Replacements of Leucine 87 in Human Insulin Receptor Alter Affinity for Insulin*

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In a previous analysis, we identified a point mutation that substituted Pro (CCG) for Leu (CTG) at amino acid 87 in the α-subunit of the insulin receptor (IR) in a Japanese patient with leprechaunism. In the present study, we transfected either the wild type (Leu-87) or the mutant (Pro-87) IR cDNA into NIH3T3 cells. Pulse-chase in nonreducing conditions revealed that the dimerization of Pro-87 IR was slightly impaired. However, cell surface biotinylation showed that Pro-87 IR was transported to the cell surface. The Pro-87 IR reduced the insulin binding affinity to about 15% of Leu-87 IR, and the dissociation of insulin in Pro-87 IR was more rapid than in Leu-87 IR. The autophosphorylation of Pro-87 IR was less sensitive to insulin than that of Leu-87 IR, suggesting the reduced insulin binding affinity. Site-directed mutagenesis at amino acid 87 was performed to substitute Ile or Ala for Leu. Both mutant IRs were transported to the cell surface and labeled by cell surface biotinylation. The Ile-87 IR enhanced the insulin binding affinity about 4-fold. The insulin binding affinity of Ala-87 IR was reduced by 85% relative to that of Leu-87 IR. In addition, the dissociation of insulin in Ile-87 IR was slower than in Leu-87 IR, but in Ala-87 IR it was more rapid. These results provide the first direct evidence for a critical role of Leu-87 in binding insulin.

Insulin receptor (IR) is a cell surface glycoprotein with heterotetrameric structure consisting of two α-subunits (135 kDa) and two β-subunits (95 kDa) linked with disulfide bonds. The α-subunit is an extracellular domain including insulin binding sites. The β-subunit consists of extracellular, transmembrane and cytoplasmic domains, the last of which has autophosphorylation sites (1-5). Autophosphorylation at these sites activates tyrosine kinase in the IR, triggers phosphorylation of intracellular substrates, and transduces intracellular signaling (6-9). Insulin binding to the IR is the first step and is essential for inducing many biological actions, such as mitogenic and metabolic actions, on the target cells.

Cloning human IR cDNA (4, 5), molecular analyses of patients with genetic forms of severe insulin resistance, including type B insulin-resistant syndrome, leprechaunism, and Rabson-Mendenhall syndrome, have been reported (10). Most cases of mutations in the α-subunit of the IR affected its intracellular transport by change of folding and reduced the amount of IR on the cell surface (10). Thus, it is difficult to assess the insulin binding affinity of these mutant IRs.

Although site-directed mutagenesis, photoaffinity labeling studies, and the construction of an IR-insulin-like growth factor-1 receptor chimera have identified the presumptive insulin binding domains (11-15), the exact contact sites of insulin in the IR are still unknown. In addition, crystallization of the IR has not been done, and its three-dimensional structure is not clear. Thus, the genetic analysis of mutation of the α-subunit without impairment of the post-translational processing of the IR and the site-directed mutagenesis at the sites of amino acid replacement might suggest the significance of these amino acids in binding insulin.

In a previous study, we identified a point mutation that substituted Pro (CCG) for Leu (CTG) at amino acid 87 of the IR in a Japanese girl with leprechaunism. Another mutation in her case was a 1.3-kilobase pair deletion in the region between exon 4 and exon 6 on the genomic DNA, which induced a skipping-out of exon 5 of the IR and produced a premature stop codon at amino acid 356 in exon 6. We reported that this deletion of maternal allele reduced the amount of IR on the cell surface. Leprechaunism is a rare congenital syndrome associated with severe insulin resistance and multiple anomalies including intrauterine growth retardation (16). Previous studies on molecular analyses of the human IR gene in leprechaunism disclosed that most cases of leprechaunism are homozygotes or compound heterozygotes of the mutant IR gene (10). Therefore, to investigate how the substitution of Pro for Leu at amino acid 87 impairs the biological actions of insulin on the target cells, we constructed an expression vector of human IR cDNA and transfected either the wild type (Leu-87) or the mutant type (Pro-87) IR cDNA into NIH3T3 cells, obtained stably expressing clones, and assessed the biosynthesis and insulin binding affinity of these receptors. In addition, because amino acid 87 is located in the presumptive insulin binding domain (11-15), the assessment of its effect on IR might give a clue to the insulin contact sites of IR. Thus, to investigate the significance of Leu at amino acid 87, we performed site-directed mutagenesis at this position, substituting Ile or Ala for Leu, and assessed the effects of these substitutions on the insulin binding affinity of NIH3T3 cells expressing these mutant IRs in a stable manner. We present evidence supporting the possibility that Leu at amino acid 87 may play an essential role in binding insulin.

