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Insulin-Resistant Diabetes Due to a Point Mutation That Prevents Insulin Proreceptor Processing

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A point mutation in the human insulin receptor gene in a patient with type A insulin resistance alters the amino acid sequence within the tetrabasic processing site of the proreceptor molecule from Arg-Lys-Arg-Arg to Arg-Lys-Arg-Ser. Epstein-Barr virus–transformed lymphocytes from this patient synthesize an insulin receptor precursor that is normally glycosylated and inserted into the plasma membrane but is not cleaved to mature α and β subunits. Insulin binding to these cells is severely reduced but can be increased about fivefold by gentle treatment with trypsin, accompanied by the appearance of normal α subunits. These results indicate that proteolysis of the proreceptor is necessary for its normal full insulin-binding sensitivity and signal-transducing activity and that a cellular protease that is more stringent in its specificity than trypsin is required to process the receptor precursor.

THE INSULIN RECEPTOR IS A HETEROTETRAMERIC integral membrane protein composed of two α and two β subunits. The α subunit contains the insulin binding site and is disulfide-bonded to the NH_2-terminal portion of the β subunit. The receptor is anchored in the plasma membrane through a single membrane-spanning region in the β subunit (*1, 2*). The α and β subunits are generated by proteolytic processing of a single chain proreceptor. Cleavage of the proreceptor normally occurs during the intracellular migration of the newly synthesized protein from the rough endoplasmic reticulum to the plasma membrane, presumably in the Golgi apparatus or an early post-Golgi vesicular stage (*3*).

The subject described here was a 23-year-old Japanese woman who was the product of a consanguineous marriage. Diabetes was first diagnosed at age six; she exhibited many of the clinical features associated with the type A syndrome of severe insulin resistance (*4*), including insulin-resistant (nonketotic) diabetes mellitus with markedly elevated serum insulin values (rising from a fasting level of 242 to 1421 μU/ml at 2 hours after glucose administration), acanthosis nigricans, hirsutism, and virilization. Her older sister was similarly affected. In addition, they exhibited some features not normally considered part of this syndrome, including mental retardation, short stature, and dental dysplasia. The latter two features have also been reported in an unrelated subject with Rabson-Mendenhall syndrome who expressed an altered insulin receptor (*5*).

Studies with Epstein-Barr virus (EBV)–transformed lymphocytes of this individual revealed the following abnormalities: (i) markedly reduced binding of ^{125}I-labeled insulin in both intact cells and lectin-purified membrane preparations, (ii) the virtual absence of normal mature α and β subunits, (iii) increased amounts of immunoprecipitable material corresponding in size to the 210-kD uncleaved form of the fully glycosy-

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lated receptor in cells labeled by either surface iodination or continuous biosynthetic labeling, and (iv) phosphorylation only of the 210-kD protein at high insulin concentrations (*6*).

In metabolic labeling and pulse-chase studies with normal cells, a 190-kD protein corresponding to the single chain proreceptor with mannose-rich glycosyl side chains appeared at the earliest time and reached a maximum at 1 hour (Fig. 1A). During the 4-hour chase, a 210-kD form of the proreceptor, representing the fully glycosylated but uncleaved proreceptor, appeared together with increasing amounts of the 135-kD α subunit and 90-kD β subunit concomitantly with a decrease in the amount of the 190-kD component. By contrast, similar studies with cells from the patient (Fig. 1B) revealed synthesis of the 190- and 210-kD molecules with kinetics similar to those of normal cells but the absence of proteins corresponding to the mature α and β subunits. Northern blot analysis indicated the presence of essentially normal levels of insulin receptor messenger RNA (mRNA) in RNA prepared from the patient's EBV-transformed lymphocytes (Fig. 2). In addition, there were no qualitative differences in the sizes of the various transcripts that hybridized with an insulin receptor complementary DNA (cDNA) probe.

The above findings suggested that expression of the insulin receptor gene occurred normally in this patient but that proteolytic processing of the insulin proreceptor did not occur despite its normal intracellular transport, glycosylation, and plasma membrane insertion. We therefore considered several possibilities that might account for a defect in cleavage of the proreceptor, including a deficiency in the cellular protease (or proteases) that converts the proreceptor into α and β subunits, or mutations within the insulin receptor gene per se.

We elected to address the latter question first and chose to examine the portion of the insulin receptor gene of the patient that encodes the cleavage site. The 275-bp exon encoding this region [exon 12 of the human insulin receptor gene (*7*)] was isolated and sequenced. A single nucleotide substitution in codon 735 (AGG → AGT) resulted in the replacement of Arg with Ser, thereby changing the putative recognition sequence for cleavage from Arg-Lys-Arg-Arg to Arg-Lys-Arg-Ser (*8*) (Fig. 3).

