

An Insulin Receptor Mutant (Asp⁷⁰⁷ → Ala), Involved in Leprechaunism, Is Processed and Transported to the Cell Surface but Unable to Bind Insulin*

(Received for publication, February 8, 1996, and in revised form, April 23, 1996)

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We have identified a homozygous mutation near the carboxyl terminus of the insulin receptor (IR) α subunit from a leprechaun patient, changing Asp⁷⁰⁷ into Ala. Fibroblasts from this patient had no high affinity insulin binding sites. To examine the effect of the mutation on IR properties, the mutant IR was stably expressed in Chinese hamster ovary cells. Western blot analysis and metabolic labeling showed a normal processing of the mutant receptor to α and β subunits. No increase in high affinity insulin binding sites was observed on Chinese hamster ovary cells expressing the mutant receptor, and also, affinity cross-linking of ¹²⁵I-labeled insulin by disuccinimidyl suberate to these cells failed to label the mutant α subunit. Biotinylation of cell surface proteins by biotin succinimidyl ester resulted in efficient biotinylation of the mutant IR α and β subunits, showing its presence on the cell surface. On solubilization of the mutant insulin receptor in Triton X-100-containing buffers, ¹²⁵I-insulin was efficiently cross-linked to the receptor α subunit by disuccinimidyl suberate.

These studies demonstrate that Ala⁷⁰⁷ IR is normally processed and transported to the cell surface and that the mutation distorts the insulin binding site. Detergent restores this site. This is an example of a naturally occurring mutation in the insulin receptor that affects insulin binding without affecting receptor transport and processing. This mutation points to a major contribution of the α subunit carboxyl terminus to insulin binding.

Insulin induces mitogenic and metabolic responses in cells. In addition, in muscle and adipose tissues, glucose transporters become translocated to the plasma membrane (1). These responses require tyrosine kinase activity in the cytoplasmic tail of the insulin receptor, which is activated on binding of insulin (2, 3). The insulin receptor is synthesized as a proreceptor, which after proteolytic cleavage into α and β subunits and extensive glycosylation in the Golgi apparatus, appears pre-

dominantly as an $\alpha_2\beta_2$ tetramer on the cell surface (4).

A number of naturally occurring mutations in the insulin receptor have been found to associate with diseases of severe insulin resistance (5). Missense mutations in the cytoplasmic domain of the receptor are often seen in patients with type A insulin resistance. These mutant receptors are processed to $\alpha_2\beta_2$ tetramers and transported to the cell surface. Usually they retain their insulin binding properties. The impaired activation of the receptor tyrosine kinase contributes to the development of the syndrome of insulin resistance. Missense mutations in the homozygous or compound heterozygous state in the extracellular part of the receptor are often associated with syndromes of extreme insulin resistance such as leprechaunism (MIM 246200) or Rabson Mendenhall syndrome (MIM 262190) (5, 6). Many of the missense mutations involved lead to a loss of high affinity insulin binding sites on cells. These mutations are predicted to affect the folding of the α subunit and the formation of correct disulfide bonds. As a result, the proreceptor is retained in the endoplasmic reticulum, and no receptors are transported to the cell surface (7, 8). Another situation that leads to a decreased number of functional receptors on the cell surface is represented by the naturally occurring mutation Glu⁴⁶⁰. This receptor undergoes enhanced internalization and degradation on binding of insulin (9).

We have recently encountered leprechaun patient HO. This patient was found to have a mutation in the insulin receptor, which interferes with insulin binding, probably by a direct effect on the structure of the insulin binding site.

