A Leucine to Proline Mutation at Position 233 in the Insulin Receptor Inhibits Cleavage of the Proreceptor and Transport to the Cell Surface†

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ABSTRACT: We have previously shown that a homozygous mutation encoding a substitution of proline for leucine at position 233 in the insulin receptor is linked with the syndrome of leprechaunism, being a lethal form of insulin resistance in newborn children. Specific binding of insulin and insulin-stimulated autophosphorylation of the insulin receptor are nearly absent in fibroblasts from the leprechaun patient. To examine the molecular basis of the observed insulin receptor abnormalities, CHO cell lines overexpressing mutant insulin receptors were made by transfection. The results show that the mutation inhibits cleavage and transport of the proreceptor from intracellular sites to the cell surface. As the mutant receptor is poorly precipitated by two different monoclonal antibodies recognizing epitopes on undenatured wild-type α-subunits, the mutation probably affects overall folding of the α-subunit. The mutant proreceptor is unable to bind insulin and exhibits no insulin-stimulated autophosphorylation. These data explain the abnormalities seen in the patient's fibroblasts. Pulse–chase labeling experiments on transfected cells show that the mutant precursor has an extended half-life ($\sim$5 h) compared to the precursor of wild-type insulin receptors ($\sim$2 h). This mutation is the first example of a naturally occurring mutation in the insulin receptor which completely blocks cleavage of the proreceptor and transport to the cell surface.

The insulin receptor is a tetrameric cell-surface protein composed of two α- and two β-subunits, interlinked by disulfide bonds. The subunits are encoded by one single open-reading frame (Ullrich et al., 1985; Ebina et al., 1985). The α-subunit is completely located extracellularly whereas the β-subunit is a transmembrane protein. When insulin binds to the α-chain, tyrosine kinase activity is activated at the cytoplasmic part of the β-chain (White & Kahn, 1986).

A variety of naturally occurring mutations in the insulin receptor have been found which interfere with proper insulin action and which are involved in the pathogenesis of a variety of syndromes like the type A syndrome of insulin resistance, leprechaunism, lipoatrofic diabetes mellitus, and the Rabson Mendenhall syndrome, as reviewed recently (Taylor et al., 1990). Some of these mutations result in the formation of truncated receptors (Kadowaki et al., 1988, 1990a). Other mutations introduce amino acid substitutions which occur in various regions of the insulin receptor. These mutations interfere with the transport of the receptor to the cell surface (Accili et al., 1990), cause enhanced down-regulation of the insulin receptor (Taylor et al., 1990), deactivate the tyrosine kinase domain (Moller & Flier, 1988; Taira et al., 1989; Odawara et al., 1989; Moller et al., 1990), or interfere with proper proteolytic cleavage of the proreceptor (Yoshimasa et al., 1988; Kobayashi et al., 1988).

We have recently described the leprechaun patient G. being homozygous for a proline for leucine mutation at position 233 in the insulin receptor (Klinkhamer et al., 1989). Examination of fibroblasts from this patient showed a loss of high-affinity insulin binding sites, and using the glycoprotein fraction, almost no insulin-stimulated receptor β-chain autophosphorylation was seen (Maassen et al., 1988).

To investigate the molecular basis of the observed receptor dysfunctions, we have made CHO cell lines expressing elevated levels of mutant receptors. The characteristics of the receptor with the proline for leucine mutation at position 233 were studied in these cells.

Experimental Procedures

Materials and Methods. Monocomponent human insulin was obtained from Novo, Denmark; [^{35}S]methionine, ^{32}P-labeled nucleotides, Na^{125}I, and A14-mono[^{125}I]iodoinsulin were from Amersham, U.K. Lactoperoxidase, glucose oxidase, neuraminidase, and endoglycosidase H were from Boehringer, Germany; bovine serum albumin (BSA) RIA grade was from USB; Disuccinimidyl suberate (DSS)[1] was from Pierce. Other reagents were analytical grade. Immunoprecipitates were analyzed on 8% SDS–polyacrylamide gels (SDS–PAGE) using as markers myosin (220 kDa), β-galactosidase (126 kDa), phosphorylase (97 kDa), bovine serum albumin (68 kDa), and ovalbumin (45 kDa). The protein markers were obtained as a high molecular weight kit from Bio-Rad. In the case of ^{125}I or ^{32}P labeling, the dried SDS–PAGE gels were exposed to Kodak-XAR films using intensifying screens. In the case of ^{35}S labeling, the gels were impregnated with Amplify (Amersham, U.K.), prior to drying and exposure to Kodak-XAR films. Monoclonal antibodies α-HIR-538 and α-HIR-1 were from Amersham and Immunotech-Luminy, Marseille, re-