To provide further support for our tentative conclusion that defective proteolytic processing of the insulin proreceptor resulting from the Arg → Ser substitution accounts for the deficiency in insulin binding, we attempted to cleave the proreceptor by treatment with trypsin in small graded quan-

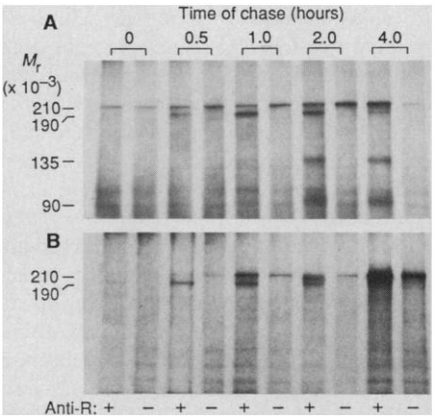

Fig. 1. Comparison of the biosynthesis and processing of insulin receptors in cells from a normal subject (**A**) and from the patient (**B**). EBV-transformed lymphocytes at stationary phase (1×10^9 cells) were sedimented by centrifugation, washed, and suspended in 50 ml of Cys- and Met-free RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum. After incubation at 37°C for 60 minutes, the cells were sedimented and suspended in 25 ml of Cys- and Met-free RPMI 1640 containing 5 mCi each of [^{35}S]Cys and [^{35}S]Met and were incubated at 37°C for 20 minutes. The cell suspensions were then divided into five tissue culture flasks containing 40 ml of complete RPMI 1640 medium with 10% fetal bovine serum, 2 m*M* Cys, and 2 m*M* Met and were incubated for the indicated chase intervals. At each time point, the cells were sedimented and solubilized in 1 ml of 50 m*M* tris-HCl (*p*H 8.0) containing 200 m*M* NaCl, 0.2% (v/v) NP40, 0.5% SDS, 0.5% sodium deoxycholate, 0.2 m*M* phenylmethylsulfonyl fluoride, pepstatin A (5 μg/ml), and leupeptin (5 μg/ml). After removal of insoluble material by centrifugation, the supernatant was applied to a 1-ml column of protein A–agarose and the flow-through was collected. For immunoprecipitation, 50 μl of protein A–agarose was added in the presence (+) or absence (−) of 5 μl of monoclonal antibody 83-14 to human insulin receptor (anti-R) (*15*). After incubation at 4°C for 4 hours, the immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis followed by fluorography.

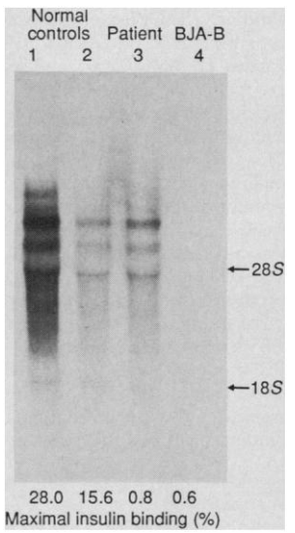

Fig. 2. Northern blot of insulin receptor mRNA. RNA was isolated from EBV-transformed lymphocytes from two normal individuals (lanes 1 and 2), the patient (lane 3), and BJA-B, a cell line established from a patient with Burkitt's lymphoma (lane 4) (*16*). Polyadenylated RNA (20 μg) was denatured with glyoxal and, after electrophoresis in a 1% agarose gel, transferred to a nylon membrane (GeneScreen). The membrane was hybridized with a nick-translated ^{32}P-labeled 4.1-kbp fragment of the human insulin receptor cDNA that includes the region downstream from the internal Eco RI site. The lymphocytes from the two normal subjects differ in mRNA content, but the patient's mRNA is within these limits of normal, whereas the non–insulin-binding cell line BJA-B expresses no detectable mRNA. The patient's insulin receptor mRNA is also normal in both size and the relative distribution of bands.

