Molecular Genetics of Severe Insulin Resistance

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Leprechaunism and type A diabetes represent inborn errors of insulin resistance whose phenotypes suggested causation by mutations in the insulin receptor gene. Cells cultured from patients with leprechaunism specifically lacked high-affinity insulin binding. Partial but different degrees of impairment were observed in cells cultured from first-degree relatives. Different mutations in the insulin receptor's α subunit were proposed in different families (Ark-1, Atl, Minn, Mount Sinai) based on phenotype, cellular insulin binding, and insulin receptor structure. Molecular cloning and sequencing of mutant insulin receptor cDNA from family Ark-1 confirmed that the proband inherited a maternal missense and a paternal nonsense mutation in the α subunit and was a compound heterozygote. The insulin receptor was immunologically present on the plasma membrane of fibroblasts cultured from patients Ark-1 and Atl but was markedly reduced in cells from patients Minn and Mount Sinai. In cells from patient Minn, but not from patient Mount Sinai, the decreased number of insulin receptors was associated with reduced insulin receptor mRNA. In two families with the less severe form of insulin resistance, type A diabetes, mutations altered post-translational processing of the insulin receptor molecule. At a cellular level, these mutations of the α subunit of the insulin receptor shared defective binding and impaired stimulation of sugar transport by insulin. In family Atl, however, glucose uptake was constitutively increased. Thus, genetic variation in the insulin receptor gene causes a spectrum of inherited insulin-resistant syndromes and altered cellular signaling.

INTRODUCTION

Inborn errors of metabolism represent a group of experiments of nature characterized by impaired metabolic flow and caused by single gene mutations. A family study of any given inborn error could clarify both the pathophysiological and normal mechanisms of complex metabolic functions in man. This philosophy led to the search described below for a discontinuous phenotype of inherited severe insulin resistance. The presumptions are that the candidate gene would be the insulin receptor and that mutations in this gene would produce insights into the complex mechanisms of cellular responses to insulin.

The recent cloning and sequencing of the insulin receptor gene encouraged the possibility of identifying human phenotypes caused by alterations in the insulin

Abbreviations: DSS: disuccinimidyl suberate  EGF: epidermal growth factor  IR: insulin receptor  NIDDM II: non-insulin-dependent diabetes mellitus  OMG: 3-O-methyl-D-glucose  PAGE: polyacrylamide gel electrophoresis  PCR: polymerase chain reaction  RFLP: restriction fragment length polymorphism

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receptor [1–3]. Although reduced cellular binding would be a genetic discriminant in pedigree evaluation, such mutations might also interfere with cellular, signal transduction mechanisms. Cellular signaling at the plasma membrane is shared by the three groups of tyrosine protein kinase receptors (Fig. 1). These receptors have in common an extracellular cysteine-rich, ligand-binding domain, a single transmembrane domain, and an intracellular tyrosine protein kinase. They are divided into three classes, based on ligand specificity and structural differences [4].

**Class I** receptors are composed of a single polypeptide chain containing two extracellular cysteine-rich regions, which produce one ligand binding pocket. This class has one intracellular tyrosine kinase domain. The receptor for the epidermal growth factor (EGF) exemplifies this class [5].

**Class II** receptors are heterotetramers composed of two α and two β subunits, linked by disulfide bonds. The two α subunits are extracellular, each contains one cysteine-rich region, and binding kinetics differ for different ligands. The insulin receptor, but not the IGF-I receptor, has complex binding kinetics, suggesting either intramolecular negative cooperativity or different classes of insulin binding sites in different tissues [6]. The β subunits span the plasma membrane and carry the kinase domains. Receptors for insulin and IGF-I typify this class [1,2,7].

**Class III** receptors are single peptides, like class I, but have two major differences. First, they have only ten cysteine residues in their extracellular domains, which presumably produce a pocket for specific ligand binding. Second, their intracellular cytoplasmic tyrosine kinase is divided by a short (77 or 107 amino acids) sequence, with the ATP binding site in the N-terminal and the phosphorylated tyrosines in the C-terminal portion. CSF-1 and PDGF receptors exemplify this class [7–9].