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[1] Abbreviations: DSS, disuccinimidyl suberate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylate gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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spectively. The latter was kindly provided by Dr. A. van Achthoven.

Insulin Receptor Expression Vectors. The expression vector containing a wild-type cDNA construct for the human insulin receptor, driven by an SV40 promotor and containing in addition the gene for dihydrofolate reductase and G-418 resistance (pWT-HIR), was previously described (Riedel et al., 1986). The pWT-HIR expression vector was converted into a vector expressing the insulin receptor with the Pro for Leu substitution at position 233 (p233-HIR) by exchange of the Not1-Bgl2 fragment (nucleotides 76–1580) from the insulin receptor cDNA with that of the leprechaun patient. The correct structure of the exchanged fragment was confirmed by DNA sequencing.

Expression of Insulin Receptor cDNA in CHO Cells. The WT-HIR and 233-HIR vectors were transfected in CHO cells by the calcium phosphate technique (Gram & Van der Eb, 1973). Colonies resistant to 400 μg/mL G-418 were picked up, and by plating at limiting dilution, single colonies were obtained.

Insulin Binding. Control CHO cells and transfected cells were grown to confluency in 24-well dishes. After the cells were washed with PBS, they were incubated for 1 h at 20 °C in PBS (pH 7.8)–1% BSA–50 pM [^{125}I]insulin (2000 Ci/mmol). Cells were washed 3 times with ice-cold PBS and lysed in 0.1 M NaOH–0.1% SDS, and radioactivity was measured by liquid scintillation counting. Nonspecific binding of insulin was determined by addition of 1 μM nonradioactive insulin to the radioactive insulin.

[^{35}S]*Methionine Labeling of Cells.* Confluent CHO cells in a 3-cm dish were labeled for 8 h with 0.15 mCi/mL [^{35}S]methionine in methionine-free DMEM (GIBCO), supplemented with 10% dialyzed fetal calf serum. Cells were washed with PBS and lysed in 0.5 mL of ice-cold lysis buffer (10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 1% Triton X-100, 0.1 mM PMSF, 1 μg/mL Trasylol, and 1 μg/mL leupeptin). Debri was removed by centrifugation for 10 min in an Eppendorf centrifuge. Supernatant was saved and used for immune precipitation.

In the case of pulse–chase labeling experiments with [^{35}S]methionine, cells were grown in 24-well dishes, and labeling with [^{35}S]methionine was for 20 min as described. The medium was changed to DMEM without [^{35}S]methionine. At time points 0, 1, 3, and 7 h, cells were harvested and lysed as described above.

Cell Surface Iodination. Cells were grown in 3-cm dishes to confluency and washed twice with PBS. Three hundred microliters of PBS was added containing 20 mM glucose, 6 units of glucose oxidase, 6 μg of lactoperoxidase, and 0.5 mCi Na^{125}I. Incubation was for 20 min at room temperature. Cells were washed with PBS. Lysis and immunoprecipitation were as described for metabolic labeling.

Immune Precipitation. To 100 μL of methionine-labeled cell lysate was added 750 μL of immune precipitation buffer (IP buffer: 50 mM Tris-HCl, pH 7.8, 200 mM NaCl, 20 mM NaF, 10 mM ATP, 10 μM sodium vanadate, 0.1 mM EDTA, 5 mM 4-nitrophenyl phosphate, 0.1% NP40, 1 μg/mL leupeptin, and 1 μg/mL PMSF). Five microliters of polyclonal αIR was added, and the mixture was incubated overnight at 4 °C. Immune complexes were isolated by *Staphylococcus aureus* and after four washes with 1 mL of IP buffer analyzed on SDS–PAGE.

