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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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Table 2. Deviations from nucleotide sequence of Ullrich *et al.* (*9*). Alleles M and P are the alleles of the insulin receptor gene that are inherited from the mother and father. Several overlapping clones were sequenced to obtain the nucleotide sequences of allele P between nucleotides 1155 and 4035, and allele M between nucleotides 557 and 3924. The last column refers to the cDNA sequence published by Ebina *et al.* (*10*). A minus sign indicates that the sequence is identical to that published by Ullrich *et al.* (*9*). A plus sign indicates that the allele has the nucleotide substitution indicated in the second column of the table.

Nucleotide	Substitution		Allele M	Allele P	Ebina (*10*)
	Nucleotide	Amino acid			
1507	A→G	Lys→Glu	+	−	−
1686	C→T	Asp→Asp	−	+	+
1698	A→G	Ala→Ala	+	+	+
2055	C→T	Phe→Phe	−	+	−
2143	C→T	Gln→STOP	−	+	−
2711	A→T	Asp→Val	+	+	+
2713	A→T	Thr→Ser	+	+	+
3846	C→G	Asn→Lys	+	+	−

were identified. These clones segregated into two categories on the basis of their sequences at positions 1507, 1686, 2055, and 2143 (Table 2). For example, the multiple differences between the sequences of clones 6.21 (nucleotides 1440–3924) and 6.13 (nucleotides 1438–4035) suggested that they arose from the two different alleles of the patient's insulin receptor gene (alleles M and P, respectively). The polymorphic sequences at positions 1686 and 2055 were silent in that both sequences encoded the same amino acids. In contrast, the differences at positions 1507 and 2143 in the nucleotide sequences are significant. Each of these mutations was confirmed by direct sequencing of two independent clones.

Position 1507. Whereas allele P had the normal sequence at nucleotide 1507, G was substituted for A in allele M. This changed the codon for lysine (AAG) at position 460 in the α subunit to a glutamic acid codon (GAG). In addition, the mutation converted the sequence GAAGA to GGAGA, thereby abolishing the pentanucleotide recognition site for the restriction endonuclease Mbo II at nucleotides 1506–1510 in the normal cDNA sequence. The substitution of the acidic amino acid Glu for the basic amino acid Lys is the type of mutation that might plausibly explain an alteration in the sensitivity of the receptor to changes in *p*H.

Position 2143. Whereas allele M had the normal sequence at nucleotide 2143, T was substituted for C in allele P. This converted the codon for glutamine (CAG) at position 672 in the α subunit to a chain termination codon (TAG). In addition, the presence of T at nucleotide 2143 in allele P converted the sequence CCAG to CTAG, thereby creating a new recognition site for the restriction endonuclease Mae I.

To determine whether allele P had been inherited from the father or the mother, we amplified the 120-bp region of genomic DNA extending from nucleotide 2119 to 2238 (Fig. 1B). Mae I did not cut amplified DNA from a normal subject (lane B) or the mother (lane H). In contrast, Mae I cleaved approximately 50% of the amplified DNA from the father (lane F) and leprechaun/Ark-1 (lane D). These data demonstrate that both leprechaun/Ark-1 and her father are heterozygous for the nonsense mutation at position 2143 in allele P. Similarly, we demonstrated that the missense mutation in allele M had been inherited from the mother by amplifying the 125-bp region of genomic DNA extending from nucleotide 1413 to 1537 (Fig. 1A). The normal Mbo II recognition site was present at position 1506 in the amplified DNA derived from both alleles of a normal subject and the father (lanes B and D) as judged by complete cleavage at that site. In contrast, with amplified DNA from leprechaun/Ark-1 and the mother, approximately 50% of the molecules resisted cleavage at the Mbo II site at nucleotide 1506 (lanes F and H). These data demonstrate that both leprechaun/Ark-1 and her mother are heterozygous for the missense mutation at position 1507 in allele M.

