Effects of Amino Acid Replacements within the Tetrabasic Cleavage Site on the Processing of the Human Insulin Receptor Precursor Expressed in Chinese Hamster Ovary Cells*

(Received for publication, May 11, 1990)

Yusunao Yoshimasa†, Jeremy I. Paul§, Jonathan Whittaker¶, and Donald F. Steiner¶

From the Department of Biochemistry and Molecular Biology and the Howard Hughes Medical Institute, The University of Chicago, Chicago, Illinois 60637 and the Division of Endocrinology, State University of New York, Stony Brook, New York 11794

We have studied the specificity requirements for processing of the human insulin proreceptor by successively replacing each basic amino acid in the tetrabasic cleavage site with alanine. These mutated receptor cDNAs have then been overexpressed in Chinese hamster ovary cells, using vectors containing the mouse dihydrofolate reductase gene to amplify the transfected cDNAs in the presence of increasing concentrations of methotrexate. High levels of expression, ranging up to 6 x 10⁴ receptors/cell were achieved in these experiments. Replacement of the P1 arginine with alanine led to the complete suppression of processing, as occurs also in a naturally occurring serine mutation at this site (Yoshimasa, Y., Seino, S., Whittaker, J., Kakahi, T., Kosaki, A., Kuzuya, H., Imura, H., Bell, G. I., and Steiner, D. F. (1988) Science 240, 783-787). A small amount of cleavage at alternative sites was detected. Replacement of the P2 arginine or P3 lysine with alanine did not in either case affect conversion to mature α and β subunits, while replacement of the P4 arginine significantly inhibited processing. The binding isotherms for the processed versions of the receptor were comparable to previously published normal values. The unprocessed proreceptor bound insulin normally but was autophosphorylated less efficiently than the mature receptor, that is, ordered as follows: signal peptide-α subunit-RKRR cleavage site-β subunit (2, 3). While the newly synthesized precursor migrates from the endoplasmic reticulum to the Golgi apparatus, it undergoes several modifications including glycosylation, assembly into oligomers, and cleavage into mature α and β subunits (5).

We previously identified a defect in the proteolytic processing of the proreceptor into mature subunits in a diabetic patient with the type A syndrome of severe insulin resistance (6). Analysis of the patient’s receptor gene showed that this defect was likely to be due to the replacement of a single basic amino acid within the cleavage site by a serine (7). Studies of the mutant proreceptor expressed on the surface of EBV-transformed lymphocytes from this patient demonstrated that, while still capable of activation, its insulin binding affinity was markedly decreased (6). This observation supports the contention that proteolytic processing is an absolute requirement for the attainment of full receptor function. The physiological importance of proteolytic processing is also suggested by the finding that the tetrabasic cleavage site is well conserved in several closely related proteins, including the insulin-like growth factor I receptor precursor (8), Drosophila insulin receptor-like protein precursor (9), and the recently identified human insulin receptor-related protein precursor (10). Furthermore, a similar sequence motif is found at the cleavage sites of the envelope glycoproteins of a number of viruses (HIV-1 gp160, influenza hemagglutinin) in which proteolytic processing at these sites is essential for full viral infectivity (11, 12). Although the sequence requirements for cleavage of several of the viral glycoprotein precursors have been studied in considerable detail (13), no studies on the requirements for cleavage of the insulin proreceptor have been reported.

Studies on the biochemical properties of the insulin proreceptor have been limited because the level of receptor expression is relatively low in EBV-virus-transformed lymphocytes or fibroblasts, the cells commonly used to characterize the biochemical phenotype of such patients. In order to characterize the properties of the insulin proreceptor in greater detail and determine the requirements for processing, we have successively replaced each of the basic residues at the RKRR

a single hydrophobic membrane-spanning segment attached to a large cytoplasmic portion. The cytoplasmic portion contains a tyrosine kinase domain whose activation occurs upon insulin binding to the α subunit and is thought to be an initiating event in cellular responses to insulin (4). The α and β subunits are derived from a single polypeptide precursor, or proreceptor, that is ordered as follows: signal peptide-α subunit-RKRR cleavage site-β subunit (2, 3). The (Y subunit is located entirely outside the cell and is linked to a large cytoplasmic portion. The cytoplasmic portion contains a tyrosine kinase domain whose activation occurs upon insulin binding to the α subunit and is thought to be an initiating event in cellular responses to insulin (4). The α and β subunits are derived from a single polypeptide precursor, or proreceptor, that is ordered as follows: signal peptide-α subunit-RKRR cleavage site-β subunit (2, 3). While the newly synthesized precursor migrates from the endoplasmic reticulum to the Golgi apparatus, it undergoes several modifications including glycosylation, assembly into oligomers, and cleavage into mature α and β subunits (5).