Although the exact mechanisms of signal transduction are not fully understood, a sequence of events is suggested. Ligand binds to the extracellular domain of the receptor, produces a conformational change, and enhances autophosphorylation. Activated receptors modulate a variety of cell functions through poorly defined intermediates, perhaps including specific G proteins [10] (Fig. 1). It should be noted that different signals are produced in a variety of differentiated cells. Some of these responses may not require either receptor autophosphorylation or generation of a kinase reaction [11]. Responses regulated by tyrosine kinase receptors include plasma membrane functions, such as nutrient and ion transport, and nuclear functions, such as gene expression and cell division [4,5]. These protein-kinase receptors control cell growth and have homology to certain oncogenes, such as the EGF receptor for v-erb B protein product [5]. The insulin receptor shares some homology with the v-ros protein [1]. This receptor controls both cell growth and a variety of anabolic cell functions [12]. Cells transfected with insulin receptor cDNAs which alter autophosphorylation of the β subunit lose insulin-responsive glucose transport [13–16]. By contrast, if the α subunit of the insulin receptor is truncated, insulin binding is lost, and a constitutive increase in glucose transport, which is not further stimulated by insulin, occurs in transfected CHO cells [17].

In man, what phenotype might be associated with specific mutations in the insulin receptor? Structural alterations in the α subunit of the insulin receptor were proposed to explain the severe heritable forms of insulin resistance associated with intrauterine growth restriction, and the syndrome called “leprechaunism” [18,19]. Patients with leprechaunism are very small for gestational age, have extreme hyperinsulinemia and altered glucose homeostasis, with fasting hypoglycemia and postprandial hypergly-
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FIG. 1. Membrane tyrosine kinase receptors, signal transduction, and membrane-responsive functions.

1. Phospholipase (IP, DAG, Inositol Glycan, Ca++)
2. Nucleotide Cyclase (cAMP, cGMP)
3. Electromotive Forces
4. Activation of Nuclear "trans" Factors
TABLE 1
Insulin and IGF-I Binding to Cells Cultured from Families with Leprechaunism

|                  | Specific Binding |             |             |
|------------------|------------------|-------------|-------------|
|                  | Insulin (\% of control) | IGF-I         |             |
| Controls         | 100 ± 15         | 100 ± 25    |             |
| Ark-I            | 11 ± 5           | 98 ± 5      |             |
| Ark-I mother     | 63 ± 4           | 78 ± 10     |             |
| Ark-I father     | 15 ± 3           | NDA         |             |
| Atl              | 11 ± 2           | 64 ± 4      |             |
| Atl mother       | 35 ± 5           | 132 ± 9     |             |
| Atl father       | 34 ± 6           | 123 ± 4     |             |
| Mount Sinai      | 2 ± 1            | 115 ± 5     |             |
| Mount Sinai mother | 56 ± 2         | NDA         |             |
| Mount Sinai father | 20 ± 2         | NDA         |             |
| Minn             | 8 ± 6            | 129 ± 8     |             |

Hormone binding was measured in fibroblasts or lymphoblasts from patients Ark-I, Atl, Minn, Mount Sinai, and their parents. Data are expressed as percentages of $^{125}I$-IGF-I (0.8 ng/ml) and $^{125}I$-insulin (0.75 ng/ml) binding to matched control cells ± SD of three independent determinations. NDA, no data available.

cemia [18,19]. Fibroblasts cultured from these patients lack high-affinity insulin binding, and partial defects in cellular binding are genetic discriminants in genotyping individuals in these families [20–24]. Some cellular functions are non-responsive to insulin, but responsiveness varies among different probands and is normal in parents' cells [22–27]. As shown in Table 1, cells from first-degree relatives express partial impairment of insulin binding. The binding defect is specific for insulin, since IGF-I binding to the same cells is within normal range. Thus, an autosomal recessive trait caused by mutations in the insulin receptor's binding region was proposed as the mechanism producing leprechaunism [19,22–24]. As evidenced by variations in insulin binding defects among probands and first-degree relatives, heterogeneity became part of this genetic hypothesis. In fact, even within these families a clinical spectrum of insulin resistance emerged. For instance, the heterozygous father of Ark-I expressed mild insulin resistance and the Ark-I proband, who is still alive, expresses a unique phenotype between leprechaunism and type A diabetes [22]. This variation in phenotypic expression was postulated as due to different mutations in the insulin receptor gene and their consequent pathophysiology [19,22].