The nonsense mutation in allele P causes translation of the receptor protein to be prematurely terminated after Asn^{671}, thereby deleting the COOH-terminal 48 amino acids of the α subunit and the entire β subunit of the receptor. As predicted by the fact that the truncated receptor lacks the transmembrane domain of the receptor located in the β subunit, the truncated receptor is not expressed at the plasma membrane (*12*).

To evaluate the significance of the substitution of Glu for Lys at position 460, we have expressed the mutant form of the insulin receptor cDNA in cultured cells. The transfection experiment described below demonstrates that substitution of Glu for Lys at position 460 accounts for the *p*H insensitivity originally observed with the mutant receptors on EBV-lymphocytes of leprechaun/Ark-1 and her mother (*4, 6*). With insulin receptors on EBV-lymphocytes from normal subjects, decreasing the *p*H from 7.8 to 6.8 causes a five- to tenfold increase in the dissociation rate for the ^{125}I-labeled insulin-receptor complex. In con-

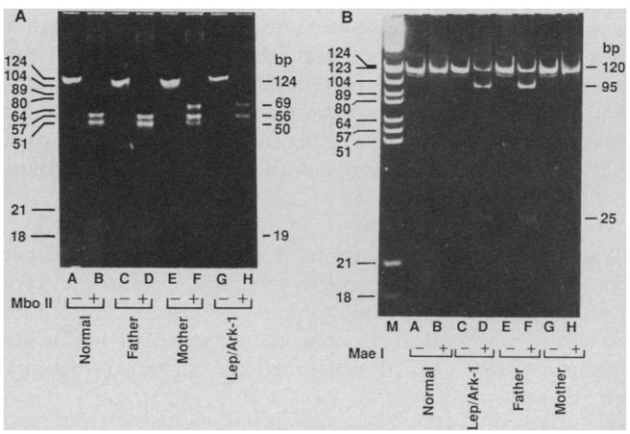

Fig. 1. Restriction endonuclease digestion of genomic DNA amplified by the polymerase chain reaction. Genomic DNA (1 μg) was prepared from EBV-lymphocytes of a normal subject, leprechaun/Ark-1, the father, and the mother. The regions of interest were amplified using 30 cycles of the chain reaction catalyzed by Taq DNA polymerase (Perkin-Elmer–Cetus) (*11*) with 20-mer synthetic oligonucleotide primers. The amplified DNA (100 ng per lane) was analyzed by electrophoresis through a 20% polyacrylamide gel. Undigested samples are shown in lanes A, C, E, and G. Digested samples are shown in lanes B, D, F, and H [Mbo II (A) and Mae I (B)]. Photographs of the gels stained with ethidium bromide are shown. (**A**) The nucleotide sequence of this region (nucleotides 1413–1537) of the normal insulin receptor gene contains two Mbo II recognition sites at nucleotides 1456 and 1506. In contrast, the missense mutation at nucleotide 1507 in allele M destroys the Mbo II recognition site at nucleotide 1506. Thus, the normal sequence is predicted to yield three fragments that are 19, 50, and 56 bp in length upon digestion with Mbo II. Allele M would be predicted to yield two fragments that are 56 and 69 bp in length. (**B**) The nucleotide sequence of this region (nucleotides 2119–2238) of the normal insulin receptor gene does not contain a recognition site for Mae I. In contrast, the nonsense mutation at nucleotide 2143 creates a site for Mae I so that amplified DNA derived from allele P is cleaved into two fragments (25 and 95 bp in length) upon digestion with Mae I. Because of their shorter lengths, the 19- and 25-bp fragments [(A) and (B), respectively] are less intensely stained by ethidium bromide and are not well seen in the photographs.

