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A mutation in the insulin receptor gene that impairs transport of the receptor to the plasma membrane and causes insulin-resistant diabetes

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Insulin binds to a receptor on the cell surface, thereby triggering a biological response within the target cell. Mutations in the insulin receptor gene can render the cell resistant to the biological action of insulin. We have studied a family in which two sisters have a genetic form of insulin-resistant diabetes mellitus. The technique of homozygosity mapping has been used to demonstrate that the mutation causing diabetes in this consanguineous family is genetically linked to the insulin receptor gene. The two insulin-resistant sisters are homozygous for a mutation encoding substitution of valine for phenylalanine at position 382 in the α-subunit of the insulin receptor. Transfection of mutant insulin receptor cDNA into NIH3T3 cells demonstrated that the Val^{382} mutation impaired post-translational processing and retarded transport of the insulin receptor to the plasma membrane. Thus, the mutation causes insulin resistance by decreasing the number of insulin receptors on the surface of the patients' cells.

Key words: consanguinity/diabetes mellitus/insulin receptor gene/mutation

Introduction

The insulin receptor is a cell surface glycoprotein that mediates the biological actions of insulin upon the target cell. The receptor, which is the product of a single gene, undergoes several post-translational processing steps including glycosylation and proteolytic cleavage into two subunits (Hedo *et al.*, 1983; Ebina *et al.*, 1985; Ullrich *et al.*, 1985). Subsequently, the receptor is transported to the plasma membrane. When insulin binds to the extracellular domain of the receptor, this activates a tyrosine kinase activity associated with the intracellular domain of the receptor thereby triggering the biological response of the target cell (Kasuga *et al.*, 1982; Yarden and Ullrich, 1988).

In the present work, we have identified a structural gene mutation that retards post-translational processing and impairs transport of the insulin receptor to the plasma membrane, thereby causing a form of non-insulin-dependent diabetes mellitus (NIDDM) associated with a particularly severe degree of insulin resistance (Barnes *et al.*, 1974). We have studied a family in which a marriage between first cousins produced two daughters having type A insulin-resistant diabetes mellitus (Kahn *et al.*, 1976) and four unaffected children (Figure 1). Epstein–Barr virus (EBV)-transformed lymphoblasts from both patients contain normal levels of insulin receptor mRNA and synthesize the proreceptor at a normal rate (Hedo *et al.*, 1985; Ojamaa *et al.*, 1988). Nevertheless, the number of insulin receptors on the surface of circulating monocytes and EBV-transformed lymphoblasts is only 10–20% of normal levels. The defect appeared to result from impaired transport of receptors to the plasma membrane. Three complementary approaches have been used to identify the mutation causing insulin resistance in these two sisters. Firstly, by analysis of the inheritance of restriction fragment length polymorphisms (RFLPs), we have shown that the mutation causing insulin resistance is genetically linked to the insulin receptor gene. Because the two sisters were the products of a consanguineous marriage, it was possible to apply the newly developed technique of homozygosity mapping (Lander and Botstein, 1987). Secondly, we have cloned insulin receptor cDNA from one of the affected sisters and have identified a single missense mutation encoding the substitution of valine for phenylalanine at position 382 in the α-subunit of the insulin receptor. The two sisters are