Structure of the Insulin Receptor

Several techniques were developed to study the protein structure of the insulin receptor on plasma membranes of normal and abnormal cultured fibroblasts. Membrane fractions are obtained from cultured cells by differential centrifugation and the insulin receptor (IR) is then extracted by non-ionic detergents [29]. After this partial purification, $^{125}I$-insulin can be cross-linked to the α subunit of the receptor with
disuccinimidyl suberate (DSS) [22,28,29]. Following separation of membrane proteins by polyacrylamide gel electrophoresis (PAGE), the $\alpha$ subunit of the insulin receptor was visualized by autoradiography. In non-reducing conditions, the heterotetramer (350 kDa) was visualized. In reducing conditions, the monomeric $\alpha$ subunit (125–135 kDa) was seen both in control and in leprechaun cells [29]. Higher concentrations of DSS cross-linked two $\alpha$ subunits, which appeared as a dimeric band of 250 kDa [29]. In cells cultured from patient Ark-1 with leprechaunism, DSS failed to cross-link $\alpha$ subunits [29]. In cells from patient Ark-1, Kadowaki et al. subsequently demonstrated a mutation in bp 1507 of the insulin receptor cDNA, which resulted in a conversion Lys460 to Glu [30]. The loss of an $\epsilon$ amino group and altered conformation caused by charge differences probably prevented dimeric cross-linking by DSS [29]. Abnormal cross-linking was not observed in cells derived from other patients with leprechaunism, again suggesting that different mutations in the insulin receptor caused these phenotypes of severe insulin resistance [29].

The $\beta$ subunit of fibroblasts' insulin receptors was studied by evaluating insulin-stimulated autophosphorylation. Insulin binding to its receptor stimulates the incorporation of $^{32}$P, derived from gamma-$^{32}$P-ATP, into tyrosine residues in the $\beta$ subunit [31]. This procedure evaluates not only the size of the $\beta$ subunit (90–95 kDa) of the insulin receptor, but also an early response to insulin. The degree of insulin-enhanced autophosphorylation by cells from patient Ark-1 was reduced concomitantly with insulin binding, suggesting that there was a reduced number of receptors on the plasma membrane, whose autophosphorylation properties were unaffected by the mutation [29]. Impairment of insulin-enhanced phosphorylation has been associated with other insulin-resistant syndromes. In some patients with type A diabetes and acanthosis nigricans, insulin binding was not impaired, but defective insulin-stimulated tyrosine kinase was found [32]. Recently, mutations were identified in the $\beta$ subunit of the insulin receptor of these patients, mutations which either truncate the $\beta$ subunit [33] or alter the ATP binding site of the $\beta$ subunit [34]. In contrast to mutations in the $\alpha$ subunit, at least one mutation in the $\beta$ subunit of the insulin receptor caused insulin resistance, which was inherited as an autosomal dominant trait [33]. In some other patients with type A diabetes, both insulin binding and $\beta$ subunit autophosphorylation were impaired [35]. Defined mutations in the $\beta$ subunit of the insulin receptor do not interfere with insulin binding either in CHO cells or in human fibroblasts [13,33,34], and further molecular studies are required to define the precise mutation in these patients.