**EXPERIMENTAL PROCEDURES**

Construction of Expression Vector—The human IR cDNA was divided into two fragments. One fragment (p13-1) was about 1.0 kilobase pairs of the 5' portion of human IR cDNA cloned into the EcoRI site of a pUC12 vector provided by I. Smith (Genentech, Inc., San Francisco, CA). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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CA). Another fragment (p12-1) was the 3′ fragment of human IR cDNA cloned into the EcoRI site of a pUC12 vector provided by American Type Culture Collection. After digestion with EcoRI and SpeI, the 3′ human IR cDNA p12-1 (nucleotides 1102-4400) was ligated into EcoRI/SpeI-digested pCMV (described above) with p13-1 (pHSIKSHIR) Expression plasmid (pCMV, CLONTECH Laboratories, Inc., Palo Alto, CA) contains the origin of replication and the ampicillin-resistance gene of the E. coli plasmid pUC19, the cytoleukemia virus early promoter/enhancer, and the SV40 polyadenylation signal. After digestion of pCMV with NotI, NotI linker 5′-GGCCGCGATCATCC- TCAATGAT1-4′ and SpeI sites (underlined) was ligated into NotI-digested pCMV.

Because p13-1 had one more translation-initiation sequence ATG in the 5′ region of the true translation-initiation site, pHSIKSHIR was digested with Sall to delete the 5′ end of the true translation-initiation site. After ligation of Sall linker 5′-TGGCAATCTGATGTTG-3′ containing a ClaI site (underlined) with ClaI-digested pHSIKSHIR and digestion with ClaI and SpeI, the 4.4-kilobase pair fragment containing the whole human IR cDNA was ligated into ClaI/SpeI-digested pCMV (PCMVHIR).

For the construction of expression vector of Pro-87 IR cDNA, the patient's IR cDNA was amplified with the following primers: 5′-CCCGGCGATCATCCGAAACCC-3′ (nucleotides 243-269 of the sequence 1260-1284 of the sense strand) and Primer 2 (nucleotides 1-100 of the antisense strand). Amplified cDNA was digested with EcoRV and Kpn1 to yield a 494-base pair fragment (nucleotides 256-704). This EcoRV/Kpn1 fragment was substituted for the comparable segment of pCMVHIR. The correct structure of the exchanged fragment was confirmed by DNA sequencing using Sequenase R Version 2.0 (U.S. Biochemical Corporation). Biosynthesis of Leu-87 and Pro-87 IR—NIH3T3 cells (5 × 105 cells) were transfected with a calcium phosphate precipitate containing a mixture of 20 µg of expression vector and 0.5 µg of a vector containing the neomycin resistance gene, which is located downstream of the Harvey murine sarcoma virus long terminal repeat (pHSVneo) (17). After selection for resistance to the antibiotic G418 (600 µg/ml; Life Technologies, Inc. and Bethesda Research Laboratory), stable transfec-
tants were isolated, cloned, and cultivated in 24-well plates. IR expression was assayed by measuring [125I]insulin binding for Leu-87 IR and by the biosynthesis of IR labeled with [35S]methionine for Pro-87 IR.

