduction level varied among independent experiments and the difference did not reach statistical significance. This mutation is hypothesis derived and not observed in a patient with inherited insulin resistance. Therefore, we cannot use patient's insulin resistance as supportive data of dominant negative effect. We conclude that this mutant is kinase negative and suggest dominant negative effect of this mutation. 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 that supports the nucleotide binding

loop. Therefore, deletion of one residue in this strand may result in distortion of the positioning of ATP. Further studies on a series of deletion mutants are needed to confirm this hypothesis.

E. Declaration of interest

There is nothing to declare.

F. Acknowledgements

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インスリン受容体のキナーゼドメインの第１βストランドの長さの重要性

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要　　約

インスリン受容体のキナーゼドメインの第１βストランドに含まれるLeu^{999}の欠失変異をインスリン抵抗性患者から発見した。この変異はキナーゼ活性を失うと既報であるが，同じロイシンをアラニンに置換した変異体はキナーゼ活性を保つと報告されている。第１βストランドの１残基欠失の意義を明らかにするため，近傍の$Arg^{1000}Glu^{1001}$を単一のグルタミン酸に置換した変異受容体を人工的に作成し，CHO-K1細胞に発現させた。この変異体（$R^{1000}E^{1001}$>Q）は正常インスリン受容体と同程度に発現されたがキナーゼ活性を持たず，ドミナントネガティブ効果が示唆された。第１βストランドはATP結合部位の直上にあり，その正確な長さがキナーゼ活性に必須であると示唆された。
（キーワード：チロシンキナーゼ，インスリン受容体，変異，インスリン抵抗性）

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