EXPERIMENTAL PROCEDURES

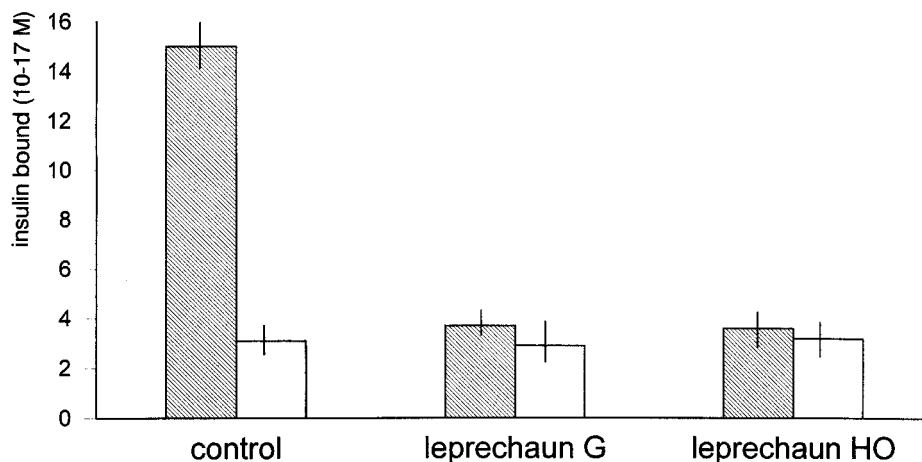
Reagents, Antibodies, and General Procedures—Cultures of fibroblasts and CHO¹ cell lines were as described (7). *Taq* polymerase was from Perkin-Elmer; other enzymes for DNA manipulation were from New England Biolabs, Promega, and Boehringer Mannheim. Other reagents were of analytical grade. Moniodo-A14 insulin (2000 Ci/mmol), a monoclonal antibody against the insulin receptor and biotin succinimidyl ester were from Amersham Corp. Protein A-Sepharose and wheat germ agglutinin-Sepharose were from Pharmacia Biotech, Inc. Disuccinimidyl suberate was from Pierce. Preparation of polyclonal antibodies against the insulin receptor, isolation of DNA from cultured fibroblasts, and amplification of the coding parts of exons 1–22 of the insulin receptor were described previously (7). Monoclonal antiphosphotyrosine antibody (PY20) was from Upstate Biotechnology, Inc. Glycoprotein from CHO cells was isolated by chromatography on wheat germ agglutinin-Sepharose, as described previously, using Hepes-containing buffers instead of Tris. Protein content was determined using the Bio-Rad DC protein assay kit.

* This work was supported by grants from the Diabetes Fonds Nederland and the Netherlands Organization for the Advancement of Pure Research through the foundation of Medical Research (NWO-Medische Wetenschappen). Additional support was provided by the European Union Cost B5 program. 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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¹ The abbreviations used are: CHO, Chinese hamster ovary; IR, insulin receptor; WT, wild type; ECL, enhanced chemiluminescence.

FIG. 1. Binding of ¹²⁵I-insulin to cultured fibroblasts. Hatched bars, total binding; open bars, binding in the presence of 1 μM nonradioactive insulin. Binding values are to 10⁶ fibroblasts.



Patient—The child, a boy, was born from first cousin parents. He had the phenotypic characteristics of leprechaunism, including marked lack of adipose tissues, prominent eyes and lips, wrinkled loose skin, acanthosis nigricans, abdominal distention, prominent nipples, and large phallus. Insulin levels varied during the day between 237 and 424 milliunits/liter (normal value, <25 milliunits/liter). The child died at the age of 8 months.

DNA Sequencing—The exons for the insulin receptor gene were amplified using DNA from the patient's fibroblasts (10). Amplified fragments were purified on low melting point agarose and sequenced using the DNA sequencing kit from Pharmacia as described previously (7).

Construction of Insulin Receptor (Ala⁷⁰⁷) cDNA in the Expression Vector SV40HIR—A DNA fragment containing exon 10 was prepared by polymerase chain reaction from the patient's DNA (10). This fragment was digested by *Dra*III and *Bsm*I and ligated into a plasmid (pMK5) containing the *Bam*HI-*Hinc*II fragment of the human IR (nucleotides 1926–3187; numbering according to Ref. 2). An *Xho*I-*Xho*I fragment (nucleotides 2091–3068) was isolated and exchanged for the corresponding *Xho*I-*Xho*I fragment in the WT IR cDNA, cloned in the pUC12 vector. A *Bst*EII-*Bst*EII fragment (nucleotides 1836–4321) was exchanged for the corresponding fragment in the expression vector carrying the WT IR cDNA, driven by the SV40 promoter (11). The correct structure of the exchanged fragment was confirmed by DNA sequencing.

Transfection of CHO Cells and Selection of Clones Expressing Ala⁷⁰⁷ IR—Construction of clonal CHO cell lines expressing mutant insulin receptors was done by using lipofectamine (Life Technologies, Inc.), and clones expressing insulin receptors were identified as described previously (7).

Cell Surface Labeling—Cells were grown to confluency in 3-cm dishes and subsequently washed three times with 1 ml of 10 mM sodium phosphate, pH 8.6, 150 mM NaCl, 0.1 mM MgCl₂, and 0.1 mM CaCl₂. Cells were kept in 1 ml of this buffer. Biotin succinimide ester (in dry dimethyl formamide) was added to the cells according to the instructions provided by the manufacturer (Amersham Corp.). Incubation was for 1 h at room temperature. Cells were washed, lysed in 0.5 ml of ice-cold lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 7.60, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, Trasylol, and soybean trypsin inhibitor; 1 μg/ml each). Cells were kept at 0 °C for 30 min, and the lysate was centrifuged for 10 min at 14,000 rpm to remove nuclei. The supernatant was diluted to 1.5 ml with immune precipitation buffer (0.1% Nonidet P-40, 20 mM Tris-HCl, pH 7.60, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, Trasylol, and soybean trypsin inhibitor; 1 μg/ml each), and 750 μl was subjected to immune precipitation with polyclonal anti-IR (5 μl) or monoclonal anti-IR (3 μl). After incubation for 16 h at 4 °C, immune complexes were allowed to bind to protein A-Sepharose beads for 30 min at 4 °C. The beads were washed five times with immune precipitation buffer, and bound proteins were eluted by SDS sample buffer. The proteins were separated on a 7% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane, which was probed with avidin-peroxidase conjugate according to the protocol provided by the manufacturer (Amersham). Proteins were visualized by ECL (Amersham).