NIH3T3 cells expressing either Leu-87 or Pro-87 IR cDNA were incubated for 1 h at 37 °C in methionine- and cysteine-free Dulbecco's modified Eagle's medium (6 ml; Life Technologies, Inc.) including 10% fetal bovine serum. Thereafter, cells were pulse-labeled for 1 h with [35S]methionine + [35S]cysteine (DuPont NEN). In the media used in the subsequent chase periods (0-20 h), the radioactive amino acids were omitted and replaced by complete Dulbecco's modified Eagle's medium at 37 °C for 2 h, cells were incubate
d with insulin (100 nM) at 37 °C for 1 min. After aspirating the medium, cells were frozen because based on [125I]insulin binding assay, the cells expressing Leu-87 IR had approximately 4-fold more receptors than those expressing Pro-87 IR. After incubation in serum-free Dulbecco's modified Eagle's medium at 37 °C for 2 h, cells were incubated with insulin (100 nM) at 37 °C for 1 min. After aspirating the medium, cells were frozen with liquid nitrogen, solubilized, and immunoprecipitated with Ab-3 at a dilution of 1:100 and protein assay as described above. After separating in 6.5% SDS-PAGE, the samples were electroblotted onto nitrocellulose filters. Then, the filters were incubated with a monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY) and analyzed according to ECL Western blotting protocol (Amersham Corp.).

Site-directed Mutagenesis at Amino Acid 87—Polymerase chain reaction was used to create the Ile-87 or Ala-87 IR from pCMVHIR. Two primer sets (A and B), which contained the mutation, were amplified with two specific oligonucleotides. In constructing the expression vector of Ile-87 IR, fragment A was amplified with primer 1 and 2. Primer 1 was 5′-TCGCGGATCCGATCATCCGAAACCC-3′, consisting of nucleotides 243-269 of the sense strand and including the EcoRV site (underlined). Primer 2 was 5′-TAGTTAAAGAAGATTCGTGATCC-3′, consisting of nucleotides 493-468 of the antisense strand, which were complementary with the mutated nucleotides (underlined). Fragment B was amplified with primer 3 and 4. Primer 3 was 5′-GGGATCAGAATCTTTCTTTAACAATACT-3′, consisting of nucleotides 468-491 of the sense strand and complementary with the mutated nucleotides (underlined). Primer 4 was 5′-CTTCCGCGATCAGGAAACCC-3′, consisting of nucleotides 711-691 of the antisense strand including the Kpn1 site (underlined). These two fragments were purified by low melting agarose gel electrophoresis and reamplified with primers 1 and 4. This PCR-fragment was digested with EcoRV and Kpn1 and ligated into EcoRV/Kpn1-pCMVHIR. In constructing Ala-87 expression vector, primer 5, which was 5′-TAGTAAAGAAGATTCGTGATCC-3′ complementary with the mutated nucleotides (underlined), was substi-
tuted for primer 2, and primer 6, which was 5′-GGGATCAGAATCTTTCTTTAACAATACT-3′ and included the mutated nucleotides (underlined), was substituted for primer 3. Transfection and insulin binding studies of these mutant expression vectors were performed as described above. To ascertain the presence of these mutant IRs on the cell surface, we performed biotinylation of the IRs on the cell surface as described above.

RESULTS
BioSynthesis of Wild Type (Leu-87) and Pro-87 IR—The posttranslational processing and intracellular transport of Pro-87 IR were investigated with pulse-chase labeling studies in the presence of [35S]methionine in reducing and nonreducing conditions, because most mutations of the α-subunit of IR caused impairment of these processes (10). In reducing conditions, the 190-kDa band corresponding to the proreceptor of Leu-87 IR was diminished more quickly than that of Pro-87 IR (Fig. 1). The half-life of the proreceptor of Leu-87 IR was 1 h. However, that of Pro-87 IR was longer, 9 h. The bands corresponding to the α- and β-subunits in Leu-87 IR began to appear 4 h after the beginning of pulse-chase study. However, those of Pro-87 IR did not appear until 6-10 h. To investigate whether the delay of the proteolytic cleavage of the proreceptor was due to the impairment of dimerization or of proteolytic cleavage from dimer to tetramer, we performed 3-10% gradient SDS-PAGE electrophoresis of pulse-chase labeled samples in nonreducing conditions. The dimerization of the proreceptor of Leu-87 IR
Intact Cells — Because one naturally occurring mutation that we assessed the autophosphorylation ability of the β-subunit of insulin more unlabeled insulin, Leu-87 IR dissociated [126] and performed dissociation kinetic studies. In the absence of unlabeled insulin, the IR have been reported to substitute Pro for Arg at amino acid 86 constitutively acti-

