Congenital Insulin ResistanceAssociated with a
Conformational Alteration in aConserved β-Sheet
in the Insulin Receptor L1 Domain*

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The hormone binding site of members of the insulin receptor family is contained within a highly conserved extracellular region of the receptor. Recent crystallization of the N-terminal region of the binding site revealed two large domains (L1, L2), each organized as a single-stranded right-handed β-helix, connected by a rod-shaped cysteine-rich domain. Here, we analyze two new naturally occurring mutations in a single β-sheet within L1, D59G and L62P, that we previously identified in a young woman with classic congenital insulin resistance (type A). Substitution of D59G, a β-sheet connecting loop residue, caused decreased hormone binding but did not disrupt overall folding, assembly, or movement to the cell surface. In contrast, replacement of the adjacent residue L62P, which is located within the β-sheet, and positioned in a hormone binding surface, completely disrupted intracellular folding, oligomerization, and trafficking and resulted in aberrant proteolytic degradation. Immunohistochemistry in combination with biochemical studies showed that misfolded receptors were retained in an incorrect cellular location and that they colocalized with the resident endoplasmic reticulum chaperone calnexin. This study, together with other mutagenesis data, shows that formation of β-sheet elements within the L1 β-helix are critical for the folding of the entire extracellular domain of the receptor and that the hormone contact site is composed in part by residues in this domain.

Members of the tyrosine kinase receptor superfamily regulate central metabolic and growth processes through binding to their corresponding ligands (1, 2). Progress in defining the molecular basis of receptor activation has been hampered by a lack of detail regarding binding site structure. The recent determination of the crystal structure of three highly conserved domains in the N terminus of the IGF-I receptor has begun to define the geometry of the unoccupied binding site and allows more precise biochemical testing to understand ligand-receptor interactions (3).

The two flanking domains in the crystal (L1, L2) were previously identified through comparative sequence analysis and are organized as β-sheet segments that assume the overall shape of a loaf of bread (3–5). The two domains are joined by a rod-shaped cysteine-rich region creating a central cavity with three putative hormone binding surfaces directed internally. Rotations about the connecting segment of the cysteine-rich region might reposition the L1 and L2 domains following hormone binding. Additional segments in the C terminus of the receptor extracellular domain implicated in hormone binding might also contribute to the final organization of the binding site (6–8).

Facing the central space formed by the L1-Cys-rich-L2 domains is a surface of the L1 domain containing a patch of residues that form a hormone-binding “footprint” based on alanine scanning mutagenesis (9). The residues are located in four discontinuous positions in the primary sequence that occur on successive parallel β-strands of the β-sheet forming the base of the loaf of bread. The overall L1 domain architecture consists of a single-stranded sequence of β-sheets that assume a novel right-handed helical supersecondary structure, termed a β-helix (3). The physicochemical properties of many of the amino acids comprising the β-strands are conserved, and several are invariant across the receptor tyrosine kinase family. Although the crystallized fragment did not bind ligand, evidence from both alanine scanning mutagenesis and naturally occurring mutations in the receptor, are consistent with the structure (9, 10).

Recently, we identified two new naturally occurring mutations occurring in the third β-strand of the important ligand binding surface within the major L1 β-sheet (11). The maternally inherited allele, D59G, is located in a connecting segment that forms a right angle bend between the side walls of the helix, whereas the paternally inherited allele, L62P, is the central residue (and invariant) within the adjacent β-strand. Whereas Gly59 mutant receptors assemble normally and have slightly decreased affinity for insulin, Pro62 mutant receptors are completely misfolded and undergo intracellular proteolytic degradation. The positions of these two residues, and the char-

IGF-IR, insulin-like growth factor I receptor; ER, endoplasmic reticulum; HEK, human embryonal kidney; HR, human insulin receptor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; WT, wild-type; FTTC, fluorescein isothiocyanate; CNX, calnexin.
acetamides of the substituting amino acids, account for the differential effects on receptor folding, trafficking, and function. These mutations therefore provide a further test of the current structural and biochemical data that have contributed to the emerging precision in our view of receptor tyrosine kinase architecture.