Cross-linking of Monoiodo-A14 Insulin to Insulin Receptors—Cells were grown to confluency in 3-cm dishes and subsequently washed with phosphate-buffered saline, pH 7.8, containing 1 mg/ml bovine serum

albumin. Cells were kept in 300 μl of this buffer, and ¹²⁵I-monoiodo-A14 insulin (2000 Ci/mmol) was added to a concentration of 100 pM. Incubation was for 1 h at room temperature. Subsequently, the mixture was cooled to 0 °C; disuccinimidyl suberate (50 mM in Me₂SO) was added to a final concentration of 0.2 mM; and incubation was continued for 20 min. The mixture was made 50 mM in Tris-HCl, pH 7.8, and cells were washed with 20 mM Tris-HCl, pH 7.6, and 150 mM NaCl. Cell lysis and immune precipitations were as described under "Cell Surface Labeling."

Cross-linking to partially purified insulin receptors was performed using glycoprotein (2 μg) in 15 μl of 10 mM Hepes, 5 mM MgCl₂, and 0.3 M *N*-acetylglucosamine. ¹²⁵I-Insulin was added to a concentration of 700 pM, and incubation was for 16 h at 0 °C. Cross-linking by disuccinimidyl suberate and immune precipitation was performed as described above.

Analysis of Insulin Receptor Expression and Tyr Phosphorylation Status by Western Blot—Cells were grown to confluency and placed for 16 h in Dulbecco's modified Eagle's medium, containing 1% bovine serum albumin, to bring them in a quiescent state. Cells were incubated for 15 min in this medium, containing, in addition, 1 μM insulin, and lysed in SDS sample buffer. Lysate (25 μg of protein) was electrophoresed on SDS-polyacrylamide gels and blotted on polyvinylidene difluoride membranes. Visualization of the insulin receptor was achieved using a polyclonal peptide antiserum, raised in rabbits, recognizing amino acids 1328–1343 at the carboxyl terminus of the receptor β chain and peroxidase-conjugated anti-rabbit IgG. Tyrosine-phosphorylated proteins were visualized using peroxidase-conjugated PY20 antiserum. Detection was by ECL.

In another set of experiments, cells were incubated in isotonic autophosphorylation buffer (20 mM Tris-HCl, pH 7.60, 140 mM NaCl, 5 mM MnCl₂, and 25 μM ATP) in the absence or presence of 1 μM insulin for 5 min at 37 °C. Cells were lysed either by addition of SDS or by addition of Triton X-100 to a 1% final concentration. Subsequently, the lysate was analyzed by Western blot for phosphotyrosine content using the monoclonal antibody PY20.

RESULTS

Insulin Binding to the Patient's Fibroblasts—Insulin binding to cultured fibroblasts from leprechaun patient HO and to fibroblasts from a healthy control is visualized in Fig. 1. For comparison, results of an insulin binding experiment using fibroblasts from leprechaun G are included. Leprechaun G was described by us previously (12) and is homozygous for a mutation in the insulin receptor, changing Leu²³³ into Pro. High affinity insulin binding sites were nearly absent on fibroblasts from patient HO and leprechaun G. The absence of high affinity insulin binding sites is a characteristic property of fibroblasts from most patients with leprechaunism.

DNA Sequence Analysis—DNA was isolated from cultured fibroblasts of patient HO and from peripheral blood of both parents. Exons encoding the insulin receptor were amplified (10) and analyzed by DNA sequence analysis, directly on amplified DNA. Apart from known polymorphisms, the patient exhibited an additional mutation in homozygous form, which leads to replacement of the Asp codon GAT at position 707 by the codon GCT for Ala. Both parents were found to be heterozy-

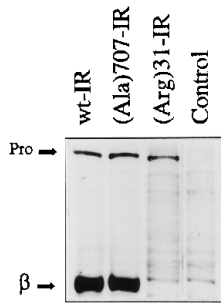


FIG. 2. Analysis of IR expression in transfected CHO cells by Western blot. CHO cells were transfected by cDNA encoding the WT IR, Ala⁷⁰⁷ IR, Arg³¹ IR, or expression vector alone (control). Clonal cell lines were isolated, and total cell lysate was analyzed by Western blotting using an antiserum recognizing a peptide epitope on the carboxyl terminus of the β chain. Arrows, positions of the β subunit (95 kDa) and the proreceptor (200 kDa).

gous for this mutation. No other mutations were detected in the coding or consensus sequences at the splice junctions. Analysis by polymerase chain reaction of cDNA prepared from the patient's fibroblasts and from fibroblasts of a control individual indicated approximately normal levels of IR mRNA (not shown).