Pro-87 IR. These findings confirm the reduced insulin binding affinity of Pro-87 IR (Fig. 5). Site-directed Mutagenesis at Amino Acid 87 — To investigate the significance of amino acid 87 (Leu) of the IR, we performed site-directed mutagenesis at amino acid 87. We constructed vector expressing either Ile-87 or Ala-87 IR cDNA, transfected each vector into NIH3T3 cells, and obtained stably expressing clones. We showed that both mutant IRs were transported to the cell surface with cell surface biotinylation (Fig. 3). In addition, insulin binding to these mutant IRs was examined by Scatchard analysis. Insulin binding to Ile-87 IR at tracer concentration of [125] insulin (0.2 ng/ml) was increased to a level approximately 2.5-fold more than that observed with Leu-87 IR-2. As judged by the intercepts of the Scatchard plot with the horizontal axis, there were approximately 45% fewer receptors on Ile-87 IR. These data thus suggested that the substitution of Ile for Leu caused an increase of about 4-fold in insulin binding affinity (Fig. 6A). In contrast, insulin binding to Ala-87 IR at a tracer concentration of [125] insulin (0.2 ng/ml) decreased to a level approximately 13-fold less than that observed with Leu-87 IR-1. As judged by the intercepts of the Scatchard plots with the horizontal axis, there were approximately 50% fewer receptors of Ala-87 IR. These data suggested that the insulin binding affinity of Ala-87 IR was reduced by 85% relative to that of Leu-87 IR (Fig. 6B). Furthermore, we performed a dissociation kinetic study of these mutant IRs. The cumulative percentages of [125]insulin dissociated from Ile-87 IR at 5 and 10 min in the absence of unlabeled insulin were 44.4 and 17.7%, less than those from Leu-87 IR. In contrast, those dissociated from Ala-87 IR at 5 and 10 min in the absence of unlabeled insulin were 59.1 and 75.3%, more than those from Leu-87 IR (Table I). These findings indicate that the substitution of Ile for Leu at amino acid 87 increases the insulin binding affinity, but the substitution of Ala for Leu at this position decreases it.

DISCUSSION

Insulin binding to the IR is the first essential step for inducing the biological actions of insulin. However, the mechanism of binding of the IR to insulin and the precise structure of the IR are not known. The investigation of the structure and function of transmembrane receptor glycoproteins is largely dependent on the genetic analysis of naturally occurring mutations and site-directed mutagenesis.

In a previous study we identified a point mutation of the IR gene in a Japanese girl with leprechaunism whose Epstein-
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Fig. 2. Pulse-chase study of IR with [35S]methionine in nonreducing condition. NIH3T3 cells expressing either wild type (Leu87IR) or mutant type (Pro87IR) receptor were pulse-labeled for 1 h, followed by chase periods (0–20 h) in the absence of [35S]methionine. Thereafter, receptors were immunoprecipitated with Ab-3. The immune complexes were analyzed by 3–10% gradient SDS-PAGE followed by autoradiography.

Fig. 3. Cell surface biotinylation of wild type and mutant IRs. Confluent monolayers of NIH3T3 cells expressing Leu-87 (lane 1), Pro-87 (lane 2), Ile-87 (lane 3), or Ala-87 (lane 4) IR and nontransfected NIH3T3 cells (lane 5) were biotinylated as described under “Experimental Procedures.” Each IR was immunoprecipitated and electroblotted onto nitrocellulose filter and detected with horseradish peroxidase-labeled streptavidin.

Barr virus-transformed lymphoblasts showed extremely reduced insulin binding. This point mutation was the substitution of C for T at nucleotide 479. It resulted in the substitution of Pro for Leu at amino acid 87. We considered that this substitution reduced the insulin binding affinity. However, most mutations in the α-subunit of the IR impair the intracellular transport to the cell surface and reduce the number of IRs on the cell surface (10).