MATERIALS AND METHODS

Construction of cDNA Expression Plasmids Encoding Mutant Receptors—Overlap polymerase chain reaction was used to generate both Gly62 and Pro62 exon 11- mutant HIRs (two overlapping fragments, bases 127–405 and 385–713, were used to make HIR cDNA, purified from low melting point agarose gels, and an overlapping fragment of 586 base pairs was generated by reamplification). The sequence of the 586-base pair fragment was confirmed and the Gly62 and Pro62 HIR inserts were cloned into pcDNA 3.1 and pREP4 (Invitrogen), respectively. This allowed coselection with Genitin for Gly62 (pcDNA 3.1) and hygroycin for Pro62 (pREP4).

Cell Transfection—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. 12–24 h prior to transfection cells were seeded to 20–40% confluence. Transfection was performed using the polycationic liposome Dsper (Roche Molecular Biochemicals) according to the recommendations of the manufacturer, using 6 μg of DNA and 1 μl of Dsper for each 6-cm cell plate. Following transfection, cells were grown in regular medium minimal essential medium supplemented with 10% serum for 24 h, prior to the addition of selection medium. Cells expressing Gly62 and Pro62 HIRs were selected for growth in the antibiotic Genitin (800 μg/ml, Life Technologies, Inc.), and resistant colonies were then cloned and expanded. Cells expressing both Gly62 and Pro62 HIRs were selected for growth in both Geneticin (300 μg/ml) and hygroycin B (300 μg/ml). HEK 293 cells expressing wild-type receptors were a generous gift of Dr. Jonathan Whittaker (Hage- dorn Research Institute, Gentofte, Denmark) and were maintained in both Geneticin (800 μg/ml) and hygrocin B (300 μg/ml).

Metabolic Labeling—Metabolic labeling was performed in poly-L-lysine-treated culture dishes (Sigma, P1274; 0.1 mg/ml in PBS). Cell labeling was performed following 1–2 h preincubation in methionine and cysteine-free Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin, 100 units/ml penicillin and 100 μg/ml streptomycin. Labeling was then performed by addition of methionine/cysteine, and excess unlabeled methionine and cysteine (2 mM of each). At the completion of chase, cells were pelleted at 14,000 × g in an Eppendorf bench top microcentrifuge, and the soluble lysate fraction was processed for immunoprecipitation. The postnuclear supernatant was precleared with nonimmune serum followed by immunoprecipitation with anti-receptor monoclonal antibody 8314 kindly provided by Dr. Kenneth Siddle (Cambridge Univer- sity, Cambridge, United Kingdom). The immune complex was sedi- mented with protein A-agarose (Pierce), washed twice in high salt buffer (300 mM NaCl, 1 mM CaCl2, 25 mM Tris (pH 7.4), 1% Triton X-100, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and once in the same buffer containing low salt (140 mM NaCl). The bound complex was either eluted with glycine (0.2 M (pH 2.5)) prior to dissolution in Laemmli sample buffer or directly redissolved in Laemmli sample buffer in the presence of 10 mM dithiothreitol or 10% β-mercap- toethanol, heated to 95 °C for 5 min, and then analyzed either by 3–10% linear gradient SDS-PAGE (nonreducing gels) or 5/8% SDS-PAGE (re- ducing gels). For analysis under reducing conditions, washed immunoprecipitates were boiled in Laemmli buffer prior to application to the gels. After electrophoresis at 50 volts for 18–20 h, the gels were fixed in 10% acetic acid and 25% isopropanol alcohol for 30 min, treated with Amplify (Amersham Pharmacia Biotech), dried, and exposed to Kodak X-OMAT film. For mass estimates were made by superimposing the relative mobility of the receptor bands to molecular mass standards (Amersham Pharmacia Biotech).

Biotinylation—Transfected cells were grown to confluence in 10-cm plates, washed twice in Hanks’ balanced salt solution and incubated with 1 μg/ml of Sulfo-NHS-Biotin (Pierce catalog number 21217) for 30 min on ice. Monolayers were washed in PBS containing 15 mM glycine and lysed in Triton X-100 lysis buffer. Receptors were immunoprecipi- tated, electrophoresed through 5/8% SDS-PAGE, and transferred to Immobilon membranes (Millipore) for blotting with streptavidin-horse- radish peroxidase (Amersham Pharmacia Biotech, 1:750 dilution). ECL was used to visualize the biotinylated receptors, and blots were subse- quently stripped (at 55 °C for 1 h in 0.1 M NaOH) and reprobed with an anti-HIR β-subunit polyclonal antibody (Upstate Biotechnology, Inc.).