Expression of Ala⁷⁰⁷ IR in CHO Cells—To examine the effect of the mutation on properties of the insulin receptor, we expressed the mutant receptor in CHO cells. IR cDNA constructs carrying the mutation were ligated into an SV40-driven expression vector carrying the *neo* resistance gene (11). CHO cells were transfected with the construct and, G418-resistant cell lines were selected. In addition, we transfected CHO cells by this vector carrying cDNA for the WT IR. Expression of the IR protein was monitored by Western blot analysis using a polyclonal antibody against a peptide sequence (amino acids 1330–1343) in the carboxyl-terminal region of the receptor β chain. Clonal CHO cell lines, transfected by Ala⁷⁰⁷ IR cDNA, showed expression of the β chain. Expression levels showed some variations in different clones. Fig. 2 represents the data on a clone with an average expression level. In addition, the data in Fig. 2 include parental CHO cells and transfected CHO cells expressing WT IRs. The latter cell line expresses approximately 250,000 high affinity insulin binding sites per cell, based on Scatchard analysis. We also included in the Western blot analysis CHO cells expressing the transport-defective receptor Arg³¹ IR, originating from leprechaun H (7). We showed previously that this transport-defective receptor is retained in the endoplasmic reticulum as a proreceptor and not processed into α and β subunits. It is obvious that Ala⁷⁰⁷ IR is proteolytically processed like WT IRs, as judged from the predominant staining of the β subunit on the Western blot. In contrast, the transport-defective receptor Arg³¹ IR from leprechaun H only exhibits staining of the proreceptor by the antibody against the peptide epitope on the β subunit carboxyl terminus.

Data obtained by metabolic labeling of these CHO cell lines by [³⁵S]methionine and immune precipitation of the insulin receptor followed by analysis of the immune precipitate by SDS-polyacrylamide gel electrophoresis and autoradiography confirmed that Ala⁷⁰⁷ IR is processed into α and β subunits, like WT IRs. These data are presented in Fig. 3. The electrophoretic mobilities of α and β subunits from WT and Ala⁷⁰⁷ IRs were similar, suggesting a similar degree of glycosylation. This notion is corroborated by the normal mobilities of mutant α subunits, visualized by biotinylation (Fig. 4) and iodination (Fig. 6).

Biotinylation of Cell Surface Proteins—To examine whether Ala⁷⁰⁷ IR is transported to the surface of cells, we labeled cell surface proteins of the various CHO cell lines with biotin suc-

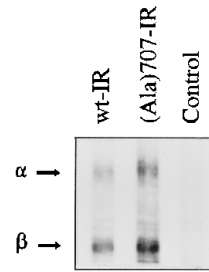


FIG. 3. Analysis of IR expression in transfected CHO cells by metabolic labeling and autoradiography. CHO cells were transfected by cDNA encoding the WT or Ala⁷⁰⁷ IR or mock transfected (control). Cells were grown in medium containing [³⁵S]methionine, followed by lysis of the cells and immune precipitation of the insulin receptor. The immune precipitated receptor was subjected to electrophoresis on SDS-polyacrylamide gels, and the gels were analyzed by autoradiography.

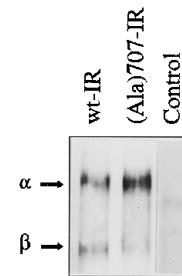


FIG. 4. Biotinylation of cell surface proteins. CHO cells expressing WT or Ala⁷⁰⁷ IR and control CHO cells were incubated with biotin succinimidyl ester. After lysing the cells, insulin receptors were immune precipitated by a polyclonal antibody, and the immune precipitate was subjected to Western blot analysis. Visualization of biotinylation was by streptavidin-peroxidase conjugate and ECL.

cinimidyl ester, a protein reagent that couples biotin moieties to exposed lysine residues of proteins on the cell surface. Parental CHO cells, and CHO cells expressing WT and Ala⁷⁰⁷ IRs, respectively, were incubated with the reagent. Subsequently, cells were lysed, and insulin receptors were immune precipitated with a polyclonal antibody against the human insulin receptor. The immune precipitates were electrophoresed on SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Biotinylated proteins were visualized by peroxidase-conjugated streptavidin and ECL (Fig. 4). CHO cells expressing the WT receptor and Ala⁷⁰⁷ IR gave a similar picture of α and β chain labeling, although when the ratio of α/β subunit labeling was considered, the mutant receptor tended to show a higher ratio of α/β chain labeling. Using parental CHO cells, no signal was obtained at these exposure times. These findings indicate that the Ala⁷⁰⁷ mutant is transported to the cell surface to a similar extent as WT IRs.