Generally, IR is translated as a 190–210-kDa proreceptor that is glycosylated with N-linked oligosaccharides, dimerized in endoplasmic reticulum, and transported to the Golgi apparatus, in which its glycosylated chains are matured and the proreceptor is cleaved into the α- and β-subunits. The IR is then transported to the cell surface (24). The Pro-233 mutation inhibits the transport of the proreceptor from intracellular sites to the cell surface due to the inhibition of cleavage of the proreceptor and of the addition of sialic acid (25). The Val-382 mutation inhibits the final processing of the N-linked oligosaccharides of the proreceptor in the Golgi apparatus and their transport to the cell surface (26). The Arg-209 mutation impairs dimerization of the proreceptor into a disulfide-linked structure, proteolytic cleavage of the proreceptor into the α- and β-subunits and terminal processing of the high mannose form of N-linked oligosaccharide impairs dimerization of the proreceptor into a complex carbohydrate (27). The Arg-31 mutation inhibits the transport of the proreceptor to the Golgi compartment, where proteolytical processing occurs (28). These mutations all result in a decreasing amount of IR on the cell surface. In the present study, the glycosylation in Pro-87 IR was normal, for the apparent molecular size of the proreceptor and the α-and β-subunits of Pro-87 IR were the same as those of Leu-87 IR. However, the post-translational processing of Pro-87 IR was slightly impaired, for the disappearance of the proreceptor and the appearance of the α- and β-subunits of Pro-87 IR were slower than in the case of Leu-87 IR in a pulse-chase study using [35S]methionine in a reducing condition. In addition, the dimerization of the monomeric proreceptor of Pro-87 IR was also slightly impaired, for the appearance of the dimeric form of the proreceptor was slower than that of Leu-87 IR in a pulse-chase study using [35S]methionine in nonreducing conditions. The delayed disappearance of the proreceptor in the pulse-chase study in reducing condition may be due to the impaired dimerization of the monomeric proreceptor. The conformational change resulting from the substitution of Pro for Leu at amino acid 87 may impair the intracellular transport and especially the dimerization of the proreceptor (monomer) in the endoplasmic reticulum. But this impairment of the intracellular transport of Pro-87 IR may be incomplete, because Pro-87 IR can be cleaved into the α- and β-subunits...
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TABLE I
The percentage of bound \(^{125}\)I-insulin that dissociated from NIH3T3 cells expressing Leu-87, Pro-87, Ile-87, and Ala-87 IR at 5 and 10 min

Dissociation kinetics was performed as described under “Experimental Procedures.” The cumulative percentages of \(^{125}\)I-insulin dissociated at 5 and 10 min were calculated as described elsewhere (21). Data were presented as the means of three replicate experiments.

| IR     | 5 min | 10 min |
|--------|-------|--------|
| Leu-87 | 29.8  | 26.7   |
| Pro-87 | 40.5  | 43.8   |
| Ile-87 | 14.4  | 17.7   |
| Ala-87 | 59.1  | 75.3   |

Fig. 5. Insulin-stimulated phosphorylation of IRs in intact cells. After incubation in serum-free Dulbecco's modified Eagle's medium at 37 °C for 2 h, NIH3T3 cells expressing either Leu-87 IR (WT(Leu87)) or Pro-87 IR (Pro87) were left untreated or stimulated with insulin (100 nm) at 37 °C for 1 min. The number of NIH3T3 cells expressing Pro-87 IR was about 4-fold of NIH3T3 cells expressing Leu-87 IR. The cells were solubilized and immunoprecipitated with Ab-3. After being separated in 6.5% SDS-PAGE, the samples were electrophoretically transferred to nitrocellulose filters. The filters were probed with anti-phosphotyrosine monoclonal antibody and analyzed according to ECL Western blotting protocol (Amersham Corp.).

Fig. 6. A, Scatchard plot analysis of Ile-87 (■) and Leu-87 IR-2 (▲). B, Scatchard plot analysis of Ala-87 (○), Leu-87 IR-1 (○), and nontransfected NIH3T3 cells (△). \(^{125}\)I-Insulin binding assay was performed as described under “Experimental Procedures.” Data were presented as the means of three replicate experiments.

and transported to the cell surface. In addition, Scatchard plot analysis of Epstein-Barr virus-transformed lymphoblasts of the patient’s father showed that the amount of IR on the cell surface was almost normal (data not shown). Thus, the substitution of Pro for Leu does not reduce the amount of IR on the cell surface.