Immunological methods, such as immunoblotting and radiodispacement of insulin receptors, are used to evaluate qualitatively and quantitatively the insulin receptor of cells derived from patients with severe insulin resistance. Using immunoblotting techniques, both the $\alpha$ and the $\beta$ subunits were present in fibroblasts' membranes from patients Ark-1 and Atl (Fig. 2). The $\alpha$ subunit band (125 kDa) appeared lighter than the $\beta$ subunit band (90 kDa) with this polyclonal antibody. More quantitative approaches used the radioreceptor displacement assay [36]; by this technique, heterogeneous levels of insulin receptors were quantified in cell membranes from different patients with leprechaunism (Table 2). Fibroblasts from patients Minn and Mount Sinai had the fewest immunoreactive insulin receptors. A partial decrease was present in cells from patient Ark-1, whereas fibroblasts from patient Atl had a normal number of receptors. Thus, variation in the number of immunoreactive insulin receptors further suggested different mutations in the insulin receptor gene. Some may be point mutations which do not interfere with the formation of a mature receptor,
whereas other mutations may impair gene expression, or post-transcriptional or post-translational processing.

**Insulin Receptor Regulation of Membrane Functions**

Insulin regulates amino acid, hexose, and ion transport in target cells [37–43]. In human fibroblasts, insulin regulates amino acid and sugar transport [41–43]. Biochemical studies in muscle and adipocytes indicated that insulin regulation of

| Table 2: Immunoreactive Insulin Receptors on Membranes of Fibroblasts Cultured from Patients with Leprechaunism |
|---------------------------------------------------------------|
| Immunoreactive Insulin Receptors (ng/mg of membrane protein) | % of Controls |
| Controls | 9.49 ± 2.82 | 100 |
| Ark-1 | 2.77 | 29 |
| Atl | 9.08 | 96 |
| Minn | 0.75 | 8 |
| Mount Sinai | 0.61 | 6 |

Membrane proteins solubilized from human fibroblasts were used to displace [125I]-labeled insulin receptor from insulin receptor-specific antibodies [46]. Human insulin receptors were purified from placenta and used as standards (courtesy of Dr. Ira Goldfine).
amino acid and sugar transport involved two different mechanisms. Insulin stimulation of amino acid transport in muscle [37-39] and in human fibroblasts [43] required new protein synthesis. By contrast, insulin stimulation of sugar transport was rapid, independent of protein synthesis [42], and involved the translocation of preformed intracellular transporters to the plasma membrane [44] (Fig. 3). In muscle and fat cells, insulin recruited to the plasma membrane a class of glucose transporters which were uniquely defined immunologically and molecularly (insulin-responsive glucose transporters [45,46]).

Insulin stimulation of sugar transport was studied in cells from patients with leprechaunism (Table 3). Insulin failed to stimulate sugar transport in patient Ark-I cells, produced attenuated responses in fibroblasts from patient Minn, and fully stimulated glucose uptake by cells from patient Mount Sinai. Glucose uptake by fibroblasts cultured from patient Atl was unexpectedly different from controls' and other patients' cells. OMG transport was markedly increased under basal conditions and non-responsive to insulin. Increased glucose transport was due to a post-transcriptional enhancement in the number of sugar transporters on fibroblasts' membrane [23,24]. Basal OMG transport and response to insulin was normal in fibroblasts cultured from both Atl parents (Table 3). Therefore, partial reduction in insulin binding to parental fibroblasts did not affect their responsiveness. The constitutive increase in glucose transport by fibroblasts cultured from patient Atl was similar to that observed in CHO cells transfected with a truncated insulin receptor cDNA [17]. In these experiments, 70 percent of the α subunit cDNA was deleted, but all of the β subunit was left intact [17]. CHO cells transfected with this truncated α subunit receptor exhibited low insulin binding and very high basal glucose uptake which was insulin-insensitive. These observations suggest that the natural mutation in patient Atl produced a functionally similar effect on glucose transport to that artificially introduced in CHO cells, but without a major deletion in the α subunit of the insulin receptor [29].
Insulin stimulation of amino acid transport was normal in cells cultured from patients Ark-1, Atl, Minn, and Mount Sinai ([22–24], and unpublished results). The discrepancy between insulin regulation of amino acid and sugar transport adds genetic evidence to the biochemical observation that the two membrane functions were regulated by different signaling pathways. These mutations in the insulin receptor’s binding domain apparently affected only glucose transport stimulation. With specific monoclonal antibodies it was possible to activate specific responses to insulin. Monoclonal antibodies that blocked insulin binding to the α subunit of the insulin receptor stimulated glucose transport without affecting other cellular functions, such as insulin receptor autophosphorylation [47]. Antibodies directed toward different epitopes of the α subunit of the insulin receptor enhanced antilipolysis, without affecting insulin binding [48]. These results suggested that different cellular functions were sensitive to different conformations of the α subunit both before and after occupancy by insulin. Different conformational changes may explain why mutations in the α subunit of the insulin receptor of patients with leprechaunism altered insulin control of glucose transport, but left regulation of amino acid transport unaffected.