We previously identified a defect in the proteolytic processing of the proreceptor into mature subunits in a diabetic patient with the type A syndrome of severe insulin resistance (6). Analysis of the patient’s receptor gene showed that this defect was likely to be due to the replacement of a single basic amino acid within the cleavage site by a serine (7). Studies of the mutant proreceptor expressed on the surface of EBV-transformed lymphocytes from this patient demonstrated that, while still capable of activation, its insulin binding affinity was markedly decreased (6). This observation supports the contention that proteolytic processing is an absolute requirement for the attainment of full receptor function. The physiological importance of proteolytic processing is also suggested by the finding that the tetrabasic cleavage site is well conserved in several closely related proteins, including the insulin-like growth factor I receptor precursor (8), Drosophila insulin receptor-like protein precursor (9), and the recently identified human insulin receptor-related protein precursor (10). Furthermore, a similar sequence motif is found at the cleavage sites of the envelope glycoproteins of a number of viruses (HIV-1 gp160, influenza hemagglutinin) in which proteolytic processing at these sites is essential for full viral infectivity (11, 12). Although the sequence requirements for cleavage of several of the viral glycoprotein precursors have been studied in considerable detail (13), no studies on the requirements for cleavage of the insulin proreceptor have been reported.

Studies on the biochemical properties of the insulin proreceptor have been limited because the level of receptor expression is relatively low in EBV-virus-transformed lymphocytes or fibroblasts, the cells commonly used to characterize the biochemical phenotype of such patients. In order to characterize the properties of the insulin proreceptor in greater detail and determine the requirements for processing, we have successively replaced each of the basic residues at the RKRR

The insulin receptor is a heterotetrameric integral membrane protein made up of two α and two β subunits (1-3). The α subunit is located entirely outside the cell and is linked by disulfide bonds to the extracellular portion of the β subunit. The β subunit is anchored to the plasma membrane through

* This study was supported by National Institutes of Health Grants DK 13914 and DK 20595. 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.

† Present address: Dept. of Endocrinology, Tenri Hospital, Nara 632 Japan.
§ Supported by the Howard Hughes Medical Institute and by JDF Grant 634643. Present address: Progenics Pharmaceuticals, Old Saw Mill River Rd., Tarrytown, NY 10591.
¶ To whom correspondence should be addressed: The Howard Hughes Medical Institute, The University of Chicago, Box 23, 5841 S. Maryland Ave., Chicago, IL 60637.
cleavage site with alanine and overexpressed these mutated receptor proteins in Chinese hamster ovary (CHO) cells.

Experimental Procedures

Materials—Crystalline porcine insulin was from Novo (Copenhagen) and bovine insulin at -20°C as a stock solution in 0.1% HCl (6 mg/100 ml) (10). E. coli or salmon testes DNA for gene transfer was isolated from Boehringer Mannheim, New England Biolabs, or Pharmacia LKB Biotechnology Inc. [1-Tyrosine-A14]-monoiodoinsulin was a gift from Dr. Bruce Frank, Lilly Co., and [1-Tyrosine-B26]-monoiodoinsulin, [125I]methionine, and [125I]sulfate were purchased from Amersham Corp. or Du Pont-New England Nuclear. Agarose-bound wheat germ agglutinin- (WGA) agarose were purchased from Vector Laboratories, Inc. and N-acetylglucosamine from Sigma. Diisuccinimidyl suberate and protein A-garose were purchased from Pierce Chemical Co. and protease inhibitors from Boehringer Mannheim and Sigma. α-Minimum essential medium without deoxynucleosides and fetal bovine serum were purchased from Gibco-Bethesda Research Laboratories and Met- and Cys-deficient Dulbecco’s modified Eagle’s medium was from Irvine Scientific. Lipofectin™ was purchased from Gibco-Bethesda Research Laboratories.

In Vitro Mutagenesis—A full-length human kidney cDNA (13-1), identical to that which we have described previously (14) except that the 3' Sst site is in the position of the AJ1 site in the 3'-untranslated region, was subcloned into the EcoRI and Sst site of M13 mp8. The cloning strand was used as a template for the two-primer methods of Zoller and Smith (15). Synthetic oligonucleotides were employed as mismatch primers to change Arg-732, Lys-735, Arg-734, and Arg-735 to alanines (numbering according to Ebina et al., Ref. 3). They were 5'-CGGCGCTTGGCAAGATGCC-3' , 5'-GAACTCTGGGCGCCG-3', 5'-GGAGACGTGCTTGCAAGATGCCG-3', and 5'-GCCAGGGAGATGCCGCTTGCAAGATG-3', respectively. After confirmation of the mutations by DNA sequencing using an oligonucleotide, 5'-CTCTCCGGACTCTGGGAC-3' as a primer, the double-stranded replicative form of the mutated M13 clones were prepared. EcoRI-SstⅡ fragments of mutant double-stranded M13 were excised and subcloned into an expression vector containing a full-length copy of the HIR coding region (pRT3HIR) which had been linearized by EcoRI digestion and partial SstⅡ digestion (29). The resulting plasmids were used to make the final constructs for expression.

Construction of Expression Plasmids—To overexpress the wild type and mutated insulin receptors we used the pMT3DSV2 expression vector which was kindly provided by Dr. D. J. Kaufman, Genetics Institute (Andover, MA). This vector contains the mouse dihydrofolate reductase gene under the transcriptional regulation of the adenovirus major late promoter and SV40 enhancer. It also contains unique EcoRI and NotⅠ sites for expressing cDNAs under the transcriptional control of the SV40 early promoter (16). The pRT3 vectors containing receptor inserts were digested with SstⅡ and filled in with Klenow fragment of DNA polymerase I. The filled in fragments were then ligated into the EcoRI site of pMT3DSV2. After the plasmids were transformed into competent DH5α cells, the orientation of the receptor inserts were confirmed by digestion with EcoRI. The resulting plasmids were designated pMT3DSV2/HIR, pMT3DSV2/P2-Α, pMT3DSV2/P2-Α, pMT3DSV2/P2-Α, and pMT3DSV2/P2-Α, respectively.