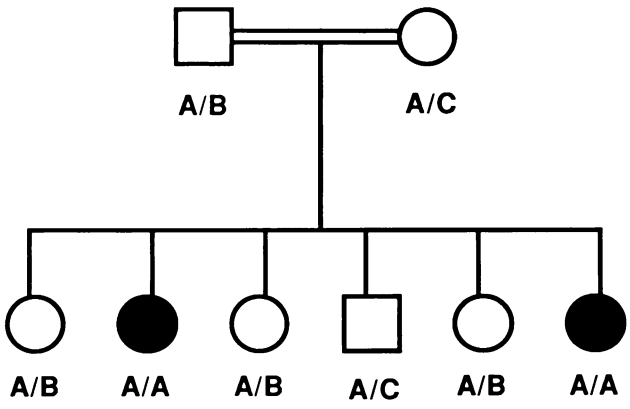

Fig. 1. Pedigree of family of insulin-resistant patients. This pedigree is modified from Barnes *et al.* (1974). The two insulin-resistant sisters are denoted by the filled circles. The alleles are described as [−,−,+,+,+,−,−], [−,+,+,+,+,−,−] or [+,+,+,+,+,−,−]; in each case, the first sign within the brackets refers to the *Sac*I (2) polymorphism, the second sign refers to the *Eco*RI (1) polymorphism, the third to the *Sac*I (1), the fourth to the *Rsa*I (1), the fifth to the *Bgl*II, the sixth to the *Hin*dIII and the seventh to the *Eco*RI (2) polymorphisms (see Table I). We have denoted alleles [−,−,+,+,+,−,−], [−,+,+,+,+,−,−] and [+,+,+,+,+,−,−] as A, B and C, respectively.

Table I. Restriction fragment length polymorphisms of the A allele of the insulin receptor gene inherited by the two insulin-resistant sisters

Restriction endonuclease	RFLP	Hybridization probe	Bands on blot	Frequency in normal population	References
*Eco*RI (1)	−	1599−2961	* 5.5 kb	0.44	Accili *et al.* (1989)
	+		5.8 kb	0.56	
*Sac*I (2)	−	2742−4341	* 9.4 kb	0.88	Elbein *et al.* (1986)
	+		7.0 kb	0.12	
*Sac*I (1)	−	2742−4341	5.8 kb	0.12	Elbein *et al.* (1986)
	+		*‡ 5.3 kb	0.88	
*Rsa*I (1)	−	1599−2961	6.8 kb	0.52	Elbein *et al.* (1986)
	+		*‡ 6.2 kb	0.48	
*Bgl*II	−	1011−5200	23 kb	0.83	Elbein *et al.* (1986)
	+		*‡20 kb	0.17	
*Hin*dIII	−	0−1011	*‡ 1.4 kb	0.91	Sanna *et al.* (1986)
	+		6.5 kb	0.09	
*Eco*RI (2)	−	0−1011	*‡22 kb	0.88	Sanna *et al.* (1986)
	+		20 kb	0.12	

This table summarizes the investigations of the RFLPs in the genomic DNA of patients A-5 and A-8. For each restriction endonuclease, the hybridization probe employed is identified by reference to the nucleotide sequence in the publication of Ullrich *et al.* (1985). The polymorphic bands are identified, together with the estimates of the allelic frequency observed in the normal Caucasian population as published previously. In each case, the fragment observed in the DNA of patients A-5 and A-8 is identified by an asterisk. For five of the RFLPs, the same pattern was observed for all three alleles in the family (denoted by ‡). The frequency, q, of the haplotype corresponding to allele A can be calculated as follows:

$$(0.91)(0.88)(0.88)(0.48)(0.17)(0.44)(0.88) = 0.02$$

We have used data derived from the Caucasian population in the United States and Italy, and have implicitly assumed that these data can be applied to the Caucasian population of Venezuela. Furthermore, we have assumed that all of these RFLPs are in linkage equilibrium with one another. This assumption has been validated for four of the RFLPs [i.e. *Sac*I (1), *Sac*I (2), *Bgl*II and *Rsa*I; Elbein *et al.*, 1986].