The possibility exists that Ala⁷⁰⁷ IR undergoes a rapid internalization and degradation on incubation with insulin. Such a situation could also deplete the cell surface of insulin receptors, thereby leading to a state of insulin resistance. This situation is exhibited by the mutant insulin receptor Glu⁴⁶⁰ IR. This particular mutant originates from a leprechaun patient (8). As this mutation results in a decreased rate of the dissociation of the receptor-ligand complex, the presence of insulin leads to enhanced internalization and degradation. To examine whether Ala⁷⁰⁷ IR exhibits pronounced insulin-induced receptor internalization, CHO cells expressing the mutant receptor were incubated in medium containing 1 μ M insulin for up to 60 min at 37 °C, followed by biotinylation of the cell surface proteins and analysis of the mutant receptor for biotinylation. As a control, CHO cells expressing WT IRs were used. Fig. 5 shows that incubation of mutant receptor-expressing cells with insu-

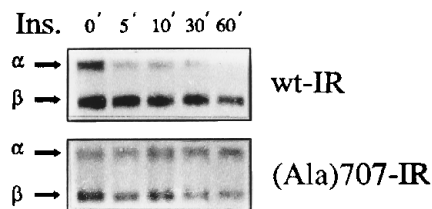


FIG. 5. **The effect of insulin on internalization of insulin receptors.** CHO cells expressing WT or Ala⁷⁰⁷ IR were incubated for the indicated time points (min) in medium with 1 μ M insulin. Subsequently, cell surface proteins were biotinylated, and insulin receptor biotinylation was assessed by Western blot, as described in the legend of Fig. 4.

lin does not markedly decrease the level of receptor biotinylation, indicating that a situation of enhanced insulin-induced receptor internalization does not take place. In the case of WT IR, incubation with insulin decreases the ratio of α subunit labeling over β subunit labeling. This lower level of α subunit labeling may result from binding insulin to the WT α subunit, thereby reducing the number of accessible Lys residues on the subunit for biotinylation or by inducing a conformational change. The mutant receptor, which does not show insulin binding, does not exhibit a decreased ratio of α/β subunit labeling on addition of insulin.

When β subunit labeling is considered in multiple experiments, an approximately 25% decrease is seen at 60 min of incubation time in the case of cells expressing WT IRs, reflecting the degree of internalization. No significant decrease is seen in the case of the receptor mutant.

Insulin Binding and Affinity Cross-linking—The various CHO clonal lines overexpressing the Ala⁷⁰⁷ IRs showed no increase in the level of high affinity binding of ¹²⁵I-insulin when compared with the parent CHO cell line (Table I). These binding experiments were performed at a tracer concentration of 30 pM ¹²⁵I-monoiodo-A14 insulin. We subsequently performed insulin binding and ¹²⁵I-insulin cross-linking experiments using insulin receptors partially purified from transfected CHO cells by chromatography on wheat germ agglutinin-Sepharose. In those experiments, insulin receptors are solubilized in a Hepes buffer containing 0.1% Triton X-100. It was found that in such a buffer ¹²⁵I-insulin (700 pM) in the presence of the protein cross-linker disuccinimidyl suberate was efficiently cross-linked to the α subunit of both WT and Ala⁷⁰⁷ IR. Judged from the degree of α subunit labeling, the efficiency of cross-linking seems similar (Fig. 6).

We also carried out cross-linking of ¹²⁵I-insulin to intact CHO cells either expressing WT or Ala⁷⁰⁷ IRs by disuccinimidyl suberate. Those experiments only showed labeling of the insulin receptor α subunit in the case of CHO cells expressing WT IR. Cells expressing the mutant receptor showed no detectable affinity labeling of the α subunit of the mutant receptor (not shown). These experiments indicate that in the case of intact cells, no transient interaction between insulin and Ala⁷⁰⁷ IR occurs, e.g. due to a high K_{off} rate. Only when detergent-solubilized insulin receptors were used, as present in the glycoprotein fraction, a detectable interaction between the mutant insulin receptor and insulin occurs.

Insulin-induced Autophosphorylation of Ala⁷⁰⁷ IR—Initially we examined whether insulin was able to induce autophosphorylation of the mutant receptor on transfected CHO cells. Cells expressing mutant IRs were incubated for 5 min in medium containing 1 μ M insulin, followed by removal of insulin by washing the cells with ice-cold phosphate-buffered saline. Subsequently, cells were lysed, the insulin receptor was immune precipitated, and the immune precipitate was examined by Western blotting for Tyr phosphorylation. As a control experiment, transfected CHO cells expressing a similar level of WT

TABLE I
Specific binding of insulin to various CHO cell lines

| Cell line | Binding ^a |
|--|------------------------------|
| Parental CHO | 10^{-17} mol/ 10^6 cells |
| CHO Ala ⁷⁰⁷ IR ^b | 2.2 ± 0.2 |
| CHO WT IR ^b | 2.3 ± 0.3 |
| | 247 ± 28 |

^a Values are mean \pm S.D.