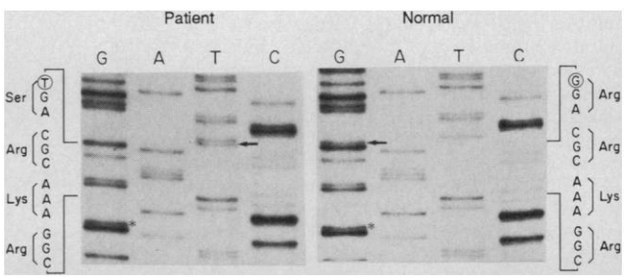

Fig. 3. Sequence of the region of the human insulin receptor gene encoding the proteolytic processing site between the α and β subunits. The sequence of this region of an insulin receptor allele from the patient is compared with that of a normal allele. A mutation (G → T) in both alleles of the patient's insulin receptor gene results in the substitution of Ser for Arg (*17*). Genomic DNA was prepared from EBV-transformed lymphocytes of the patient. A portion of the DNA (1 μg) was digested with Eco RI and ligated to Eco RI arms of λ EMBL-4. After in vitro packaging, phage containing the exon encoding the proteolytic processing site were identified by hybridization with a 1014-bp Xho I fragment of the human insulin receptor cDNA encoding amino acids 655 to 993 (*2*). Four clones of ~7×10^5 recombinants hybridized with this probe. One of these, λ YTIR1, also hybridized with a synthetic oligonucleotide, 5′-GGCGATGTTGGAATGTGACGGTGGCCGTGCCCACGGTGGCAGCT-3′, encoding amino acids 738 to 752 of the β subunit. This clone contained a 15-kbp Eco RI insert. The partial sequence of a 1.5-kbp Pst I fragment and a 0.3-kbp Sau 3A fragment encoding the processing site were determined by the dideoxy method (*18*). Both strands of the exon region were sequenced. The corresponding region of a normal insulin receptor allele isolated from the Hae III–Alu I fetal human liver DNA library of Lawn *et al.* (*19*) was sequenced for comparison. Arrow indicates the one-base mutation, asterisk indicates the intron-exon junction.

tities. We assumed that cleavage within the residual Arg-Lys-Arg sequence might occur with sufficient rapidity (as compared with cleavage at other trypsin-sensitive sites that might lead to receptor inactivation) to allow us to detect some functional activation of the proreceptor. Trypsin treatment at a concentration of 10 μg/ml for 10 minutes led to a four- to fivefold increase in insulin binding in cultured lymphocytes from the patient (Fig. 4). This concentration of trypsin did not cause a significant increase in insulin binding to IM-9 cells. Higher concentrations of trypsin significantly decreased insulin binding in the control IM-9 cells and reduced the extent of binding activation of the patient's cells.

To determine whether functional α subunits were produced by the mild trypsinization of the patient's cells, we used ^{125}I-labeled insulin for affinity labeling of the insulin receptors on control cells and cells treated with trypsin. The 210-kD proreceptor was barely labeled in the patient's cells before trypsinization, whereas in trypsin-treated cells apparently normal α subunits, as well as several lower molecular size bands representing proteolytic fragments of this subunit, were clearly labeled (Fig. 5). A similar pattern of bands was observed after mild trypsin treatment of control IM-9 cells. Thus, the relatively low trypsin concentration used in this experiment resulted in the preferential cleavage of the altered processing site of the mutant proreceptor.

These results, together with previous observations that this mutant insulin receptor is capable of binding insulin and undergoing insulin-stimulated phosphorylation albeit weakly and only at high insulin concentrations (*6*), suggest that normal insulin affinity and sensitivity are conferred only after proteolytic separation of the α and β subunits. The conservation of the tetrabasic amino acid cleavage site in both the insulin-like growth factor–I (IGF-I) receptor (*9*) and the *Drosophila* insulin receptor-like protein (*10*) also strongly argues that cleavage is a prerequisite for normal function in these structurally related proteins. Moreover, just as in the case of other precursors such as proinsulin (*11*), the proreceptor retains some weak biological activity and is thus not an inactive zymogen in the usual sense.

A similar cleavage site having the canonical sequence Arg-X-Lys/Arg-Arg occurs in the envelope protein precursor of the human immunodeficiency viruses HIV-1 and HIV-3 as well as in the influenza virus hemagglutinin glycoprotein (*12*) and certain flaviviruses (*13*). Cleavages at these sites are known to increase the virulence of these viruses. Whether the insulin receptor precursor and these viral precursors are processed by the same cellular protease is an interesting question that might have a bearing on any mode of antiviral therapy based on the principle of inhibiting such maturation events in the viral reproductive cycle.