Autophosphorylation and Affinity Purification of WGA-Purified Receptors. Glycoprotein was prepared as previously described (Klinkhamer et al., 1989). Autophosphorylation was without insulin or in the presence of 50 nM insulin. Conditions were as previously described (Klinkamer et al., 1989). Affinity labeling of the insulin receptor by [^{125}I]insulin was by cross-linking using 0.2 mM disuccinimidyl suberate as earlier described (Klinkhamer et al., 1989).

Enzymatic Deglycosidation. Immune precipitates of ^{35}S-labeled cell lysates were prepared as described above with the modification that Sepharose-G was used instead of *Staphylococcus aureus.* The resin–immune precipitate was suspended in 50 μL of neuraminidase buffer (40 mM Tris-HCl, pH 7.8, 4 mM $CaCl_2$, 0.1 mM PMSF, and 1 μg/mL Trasylol) containing 5 milliunits of neuraminidase. Incubation was for 2 h at 37 °C. As control, an incubation without enzyme was done. In the case of endoglycosidase H incubation, the resin was incubated for 2 h at 37 °C in 50 μL of Endo-H buffer (20 mM sodium acetate, pH 5.5, 0.1 mM PMSF, 0.01% SDS, and 100 mM 2-mercaptoethanol) containing 5 milliunits of Endo-H. As control, an incubation without enzyme was performed.

Results

Cell Lines and Immune Precipitations. CHO cells were transfected with the expression vector pWT-HIR which carries a cDNA construct for the human wild-type insulin receptor and p233-HIR which encodes the insulin receptor with the proline for leucine substitution at position 233. The expression vector carries in addition a gene for resistance against the antibiotic G-418 and the dihydrofolate reductase gene which enables amplification by methotrexate. G-418-resistant colonies were examined for insulin receptor expression by measuring binding of [^{125}I]insulin and by metabolic labeling with [^{35}S]methionine followed by immune precipitation with a polyclonal antibody against the insulin receptor. The immune precipitates were analyzed by SDS–polyacrylamide gels and fluorography. In the transfection experiment of CHO cells with pWT-HIR, most of the G-418-resistant colonies showed increased binding of insulin.

Twelve cell lines were investigated, and the percentage of [^{125}I]insulin binding ranged between 0.8 and 15%, the average being 9%. Parental CHO cells bound 0.4% of added [^{125}I]-insulin. Metabolic labeling confirmed increased expression of insulin receptor α- and β-chains. In the case of transfection with 233-HIR, five G-418-resistant colonies were examined by this approach, and none of these clones showed increased binding of [^{125}I]insulin. Specific binding of [^{125}I]insulin was 0.4%, similar to binding parental CHO cells. In Figure 1, the results of the metabolic labeling experiments are shown. In this figure, CHO cells expressing wild-type and mutant receptors were selected having similar levels of insulin receptor mRNA as concluded from Northern blot analysis; however, the other clonal cell lines gave similar results. The selected HIR-CHO cell line had approximately 60 000 high-affinity insulin binding sites with a K_D of ~0.5 nM. In the case of the HIR-CHO cell line, the polyclonal antibody precipitates the α- and β-chains of the insulin receptor next to some 210-kDa α–β proreceptor. In the CHO cell line expressing the mutant insulin receptor (233HIR-CHO), only a 210-kDa band is specifically precipitated by the polyclonal antibody. No α- and β-chains were detected. The 210-kDa protein in both cell lines is indistinguishable with respect to electrophoretic mobilities on SDS–PAGE.

When two monoclonal antibodies (α-HIR-538 and α-HIR-1) recognizing epitopes on the α-subunit were used for immune precipitation experiments, the WT precursor, together with α- and β-subunits, was precipitated efficiently whereas the mutant precursor was also precipitated, albeit with a strongly