It is possible to use the presence of G at nucleotide 1273 as another marker for the A allele (see Figure 4). [In the published sequences of the insulin receptor gene (Ullrich *et al.*, 1985; Ebina *et al.*, 1985), there is a T at nucleotide 1273.] In 160 chromosomes from the normal population, we never detected G at nucleotide 1273 of the insulin receptor gene (Figure 3). This corresponds to an observed frequency of zero for this mutant allele. The 99.9% confidence limit for this allele frequency is 4.2%. If this observation is factored into the calculations outlined above, then the frequency of allele A in the gene pool is almost certainly $<1\%$.

homozygous for this mutation while the two parents are heterozygous carriers. Moreover, the mutation appears not to be a normal polymorphism in that it has not been found in any of the four published sequences of the insulin receptor cDNA (Ebina *et al.*, 1985; Ullrich *et al.*, 1985; Kadowaki *et al.*, 1988) nor in amplified genomic DNA from 80 normal individuals. Thirdly, we have expressed the mutant insulin receptor cDNA by transfection into NIH3T3 cells, and demonstrated that the mutation impairs post-translational processing and transport of the insulin receptor to the cell surface.

Results

Mutation causing insulin resistance is genetically linked to insulin receptor locus

Previous work suggested that the patients' insulin resistance resulted from impaired transport of the insulin receptor to the cell surface (Hedo *et al.*, 1985; Ojamaa *et al.*, 1988). This cellular phenotype could result from either an intrinsic defect in the receptor impairing transport to the cell surface or, alternatively, a defect in some other component of the cellular machinery required for transporting plasma membrane proteins to the cell surface. To distinguish between these two possibilities, we inquired whether the disease genetically co-segregated with RFLPs at the insulin receptor locus.

Genotypes were determined at each of seven known RFLPs in the insulin receptor locus (Table I). Under the assumption that there was no crossing over within the insulin receptor locus in the meioses producing the children, these observations enabled us to identify three haplotypes in the family: A = [−,−,+,+,+,−,−], B = [−,+,+,+,+,−,−] and C = [+,+,+,+,+,−,−] (see Table I for definition of alleles). The two affected sisters had genotype A/A; the mother and brother had genotype A/C; the father and unaffected sisters had genotype A/B (Figure 1).

The mode of inheritance of the disease is almost certainly autosomal recessive, inasmuch as the parents are first cousins and the phenotype was not observed in earlier generations. By standard linkage analysis of a recessive phenotype in a nuclear family, one obtains a LOD score of 1.10 in favor of the hypothesis that the insulin receptor locus is tightly linked to the disease-causing locus (for details of calculation, see Materials and methods). This LOD score corresponds to an 8% chance that the data might have been observed by random chance even if the disease-causing locus and the insulin receptor locus were unlinked. Thus, a LOD score of 1.10 is too low to declare linkage even when testing a single candidate locus.

Recently, however, Lander and Botstein (1987) have pointed out that considerably more linkage information can be obtained from consanguineous marriages by a procedure called homozygosity mapping. The underlying principle is as follows: in a consanguineous marriage such as this, it is predicted that one of the patients' greatgrandparents was a carrier for a mutant allele of the insulin receptor gene. Futhermore, it is predicted that this mutant allele would have been inherited by both of the patients' parents, and that the two patients should be homozygous by descent for this

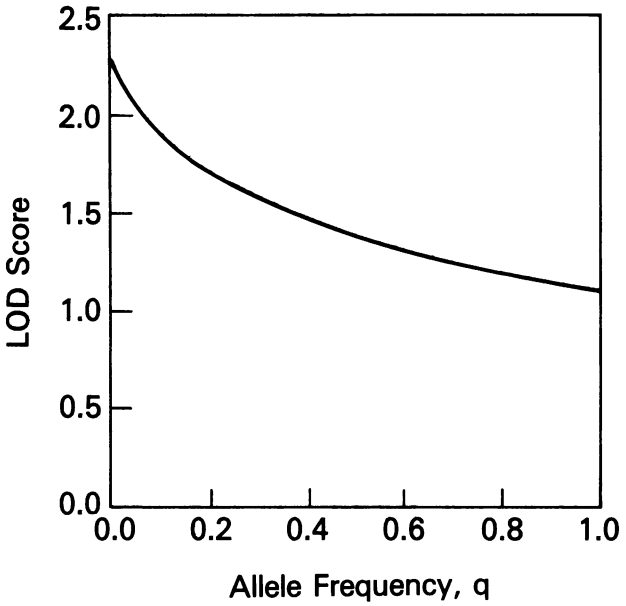

Fig. 2. Dependence of LOD score upon probability of homozygosity. For a first-cousin marriage, a rare disease and tightly linked RFLPs, the LOD score is increased by $\log_{10}[16/(1+15q)]$ where q is the frequency of the observed homozygous haplotype. See Materials and methods for the detailed calculation.