trast, the effect of this change in *p*H on dissociation rate is blunted in receptors on cells from leprechaun/Ark-1 and her mother (*4, 6*). When the expression vectors encoding the normal (WT) and mutant (KE-460) forms of the insulin receptor were transfected into NIH 3T3 cells, comparable levels of receptor expression were obtained (approximately 2×10^6 to 3×10^6 receptors per cell). We evaluated the effect of *p*H on the rate at which ^{125}I-labeled insulin dissociates from NIH 3T3 cells transfected with either the normal or the mutant form of the insulin receptor. With the transfected cells expressing the normal receptor, the dissociation rate was accelerated fourfold at *p*H 6.8 and tenfold at *p*H 6.0. In contrast, with the transfected cells expressing the KE-460 mutant receptor, lowering the *p*H to 6.8 or 6.0 had a 60 to 70% smaller effect on the dissociation rate (Table 3). This observation suggests that Lys^{460} may be involved directly in the insulin binding site.

The father exhibits a moderate degree of insulin resistance (*4, 7*), which probably results from a decrease in the number of insulin receptors on the surface of his cells. The decrease in receptor number is most likely caused primarily by a gene dosage effect. According to this hypothesis, the existence of only one (presumably normal) gene encoding a functional full-length insulin receptor leads to a 50% reduction in the number of insulin receptors on the cell surface (*13*). This, in turn, may lead to a compensatory increase in the level of insulin in plasma and, secondarily, a further decrease in receptor number as a result of down regulation (*14*). Consistent with this hypothesis, the number of insulin receptors on the surface of the father's circulating monocytes was reduced to 30 to 40% of normal (*4*).

Like her father, leprechaun/Ark-1 is heterozygous for the nonsense mutation. However, in leprechaun/Ark-1, the second allele (allele M) contains a missense mutation encoding the substitution of Glu for Lys at position 460 in the α subunit. Although the mutant form of the receptor encoded by allele M has abnormal binding properties, it appears to have normal tyrosine kinase activity as judged by studies with insulin receptors from the patient's EBV-lymphocytes (*12, 15*). Therefore, it is not yet known why allele M is less effective than a normal allele in complementing the nonsense mutation in allele P.

Syndromes such as leprechaunism are associated with a more extreme form of insulin resistance than is observed in NIDDM. It is possible that this results from a gene dosage effect similar to that observed in several genetic diseases. For example, in familial hypercholesterolemia, heterozygosity for a mutation in the low-density lipoprotein receptor gene is associated with a less severe degree of hypercholesterolemia than is observed in the homozygous state (*16*). In this regard, it is intriguing that the father of leprechaun/Ark-1 has impaired glucose tolerance and a degree of insulin resistance that is closer to that observed in obese patients or patients with NIDDM. Thus, it seems appropriate to reconsider the possibility that a significant subpopulation of patients with NIDDM may be heterozygous for mutations in the insulin receptor gene. Moreover, it is possible that some individuals may be homozygous for mutations that impair receptor function, but less severely than the type of mutation identified in allele P of leprechaun/Ark-1. The application of recombinant DNA technology may provide answers to these questions and has the potential to yield important insights into the molecular basis of the genetic defects that cause NIDDM.