Several researchers have tried to identify the insulin binding domain. A consensus that at least two separate regions of the IR are involved in binding insulin has recently emerged. They are mapped within the amino-terminal domain encoded by exon 2 (residues 1–119) and the carboxyl-terminal domain encoded by exon 6 and 7 (residues 311–428) of the extracellular domain (11–15, 29, 30). However, the exact contact sites of insulin on the IR are not known. A few cases that showed decreased insulin binding without impaired post-translational processing of the IR have been reported. The Lys-15 mutation causes a 5-fold reduction in the capacity of the IR to bind insulin. However, this mutation also retards the post-translational processing of the IR and impairs transport of the receptor to the cell surface (31). Recently, the Leu-323 mutation has been reported to cause decreased insulin binding with normal post-translational processing and cell surface expression of IR (32). These naturally occurring mutations have suggested that the sites of amino acid replacement may play an important role in binding insulin. However, to confirm that these amino acids are the insulin contact sites, site-directed mutagenesis is needed, because these substitutions may only cause conformational change of the true insulin contact sites. De Meyts et al.

showed that an aromatic side chain of Phe at amino acid 89 seemed necessary but not sufficient for high affinity interaction with insulin, but Phe-88 was not involved in binding insulin by site-directed mutagenesis (12). In addition, site-directed mutagenesis at amino acids 85 and 86 showed that these positions were probably not directly involved in ligand binding (22). Leu at amino acid 87 is placed in the insulin binding domain described above and is also adjacent to Phe at amino acid 89. In addition, Pro is often found at sites of β-turn of polypeptides, and the introduction of Pro does bend the polypeptides and induce the conformational change of the protein. Several naturally occurring mutations in patients with insulin resistance, site-directed mutagenesis, photoaffinity cross-linking studies, and the construction of IR/insulin-like growth factor-1 receptor chimeras give insight into the mechanism of insulin binding and the insulin binding domains. Indeed, both Scatchard plot analysis and dissociation kinetics reveal that the substitution of Pro for Leu at amino acid 87 results in reduced binding affinity. This substitution may thus induce conformational change of the insulin binding domain and reduce the insulin binding affinity without gross impairment of post-translational processing of the IR. In addition, the capacity for autophosphorylation of the β-subunit of Pro-87 IR is depressed compared with that of Leu-87 IR, probably because of decreased insulin binding affinity. However, Pro-87 IR preserves the capacity for
autophosphorylation in a dose-dependent manner.

Furthermore, Leu at amino acid 87 is conserved among other kinds of receptors, such as human insulin-like growth factor-1 receptor, Drosophila epidermal growth factor receptor, and c-erb-B2 receptor (29). Thus, Leu at amino acid 87 may play an important role in binding insulin. Site-directed mutagenesis at amino acid 87 also suggests the importance of Leu at amino acid 87 in binding insulin. The substitution of Ile for Leu decreases the insulin binding affinity. In contrast, the substitution of Ala for Leu decreases the insulin binding affinity. These findings identify the substitution of Ile for Leu at amino acid 87 as being of special significance and suggest that at amino acid 87 the β- or γ-branched side chain is needed for binding insulin and that the γ-branched side chain of Leu results in a more effective binding than the β-branched side chain of Ile. Similarly, the replacement of the β-branched side chain of Val with the γ-branched side chain of Leu at position A3 of human insulin results in a naturally occurring insulin variant (Insulin Wakayama) possessing the lowest receptor binding potency of any abnormal human insulin studied to date (33). Because the side chain is important for the interaction between the ligand and its receptor, these modifications at amino acid 87 may be associated with a change of insulin binding affinity. These findings suggest that at amino acid 87 of the IR, the β-branched side chain is more suitable than the γ-branched side chain for binding insulin. In conclusion, the substitution of Pro for Leu at amino acid 87 of the IR reduces the insulin binding affinity without gross impairment of intracellular transport. In addition, Leu at amino acid 87 may play an important role in binding insulin, such as providing the insulin contact site. Crystallization of the IR may reveal this in the future.

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