# Hybrid Insulin Receptors
## Molecular Mechanisms of Negative-Dominant Mutations in Receptor-Mediated Insulin Resistance

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**Certain syndromes of extreme insulin resistance are the result of negative-dominant mutations of the insulin receptor. The insulin-receptor heterotetramer appears to be the minimal functional unit for insulin signal transduction probably due to a requirement for intersubunit interactions. The observation that insulin and insulinlike growth factor I receptors can be found in hybrid heterotetramers suggests that insulin receptors can be composed of heterodimers that are the products of separate genes. Such a structure provides a potential molecular mechanism for negative-dominant receptor mutations. *Diabetes Care* 13:576–81, 1990**

The first event in the action of insulin on target cells is binding to a specific cell-surface receptor. The binding leads to a conformational change in the receptor protein, which is propagated across the cell membrane and initiates the intracellular changes characteristic of the biological response to insulin in that cell. Any perturbation of this pathway will lead to insulin resistance. Such perturbations in disease states can be primary or secondary (for review, see ref. 1). In their most extreme form, these perturbations manifest themselves as the syndrome of extreme insulin resistance (2). This syndrome can arise from the development of insulin-receptor autoantibodies, which inhibit insulin binding as in the type B syndrome (3), or from a primary defect in target cells for insulin (2). In a subset of the latter disorder, the type A syndrome (4,5) and other related diseases, e.g. lipoatrophic diabetes (6), leprechaunism (7–10), and Rabson-Mendenhall syndrome (11), the defect in insulin action appears to be caused by a marked reduction in insulin receptors expressed at the cell surface or to result from the primary defects in the receptor that lead to grossly impaired function.

Recently, the application of molecular cloning techniques to the investigation of these disorders has led to the determination of insulin-receptor mutations in several affected patients. Furthermore, high-level expression of the mutated cDNAs in cultured cells has afforded considerable insights into the resulting perturbations of receptor function. In the type A syndrome, mutations have been described that impair posttranslational processing of the receptor (12,13) or produce defective insulin-stimulated autophosphorylation (14–16). In patients with leprechaunism, a nonsense mutation in the extracellular domain, a point mutation producing abnormal insulin binding, and a mutation causing impaired insertion of receptors into the cell membrane and defective signal transduction have been reported (17,18). Thus, the syndrome appears to be the consequence of a defective insulin-receptor gene product or products. Also, these patients, with the exception of those with defects in receptor autophosphorylation (14,15), are either homozygotes or compound heterozygotes. In fact, it is not clear why simple heterozygotes should be subject to extreme insulin resistance, because in principle, 50% of their insulin receptors should be functionally normal. Such decreases in insulin-receptor concentrations have been observed in normal populations without giving rise to extreme insulin resistance (19,20); therefore, the mutations seem to be negative-dominant mutations. The finding that several heterozygotes for these conditions exhibit carbohydrate intolerance (17) or de-

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creased insulin binding (18) supports this concept. In this review, we examine aspects of insulin-receptor structure, function, and biosynthesis to develop hypothetical mechanisms to explain negative-dominant effects of insulin-receptor mutations in the heterozygote.

## INSULIN-RECEPTOR STRUCTURE AND FUNCTION

The insulin receptor is an integral membrane glycoprotein composed of two α- and two β-subunits linked together by disulfide bonds (for review, see ref. 21). The cloning and sequencing of human insulin-receptor cDNA (22,23) has revealed that the α-subunit, which has a molecular mass of 135,000, exists as two isoforms, one of which contains a 12–amino acid insert at its COOH-terminal (amino acid sequence [aas] 718–729; the numbering system of Ebina et al. [23]) produced by alternative splicing of the insulin-receptor gene (24). It contains a striking cysteine-rich domain (aas155–312) homologous to that in the epidermal growth factor (EGF) receptor (25) and is heavily glycosylated (26); the deduced amino acid sequence predicts 13 potential acceptor sites for *N*-linked oligosaccharides (22,23).