Tyrosine Phosphorylation—HEK 293 cells were grown to confluence in poly-L-lysine-coated 10-cm culture plates and incubated in the presence or absence of 1 μM insulin for 5 min at 37 °C. Cells were lysed in cold lysis buffer as described above with the addition of 100 mM sodium fluoride, 2 mM vandate, and 4 μM sodium pyrophosphate. Immunoprecipitation was carried out as described above, and samples were processed by SDS-PAGE followed by electroblootning to Immobilon. Phosphorylated proteins were detected with a horseradish peroxidase-conjugated monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.) and visualized by ECL. Blots were then stripped and reprobed with an anti-HIR β-subunit antibody (Upstate Biotechnology, Inc.).

Insulin Binding—Confluent cells grown on poly-L-lysine treated 24 wells plates were washed three times with cold PBS and then incubated for 18 h at 4 °C with 20 μl [iodo-TyrA14]insulin in binding buffer (1 μM Hanks’ balanced salt solution supplemented with 25 μM HEPES (pH 7.6), and 10 μg/ml insulin). Following incubation, unbound insulin was removed, and cells were washed twice with cold PBS and solubilized in 0.25 N NaOH at room temperature for 2 h. Bound counts were counted in a γ counter and analyzed using Kaleidagraph software (Synergy Software, Reading, PA) (12).

Immunocytochemistry—20–30% confluent cells monolayers, grown on poly-L-lysine-treated glass coverslips, were washed on the day of the experiment twice with cold PBS for 10 min. Cells were fixed in 4% paraformaldehyde/PBS for 10 min at room temperature and permeabili- zed with 0.1% Triton X-100 in PBS. Cells were then washed in PBS and incubated for 30 min in a blocking solution 2% normal goat serum in PBS (NGS). Coverslips were then incubated with primary antibody diluted in 2% NGS overnight (dilution of anti-HIR 8314, 1:250 and anti-calnexin, 1:200 (Transduction Labora- tories). After three washes for 10 min in 0.2% Tween 20 in PBS (PBS-T), secondary biotinylated anti-rabbit antibody was added to the cells for 18 h at a 1:500 dilution in PBS/NGS (Jackson ImmunoRe- search). Cells were washed again with PBS-T and streptavidin-coupled FITC (for anti-calnexin primary; dilution 1:3000 in PBS/NGS) or Cy3 (for anti-HIR primary; dilution 1:3000 in PBS/NGS) was added. Cells were washed twice with PBS-T and chromomeric chromatin stain was applied (Hoechst 33342, 10 mg/ml in PBS). After two additional washes, coverslips were directly mounted on glass slides, and imaging was performed using an AxioPLAN microscope coupled to a CCD camera (Zeiss, Germany). The fluorescence intensity in both the FITC and Cy3 spectrum was pseudocolored, and montages of images were prepared by using ADOBE Photoshop software.

Molecular Modeling—Molecular modeling was carried out based on the x-ray structure of the L1 domain of the IGF-1 receptor as described previously (3). The L1 and L2 domains of human IGF-IR and HIR were aligned, and the side chains for residues of IR were modeled on the IGF-IR structure. The figures were drawn in MOLSCRIPT using RASTER3D (13).

RESULTS

Defective Conformational Maturation of Pro62 Mutant Receptors—Two new mutations in the insulin receptor L1 domain were identified in a compound heterozygote with classic type A insulin resistance and found to localize to a conserved β-strand within the insulin binding site (3, 11). To elucidate the effects of each mutation on the conformational maturation of the re- ceptor, we followed movement of newly synthesized receptors through the secretory pathway and monitored the tertiary and quaternary changes by nonreducing SDS-PAGE. Protein confor- mational intermediates are characterized by this analysis, and progress between each intermediate requires intact disulfide bond formation, exposure of a dimerization surface, and movement into the late trans Golgi apparatus for carbohydrate capping (14, 15). The appearance of these four species in cell lines expressing wild-type receptors indicates
both correct folding and movement to the cell surface (Fig. 1, WT HIR, the early monomer (EM), late monomer (LM), dimer (D), and tetramer (T)). Gly59 mutant receptors similarly demonstrated normal tertiary and quaternary conformational maturation based on the appearance of the four intermediates on nonreducing SDS-PAGE (Fig. 1, G59 HIR).