An alternative hypothesis may be the presence of more than one insulin receptor. A variant of the human insulin receptor has been described recently in human placenta [49]. This receptor, which accounts for 6–18 percent of total insulin receptors, binds insulin and IGF-I with similar affinity. Such a receptor with high affinity but low insulin-binding capacity, may be spared in some patients with severe insulin resistance, enabling insulin stimulation of amino acid transport.
The Insulin Receptor Gene in Patients with Severe Insulin Resistance

Both the α and the β subunit of the insulin receptor are coded for by a single gene located on chromosome 19 [1,2,44]. The cDNA for the insulin receptor is approximately 5.2 kb. Figure 4 schematically shows the correspondence between cDNA sequence and protein domains in the mature insulin receptor. The α subunit contains the signal peptide, the cysteine-rich, cross-linking domain, and a poorly defined ligand-binding domain. The β subunit is preceded by a consensus sequence for Arg-Lys-Arg-Arg which is the site for post-translational proteolytic cleavage. The β subunit contains a short membrane spanning domain and the tyrosine kinase domain. The latter has an ATP binding site and many tyrosine residues, some of which are autophosphorylated.

The gene for the insulin receptor is composed of 22 exons and spans more than 120 kb [3]. The 5' pre-transcription region of this gene lacks TATA or CAAT sequence but contains three GC-rich promoters, similar to genes constitutively expressed in eu-karyotic cells (housekeeping genes [3,50]). Several of the translated exons encode well-defined functional domains: exon 1, signal peptide; exons 2 and 3, the cysteine-rich region; exon 15, the transmembranal domain [3]. Exon 11 codes for only 12 amino acids and is alternatively spliced during RNA processing [51]. This alternative splicing explains the differences observed between the two original reported sequences of the insulin receptor cDNA [1,2].

Portions of this gene are polymorphic [52]. Several restriction fragment length polymorphisms (RFLPs) have been described. RFLPs are very frequent in that portion of the gene coding for the junction between α and β subunits. They are rare in the first 2,000 bp of the coding region [52]. RFLPs have been useful in studying the insulin receptor gene and its transmission in families with leprechaunism. The insulin receptor gene of patients Ark-1, Atl, and Minn with leprechaunism presented no private polymorphisms and therefore no evidence for major deletions or insertions [53].
constructing haplotypes, the transmission of the insulin receptor gene could be determined. Patient Atl, the product of a consanguineous marriage, was homozygous for all RFLPs studied and received the same allele from both parents [53]. Atl's parents differed at only one of five polymorphic sites. By contrast, patient Ark-I, whose parents were unrelated, remained heterozygous for several alleles, and her parents differed at three out of four RFLPs [52]. Thus RFLP analysis conformed to the genetic hypotheses developed by pedigree analysis and insulin binding to their cultured cells [22–24,29]. Reduced insulin binding by cells cultured from some patients also resulted from a decreased expression of the insulin receptor gene. Cells cultured from patients Ark-I, Atl, and Mount Sinai had insulin receptor mRNA comparable to controls ([23,54] and unpublished results). By contrast, cells from patients Minn and some patients with type A diabetes had decreased mRNA for the insulin receptor [54,55].