Affinity-labeling studies with $^{125}$I-labeled insulin have shown that the α-subunit contains the major insulin-binding domains (27,28). More precise localization of the binding domain has been suggested. Waugh et al. (29), using affinity labeling and proteolytic fragmentation, demonstrated that the major cross-linking/insulin-binding region resided in a 55,000-$M_r$ tryptic peptide generated from aas1 to aas150 of the receptor protein. Wedekind et al. (30), using a similar approach but a different affinity label, further localized it to a 14,000-$M_r$ peptide, whose $NH_2$-terminal corresponded to Leu-20 of the receptor. Further evidence that this region of the insulin receptor may be an important insulin-binding region comes from the results of molecular genetic studies by De Meyts et al. (31). Mutant insulin receptors with a deletion of the region encoded by exon 2 of the receptor gene, aas7–190 (24), were virtually devoid of insulin-binding activity, whereas mutants missing aas1–66 could still bind insulin. Furthermore, a β-aromatic amino acid at position 89 was necessary for high-affinity binding. In contrast, Rafaeloff et al. (32), also using molecular genetic techniques, found evidence that aas242–247 in the cystein-rich domain may be involved in insulin binding. However, these studies suggest only a preliminary localization of the binding domain, and further studies with molecular genetic and protein chemical techniques are necessary to unequivocally identify amino acids that interact with insulin.

The 95,000-$M_r$ β-subunit is bound to the α-subunit by disulfide bonds and has both extracellular and intracellular domains connected by a single helical transmembrane domain. The cytoplasmic domain is a protein tyrosine kinase (33–35) that is activated by insulin-stimulated autophosphorylation on tyrosine residues (36). Tyrosine aas1158, 1162, 1163, 1328, and 1334 have been identified as phosphorylation sites (37,38).

Insulin-stimulated receptor autophosphorylation appears to be essential for the initiation of insulin's biological effects. Microinjection of monoclonal antibodies directed against the major autophosphorylation domain of the β-subunit (aas1158, aas1162, and aas1163) into *Xenopus* oocytes inhibits the ability of insulin to stimulate their maturation (39). Similarly, their introduction into several types of mammalian cells blocks both rapid and intermediate responses to insulin (40). Transfection and stable expression of insulin-receptor cDNAs, in which tyrosines 1162 and 1163 have been mutated to phenylalanine, in eukaryotic cells produces cell lines that exhibit markedly diminished responsiveness to insulin (41). In eukaryotic cells, the expression of insulin-receptor cDNAs, mutated in vitro at their ATP-binding site, produces receptors that seem incapable of insulin-receptor signal transduction (42,43).

Although these studies provide compelling evidence for an essential role for insulin-receptor autophosphorylation in insulin signal transduction, the nature of its role and subsequent events are unclear. It could initiate a cascade of phosphorylations; an increasing number of proteins have been shown to be phosphorylated on tyrosine in response to insulin, although their functional importance has not been demonstrated (44,45). Alternatively, insulin-stimulated receptor autophosphorylation may produce conformational changes in the cytoplasmic domain of the β-subunit, permitting interaction with and consequent activation of accessory proteins, e.g., protein kinases, G proteins, or enzymes involved in the generation of second messengers.

In vitro studies have shown that the $\alpha_2\beta_2$-heterotetramer is the minimal functional unit of the insulin receptor (46–48). Under mild reducing conditions, the receptor heterotetramer may be separated into its constituent native αβ-heterodimers, causing marked changes in function. The affinity for insulin is reduced, and the characteristic feature of insulin binding to its receptor, negative cooperativity, is lost (46,47). These studies also suggest that only half the heterodimers bind insulin at maximal receptor occupancy in the heterotetrameric conformation. In contrast, all heterodimers bind insulin after separation, suggesting that, in the heterotetramer, the binding of insulin to one heterodimer produces a conformational change that is propagated between the constituent heterodimers, possibly inducing absolute negative cooperativity. The insulin-receptor heterodimer is also incapable of insulin-stimulated autophosphorylation unless it reassociates to form a heterotetramer (48). Receptor heterotetramers in which the cytoplasmic domain of one β-subunit has been proteolytically degraded during isolation are also incapable of insulin-stimulated autophosphorylation (49). Thus, interaction between the receptor kinases of the two heterodimers may be a prerequisite for autophosphorylation.