Transfection and Establishment of Cell Lines—Chinese hamster ovary (~5 × 10⁵ cells) lacking the endogenous dihydrofolate reductase gene (clone DGG4 kindly provided by Dr. L. Chassin, Columbia University) were transfected with each of the HIR constructs (10 μg) and the plasmid pSV2neo (1 μg) by the method of cationic liposome-mediated transfection (17). The cells were harvested 72 h after transfection, replated at lower density into 60 mm dishes and then selected with the antibiotic G418 (400 μg/ml), alone or together with several concentrations of methotrexate (50 and 100 nm) in a minimum essential medium (without deoxynucleosides) supplemented with 10% fetal bovine serum. After 3 weeks, colonies were harvested, pooled, replated, and amplified stepwise with increasing concentrations of methotrexate up to 2 μM. Methotrexate-resistant cell lines expressing high levels of insulin receptors were screened by binding of radiolabeled insulin in the presence or absence of unlabeled insulin.

Insulin Binding Assay—Established clonal cell lines were plated at the indicated numbers/well in 12- or 24-well dishes. After 24 h of incubation, cells were washed twice with Hank’s balanced salt solution supplemented with 10% fetal bovine serum. Cells and insulin were incubated in buffer A with 10 mg/ml bovine serum albumin containing 10-30 pmol of A-14 monoiodinated [125I]insulin and various concentrations of unlabelled insulin for 2 h at 15 °C. The cells were washed three times with ice-cold buffer A and then solubilized in 600 μl of 0.25 M NaOH for 30 min at 37 °C for measurement of radioactivity. Specific binding was determined by subtracting the amount of [125I]insulin bound in the presence of excess (10⁻⁶ M) unlabelled porcine insulin.

Metabolic Labeling of the Insulin Receptors—Prior to labeling, cells (2-5 × 10⁶ cells/100-mm dish) were washed twice with Dulbecco’s modified Eagle’s medium deficient in methionine and cysteine. The cells were then incubated with 200-400 μCi/dish each of [125I]methionine and [35S]sulfate for 16 h in deficient Dulbecco’s minimal essential medium supplemented with 10% dialyzed fetal bovine serum and 0.1 μM minimum essential medium nonessential amino acids. After incubation, cells were washed twice with ice-cold phosphate-buffered saline and then solubilized in 1 ml of 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100 containing 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, and 5 μg/ml leupeptin for 30 min on ice. After removal of insoluble material by centrifugation, metabolically labeled receptor proteins were immunoprecipitated by addition of 5 μl of anti-human insulin receptor monoclonal antibody 83-14 (18) (kindly provided by Dr. K. Siddle, University of Cambridge, United Kingdom) and 50 μl of protein A-garose. After 4 h of incubation at 4 °C, the immunoprecipitates were sedimented and washed three times with high salt buffer, (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% SDS, 0.5% Triton X-100) and were then heated for 3 min at 95 °C in 50 μl of 2× Laemmli’s sample buffer with or without 100 mM diithiothreitol just prior to electrophoresis on 7.5% polyacrylamide gels.

Insulin Receptor Phosphorylation—Cells (~5 × 10⁶) were solubilized in 5 ml of 1% Triton X-100, 50 mM Hepes, pH 7.6 (buffer A) or 30 mM Hepes, pH 7.8, 150 mM NaCl containing protease inhibitors for 30 min at 4 °C. The cell lysates were centrifuged at 12,000 rpm for 20 min at 4 °C, and the supernatant fraction was then incubated batchwise with 1 ml of wheat germ agglutinin- (WGA) agarose for 2 h at 4 °C (19). The resin was then washed three times with high salt buffer, (50 mM Tris-HCl, pH 7.4, 0.1% SDS, 0.5% Triton X-100) and were then heated for 3 min at 95 °C in 50 μl of 2× Laemmli’s sample buffer with or without 100 mM diithiothreitol just prior to electrophoresis on 7.5% polyacrylamide gels.

Phosphorylation was initiated by addition of 2 μl of [γ-32P]ATP (6000 Ci/mmol), and the reactions were incubated for 1 h at room temperature (20). The reactions were terminated by addition of EDTA to 20 mM. Immunoprecipitation of autophosphorylated receptor complexes were performed, as described above, and the immunoprecipitated proteins were analyzed by SDS-PAGE.

Cross-linking of the Insulin Receptor—[125I]-labeled insulin was cross-linked to solubilized receptor, as previously described (21). The lectin-purified receptors were incubated with 1 μCi of B-2-monoiodoinsulin insulin in a volume of 0.5 ml of buffer A (50 mM Hepes, pH 7.6, 150 mM NaCl, 10 mM MgCl₂, and 10 mM MnCl₂. Phosphorylation was initiated by addition of 2 μl of [γ-32P]ATP (6000 Ci/mmol), and the reactions were incubated for 1 h at room temperature (20). The reactions were terminated by addition of EDTA to 20 mM. Immunoprecipitation of autophosphorylated receptor complexes were performed, as described above, and the immunoprecipitated proteins were analyzed by SDS-PAGE.