Table 3. Transfection of insulin receptor cDNA: effect of substitution of Glu for Lys at position 460 in the α subunit. The normal insulin receptor cDNA (nucleotides 1–4572) was cloned into a bovine papilloma virus expression vector according to the method of Whittaker *et al.* (*17*). However, we inserted the SV40 polyadenylation signal [derived from nucleotides 3094 to 3502 of pSV2CAT (*18*)] on the 3′ side of the insulin receptor cDNA before the addition of the Sal I linkers. The mutant KE-460 expression vector was constructed by removing a 103-bp Nar–I/Bgl II fragment of cDNA (nucleotides 1478–1580) from the normal insulin receptor cDNA and substituting the corresponding region of allele M (clone 6.21). NIH 3T3 cells (5×10^5 cells) were transfected with a calcium phosphate precipitate containing 10 μg of a mixture of expression vector (either WT or KE-460) plus plasmid pRSVneo DNA in a 20:1 ratio (*19*). After selection by culture in the presence of the antibiotic G418 (600 μg/ml; Gibco), stable transfectants were isolated, cloned, and cultivated in six-well plates (3.5-cm diameter per well). In these studies, we used three cell lines transfected with WT receptor (615, 620, and 1519) and one KE-460 (1603). To assay the effect of *p*H on insulin dissociation kinetics, [^{125}I]-labeled insulin (16,000 cpm) was added to each well in 1 ml of binding buffer (*20*) at 4°C, and the incubation was continued overnight. The medium was aspirated, and the cells were washed quickly with 2 ml of fresh medium at 4°C. (The transfected cells have approximately 2×10^6 to 3×10^6 receptors per cell, whereas the nontransfected NIH 3T3 cells have approximately 10^4 receptors per cell.) Thereafter, fresh medium with a *p*H of 7.8, 6.8, or 6.0 (*21*) was added at room temperature. After incubation for 5 minutes, the medium was aspirated. The radioactivity (counts per minute) in the medium (D) was taken as a measure of the amount of ^{125}I-labeled insulin that had dissociated. The cells were then solubilized overnight in 1 ml of 1*N* NaOH to determine the counts per minute remaining associated with the cells (B). The percentage of insulin that had dissociated was calculated as 100% × D/(D + B). The results of two separate experiments are presented below. Data are presented as means of duplicate determinations ± half the difference between the duplicate values.

Experiment	Cell line	% ^{125}I-labeled insulin bound per 10^6 cells	% Bound ^{125}I-labeled insulin dissociated per 5 minutes		
			*p*H 7.8	*p*H 6.8	*p*H 6.0
1	WT (620)	48	7.4 ± 0.6	28.5 ± 1.1	
	KE-460 (1603)	60	11.8 ± 1.5	7.7 ± 0.6	
2	WT (1519)	57	4.1 ± 0.2	16.0 ± 0.5	43.0 ± 0.5
	WT (615)	37	7.0 ± 0.4	20.3 ± 3.0	46.7 ± 3.7
	KE-460 (1603)	65	2.6 ± 0.6	3.9 ± 0.3	16.2 ± 0.2

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20. The binding buffer used was 120 m*M* NaCl, 2.5 m*M* KCl, 1 m*M* EDTA, 100 m*M* Hepes (*p*H 7.8),

1.2 m*M* $MgSO_4$, 15 m*M* sodium acetate, 10 m*M* glucose, and bovine serum albumin, 10 mg/ml.

21. The medium added to vary the *p*H was 120 m*M* NaCl, 4.5 m*M* KCl, 1 m*M* EDTA, 1.2 m*M* $MgSO_4$, 10 m*M* glucose, bovine serum albumin, 10 mg/ml; buffered with 100 m*M* *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid, *p*H 7.8, or 100 m*M* *N*-(2-acetamido)-2-aminoethanesulfonic acid, *p*H 6.8, or 100 m*M* 1-(*N*-morpholino)ethanesulfonic acid, *p*H 6.0.

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Translocation and Rearrangement of Myeloperoxidase Gene in Acute Promyelocytic Leukemia

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Acute promyelocytic leukemia (subtype M3) is characterized by malignant promyelocytes exhibiting an abundance of abnormally large or aberrant primary granules. Myeloperoxidase (MPO) activity of these azurophilic granules, as assessed by cytochemical staining, is unusually intense. In addition, M3 is universally associated with a chromosomal translocation, t(15;17)(q22;q11.2). In this report, the *MPO* gene was localized to human chromosome 17 (q12–q21), the region of the breakpoint on chromosome 17 in the t(15;17), by somatic cell hybrid analysis and in situ chromosomal hybridization. By means of *MPO* complementary DNA clones for in situ hybridization and Southern blot analysis, the effect of this specific translocation on the *MPO* gene was examined. In all cases of M3 examined, *MPO* is translocated to chromosome 15. Genomic blot analyses indicate rearrangement of *MPO* in leukemia cells of two of four cases examined. These findings suggest that *MPO* may be pivotal in the pathogenesis of acute promyelocytic leukemia.