In contrast, pulse-chase studies with cell lines expressing the Pro62 HIR mutant receptors revealed marked differences from that of either the wild-type or the Gly59 HIR. Analysis of the receptor by nonreducing gels showed the initial appearance of early monomer and late monomer species, however the LM did not progress to the dimer at late chase time points (Fig. 1, P62 HIR). Since dimerization has previously been shown to be necessary for export out of the ER, the failure of Pro62 to dimerize suggests that it is trapped in the early secretory pathway (14). Additionally, in contrast to wild-type and Gly59 receptors, Pro62 mutant proreceptors did not undergo the same proteolytic cleavage and carbohydrate modification observed for wild-type receptors. Instead, Pro62 mutant proreceptors were proteolytically cleaved to generate two smaller fragments of ~120 and ~80 kDa and subsequently degraded without transport to the cell surface (see “Discussion”). HEK 293 cells expressing both Gly59 and Pro62 mutant HIRs demonstrated only partial conversion to the tetramer at late chase time points; however, it was not possible to quantitate the amount of pro-HIR processing in these cells due to the low expression level of the hybrid Gly59/Pro62 HIRs (data not shown).

**FIG. 1. Conformational maturation of wild-type insulin and mutant insulin receptors.** Overlap polymerase chain reaction was used to generate cDNA expression vectors encoding the two mutant HIRs. HEK 293 cells were stably transfected with each mutant and selected for resistance to Geneticin and/or hygromycin. Both the mutant and wild-type receptor cDNAs used to generate stable cell lines contained exon 11. Stable HEK 293 cells expressing the wild-type insulin receptor were pulse-labeled for 30 min with [35S]methionine and [35S]cysteine and chased in medium containing 2 mM amounts each of methionine and cysteine. Cell extracts were prepared at the indicated times, immunoprecipitated, and redissolved in nonreducing Laemmli sample buffer through linear gradient 3–10% SDS-PAGE. The positions of the receptor oligomeric species are indicated with arrows to the right (EM, early monomer; LM, late monomer; D, dimer; T, tetramer).

**FIG. 2. Immunolocalization of wild-type receptors.** Cells expressing wild-type and mutant insulin receptors were fixed and processed either intact (panels 1–3) or following Triton permeabilization (panels 4–6) for indirect immunofluorescence. Cells were incubated with either anti-HIR (panels 2 and 5) or anti-HIR plus anti-CNX (panels 3 and 6). Calnexin is a resident ER protein and was used as a marker of this compartment. Secondary FITC-conjugated anti-mouse antibodies and Cy3-conjugated anti-rabbit antibodies were used and fluorescence intensity in both the FITC (green; CNX) and Cy3 (red; HIR) spectrum were pseudocolored using ADOBE photoshop version 4. Hoechst staining was performed to identify the nuclei (blue). Panels 1 and 4 show phase contrast images obtained on the same cells prior to fluorescence imaging. Antibody dilutions and immunostaining were adjusted so that the low levels of endogenous HIR in HEK 293 cells were below the range of detection in both intact and Triton-permeabilized cells (data not shown).

**FIG. 3.** Insulin-induced tyrosine phosphorylation of mutant and wild-type receptors. A, equivalent numbers of HEK 293 cells were incubated for 5 min at 37 °C with culture medium containing 1 μM insulin then lysed, immunoprecipitated with an anti-HIR antibody, and analyzed by reducing 5/8% SDS-PAGE. The protein was then transferred to Immobilon-P, and the blots were processed with anti-phosphotyrosine antibody and visualized by ECL. The position of the β-subunit is indicated to the left. B, the Immobilon membranes were stripped and reprobed with an anti-β-subunit insulin receptor antibody. The positions of both the prorpeceptor (pro-HIR) and β-subunit is indicated with arrows to the left.

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**FIG. 4.** Localization of mutations in the polypeptide fold of the L1-Cys-rich-L2 structural domains. A, a cartoon of the IGF-IR backbon is shown viewed from the face of the \( \beta \)-sheet surface (L1, residues 1–150) with secondary structural elements shown (broad arrows, \( \beta \)-strands; curled ribbon, helices; color coding as in Ref. 3). The side chains for equivalent residues for IR are modeled on the IGF-IR structure and are represented as ball-and-stick (larger atoms are \( \alpha \) carbons and colors: white, carbon; blue, nitrogen; red, oxygen). B, substitutions of residues 59 and 62 of the IR are shown with the side chains. For Pro62, the framework is unmodified; however, the proline mutation would disrupt hydrogen bonding on either side of the mutated strand.