The mechanism by which cleavage of the insulin proreceptor leads to enhanced insulin binding is not known but might be considered to arise from one or more of the following factors acting singly or in combination: (i) a conformational change leading to alterations in the binding site for insulin in the α subunit, (ii) altered self-association of receptors into oligomeric structures with improved binding characteristics, (iii) enhanced association of receptor with other cell surface components that normally regulate its affinity. Methods are now available for expressing receptor precursors having this Arg → Ser mutation at increased levels (*14*) in order to study in a more systematic fashion both the kinetics of tryptic activation and the structural concomitants of proreceptor activation. Related studies are needed to determine the precise structural requirements for cleavage of the proreceptor and whether the same processing enzyme may also participate in some of the viral protein cleavages described above.

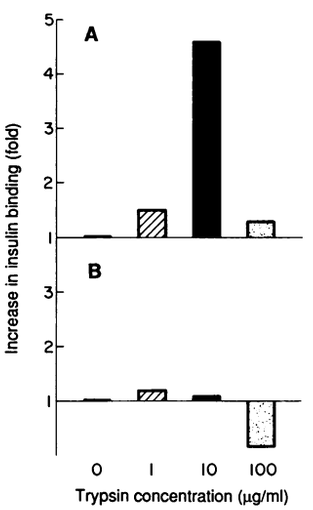

Fig. 4. Effect of trypsin treatment on ^{125}I-labeled insulin binding to (**A**) the patient's cultured lymphocytes and (**B**) IM-9 lymphocytes. Cells were suspended in 50 m*M* Hepes (*p*H 7.8), containing 120 m*M* NaCl, 1.2 m*M* $MgSO_4$, 15 m*M* sodium acetate, 10 m*M* glucose, and bovine serum albumin (10 mg/ml) and were treated with the indicated concentrations of trypsin for 10 minutes at 37°C. A twofold excess of bovine pancreatic trypsin inhibitor was then added and the cells were sedimented. The cells were resuspended in the above buffer (10^7 cells per milliliter) and incubated for 2 hours at 15°C with a tracer amount of insulin radioiodinated at Tyr^{14} of the A polypeptide chain (^{125}I-labeled tyrosyl A-14 insulin). Nonspecific binding was estimated by the addition of excess insulin (1×10^{-5} *M*). The cells were then sedimented and washed twice with phosphate-buffered saline; the radioactivity in the pellets was measured in a gamma spectrometer. ^{125}I-labeled insulin binding to EBV-transformed lymphocytes from normal subjects was 9.0 ± 1.7% per 10^7 cells per milliliter (mean ± SEM). After mild trypsinization, insulin binding to the patient's cells was increased to the low normal range. The values for stimulation of binding from two independent assays were 4.6- and 5.2-fold.

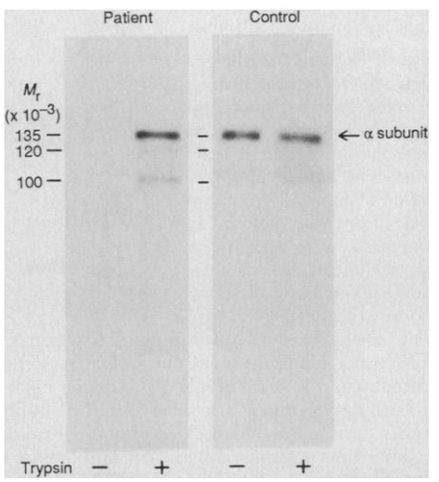

Fig. 5. Effect of mild trypsinization on affinity labeling of the insulin receptor in the patient's cultured lymphocytes and IM-9 lymphocytes. The cultured lymphocytes (~1×10^9 cells) and IM-9 lymphocytes (~5×10^8 cells) were suspended in 2 ml of Hanks balanced salt solution, and trypsin was added at a concentration of 10 μg/ml. After incubating for 10 minutes at 37°C, a twofold excess of bovine pancreatic trypsin inhibitor was added. The cells were sedimented, resuspended in 1 ml of 50 m*M* Hepes (*p*H 7.8) containing 120 m*M* NaCl, 1.2 m*M* $MgSO_4$, 15 m*M* sodium acetate, and 10 m*M* glucose and were incubated with 5 μCi of insulin radioiodinated at Tyr^{26} of the B polypeptide chain (^{125}I-labeled tyrosyl-B-26 insulin) (Amersham) for 2 hours at 15°C. The cells were then sedimented and resuspended in 1 ml of the above buffer. Cross-linking was carried out by incubating the cells with 0.2 m*M* disuccinimidyl suberate for 15 minutes at 4°C. The reaction was stopped by the addition of tris-HCl (*p*H 7.6) to a final concentration of 10 m*M*. The cells were then solubilized by the addition of Triton X-100 to a final concentration of 1%. After removal of insoluble material by centrifugation, immunoprecipitation was performed by adding rabbit anti-human insulin receptor serum (1:200 dilution) and 50 μl of protein A–agarose and incubating for 4 hours at 4°C. The immunoprecipitates were washed with 50 m*M* Hepes (*p*H 7.8), 120 m*M* NaCl, and 0.1% Triton X-100 and then were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography.