^b Western blot data indicate similar levels of insulin receptor expression.

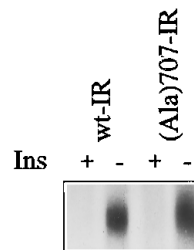


FIG. 6. **Cross-linking of ¹²⁵I-insulin to the insulin receptor α -subunit.** Glycoprotein was prepared from CHO cells expressing WT or Ala⁷⁰⁷ IR. Glycoprotein was incubated with 700 pM ¹²⁵I-insulin alone (–) or together with 1 μ M nonradioactive insulin (+). Cross-linking to the α subunit was by disuccinimidyl suberate. Subsequently, proteins were electrophoresed on SDS-polyacrylamide gels and visualized by autoradiography.

IRs were included. Incubation of cells expressing the WT IR showed a pronounced increase in Tyr phosphorylation of the β subunit, whereas cells expressing Ala⁷⁰⁷ IR showed no detectable β subunit Tyr phosphorylation (Fig. 7). When the phosphotyrosine phosphatase inhibitor phenyl arsenic oxide was included, no β subunit labeling was detected in the case of Ala⁷⁰⁷ IR (Fig. 7). Also, when cells were directly lysed in SDS sample buffer after incubation with insulin, Western blot analysis did not show any increase in phosphotyrosine labeling of cellular proteins in the case of the mutant receptor (not shown). These observations argue against a situation in which the mutant receptor is highly susceptible to dephosphorylation by a phosphotyrosine phosphatase.

We also examined insulin-induced receptor autophosphorylation using partially purified insulin receptors, obtained by chromatography on wheat germ agglutinin-Sepharose. Insulin receptors isolated in this way are solubilized in a buffer containing 0.1% Triton X-100. Insulin (1 μ M) was found to induce β chain phosphorylation in the case of WT and mutant receptors. Absolute levels of phosphate incorporation were approximately 15% lower in mutant receptors compared with WT IRs, although this difference did not reach statistical significance. When a dose-response relationship was determined, the ED₅₀ was the same for WT and mutant receptors. The only difference that showed statistical significance was a lower level of phosphate incorporation in mutant receptors at low insulin concentrations (Fig. 8).

To further examine the requirement for Triton X-100 for insulin-induced receptor autophosphorylation and to exclude the possibility that other components of the glycoprotein buffer, such as *N*-acetylglucosamine, are responsible for the restoration of the interaction between Ala⁷⁰⁷ IR and insulin, in a parallel experiment we incubated CHO cells expressing WT and mutant IRs for 5 min with insulin. This incubation occurred in an isotonic buffer, containing 1 μ M insulin, ATP, and MnCl₂, reagents required for *in vitro* autophosphorylation. As a control, cells were incubated in this buffer without insulin. Receptor autophosphorylation was analyzed by lysing the cells by addition of SDS sample buffer, and the lysate was analyzed for the presence of Tyr-phosphorylated proteins by Western

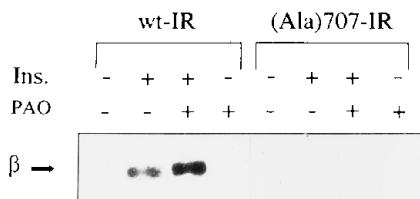


FIG. 7. **Insulin-induced Tyr phosphorylation of insulin receptors.** CHO cells expressing WT or Ala⁷⁰⁷ IR were kept quiescent or incubated with 1 μ M insulin for 10 min. Cells were lysed, and IRs were immune precipitated by a monoclonal antibody. The immune precipitate was subjected to Western blot analysis, and visualization of Tyr-phosphorylated protein was by monoclonal antiphosphotyrosine antibody and ECL. To enhance a possibly low level of Tyr phosphorylation of the insulin receptor β chain, phenylarsenic oxide (PAO, +) was included in some experiments.