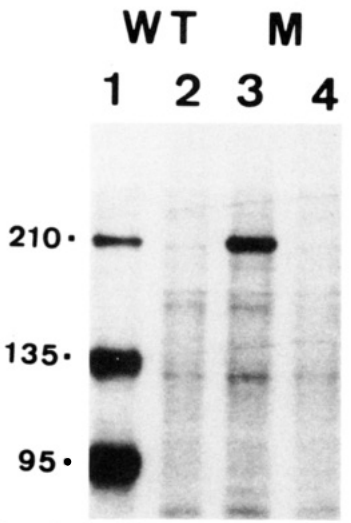

FIGURE 1: Biosynthesis of insulin receptors by HIR-CHO and 233CHO cells assessed with [^{35}S]methionine labeling. Cells were labeled for 8 h with [^{35}S]methionine, and proteins were immunoprecipitated from each cell lysate. Lanes 1 and 2, HIR-CHO cells (WT); lanes 3 and 4, 233HIR-CHO cells (M). Lanes 1 and 3, precipitation with polyclonal α-IR; lanes 2 and 4 with nonimmune rabbit serum. Samples were analyzed by SDS–PAGE using reducing sample buffer. Positions of β- and α-subunits and proreceptor are indicated at respective positions of 95, 135, and 210 kDa. Exposure was for 16 h.

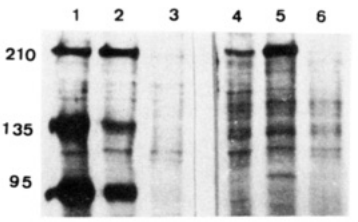

FIGURE 2: Immune precipitation of the wild type and mutant 210-kDa precursor by a polyclonal and a monoclonal antibody. Cells were metabolically labeled with [^{35}S]methionine as described in the legend of Figure 1. Lanes 1–3, HIR-CHO cells; lanes 4–6, 233HIR-CHO cells. Lanes 1 and 4, immune precipitation with monoclonal α-HIR538; lanes 2 and 5 with polyclonal α-IR; lanes 3 and 6 with rabbit nonimmune serum.

reduced efficiency. Figure 2 shows a comparison of immune precipitation of the wild-type and mutant 210-kDa precursor by polyclonal and monoclonal α-HIR-538. Similar results were obtained using monoclonal α-HIR-1. Both monoclonal antibodies do not recognize the wild-type insulin receptor after denaturation by SDS followed by renaturation in immune precipitation buffer. The polyclonal antiserum does recognize the receptor after the denaturation–renaturation step (not shown).

The 210-kDa Protein Is a Precursor of the Insulin Receptor. The 210-kDa protein is synthesized in 233HIR-CHO cells, and its size and recognition by antibodies against the insulin receptor suggest that it is the high mannose form precursor of the insulin receptor. This was substantiated by enzymatic deglycosylation with endoglycosidase H. The mutant and wild-type 210-kDa bands were both converted into a 180-kDa band (Figure 3). Both 210-kDa bands were resistant to treatment with neuraminidase under conditions where the mature α- and β-subunits are shifted to a lower molecular weight form (Figure 3).

Both 210-kDa precursors also behaved similarly with respect to binding to wheat germ agglutinin–Sepharose. When ^{35}S-labeled cell lysates of the HIR-CHO and 233HIR-CHO cells were chromatographed on wheat germ agglutinin–Sepharose followed by immune precipitation with antibodies against the

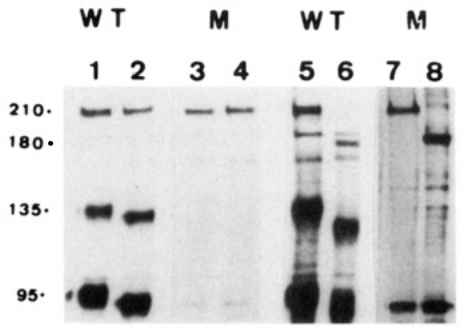

FIGURE 3: Enzymatic deglycosylation of wild-type and mutant insulin receptors. HIR-CHO and 233HIR-CHO cells were labeled biosynthetically with [^{35}S]methionine for 8 h. Cells were lysed, and the lysate was immune-precipitated with polyclonal αIR. The immune precipitate was incubated with neuraminidase or endoglycosidase H and analyzed by SDS–PAGE using reducing sample buffer. Positions of β- and α-subunits at 95 and 135 kDa and of the 180- and 210-kDa precursors of the insulin receptor are indicated. Lanes 1, 2, 5, and 6 represent immunoprecipitates from HIR-CHO cells (WT); lanes 3, 4, 7, and 8 from 233HIR-CHO cells (M). Lanes 2 and 4 are precipitates after neuraminidase incubation; lanes 6 and 8 after endoglycosidase H treatment. Exposure was for 72 h. The proteins at 95, 135, 180, and 210 kDa are absent in precipitations using nonimmune serum.