mutant allele. Therefore, if one can demonstrate that the two parents share an allele in common and that it is precisely this common allele for which the patients are homozygous, then these observations provide additional evidence that the insulin receptor locus is linked to the disease-causing locus. The amount of additional linkage information depends upon the frequency of the haplotype in the general population. While homozygosity for a common haplotype might occur at random even in a region that is not homozygous by descent, homozygosity for a very rare haplotype provides strong evidence that the region is actually homozygous by descent (Figure 2). According to the method of homozygosity mapping, the LOD score for the family is approximately 1.9−2.3, based on our estimate of the frequency of the allele A haplotype. Such an LOD score exceeds the threshold for declaring linkage when studying a single candidate locus (see Materials and methods and Discussion).

Missense mutation identified in insulin receptor cDNA

Since the disease-causing mutation appeared to be linked to the insulin receptor locus, we undertook to identify the mutation by cloning the patients' insulin receptor cDNA. We constructed a random-primed cDNA library from mRNA from cultured lymphoblastoid cells derived from patient A-8. Ten overlapping cDNA clones were sequenced to yield the nucleotide sequence extending from 535 nucleotides upstream of the ATG translation start site to nucleotide 3846 (according to the numbering system of Ullrich *et al.*, 1985). To obtain the remainder of the coding sequence (nucleotides 3846−4158), which was not obtained from our cDNA library, we used the polymerase chain reaction to amplify that region of cDNA.

The sequence of the 5′-untranslated region of the patient's mRNA agreed closely with published sequences in this region (Araki *et al.*, 1987; Mamula *et al.*, 1988; Seino *et al.*, 1989). In any case, because insulin receptor mRNA levels and the rate of proreceptor biosynthesis are normal in the patients (Hedo *et al.*, 1985; Ojamaa *et al.*, 1988), it seemed unlikely that the disease would be caused by a mutation in this region. (For discussion of the few differences in the nucleotide sequence in this region, see the legend to Table II.) The sequence of the coding region of mRNA was identical to that of Ullrich *et al.* (1985) with five exceptions (Table II). All but one of the deviations from the sequence of Ullrich *et al.* (1985) have been observed in other published sequences (Table II) and are, therefore, unlikely to be disease-causing mutations. However, at nucleotide 1273 in the patient's insulin receptor cDNA, a G was substituted for the T in the normal sequence. This mutation (substitution of valine for phenylalanine-382 in the α-subunit of the patient's insulin receptor) was confirmed by sequencing three independent cDNA clones and appears to be responsible for causing the disease in this family.

Table II. Deviations from nucleotide sequence of Ullrich *et al.* (1985)

	Substitution			
Nucleotide no.	Nucleotide	Amino acid	Ebina *et al.*	Kadowaki *et al.*
1273	T → G	Phe → Val	−	−
1698	A → G	Ala → Ala	+	+
2711	A → T	Asp → Val	+	+
2713	A → T	Thr → Ser	+	+
3846	C → G	Asn → Lys	+	+

The last two columns refer to the cDNA sequences published by Ebina *et al.* (1985) and Kadowaki *et al.* (1988). A plus sign indicates that the allele has the nucleotide substitution indicated in the second column of the table. A minus sign indicates that the sequence is identical to that published by Ullrich *et al.* (1985).