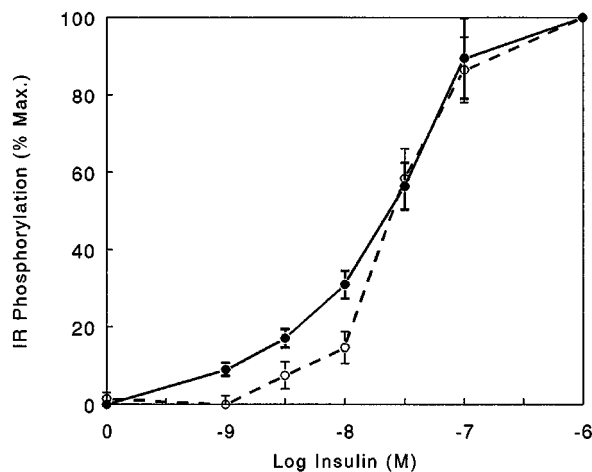


FIG. 8. **Insulin-induced β chain phosphorylation using partially purified insulin receptors.** Glycoprotein was prepared from CHO cells expressing WT or Ala⁷⁰⁷ IR. Glycoprotein was incubated with increasing concentrations of insulin for 30 min, followed by addition of [γ -³²P]ATP to induce autophosphorylation. Proteins were electrophoresed on SDS-polyacrylamide gels, and incorporation of ³²P in the insulin receptor β chain was quantitated by PhosphorImager analysis. Incorporation is expressed as percentage of maximum incorporation. ●, WT IRs; ○, Ala⁷⁰⁷ IRs. Values are averages of a triplicate experiment. Bars, S.D.

blot. Fig. 9 shows that under these conditions, insulin is capable of inducing receptor autophosphorylation only in CHO cells expressing WT IRs. However, when cells were incubated in the isotonic autophosphorylation buffer and Triton X-100 was added to lyse the cells, insulin induced a marked autophosphorylation of the mutant insulin receptor.

DISCUSSION

Leprechaun patient HO is homozygous for a mutation in the insulin receptor, which replaces Asp⁷⁰⁷ by Ala. This mutant receptor is normally processed into α and β subunits and transported to the cell surface. In the α chain, the proper positioning of Cys residues is essential for correct disulfide bond formation and for the formation of functional insulin receptors, which can be transported to the cell surface. A number of mutations in the α chain, found in patients with leprechaunism or Rabson Mendenhall syndrome, lead to transport-defective receptors, which are retained in the endoplasmic reticulum. Those mutations often involve amino acid residues near Cys residues, and the nature of the amino acid substitution is such that it is likely that they affect the folding of the polypeptide chain (5). Residue 707 is located near the carboxyl terminus of the α chain. This region has a high probability of existing in an α helical conformation. No Cys residues involved in disulfide bond formation are found in this part of the receptor. Because Ala⁷⁰⁷ IR is

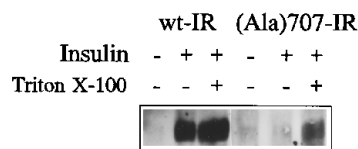


FIG. 9. **Effect of Triton X-100 on insulin-induced receptor β chain autophosphorylation.** CHO cells expressing WT or Ala⁷⁰⁷ IR were incubated in isotonic autophosphorylation medium without (–) or with (+) insulin, and incubation was for 5 min. Subsequently, Triton X-100 was added to indicated cells (+) to a concentration of 1%. Incubation was continued for another 3 min. Subsequently, SDS sample buffer was added, and the lysate was subjected to Western blot analysis and stained with antiphosphotyrosine antibodies. Visualization was by ECL. The position of the insulin receptor β chain is presented.

transported to the cell surface, it is expected that the mutation has no major effect on the overall folding of the α subunit. This situation is corroborated by protein structure predictions, which indicate that substituting Ala for Asp has no major effect on the probability of the carboxyl terminus adopting an α helical configuration. Because of the smaller side chain of Ala compared with Asp, the helix may become more flexible at that site.

Despite the predicted absence of a major effect on protein folding, Ala⁷⁰⁷ IR is unable to bind insulin when expressed on cells. The absence of insulin binding to cells expressing the mutant insulin receptor is not the result of enhanced internalization and receptor degradation, a situation seen in case of the Glu⁴⁶⁰ mutant (9). We conclude that the loss of insulin binding to cells expressing Ala⁷⁰⁷ IR is due to a direct effect of the mutation on the process of insulin binding. This loss of binding may, in principle, result from an essential contribution of Asp⁷⁰⁷ to the interaction of the receptor with insulin or to a conformational change induced by Ala⁷⁰⁷, which affects the structure of the insulin binding pocket.

When insulin binding to Ala⁷⁰⁷ IR is determined in the presence of Triton X-100, binding of insulin to the mutant receptor is seen, and this binding induces receptor autophosphorylation. The dose-response relationship for insulin-induced receptor autophosphorylation shows an unchanged ED₅₀ and a slightly, but significantly, lower autophosphorylation at low insulin concentrations. These observations indicate that in the presence of detergent, the mutation has a minor effect on the kinetics of insulin binding and that Asp⁷⁰⁷ does not provide interactions that are essential for insulin binding. Rather, the data suggest that the introduction of Ala⁷⁰⁷ induces a change in the positioning of the carboxyl-terminal α helix, which prevents insulin from binding when the receptor is on the cell surface. Detergent somehow is able to restore insulin binding. It may do so by making the structure of the carboxyl-terminal part of the receptor α subunit more flexible, thereby allowing the carboxyl terminus of Ala⁷⁰⁷ IR to adopt a position that allows insulin to bind. Another possibility is that the mutant receptor has gained the ability to bind to another protein, which prevents insulin from binding. We think that this situation is unlikely, because we do not observe coprecipitating proteins in insulin receptor immune precipitates from metabolically labeled Ala⁷⁰⁷ IR-expressing cells that are absent in cells expressing WT IRs.