MYELOPEROXIDASE (MPO) IS A critical bacteriocidal protein with molecular mass of approximately 150 kD (*1*). MPO is the most abundant protein of the mature human polymorphonuclear neutrophil, accounting for 3 to 5% of the dry weight of the cell (*2*). We (*3*) and others (*4, 5*) have demonstrated that *MPO* is abundantly expressed only at the promyelocyte stage; the amount of *MPO* rapidly declines as myeloid cells proceed toward terminal differentiation. The appearance of MPO in early granulocytes (*6–8*) serves as a lineage-specific marker of both normal and malignant myeloid differentiation. As a clinical diagnostic tool, MPO cytochemical activity is critical in distinguishing poorly differentiated malignant blast cells of acute myelogenous leukemia (AML), which have MPO activity, from the blast cells in acute lymphoblastic leukemia (ALL), which do not have MPO activity (*9*), and in assigning acute leukemia cases to one of several subtypes according to the French-American-British (FAB) system.

Acute promyelocytic leukemia (APL) (FAB classification, M3) is a distinct, well-characterized, clinical, morphologic, and cytogenetic subtype of AML. Morphologically, intense granulation with abnormally large or bizarre primary granules, kidney-shaped nuclear morphology, and abundant Auer bodies are characteristic of the malignant promyelocytes associated with this disorder. Intensely positive MPO activity in APL, as assessed by cytochemical staining, suggests an abundance of functional MPO. Cytogenetic analysis consistently reveals a translocation involving chromosomes 15 and 17 in M3 cells. This specific translocation, t(15;17)(q22;q11.2), has not been observed in any other subtype of AML or in any other malignant disease (*10*). The apparently high levels of MPO seen in APL raise the possibility that *MPO* may be affected by this translocation and, therefore, may be involved in the pathogenesis of this malignancy. We describe herein the use of *MPO* complementary (cDNA) clones as hybridization probes to localize *MPO* to normal metaphase chromosomes, to examine its location in metaphase cells from M3 leukemia, and to analyze its genomic organization by Southern blotting of DNA from normal and M3 cells.

A cDNA clone (pHMP7) was isolated by immunologic screening with rabbit antiserum raised against purified MPO and characterized as an authentic *MPO* cDNA clone as described (*3*). This clone was subsequently used to isolate two longer cDNA clones by nucleic acid hybridization to the original cDNA libraries; these were subcloned into the plasmid vector pGEM-3 blue (Promega Biotech). Comparison of the restriction endonuclease maps of these clones (Fig. 1) with published data (*11*) indicates that our clones pHMP10A and pHMP2E cover the 3′ end of *MPO*. Our clones represent both

Table 1. Synteny test of the *MPO* gene and human chromosomes in rodent × human hybrid clones. Somatic cell hybrids were scored for the presence (+) or absence (−) of specific human chromosomes by gene-enzyme and cytogenetic analysis and the presence or absence of human *MPO* coding sequences by hybridization or radiolabeled *MPO* cDNA to Southern blots as described (*12*).

Human chromosome	*MPO* gene/chromosome				% Asynteny
	+/+	+/−	−/+	−/−	
1	16	14	3	1	50
2	9	18	3	0	70
3	1	9	0	1	82
4	7	3	1	0	57
5	15	11	1	2	41
6	17	10	3	1	42
7	0	9	0	1	90
8	15	12	3	0	50
9	7	2	4	0	46
10	6	6	0	0	50
11	21	3	5	0	28
12	15	9	3	1	43
13	21	5	2	1	24
14	20	7	3	0	33
15	19	13	2	2	42
16	11	0	8	1	40
17	28	0	0	19	0
18	1	11	0	0	92
19	16	11	1	3	39
20	10	8	0	4	36
21	0	11	0	0	100
22	0	12	0	1	92
X	10	12	1	2	52

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