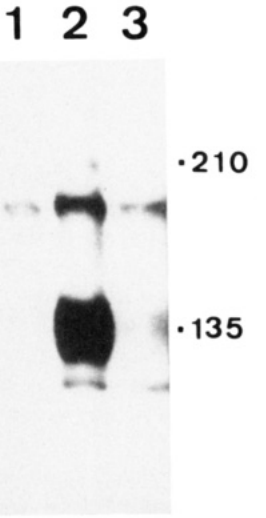

FIGURE 4: Affinity cross-linking of [^{125}I]insulin to its receptor. Glycoprotein was prepared from parental CHO cells and from HIR-CHO and 233HIR-CHO cells. Ten micrograms of glycoprotein was incubated with 50 pM [^{125}I]insulin, and cross-linking was by 0.2 mM DSS. The reaction mixture was analyzed by SDS–PAGE autoradiography. Exposure was such that endogenous insulin receptor α-chain from CHO cells was detectable. Lane 1, parental CHO cells; lane 2, HIR-CHO cells; lane 3, 233HIR-cells. Positions of the α-subunit and the precursor at 135 and 210 kDa, respectively, are indicated. Exposure was for 96 h.

insulin receptor, both the wild type and the mutant α–β precursor were only detected in the *N*-acetylglucosamine fraction (not shown).

The Mutant α–β Precursor Is Unable To Bind Insulin and To Undergo Insulin-Stimulated Autophosphorylation. Glycoprotein was prepared from cells expressing similar levels of either wild-type or mutant insulin receptors and from parental CHO cells. Insulin binding was determined by affinity cross-linking of [^{125}I]insulin to its receptor by DSS. Figure 4 shows the results. In HIR-CHO cells, enhanced labeling of α-chain occurs when compared to parental CHO cells. The increase is approximately 35-fold and agrees with the increase in binding of [^{125}I]insulin to this cell line compared to parental CHO cells. In 233HIR-CHO cells, only weak α-chain labeling occurs comparable to the signal of the parental CHO cells.

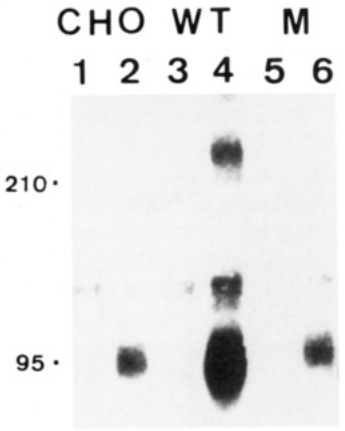

FIGURE 5: Insulin-stimulated autophosphorylation of insulin receptors synthesized by HIR-CHO and 233HIR-CHO cells. Glycoprotein was prepared, and 3 μg was incubated with or without 50 nM insulin. Autophosphorylation was started by addition of [γ-^{32}P]ATP. The insulin receptor was immunoprecipitated by polyclonal α-IR, and the immunoprecipitate was analyzed by SDS–PAGE and autoradiography. Exposure of the film was so long that autophosphorylation of endogenous insulin receptors from CHO cells was clearly visible. Lanes 1, 3, and 5, no insulin; lanes 2, 4, and 6, 50 nM insulin. Lanes 1 and 2, glycoprotein from parental CHO cells; lanes 3 and 4, HIR-CHO cells; lanes 5 and 6, 233HIR-CHO cells. Positions of the β-subunit and the proreceptor are indicated at respectively 95 and 210 kDa. Exposure was for 96 h.