Results

Selection of Cell Lines to Overproduce Wild Type and Mutated Insulin Receptors—Several cell lines that became resistant to high concentrations of methotrexate (up to 2 μM) were screened for the presence of insulin binding activity. Not

The abbreviations used are: CHO, Chinese hamster ovary; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; WGA, wheat germ agglutinin; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; IFG, insulin-like growth factor; HA, hemagglutinin.
only the cell lines transfected with wild type insulin receptors but all cell lines expressing mutated insulin receptors showed significant increases in specific insulin binding as compared with control CHO cells (data not shown).

Biosynthetic Labeling—Since studies of the binding of insulin to the mutant proreceptor (P1 serine substitution for arginine) on EBV-transformed lymphocytes from the patient showed that it was markedly reduced (6), we carried out biosynthetic labeling studies on cells expressing mutated insulin receptor precursors followed by immunoprecipitation with antireceptor antibodies to detect proreceptors and/or subunits. As demonstrated in Fig. 1, cell lines expressing insulin receptors with mutations within the cleavage site (designated P4-A, P3-A, P2-A, and P1-A) all synthesize receptor precursors having a molecular mass of 190 kDa in greatly increased amounts. Indeed, we found that receptor proteins solubilized from cell lines expressing the P3-A and P1-A mutations could be identified by Coomassie Blue staining of the SDS-polyacrylamide gels after immunoprecipitation, even though only a relatively small number of cells (~5 x 10⁶) were analyzed. However, the proteolytic processing of the respective receptor precursors during the chase period differs significantly among these lines (see below).

While the precursors with P3-A and P2-A substitutions are processed almost completely into mature α and β subunits (Fig. 1, lanes 2 and 3), the P1-A-substituted HIR gives rise predominantly to intact proreceptor, although a faint band is also evident that migrates slightly faster than the authentic α subunit (Fig. 1, lane 4). Further biosynthetic labeling experiments using CHO P1-A cells revealed that about 5% of the precursor (by densitometric scanning of the autoradiogram) is cleaved, giving rise to two proteins having molecular weights close to but not identical with those of normal antigen and β subunits. These results suggest that cleavage of this mutated HIR occurs at an alternative site(s) close to the normal processing site but that essentially no cleavage occurs at the normal site. These studies also revealed the presence of an additional band with a molecular mass of approximately 180 kDa in the CHO P1-A1 cells which could be cross-linked to insulin (see Fig. 5). The site of cleavage to produce this band is unknown but it probably contains the entire α subunit and much of the external portion of the β subunit. The receptor precursor having alanine at P4 is processed into subunits less efficiently than the processed versions, as shown by the relatively large amount of the proreceptor in relation to mature subunits (Fig. 1, lane 1).

High Level Expression of Receptor Proteins in CHO Cell Lines—When the insulin receptor from P3-A cells was solubilized, enriched by WGA-agarose chromatography and subjected to SDS-PAGE without immunoprecipitation, polypeptides migrating with the M, of HIR α and β subunits were visible after staining with Coomassie Blue (Fig. 2). This finding suggests that HIR subunits comprise a substantial portion (up to 50%) of the total protein present in WGA eluates from the P3-A cell line. We therefore estimate that 30–60 μg of insulin receptor is present in the WGA eluate from 5 x 10⁶ CHO P3-A cells (approximately 10 pg/cell; 10⁷ molecules/cell). It should be noted also that the integrity of the receptor protein is well maintained, in that the β subunit, which often appears to be degraded such that it exhibits a smaller than expected size as compared with the α subunit in purified receptor preparations, is comparable in amount to the α subunit. We have confirmed the identities of these two Coomassie Blue-stained bands by demonstrating that the M, = 135,000 band can be affinity labeled with [125I]tyrosine-BSH insulin and the M, = 96,000 band incorporates 32P04 in an insulin-dependent fashion in an in vitro autophosphorylation reaction (data not shown).

We compared the levels of expression attained in some of these cell lines with those achieved with other expression systems that have been used in this laboratory (14, 22). Equal amounts (10 μg) of WGA eluate were prepared from CHO P3-A cells, the fibroblast cell line (HIR2/S3 3.5) and Sf9 insect cells infected with a recombinant baculovirus encoding the human insulin receptor and were analyzed by cross-linking and autophosphorylation. The results showed that the highest level of expression was attained in the present study (data not shown).

For further biochemical analyses of the mutant receptors, we chose the CHO P1-A cell line as a source of the uncleaved proreceptor and the CHO P3-A cell line as a source of a processed version of the receptor.