Whereas the minimal structural unit of the insulin re-

ceptor is the $\alpha_2\beta_2$-heterotetramer, recent studies suggest it can exist as a higher-order oligomer. Kubar and Van Obberghen (50) and Fujita-Yamaguchi et al. (51) have shown that partially purified receptors can exist as three distinct oligomeric species with different functional properties: *1*) a low-molecular-weight form that exhibits high-affinity insulin binding and insulin-stimulated autophosphorylation, *2*) an intermediate form that binds insulin but does not autophosphorylate, and *3*) a high-molecular-weight species with decreased affinity for insulin but a hyperactive insulin-stimulated tyrosine kinase. Although it was not possible to identify the nature of these molecules, it has been suggested that the low-molecular-weight form represents the $\alpha_2\beta_2$-monomer, the intermediate form an $(\alpha_2\beta_2)_2$-dimer, and the high-molecular-weight form an $(\alpha_2\beta_2)_n$-oligomer, where $n > 2$ (50). Note that such oligomeric forms have only been demonstrated in solubilized receptor preparations in vitro. Although their existence in intact cells is unconfirmed, a recent report suggesting the occurrence of intermolecular receptor phosphorylation in intact cells may represent the functional consequence of this kind of structural organization (52). Such a hierarchy of structures may indeed be essential for the coordinate amplification of insulin signaling.

## INSULIN-RECEPTOR BIOSYNTHESIS

Biosynthetic studies (53–56) and the cloning and sequencing of human insulin-receptor cDNA (22,23) have provided considerable insights into the biosynthesis and posttranslational processing of the insulin-receptor protein. Initial biosynthetic studies showed that the receptor was synthesized as a 190,000-$M_r$ core glycosylated single-chain precursor, which is subsequently cleaved and terminally glycosylated to form the mature α- and β-subunits inserted into the plasma membrane of the cell. The precise order of these events may depend on cell type. The $NH_2$-terminal sequencing of the α- and β-subunits and the availability of the deduced amino acid sequence of the whole precursor from human placental cDNA clones identified the site of cleavage of the proreceptor into its constituent α- and β-subunits to be a tetrabasic amino acid sequence (Arg-Lys-Arg-Arg, aas732–735; 22). This has subsequently been confirmed by the demonstration of an arginine-to-serine mutation at position 750 in a type A patient with defective cleavage of the proreceptor (12). The cleavage has also been shown to be essential for the generation of a high-affinity insulin-binding site (12). By subcellular fractionation, Hedo and Simpson (57) have shown that these processes take place in the Golgi apparatus.

More recent biosynthetic studies in NIH/3T3-L1 fibroblasts have provided insights into some of the processes involved in the assumption of the final tertiary structure of the receptor precursor molecule (58). The initial translation product in these cells has a molecular mass of 190,000 and is monomeric when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The molecular mass then increases to 210,000, although the molecule remains monomeric. The increase is due to the formation of intramolecular disulfide bonds. Shortly thereafter, the formation of an insulin-binding site and epitopes recognized by a polyclonal antibody specific for the native conformation of the receptor takes place without further detectable structural change. A disulfide-bonded dimer is then formed that is exported from the endoplasmic reticulum to the Golgi apparatus for further processing. These events are similar to those taking place during the biosynthesis of viral glycoproteins (59,60), although whether noncovalent dimers of the insulin-receptor precursor are formed before disulfide bond formation, as has been described for the latter, is unclear. The formation of disulfide-bonded dimers may be a prerequisite for transfer of the receptor precursor from the endoplasmic reticulum to the Golgi apparatus, as in mutant receptors, in which the folding pathway is perturbed, dimerization is inefficient, and the major part of the precursor remains in the endoplasmic reticulum where it is degraded (J.W., unpublished observations).

This complex biosynthetic pathway can be summarized in terms of the functional maturation stages of the receptor. The first stage takes place in the endoplasmic reticulum and involves folding of the 190,000-$M_r$ translation product into a functional conformation. The formation of a disulfide-bonded dimer appears to initiate its export to the Golgi apparatus. Here, the second stage of the functional maturation of the receptor takes place; conformational changes resulting from proteolytic cleavage and possibly terminal glycosylation lead to the generation of a high-affinity insulin-binding site. Finally, the mature heterotetramer is transferred to the plasma membrane, where it may assemble into higher-order oligomers.