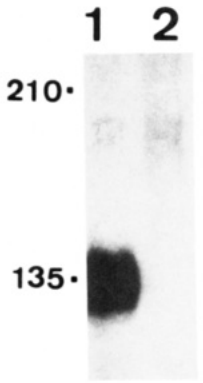

FIGURE 6: ^{125}I cell surface iodination of HIR-CHO and 233HIR-CHO cells. After lactoperoxidase-catalyzed cell surface iodination, cell lysate was immunoprecipitated with polyclonal α-IR. Lane 1, HIR-CHO cells; lane 2 233HIR-CHO cells. Positions of the α-subunit and proreceptor at 135 and 210 kDa are indicated. Exposure was for 40 h.

No additional band at 210 kDa is seen in 233HIR-CHO cells.

The glycoprotein fraction was also examined for insulin-stimulated receptor autophosphorylation (Figure 5). Similar to the affinity cross-linking data, the HIR-CHO cells exhibited an approximately 35-fold increase in β-chain labeling when compared to parental CHO cells. The 233HIR-CHO cells had β-chain labeling similar to the parental CHO cells. However, on overexposed original autoradiographs, in addition a weak signal is detected at the position of the α–β precursor, both in the absence and in the presence of insulin, suggesting a low constitutive activity of the mutant precursor. The bands seen in lane 4 at positions of 130 and 240 kDa are of unknown origin.

The Mutant Insulin Receptor Is Not Transported to the Cell Surface. As the mutant α–β precursor is unable to bind insulin, the lack of insulin binding to 233HIR-CHO cells gives no information whether the precursor is transported to the cell surface. To investigate the presence on the cell surface, HIR-CHO cells and 233HIR-CHO were radioiodinated by lactoperoxidase-catalyzed cell surface iodination, and radiolabeling of the insulin receptor was measured by immune precipitation, SDS–PAGE, and autoradiography (Figure 6). In the HIR-CHO cells, the α-chain is clearly labeled whereas in 233HIR-CHO cells no labeling of either the α-chain or the 210-kDa precursor occurred.

Table I: Specific Binding of [^{125}I]Insulin to Parental CHO Cells and HIR-CHO and 233HIR-CHO Cells[a]

cell	insulin bound (10^{-17} mol/10^6 cells)
CHO	9.5 ± 1.3
HIR-CHO	610 ± 33
233HIR-CHO	16.0 ± 1.0

[a] HIR-CHO and 233HIR-CHO expressed similar levels of IR-mRNA.

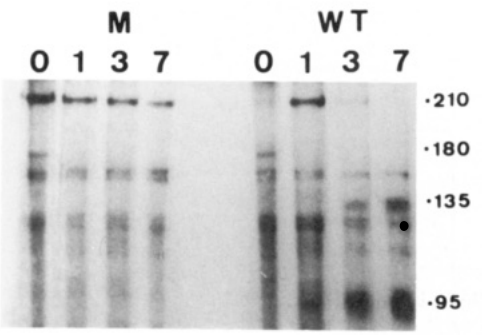

FIGURE 7: Pulse–chase labeling of HIR-CHO and 233HIR-CHO cells. HIR-CHO (WT) and 233HIR-CHO (M) cells were biosynthetically labeled for 20 min with [^{35}S]methionine followed by a further incubation with nonradioactive methionine for 0, 1, 3, and 7 h. Cell lysate was prepared and immunoprecipitated with polyclonal α-IR. The immune precipitate was analyzed by SDS–PAGE and fluorography. The positions of the α- and β-subunits at 135 and 95 kDa and both insulin receptor precursors at 180 and 210 kDa are indicated. These bands are only precipitated by polyclonal α-IR serum and not by nonspecific serum. Exposure was for 72 h.

Expression of the Mutant Receptor in CHO Cells Does Not Interfere with Insulin Binding to Endogenous Insulin Receptors. The tetrameric insulin receptor is composed of two individual gene products, and a situation can arise in which tetramers containing combinations of WT and mutant receptors are formed. We have examined whether the overexpression of the mutant receptor had an effect on the number of endogenous insulin receptors on CHO cells. For that, the number of high-affinity insulin binding sites was determined on parental CHO and 233HIR-CHO cells. The data are shown in Table I. No effect of the expression of the mutant insulin receptor was seen on the number of endogenous high-affinity insulin binding sites.