Cells cultured from most patients with leprechaunism express the insulin receptor gene, but produce a mature protein which is unable to bind insulin. This result suggested a mutation producing structural alteration in the insulin receptor protein; this hypothesis was recently confirmed in several families. In family Ark-1, the proband inherited two noncomplementing mutations in the insulin receptor from her unrelated parents (Fig. 4) [30]. The father had a mutation at bp 2143 which converted Gln672 to a STOP codon. It must be noted that the heterozygous father of patient Ark-I expressed a mild form of insulin resistance without leprechaunism [22]. The mother of
| Family     | Total Insulin Binding(%) | Membrane IR | Glucose Transport Insulin | IR mRNA | Parents' cDNA | Proband Genotype |
|------------|--------------------------|-------------|---------------------------|---------|---------------|-----------------|
|            |                          | Structure   | Immuno-reactivity         | Basal   | Response      |                 |
| Ark-1      | 13                       | Decreased X-linking by DSS | Partially decreased | Normal | Absent | +   | Father Gln477→STOP Mother Lys460→Glu | IR1/IR2 |
| Atl        | 10                       | Normal      | Normal                    | High    | Absent | +   | NDA               | IR3/IR3 |
| Minn       | 7                        | Normal      | Severely decreased        | Normal  | Reduced | 0   | NDA               | IR4/?  |
| Mount Sinai| 2                        | NDA         | Severely decreased        | Normal  | Reduced | +   | NDA               | IR4/IR4 |

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| Jap-1      | Decreased α β        | Uncleaved Partially decreased | NDA | NDA | +   | Father/Mother Arg375→Ser | IR5/IR7 |
| A Sisters  | Decreased            | NDA                        | NDA | NDA | +   | Father/Mother Phe347→Val | IR6/IR8 |

NDA, no data available.
Ark-1 had a mutation in bp 1507 converting Lys460 into Glu. The proband expressed only the insulin receptor allele inherited from the mother which lacked Lys460 [30]. This mutation resulted in the loss of a Mbo II restriction site [30]. To determine whether a similar mutation was present in patient Atl, we amplified bp 1413-1537 of the insulin receptor gene. The results, shown in Fig. 5, indicated that the insulin receptor gene of patient Atl was different and retained all Mbo II cut sites.

Some patients with type A diabetes also have mutations in their insulin receptor gene [56,57]. Siblings from Japan who were products of a consanguineous mating were homozygous for a mutation in bp 2424 which converted Arg735 into Ser [56]. This substitution produced a loss of the proteolytic cleavage site between the α and the β subunit. It generated a defective, unprocessed polypeptide chain. In another consanguineous family, the mutation at bp 1363 of the insulin receptor cDNA converted Phe382 into Val. This substitution caused impaired post-translational receptor processing and markedly reduced the number of insulin receptors on the plasma membrane [57].

**Heterogeneity of Defects in the Insulin Receptor**

Table 4 summarizes the results obtained in different patients with a spectrum of familial severe insulin resistance. At least eight different mutations in the insulin receptor gene are likely, based on binding, structure, function, and sequence data from these families. Abnormalities in the insulin receptor arise from decreased gene expression, from altered post-translational processing, and from point mutations which produce proteins with impaired insulin binding. With further application of these molecular techniques, a much larger number of mutations and pathophysiological sequelae are expected.

The studies described above indicate that the insulin receptor is altered in patients with a wide variety of clinically expressed insulin resistance. Different mutations in the α subunit of the insulin receptor cause different phenotypes. Mild insulin resistance is observed in heterozygotes with one mutant allele; however, the heterozygotes do not have growth restriction or other somatic signs of leprechaunism. Mutations in the insulin receptor gene may contribute to the development of common forms of insulin resistance, non-insulin-dependent diabetes mellitus (NIDDM II). In some families, RFLPs in the insulin receptor gene co-segregate with maturity onset diabetes of the young [58]. Furthermore, some RFLPs in the insulin receptor gene are in linkage disequilibrium with a Chinese population expressing NIDDM II [59]. NIDDM II is not a single gene disorder and will not be explained by genetic variations in the insulin receptor gene alone; however, such genetic variation may produce susceptibility to other environmental and genetic factors. Further molecular and physiological studies of families with inborn errors of the insulin receptor will clarify both the normal mechanisms by which insulin signals cellular responses and shed light on common disorders characterized by insulin resistance.

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