Insulin Binding: Intact Cells—Fig. 3 shows the results of competitive binding experiments performed on whole cells and with partially purified HIR complexes from the P1-A and P3-A cell lines. In all instances, whether with intact cells or

![Fig. 1. Biosynthesis of insulin receptors with alanine replacements in each position of the tetrasgenic cleavage site. CHO cell lines were grown, labeled, and prepared for SDS-PAGE as described under "Experimental Procedures." Designations P1-P4 indicate positions of alanines relative to the site of cleavage (N terminus of β subunit). Note extensive processing to mature subunits in the P2-A and P3-A cell lines.](http://www.jbc.org/)

![Fig. 2. Coomassie Blue staining of HIR subunits enriched by WGA-agarose chromatography from overproducing P3-A cell line. Note low level of background staining. The estimated level of expression is in the range of 20–50 million receptors/cell.](http://www.jbc.org/)
Insulin Proreceptor-processing Site Mutants

Fig. 3. Comparison of competitive binding curves for PI-A (proreceptor) versus P3-A (processed receptor) in whole cells (left panels) or wheat germ agglutinin purified preparations (right panels). Insets show Scatchard transformations of the binding data (35). See "Experimental Procedures" for details.

solubilized HIR complexes, insulin binding to HIR yielded biphasic displacement curves typical of insulin binding to placental HIR. Cells expressing both the PI-A mutation (PI-A cells, upper left panel) and the P3-A mutation (P3-A cells, lower left panel) bound insulin with high affinity; ED50 for both cell lines ranged from 2–5 × 10⁻⁸ M. Upon transformation by the method of Scatchard (23), the PI-A cell line yielded a curvilinear plot, with Kd₁ = 6.5 × 10⁻⁹ M and Kd₂ = 1.4 × 10⁻⁷ M. The number of high and low affinity sites were estimated to be 9.6 × 10⁵ and 3.4 × 10⁶/cell, respectively, for the PI-A line. In contrast, transformation of the binding data obtained with the P3-A cell line yielded a linear plot characteristic of a single high affinity binding site with Kd = 5 × 10⁻⁷ M. The number of high affinity sites on these cells was estimated to be 6.2 × 10⁷/cell. After partial purification (see next section and Fig. 3), receptors from the P3-A cells bound insulin essentially normally. Hence, the linear Scatchard behavior of the receptor on intact cells may reflect either crowding due to the very high level of expression or an aberrant interaction of receptors with other cellular components.

Insulin Binding: Partially Purified Preparations—To compare the insulin binding ability of solubilized processed receptor and proreceptor, we first adjusted the amount of protein of WGA eluates prepared from CHO PI-A cells and CHO P3-A cells so as to give similar levels of specific insulin binding. These were 1 μg for CHO PI-A cells and 50 ng for CHO P3-A cells, respectively. Using these amounts, competitive insulin binding assays were performed and the data analyzed by the method of Scatchard (23). The results show that the ED50 for insulin binding of both receptor forms are almost identical, approximately 5–8 × 10⁻⁷ M. Transformation of these data by the method of Scatchard revealed complex curvilinear plots. These were interpreted in terms of a two-site model yielding a Kd₁ = 1.3 × 10⁻⁸ M and Kd₂ = 1.7 × 10⁻⁶ M for the processed receptor as against Kd₁ = 1.8 × 10⁻⁹ M and Kd₂ = 1.0 × 10⁻⁷ M, respectively, for the proreceptor. The maximal insulin binding capacity (Bmax) was also parallel in both receptor preparations, indicating that the insulin binding activity present in the WGA eluate from CHO PI-A cells can be attributed to the uncleaved proreceptor. (The small amounts of cleaved precursor present in this preparation could not account for this essentially normal binding behavior.)

The ratio of the amounts of proreceptor in CHO PI-A cells versus processed receptor in CHO P3-A cells observed in the foregoing insulin binding experiments was substantiated by the following data. When identical numbers of CHO PI-A and CHO P3-A cells were labeled biosynthetically and the immunoprecipitated receptor proteins were measured by densitometry of electrophoretograms (Fig. 4), 8-fold more processed receptor was produced in CHO P3-A cells as opposed to the amount of proreceptor produced in CHO PI-A cells. Comparison of the Coomassie Blue staining intensities after SDS-PAGE of WGA eluates from these two cell lines revealed that the background of absorbed glycoproteins other than
specifically. This finding is in agreement with the data from metabolic labeling, which suggested that two alternate processing sites appear to be used at low levels when cleavage at the normal processing site is prevented.

**Autophosphorylation of the Proreceptor**—Fig. 6 shows that the proreceptor becomes phosphorylated in an insulin-dependent fashion similarly to the β subunit. However, densitometric scanning of the autoradiogram shows a significant difference in dose dependence. Autophosphorylation of the proreceptor occurs more gradually with an E_{D50} about 10-fold higher than that for autophosphorylation of the mature β subunit. Fig. 6 also shows that the small amount of β subunit-like material derived from cleavage of the precursor having P1-A also becomes phosphorylated with a dose dependence similar to that of the β subunit of the processed receptor.

**Oligomeric State of the Proreceptor**—As shown in Fig. 7A, metabolic labeling of HIR subunits followed by SDS-PAGE under nonreducing conditions shows a single band of dimeric proreceptor from CHO P1-A cells with a molecular mass of approximately 380,000. In contrast, a more complicated pattern consisting of at least three major and two minor bands is derived from the processed receptor from the CHO P3-A cells. Three of the bands have high molecular weights and appear to represent various states of processing of the dimeric proreceptor. The two minor bands having M_{r} values of 230,000 and 95,000 correspond to the monomeric receptor precursor and a small amount of free β subunit, respectively.