**Immunohistochemistry and Biotinylation Demonstrate Mislocalization of Pro62 Mutant Receptors—While biosynthetic studies provided biochemical evidence that Pro62 mutant receptors failed to undergo dimerization and ER export, direct visualization of the receptors was used to further examine their fate in the cell. Cell surface and intracellular receptor pools were distinguished by performing immunohistochemistry either in intact or permeabilized cells. Cells expressing the wild-type receptor showed abundant cell surface insulin receptors as detected by the red fluorescence signal in intact cells following incubation with a primary antireceptor antibody followed by a Cy3-conjugated secondary antibody (Fig. 2, WT HIR, panel 2). Similarly, in nonpermeabilized cells expressing Gly59 mutant receptors, the receptor was detected both on the cell surface in intact cells and intracellularly following permeabilization (Fig. 2, G59 HIR, panels 2 and 5).

However, the pattern of immunofluorescence in nonpermeabilized cells expressing Pro62 mutant receptors was markedly different. There was no fluorescence detected for the receptor in intact cells, rather, following permeabilization a robust receptor signal was visible (Fig. 2, P62 HIR, panel 2 versus panel 5). Following permeabilization, the FITC (green) CNX signal was detected in a diffuse reticular pattern throughout the cytoplasm, with the most intense signal found in the perinuclear area (Figs. 2, panel 6). Double immunofluorescence with both CNX and HIR antibodies indicated colocalization of Pro62 mutant receptors with CNX, consistent with retention of the receptor in the ER (Fig. 2, P62 HIR, panel 6). Overall, the immunocytochemical experiments provide visual evidence that Pro62 HIRs are trapped intracellularly and fail to progress to the cell surface.

Biotinylation was used as an additional test to determine whether Pro62 mutant receptors were present on the cell surface. Intact cells expressing either Pro62 mutant or wild-type receptors were biotinylated, and cell lysates were immunoprecipitated when analyzed by SDS-PAGE and immunoblotting with streptavidin-horseradish peroxidase and anti-\( \beta \)-subunit antibodies (data not shown). Densitometry of the blots was used to compare the levels of cell surface-biotinylated receptor with the total lysate receptor pool. Cells expressing Pro62 mutant receptors were found to have a 97% reduction in the relative ratio of cell surface to intracellular receptors compared with cells expressing the wild-type receptor.

**Reduced Affinity for Insulin of Gly59 HIR—**We previously showed that inheritance of a single Gly59 mutant receptor allele in the proband’s mother resulted in marked insulin resistance, yet the biosynthetic and immunohistochemical studies reported here did not indicate any major abnormalities in conformational maturation. To assess Gly59 HIR function in more detail, we performed both insulin binding studies and analyzed insulin-induced tyrosine autophosphorylation in intact cells expressing Gly59 mutant receptors. 

**EC50 values were similar for both the wild-type (–2 nM) and Pro62 (–3 nM) mutants. In contrast, the EC50 value for Gly59 HIR was decreased to ~8 nM, showing a reduced affinity for ligand compared with the recombinant wild-type receptor.**

We used insulin-induced autophosphorylation to further examine the insulin sensitivity of mutant and wild-type receptors (Fig. 3). Cells were stimulated with 0.1 \( \mu \)M insulin for 5 min, lysed in the presence of phosphatase inhibitors, immunoprecipitated, and electrophoresed on reducing gels. Immunoblotting was first performed with an anti-phosphotyrosine antibody followed by an anti-HIR \( \beta \)-subunit antibody. As seen in Fig. 3, a robust signal was obtained for the wild-type receptor, indicating full hormone responsiveness. The same membranes were stripped and reprobed with an anti-HIR \( \beta \)-subunit antibody, which allowed comparison of the relative autophosphorylation of Gly59, Pro62, and recombinant wild-type receptors. Phosphorylation of wild-type receptors was quantitative with 0.1 \( \mu \)M insulin, a concentration that maximally stimulates the receptor. In contrast, autophosphorylation of the Pro62 HIR was reduced following insulin stimulation, while total cellular receptor levels were equivalent to HIR levels in cells expressing recombinant wild-type HIR. The amount of pro-HIR was also higher in cells expressing the Pro62 HIR compared with the wild-type HIR (Fig. 3). Very little mature \( \beta \)-subunit was present in cells expressing Pro62 HIR, instead, multiple proteolytic fragments were detected that were immunoreactive with the anti-HIR antibody (Fig. 3, lower panel, lanes 5 and 6). One of the proteolytic fragments of Leu62 (~80 kDa, Fig. 3, panel B, lanes 5 and 6) was close in position to that of the \( \beta \)-subunit of recombinant wild-type HIR (Fig. 3, panel B, lanes 1 and 2). Although cells expressing Gly59 mutant receptors produced more mature \( \beta \)-subunit than Pro62 HIR, insulin-induced tyrosine phosphorylation of Gly59 HIR \( \beta \)-subunit was reduced compared with wild-type HIR based on densitometry of blots in Fig. 3, panel A. Together, these studies indicated decreased affinity of Gly59 HIR for insulin and correspondingly decreased insulin-induced autophosphorylation.
DISCUSSION