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Two Mutant Alleles of the Insulin Receptor Gene in a Patient with Extreme Insulin Resistance

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Insulin receptor complementary DNA has been cloned from an insulin-resistant patient with leprechaunism whose receptors exhibited multiple abnormalities in insulin binding. The patient is a compound heterozygote, having inherited two different mutant alleles of the insulin receptor gene. One allele contains a missense mutation encoding the substitution of glutamic acid for lysine at position 460 in the α subunit of the receptor. The second allele has a nonsense mutation causing premature chain termination after amino acid 671 in the α subunit, thereby deleting both the transmembrane and tyrosine kinase domains of the receptor. Interestingly, the father is heterozygous for this nonsense mutation and exhibits a moderate degree of insulin resistance. This raises the possibility that mutations in the insulin receptor gene may account for the insulin resistance in some patients with non–insulin-dependent diabetes mellitus.

INSULIN RESISTANCE IS A KEY FACTOR in the pathogenesis of non–insulin-dependent diabetes mellitus (NIDDM) (*1*). Despite extensive in vivo and in vitro studies, the molecular mechanisms underlying the insulin-resistant state have not been defined precisely. Nevertheless, considerable epidemiological evidence suggests that genetic factors contribute significantly to the etiology of NIDDM (*2*). Thus, it is important to identify the genes that are targets for mutations causing NIDDM. Patients with genetic forms of extreme insulin resistance are particularly interesting because they provide insights into the disease mechanisms that can cause insulin resistance in humans (*3*). Previous studies suggested that one such patient (leprechaun/Ark-1) is a compound heterozygote with two different mutant alleles of the insulin receptor gene (*4, 5*). We have now cloned insulin receptor complementary DNA (cDNA) from this patient and have identified the point mutations in both alleles.

Leprechaun/Ark-1 is a patient with extreme insulin resistance in association with the syndrome of leprechaunism (*4–7*). She has diabetic glucose tolerance and severe hyperinsulinemia, with levels of insulin in plasma elevated 10- to 100-fold above the normal range (Table 1) (*4, 7*). The number of insulin receptors on the surface of her circulating monocytes is reduced to 15 to 20% of normal (*4*). In contrast, when the patient's lymphocytes were transformed with Epstein-Barr virus (EBV-lymphocytes) and cultured in vitro, the number of insulin receptors expressed on the surface of these cells was in the low normal range. However, there were multiple qualitative abnormalities in insulin binding to receptors on the surface of the patient's EBV-lymphocytes. For example, binding of insulin to the patient's receptors is relatively insensitive to changes in *p*H and temperature (*4, 6*).

Insulin receptors on the surface of EBV-lymphocytes from the patient's mother exhibited qualitative abnormalities in insulin binding that were similar to those observed in leprechaun/Ark-1 (*4, 6*). However, the mother is neither insulin-resistant nor diabetic, and her insulin levels are normal (Table 1) (*4, 7*). In contrast, as reflected by the elevated level of insulin in his plasma, the father is insulin-resistant—although less severely so than leprechaun/Ark-1. The number of insulin receptors on the surface of the father's circulating monocytes is decreased to 30 to 40% of normal. However, insulin binding to his EBV-lymphocytes appears normal both quantitatively and qualitatively (Table 1) (*4*).

A random-primed cDNA library was constructed in λgt10 from polyadenylated RNA from EBV-lymphocytes of leprechaun/Ark-1 (*8*). When 2×10^6 independent clones were screened with a 5167-bp human insulin receptor cDNA (*9*), 40 positive clones

Table 1. Characteristics of patients (*4, 7*). All three patients were given oral glucose tolerance tests in which plasma levels of glucose and insulin were measured. Leprechaunism resembles NIDDM in that both syndromes are associated with insulin resistance and glucose intolerance. However, unlike NIDDM, leprechaunism is frequently associated with fasting hypoglycemia (*7*).

Patients	Glucose (mg/dl)		Insulin (μU/ml)		Insulin binding (% per 10^7 cells)	
	Fasting	2-hour	Fasting	Peak	Monocytes	EBV-lymphocytes
Lep/Ark-1	18	480	125	10,000	2	17
Father	71	130	90	485	4	31
Mother	71	73	6	110	16	42
Normal range	60–105	<140	<20	<200	10–18	16–62

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