To further examine their oligomeric states, the labeled receptor proteins were dissociated by graded concentrations of dithiothreitol and analyzed by SDS-PAGE under nonreducing conditions. As shown in Fig. 7B, the major bands with high molecular weights derived from processed receptor from the CHO P3-A cells are dissociated into four proteins. Among these are the receptor precursor and the native α and β subunits; the identity of the fourth receptor-related band having a M_{r} of 280,000, which is consistently found in the metabolic labeling studies, is unknown.

It is of interest that under the conditions used for reduction in these experiments (95 °C, 3 min) reduction of the inter-α-β disulfide bonds occurred more rapidly than reduction of inter α bonds, as occurs under milder conditions (24). In the case of the unprocessed form of receptor from CHO P1-A cells, the single band of M_{r} 380,000 seen in Fig. 7A yields two proteins upon reduction, the precursor and the proreceptor.

---

**Insulin Proreceptor-processing Site Mutants**
creasing concentrations of dithiothreitol
A
duction on the SDS-PAGE analysis
of metabolically labeled receptors in
as follows: lane 6, 0; lanes 1 and 7, 0.2
mM; lanes 2 and 8, 0.5 mM; lanes 3 and
9, 1.0 mM; lanes 4 and 10, 3.0 mM; lanes
5 and 11, 10 mM. Labeling, immunoprecipita-
tion, and SDS-PAGE radioautography were carried out as described un-
der "Experimental Procedures."

(Fig. 7B). Interestingly, with increasing concentrations of dithiothreitol the apparent molecular size of the 380-kDa band increases significantly. This change in mobility may result from partial reduction of disulfide bridges, allowing the molecule to become more asymmetric. Taken together, these data indicate that the proreceptor is inserted into the plasma membrane as an organized (α-β)2 dimer.

DISCUSSION

The high degree of conservation of the tetrabasic sequence at the cleavage site separating the α and β subunits in the precursors of both the insulin (human, Drosophila) and IGF-
I (human) receptors (3, 8, 9, 19) suggests that these sites would be likely to exhibit stringent requirements for processing by a special cellular trypsin-like protease. However, the results of the study reported here, in which we have systematically replaced all 4 of these residues individually with alanine, a small amino acid having a neutral side chain consisting of a single methyl group, indicate that this is not the case.

This study was prompted by our analysis of a mutation in the insulin receptor gene of a patient with severe insulin resistance due to defective insulin binding. Various studies revealed the presence of essentially normal numbers of un-
cleaved proreceptors on the surface of EBV-transformed lymphocytes from this patient (6, 7). No mature α or β subunits could be identified on these cells. Sequence analysis of HIR exon 12 (25) from this patient's genomic DNA revealed a point mutation in both copies of the gene that changed the P1 arginine residue at the cleavage site to serine. It was further shown that graded trypsin treatment resulted in the appearance of some normal subunits accompanied by partial restoration of binding activity (7). We were interested in further characterizing the effects of this mutation on receptor function and also in defining more clearly the requirements for cleavage. The amino acid sequences of the cleavage sites of some viral glycoproteins and the influenza hemagglutinin (HA) are also similar to that of the insulin receptor. Studies of viral variants have indicated that a canonical sequence, R-
X-R/K-R, is required for efficient processing of these pro-
tins (13). Thus, cellular proteases having similar specificities may be involved in the proteolytic processing of all of these proteins.

The present study demonstrates that in CHO cells stably expressing insulin receptor constructs at high levels 1) alanine substitution at P1 profoundly impairs processing, 2) alanines at positions P2 and P3 have no effect on cleavage, and 3) alanine at P4 reduces the efficiency of processing. These observations are thus consistent with the existence of a cellular protease with restricted trypsin-like specificity. Since mutational analyses of the cleavage sites of membrane glycoproteins similar to the insulin receptor are limited, the specificity requirements of these processing enzymes remains undefined. However, a study examining the requirements for cleavage of the influenza HA suggests that these proteases require the presence of several basic amino acids at the cleavage sites, preceded by a β turn (12, 13). The insulin and IGF receptors also have a region with a high predicted probability for a β turn just upstream of the cleavage site as shown in Fig. 8. A unique protease with a specificity similar to that of the insulin proreceptor convertase has also been reported to be involved in processing several peptide precursors in Xenopus laevis skin (26). These observations, along with ours, suggest that protease(s) with specificities for clusters of basic amino acids may be widespread in cells and probably differ in specificity and other properties, such as pH optimum and subcellular localization, from the processing enzymes that cleave prohormones which usually recognize single or adjacent pairs of basic amino acids.

Our experiments indicate, in addition, that the cellular protease(s) is capable of processing receptor precursor with very great efficiency, as exemplified by the experiments using CHO P3-A in which essentially complete proteolytic process-

![Fig. 7](#)

**FIG. 7.** Effects of progressive reduction on the SDS-PAGE analysis of metabolically labeled receptors in the P3-A and P1-A cell lines. Panel A shows results obtained in the absence of reductant; lane 1, P3-A cells; lane 2, P1-A cells. Panel B shows effects of increasing concentrations of dithiothreitol as follows: lane 6, 0; lanes 1 and 7, 0.2 mM; lanes 2 and 8, 0.5 mM; lanes 3 and 9, 1.0 mM; lanes 4 and 10, 3.0 mM; lanes 5 and 11, 10 mM. Labeling, immunoprecipitation, and SDS-PAGE radioautography were carried out as described under "Experimental Procedures."