## HYBRID RECEPTORS

From the description of the pathway of insulin-receptor biosynthesis, it is apparent that receptor precursors translated from separate polysomes may associate and then be processed into hybrid heterotetrameric molecules. Recent studies of insulinlike growth factor I (IGF-I) receptors by Soos and Siddle (61) and Moxham et al. (62) support this concept. The IGF-I receptor has the same structural organization as the insulin receptor; i.e., it is an $\alpha_2\beta_2$-heterotetramer (63). The cloning and sequencing of the human placental IGF-I receptor revealed >50% sequence homology with the human insulin receptor (64). Soos and Siddle (61) demonstrated that several species-specific monoclonal antibodies directed toward different epitopes of the human insulin receptor can cross-react with the human IGF-I receptor; i.e., they can inhibit IGF-I binding and immunoprecipitate IGF-I receptors. However, the immunoreactivity is indirect in that it is abolished by prior exposure of the

receptor to the reducing agent dithiothreitol; in contrast, recognition of the insulin receptor by the antibodies is unaffected by this treatment. These findings indicate that IGF-I receptors "reacting" with the insulin-receptor monoclonal antibodies must exist in hybrid heterotetramers with insulin receptors, i.e., an IGF-I–receptor heterodimer disulfide bonded to an insulin-receptor heterodimer, and only the insulin receptor is recognized by the antibodies. It was also demonstrated that hybrid receptors exist in intact cells. In a similar study, Moxham et al. (62) also demonstrated that insulin and IGF-I receptors exist as hybrids by use of a species-specific monoclonal antibody to the IGF-I receptor to immunoprecipitate insulin receptors in the absence but not presence of reductant.

More recently, Treadway et al. (65) showed that insulin- and IGF-I–receptor heterodimers can associate in vitro to form hybrid heterotetramers (65). Therefore, the hybrid receptors found in intact cells could be formed comparatively late in biosynthesis, and even mature receptors might dissociate and reassociate to form hybrids if exposed to the appropriate redox environment.

Studies in cultured cells suggest the amount of hybrid receptor depends on the relative amount of each receptor that is expressed; i.e., in cells with equal levels of expression, 50% of each type will be present as hybrids, and where one receptor is dominant over the other, the minority receptor will be found almost entirely in hybrid structures (61).

## IMPLICATIONS FOR INSULIN-RECEPTOR MUTATIONS AND INSULIN RESISTANCE

The observations that *1*) the $\alpha_2\beta_2$-receptor heterotetramer is the minimal functional unit of the insulin receptor, *2*) hybrid heterotetramers can be formed from separate gene products, and *3*) insulin receptors may be found in higher-order oligomeric structures suggest possible mechanisms by which receptor mutations resulting in abnormal structure or function can cause extreme insulin resistance. These could operate either during biosynthesis or in the mature receptor. Nascent mutant and nonmutant receptor precursors may noncovalently associate early during the course of receptor biosynthesis, and the mutant could impair the coordinate folding of the heterodimeric precursor into its native conformation and lead to subsequent retention and degradation of the malfolded protein in the endoplasmic reticulum, as has been described for viral glycoproteins. Alternately, a malfolded mutant receptor precursor could form a disulfide-bonded dimer with a wild-type receptor precursor at a later stage of biosynthesis, producing a similar end result, i.e., retention and degradation in the endoplasmic reticulum. The net effect of such mutations would be a decrease in insulin-receptor concentration at the cell surface. Assuming equal rates of synthesis of the two species of receptor, this would lead to a 75% decrease in receptor number at the cell surface. Greater decreases would result if the affinity of the mutant receptor precursor for the wild-type precursor were greater than that of the wild-type precursor for itself or if the rate of biosynthesis of the mutant were greater than that of the wild type.

In the mature receptor, the mutant receptor could lead to impaired function of the hybrid receptors. It is possible that a mutant heterodimer with impaired transmembrane signal transduction could impair signal transduction in the hybrid receptor. Similarly, mutant receptors with impaired autophosphorylation would lead to impaired autophosphorylation and kinase activation of the hybrid receptors.

Finally, if noncovalently linked higher-order oligomers exist, and if intermolecular receptor interactions indeed occur, interactions between mutant and wild-type receptors could possibly occur at this level as well. Thus, the presence of mutant insulin receptors, which exhibit impaired signal transduction, in an oligomeric complex may impair the coordinate amplification of an insulin signal.

Although these proposed mechanisms of receptor-mediated insulin resistance are hypothetical, molecular genetic techniques provide the means to explore their feasibility. Both wild-type insulin-receptor cDNAs and mutant receptor cDNAs of different kinds can be coexpressed in cultured cells, and the biochemical properties of the resulting cell lines can be evaluated.

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