The N-terminal L1-Cys-rich-L2 domains of the insulin and IGF I receptors constitute a major component of the hormone binding site and are organized in an independently folded superhelical topology (9, 10, 16–22). Three hormone contact sites within the L1 domains form a central cavity that likely accommodates ligand (3). Sequence analysis and molecular modeling predicted the three-dimensional fold of this region with a high degree of accuracy (4). The model of Bajaj et al. (4) was also well supported by the finding that many naturally occurring mutations in conserved amino acids positioned in this fold caused defects in biosynthesis and function. The present study demonstrates that formation of a single β-strand within the major L1 domain β-sheet is vital for the folding and conformational maturation of the entire receptor and that its disruption results in severe clinical insulin resistance. 

Fig. 4 identifies the positions of both amino acids Asp59 and Leu62 within the L1-Cys-rich-L2 polypeptide fold. The topology of both L1 and L2 domains is that of a rectangle composed of β-sheets in the base and side walls (a β-helix). Residue Leu62 localizes to a central β-strand that forms part of the β-sheet on the base of the L1 domain, and substitution with proline alters the local conformation (Fig. 4A). Proline is absent from most β-structural segments in proteins and biochemical restrictions disfavor proline in β-sheets (23). In the case of the proline mutant, the main chain twists around forcing the previous carbonyl oxygen (residue 61) into the core. The Pro62 substitution also forces the carbonyl oxygen of the previous β-strand (residue 33) inwards, since the nitrogen of position 62 is occupied by the proline side chain. Local conformational strain in the L1 β-sheet induces misfolding throughout the entire extracellular domain and prevents dimerization or export out of the ER.

In contrast residue Asp59 is semiconserved and located in the short right angle bend connecting the side-wall and base of the L1 domain (Fig. 4). Gly59 HIR showed a small decrease in both insulin-induced autophosphorylation (Fig. 3) and in insulin binding when compared with recombinant wild-type HIR. This result was consistent with clinical studies showing decreased binding affinity in erythrocytes from the proband’s mother and with alanine substitution at the same position (9, 11). Substitution with glycine at this position does not cause stereochemical restriction, particularly since this residue is not contained within a region of defined secondary structure (Fig. 4). Moreover, both glycine and aspartic acid have a high level of mutual replacements in globular proteins (23). This might explain the normal folding and processing of Gly59 HIR, although subtle conformational changes still prevent normal insulin binding.

In summary, the effects of two new naturally occurring mutations in the insulin receptor analyzed here highlight unique aspects of the architecture of the extracellular domain and its biosynthesis. Since it was recently shown that fusion of L1-Cys-rich-L2 domains to 12 amino acids in the C terminus of the α-subunit conferred insulin binding to the three N-terminal domains, it is likely that these domains are in geometric proximity in the native receptor (8). The observation that substitution in the most N-terminal structural domain induces complete misfolding indicates that the L1 domain forms a scaffolding that is kinetically responsible for this process. In its native conformation, the L1 domain is also accessible for ligand binding, as evident from both the analysis of D59G mutant receptors and previous studies of mutant receptors (24–26). Residues in L2 have also been implicated in ligand binding, yet in the crystal structure, L1 and L2 are not close enough to bind simultaneously to IGF I or insulin (5–7, 25, 27). The possibility remains that either ligand-induced rotation repositions L2 closer to L1 or that a second α-subunit contributes an additional binding surface. Studies are currently in progress to examine how misfolding of the L1 domain induces retention and degradation of the non-native conformer. Understanding the native organization of the receptor extracellular domain in the unoccupied state and elucidating the kinetics of domain folding will provide insight into the structural basis of ligand binding and conformational changes that lead to receptor activation.

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