![Fig. 8](#)

**FIG. 8.** Amino acid sequences in the region of the cleavage site of the insulin proreceptor, the related Drosophila and IGF-I receptors, and several viral envelope glycoproteins. The residues in the insulin receptor cDNA that were replaced individually by alanines in this study are shown enclosed in the box. The position of the naturally occurring serine substitution (7) is noted above the box. The location of the putative β turn was predicted using the rules of Chou and Fasman (34).
ing of the proreceptor occurs despite the fact that it is being produced at levels that are several thousand-fold greater than normal. Conversely, it also can be concluded that the constraining effect of P1 substitutions on processing is very strict because the proteolytic processing is impaired almost completely even though the receptor precursor is being overproduced in large amounts.

This latter finding contrasts with the reported observation that only a small percentage of the envelope glycoprotein (gp160) of human immunodeficiency virus I is cleaved into gp120 and gp40 (27). The cleavage site in this protein, REKR, would appear to be a good substrate in itself but may be influenced by other features of the local environment of the precursor. Cleavage of the gp160 envelope precursor has been shown to occur predominantly in the cis or medial compartments of the Golgi and is unusually slow in that it requires several hours for its transfer from the RER to the Golgi complex (28).

We have used cells expressing the insulin proreceptor due to substitution of alanine for arginine at P1 of the cleavage site to characterize the proreceptor in greater detail. Several interesting aspects of the proreceptor have been revealed which appear to contrast with the properties of the proreceptor resulting from a naturally occurring serine substitution at this position in the patient with severe insulin resistance (6, 7). The proreceptor thus expressed possesses high insulin binding affinity and shows a complex curvilinear Scatchard plot that is almost identical to that of the normally processed receptor. Also, phosphorylation of the proreceptor is only moderately reduced in comparison with the processed receptor, implying that signal transduction through the proreceptor is not grossly impaired. These observations contrast sharply with the behavior of the proreceptor on the patient’s EBV-transformed lymphocytes, as described above.

A clue to reconciling these discrepancies may come from findings that exon 11 of human insulin receptor gene is alternatively spliced to give rise to two receptor (proreceptor) isoforms, as originally indicated by the data of Ebina et al. (3), and that the alternative splicing event occurs in a tissue-specific manner. Several groups (29, 30) have reported that a receptor isoform lacking exon 11 is expressed in a number of tissues, including cell lines of B-lymphocyte origin, whereas both receptor isoforms are expressed in liver, kidney, and placenta. Since the human insulin receptor cDNA that we used as a template for these mutagenesis studies contains exon 11, the differences in the properties of the proreceptors observed in the two kinds of cells could be ascribed to the presence or absence of exon 11.

Although exon 11 encodes only 12 amino acids, these are located immediately upstream of the cleavage site (25) and relatively distant from the putative insulin-binding domain (31) of the insulin receptor. Nonetheless, it may be possible that the presence or absence of the short stretch of amino acids encoded by exon 11 may affect the folding and/or conformation of the proreceptor so as to confer altered sensitivity to insulin. In the influenza hemagglutinin (HA), the presence or absence of a glycosylation site near the N terminus of the protein is in sufficient proximity to the cleavage site to significantly affect its processing (12). In the insulin receptor evidence suggests that the insulin binding site may include portions of the N-terminal domain of the α subunit (29, 30), and if organized like the influenza HA molecule the binding site might be directly hindered by the shorter (exon 11 minus) αβ junction. Such a difference in receptor function could be of physiological relevance. Experiments are currently underway to compare the biochemical properties of two isoforms of the proreceptor.

Some of our data on the proreceptor indicates properties that differ subtly from those of the processed receptor, e.g., the dose dependence for phosphorylation of the proreceptor is right-shifted (EDso = 1 × 10−8 M for the proreceptor and EDso = 2 × 10−9 M for the processed receptor) although the maximal incorporation of PO4 above the basal level is comparable.

Another difference is in the oligomeric state of the proreceptor as shown by SDS-PAGE under nonreducing and graded reducing conditions. The proreceptor forms an (α-β)3 dimer linked together by disulfide bonds and the dimer appears to have a more elongated shape, as indicated by the data that the proreceptor dimer migrates much more slowly when the intermolecular disulfide bonds are dissociated with graded concentrations of dithiothreitol. Since phosphorylation of the proreceptor, as well as the mature β subunit, may require a conformational change of the receptor protein after insulin binding to the α subunit, an altered oligomeric state of the proreceptor might influence the dose dependence for phosphorylation as mentioned above.

Evidence that the proreceptor with exon 11 is functional might shed light on the structural (quaternary) requirements for signal transduction through molecules that are related to the insulin receptor. With regard to this point it is of interest that the insulin receptor of the stingray, a cartilaginous fish, appears to be a proreceptor dimer like the mutated human proreceptor studied here (32). The stingray receptor binds both insulin and IGF-I with high affinity and undergoes autophosphorylation (with either ligand). It has also been observed that the wild-type growth hormone receptor must oligomerize on the cell surface in order to accomplish a full signal transduction (33). Thus, one requirement for signal transduction through the relevant receptors appears to be dimerization.