Kinetics of Biosynthesis. Pulse–chase experiments were performed to study the kinetics of the biosynthesis of the WT and mutant insulin receptors in transfected CHO cells. The transfected cells were pulsed for 20 min with [^{35}S]methionine, chased for varying periods of time with unlabeled methionine, and then solubilized, subjected to immune precipitation with a polyclonal antibody, and analyzed by polyacrylamide gel electrophoresis and fluorography (Figure 7). At zero time after the pulse, both cell lines exhibited a 180- and a 210-kDa form which were specifically precipitated by the antibody, though in the case of the mutant receptor significantly more 210-kDa precursor is already formed. After a 1-h chase, the 180-kDa form had disappeared and was converted into the 210-kDa form. The wild-type 210-kDa precursor was at the 7-h chase time point largely converted into α- and β-chains whereas degradation of the mutant 210-kDa precursor was much less pronounced. Remarkably, a gradual shift toward a slightly lower molecular weight form occurred during the

chase. Densitometry indicates that the $T_{1/2}$ of the wild-type precursor is less than 2 h whereas the $T_{1/2}$ of the mutant precursor is between 3 and 7 h. When another cell line expressing lower levels of mutant and WT insulin receptors was used for pulse–chase labeling, similar results were obtained, indicating that the level of expression is not a major determinant of the kinetics of biosynthesis.

Discussion

Fibroblasts from the leprechaun patient showed a loss of high-affinity insulin binding sites, and judged from the glycoprotein fraction from these cells, hardly an insulin-stimulated autophosphorylation of the β-chain of the insulin receptor is detected (Maassen et al., 1988). These data are consistent with the observed abnormalities of the mutant insulin receptor seen in the transfected cell line. We also reported that a protein with a molecular mass of 135 kDa could sometimes be labeled by affinity cross-linking of [^{125}I]insulin to the glycoprotein fraction of the patient's fibroblasts. In light of the present data on the transfected cell lines and considering the fact that primary fibroblasts contain a large excess of IGF-1 receptors compared to insulin receptors, we judge from the present results that the 135-kDa band seen in those experiments is the α-chain of the IGF-1 receptor.

Recently, a variety of mutations in the insulin receptor have been described. In two situations, the mutation results in accumulation of proreceptor. In one situation, this is due to a point mutation in the tetrabasic processing site of the proreceptor which results in a reduced rate of proteolytic cleavage (Kobayashi et al., 1988; Yoshimasa et al., 1988). As a result, increased levels of proreceptors are present, and these proreceptors are transported to the cell surface, indicating that proreceptors as such have the capacity to be transported. The other mutation leading to increased proreceptor formation is a valine for phenylalanine substitution at position 382 (Accili et al., 1989). This mutation retards cleavage of the proreceptor and transport to the cell surface. Very recently, Kadowaki et al. (1990b) have shown that a Lys for Arg substitution at position 15 also decreases the efficiency of transport of the receptor. The mutation substituting proline for leucine at position 233 completely blocks cleavage of the proreceptor, and also no transport of the proreceptor occurs to the cell surface as indicated by cell surface iodination.

The kinetics of biosynthesis of the insulin receptor have been thoroughly studied (Lane et al., 1985; Hedo et al., 1981). The receptor is synthesized as a 180-kDa precursor representing the primary translation product containing the high mannose oligosaccharide core. Further glycosylation converts the molecule into a 210-kDa form. In the Golgi apparatus, proteolytic cleavage takes place, and sialic acid residues are added. The pulse–chase experiments indicate that the transfected WT receptor in CHO cells has kinetics of biosynthesis similar to the situation in adipocytes, i.e., the formation of a 180-kDa precursor with a half-life of approximately 15 min and a half-life for the 210-kDa form of ~2 h. The mutant receptor shows a different situation. After a pulse of 20 min, the 210-kDa form is already the major form in the mutant situation whereas the wild-type receptor is still predominantly in the 180-kDa form. This suggests that the mutant 180-kDa precursor is more rapidly glycosylated. The decay of the 210-kDa proreceptor is slower in the mutant, which may result from its inability to be transported to the cell surface. On the basis of the observation that the electrophoretic mobility of the 210-kDa mutant precursor is not affected by neuraminidase treatment, it seems probable that the 210-kDa precursor is not transported to distal Golgi elements where attachment of sialic acid occurs. Remarkably, the mutant 210-kDa precursor is gradually converted into a form with a slightly higher electrophoretic mobility on SDS gels during the 8-h chase.