Previously, sites on the insulin receptor that are important for the binding of insulin were identified in the NH₂-terminal region, especially Phe⁸⁹ (13–15), the cysteine-rich region (16–18), and the region encoded by exons 6 and 7 (9, 19, 20). Also, an important contribution of Ser³²³ to the formation of a high affinity insulin binding site on the receptor has been detected by the study of an insulin-resistant patient. Substituting Leu for Ser³²³ leads to a severe impairment in insulin binding without significantly altering the processing or cell surface expression of the receptor (21).

Subsequently, another site on the receptor has been identified that may contribute to insulin binding. Ultraviolet light-induced cross-linking of B25 azidophenylalanine insulin was found to occur in a peptide comprising residues 704–718 (22). This finding suggests a direct interaction between insulin and the carboxyl terminus of the receptor. Also in agreement with a role of the carboxyl terminus in insulin binding is the observation that a secreted form of the extracellular part of the insulin receptor, in which the carboxyl terminus of the α subunit is lacking, has a low affinity for insulin (23).

The region comprising amino acids 700–718 of the insulin receptor has a high probability to adapt an α helical conformation. As we see a complete loss of insulin binding to the mutant receptor in the absence of detergent, whereas addition of detergent restores binding, it is likely that the precise positioning of the carboxyl terminus is essential to enable binding of insulin. In case of Ala⁷⁰⁷ IR, this positioning may be somewhat changed, either due to an increased flexibility because of an Ala residue at 707, or because Asp stabilizes the spatial positioning of the α helix in a conformation that allows binding of insulin. Somehow, detergent is able to restore the correct spatial positioning of the carboxyl terminus of the α chain, thereby restoring the insulin binding site and insulin-induced receptor autophosphorylation. Although the precise mechanism by which Ala⁷⁰⁷ induces a complete loss of insulin binding is not known, our data indicate that the carboxyl terminus of the α subunit provides an essential contribution to the process of insulin binding. Besides, our results show that solubilization by detergent can markedly affect the interpretation of data on mutated receptors when insulin binding characteristics are considered.

Other evidence suggesting a contribution of amino acid residues in the carboxyl terminus of the α subunit to the binding of insulin comes from insulin binding studies of proreceptors. When mutations are present in the tetrabasic processing site at the junction of α and β subunits, proreceptors are generated, which appear on the cell surface (24). It was found that proreceptors without exon 11 have markedly reduced insulin binding compared with proreceptors with exon 11, which bind insulin with near normal affinity. Cleavage of proreceptors without exon 11 into α and β subunits markedly restored insulin binding. These findings suggest that the presence or absence of 12 amino acids encoded by exon 11 affects the folding and/or conformation of the α subunit carboxyl-terminal region in the proreceptor to confer altered binding of insulin. In the absence of exon 11, this region is in a strained conformation that disrupts the insulin binding site. Proteolytic cleavage at the α - β junction releases this constraint (25).

After completion of the manuscript, a publication appeared describing alanine-scanning mutagenesis of carboxyl-terminal amino acids in the insulin receptor α subunit (26). This study included the replacement of Asp⁷⁰⁷ by Ala. The results show that when a secreted form of the α subunit is examined for insulin binding, replacement of Asp⁷⁰⁷ by Ala did not affect insulin binding properties. Replacements of several other amino acid residues in the carboxyl-terminal region by Ala did

affect insulin binding. These results support our conclusion that Asp⁷⁰⁷ as such is not essential for insulin binding when receptors are in the solubilized state. Rather, when the insulin receptor is present on the cell surface, Asp⁷⁰⁷ is essential in keeping the carboxyl-terminal tail in a spatial conformation, which allows insulin to bind. Ala⁷⁰⁷ IR represents the first naturally occurring missense mutation in the carboxyl-terminal part of the insulin receptor α subunit that affects binding of insulin in the absence of an effect on receptor processing or transport.

Acknowledgments—We thank Drs. A. Ullrich and R. Lammers for providing the WT IR expression vector and K. Siddle for his generous gift of antibodies against the insulin receptor.

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Protein Chemistry and Structure:
An Insulin Receptor Mutant (Asp⁷⁰⁷ → Ala), Involved in Leprechaunism, Is Processed and Transported to the Cell Surface but Unable to Bind Insulin

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J. Biol. Chem. 1996, 271:18719-18724.
doi: 10.1074/jbc.271.31.18719

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