Acknowledgments We wish to thank Paul Gardner for his assistance in preparing the oligonucleotides used for mutagenesis, Terri Reid for skillful assistance in the maintenance of cell cultures, and Florence Rosenfeld for expert assistance in preparing this manuscript.

REFERENCES

1. Czech, M. P. (1985) Annu. Rev. Physiol. 47, 357-381
2. Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petrussali, M., Oveys, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., and Ramachandran, J. (1985) Nature 313, 755-761
3. Ebina, Y., Ellis, L., Narnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Maslarz, R., Kan, Y. W., Goldfine, I. D., Roth, R. A., and Rutter, W. J. (1985) Cell 40, 747-753
4. Rosen, O. M. (1987) Science 237, 1452-1458
5. Olson, T. S., Bamberger, M. J., and Lane, M. D. (1988) J. Biol. Chem. 263, 7342-7351
6. Kakehi, R., Hisatomi, A., Kuzuya, H., Yoshimasa, Y., Okamoto, M., Yamada, K., Nishimura, H., Kosaki, A., Nawata, H., Umeda, R., Ibashishi, H., and Imura, H. (1988) J. Clin. Invest. 81, 2009-2022
7. Yoshihama, Y., Seino, S., Whittaker, J., Kakehi, T., Kosaki, A., Kuzuya, H., Imura, H., Bell, G. I., and Steiner, D. F. (1988) Science 240, 783-787
8. Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J., and Fujita-Yamaguchi, R. (1986) EMBO J. 5, 2503-2519
9. Nishida, Y., Hata, M., Nishizuka, Y., and Ebina, Y. (1986) Biochem. Biophys. Res. Commun. 141, 474-481
10. Shier, P., and Watt, V. M. (1989) J. Biol. Chem. 264, 14005-14008
11. McCune, J. M., Rabin, L. B., Feinberg, M. B., Lieverman, M.,...
12. Kawaoka, Y., and Webster, R. G. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 324–329
13. Webster, R. G., and Rott, R. (1987) Cell 50, 665–666
14. Whittaker, J., Okamoto, A. K., Thys, R., Bell, G. I., Steiner, D. F., and Hofmann, C. A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5237–5241
15. Zoller, M. J., and Smith, M. (1984) DNA 3, 479–488
16. Kaufman, R. (1990) Methods Enzymol. 185, 537–560
17. Felger, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrup, J. P., Ringold, G. M., and Danielsen, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7413–7417
18. Soos, M. A., Siddle, K., Potts, B. J., Martin, M. A., and Danielsen, M. (1987) J. Biol. Chem. 262, 8395–8401
19. Hedo, J. A., Harrison, L. C., and Roth, J. (1981) Biochemistry 20, 3385–3393
20. Tavare, J. M., and Denton, R. M. (1988) J. Biochem. (Tokyo) 252, 697–715
21. Pileh, P. F., and Czech, M. P. (1981) J. Biol. Chem. 255, 1722–1731
22. Paul, J. I., Tavare, J. M., Denton, R. M., and Steiner, D. F. (1990) J. Biol. Chem. 265, 13074–13083
23. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 190, 221–234
24. Boni-Schnetzler, M., Scott, W., Waugh, S. M., DiBella, E., and Pilch, P. F. (1987) J. Biol. Chem. 262, 8395–8401
25. Seino, S., Seino, M., Nishi, S., and Bell, G. I. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 114–118
26. Kuks, P. F. M., Creminon, C., Leseney, A.-M., Bourdais, J., Morel, A., and Cohen, P. (1989) J. Biol. Chem. 264, 14609–14612
27. Willey, R. L., Bonifacino, J. S., Potts, B. J., Martin, M. A., and Klausner, R. D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9580–9584
28. Stein, B. S., and Engleman, E. G. (1990) J. Biol. Chem. 265, 2640–2649
29. Seino, S., and Bell, G. I. (1988) Biochem. Biophys. Res. Commun. 159, 312–316
30. Moller, D. E., Yokota, A., Caro, J. R., and Flier, J. S. (1989) Mol. Endocrinol. 3, 1263–1269
31. DeMeys, P., Gu, J.-L., Shymko, R. M., Kaplan, B. E., Bell, G. I., and Whittaker, J. (1990) Mol. Endocrinol. 4, 400–416
32. Stuart, C. A. (1988) J. Biol. Chem. 16, 7881–7886
33. Schlessinger, J. (1988) Biochemistry 27, 3119–3123
34. Chou, T.-Y., and Fasman, G. D. (1979) Annu. Rev. Biochem. 47, 251–276
35. Rodbard, C., and Munson, P. J. (1980) Anal. Biochem. 107, 220–239
Effects of amino acid replacements within the tetrabasic cleavage site on the processing of the human insulin receptor precursor expressed in Chinese hamster ovary cells.

Y Yoshimasa, J I Paul, J Whittaker and D F Steiner

*J. Biol. Chem.* 1990, 265:17230-17237.

Access the most updated version of this article at [http://www.jbc.org/content/265/28/17230](http://www.jbc.org/content/265/28/17230)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/28/17230.full.html#ref-list-1](http://www.jbc.org/content/265/28/17230.full.html#ref-list-1)