The transport defect may be related to the presence of cysteine residues which are unable to form disulfide bonds within the insulin receptor. The proline for leucine mutation is within the disulfide-rich cluster and is expected to change the stereochemical configuration of the flanking cysteine residues. In case of the LDL receptor, a number of mutants have been found which interfere with the transport of the mutant receptor from the Golgi to the cell surface. It has been suggested that the transport defect results from unpaired cysteine residues in the mutant LDL receptor (Yamamoto et al., 1986). On the other hand, several examples of mutant plasma membrane receptors have been described where involvement of unpaired cysteins residues is unlikely and where the involvement of particular proteins has been proposed in retaining incorrectly folded proteins in intracellular compartments (Esser & Russell, 1988; Klausner & Sitia, 1990).

The functional insulin receptor is a tetrameric structure composed of two gene products. Thus, expression of a wild type and a mutant insulin receptor gene could lead to the formation of hybrid structures. If this situation occurs, overexpression of the mutant receptor in CHO cells is expected to result in a situation in which most of the endogenous receptors are trapped in a tetrameric structure composed of a wild type and a mutant protein chain. As the mutation interferes with transport to the cell surface, we would expect that such a hybrid structure is not transported. The insulin binding data (Table I) do not agree with such a situation. They suggest that the mutant receptor does not interfere with expression of endogenous receptors.

It is unclear how the mutation interferes with cleavage of the proreceptor. The immune precipitation data with monoclonal antibodies suggest that the folding of the α-chain is changed. This abnormal folding may result in inaccessibility of the tetrabasic cleavage site for the processing enzyme. A more likely explanation is that the mutant proreceptor is retained in early Golgi compartments where no proteolytic cleavage of the proreceptor occurs and the lack of addition of sialic acid to the 210-kDa precursor agrees with such a situation.

The affinity cross-linking experiments show that the mutant proreceptor is unable to bind insulin. The WT proreceptor acquires insulin binding properties during maturation and glycosylation. It has been shown that during maturation of glycosylation, a conformational transition is introduced in the 210-kDa precursor which generates a functional insulin binding site (Olson & Lane, 1987). The lack of insulin binding to the mutant proreceptor could suggest that the conformational transition does not occur. On the other hand, the region around Leu-233 is important for insulin binding (Yip et al., 1988; Gustafson & Rutter, 1990), and the Pro for Leu substitution is expected to change the conformation of this particular region.

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Site-Directed Mutagenesis of β-Lactamase Leading to Accumulation of a Catalytic Intermediate†

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ABSTRACT: Site-specific mutation of Glu-166 to Ala in β-lactamase causes a millionfold reduction in catalytic activity toward both penicillin and cephalosporin substrates and results in the stoichiometric accumulation of a normally transient acyl-enzyme intermediate. Kinetic analysis indicated that substitution of Glu-166 by Ala leads to negligible effect on the acylation half of the reaction but effectively eliminates the deacylation reaction. Such differential effects on the rates of formation and breakdown of an enzyme–substrate intermediate have not been previously reported. Thus, unlike the situation for most transfer enzymes, e.g., the serine proteases, acylation and deacylation in β-lactamase catalysis are not "mirror" images and must involve different mechanisms. The results suggest an explanation for the different catalytic activities between the β-lactamases and the penicillin-binding proteins involved in bacterial cell-wall synthesis.

The production of β-lactamases is the principal means by which bacteria achieve β-lactam antibiotic resistance. These enzymes catalyze the hydrolysis of penicillins and cephalosporins to their biologically inactive products. Due to the widespread use of these antibacterial agents, there has been a strong selection for microorganisms which are resistant to β-lactams. In order to overcome this trend to resistance much research has been carried out to determine the mechanisms of these enzymes. Thus, an understanding of the interactions between β-lactamases and their substrates is critical to the design of inhibitors of potential application as antibiotics.

The β-lactamase from *Bacillus licheniformis* 749C is a class A β-lactamase, exhibiting both penicillinase and cephalosporinase activities, and characterized by the presence of an active

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