The sequence of nucleotides in the 535 bp of cDNA corresponding to 5′-untranslated mRNA agreed well with previously published sequences (Araki *et al.*, 1987; Mamula *et al.*, 1988; Seino *et al.*, 1989). There were, however, at least three differences from the sequence published by Araki *et al.* (1987): (i) nucleotide −497 was G rather than A, in agreement with others (Mamula *et al.*, 1988; Seino *et al.*, 1989; Kadowaki *et al.*, unpublished results); (ii) at nucleotide −351, we found T instead of C in the only clone we sequenced in this region; (iii) at nucleotides −95 through −90, Araki *et al.* (1987) found the sequence CCCCCC whereas one of these six Cs was deleted in our sequence as has been observed by others as well (Seino *et al.*, 1989; Kadowaki *et al.*, unpublished results). There are several other differences among the published sequences of the 5′-untranslated mRNA. For example, in several sequences (Mamula *et al.*, 1988; Seino *et al.*, 1989; Kadowaki *et al.*, unpublished results) a GC dinucleotide was inserted between −291 and −292 of the Araki *et al.* (1987) sequence; however, our sequence agreed with that of Araki *et al.* (1987) and did not contain the GC insertion. There were a small number of regions where we sequenced only one strand of DNA and where our sequencing gels were ambiguous so that we are not entirely confident of the sequence in those regions.

Affected sisters are homozygous for the Val^{382} mutation which is not detected in the general population

If the T → G transversion at position 1273 is the cause of the disease, we expected that both patients would be homozygous for this mutation. To test this hypothesis, we amplified the region of the presumed mutation (nucleotides 1172−1306) in the genomic DNA. The amplified fragments of DNA were analyzed by hybridization with allele-specific oligonucleotides (Figure 3). The patients' DNA was detected by hybridization with an oligonucleotide corresponding to the mutant sequence, but not with an oligonucleotide corresponding to the wild-type sequence. Amplified DNA from the parents and the four unaffected siblings hybridized

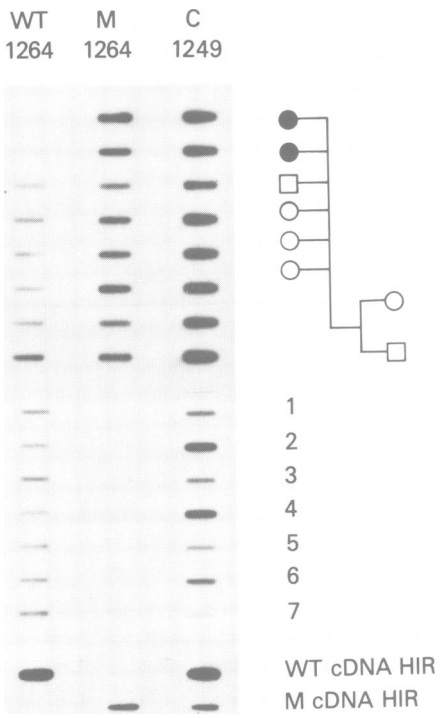

Fig. 3. Hybridization of amplified insulin receptor DNA with allele-specific oligonucleotides. The 134 bp fragment of genomic DNA corresponding to nucleotides 1172–1306 of insulin receptor cDNA was amplified with *Taq* DNA polymerase. Thereafter, one-tenth of the amplified product (~100 ng DNA) was heat denatured at 95°C for 5 min, and then applied to a nitrocellulose filter using a slot-blot apparatus. The blot was hybridized to sequence-specific oligonucleotides corresponding to nucleotides 1264–1281 of either the wild-type sequence (WT 1264), the mutant sequence (M 1264) or a control sequence (nucleotides 1249–1266) located upstream from the mutation (C 1249) (Table IV). Amplified genomic DNA from the family members is located in the rows denoted by the symbols of the pedigree at the top of the figure; rows 1–7 represent amplified DNA from seven representative normal individuals. As a control, hybridization to clones containing either the wild-type (WT) or the mutant (M) sequences is shown in the two rows at the bottom of the figure. Seventy-three additional normal individuals were studied (data not shown). None of the 80 normal individuals had the Val^{382} mutation. Thus, the observed frequency of this mutation in 160 alleles from the normal population was zero. The 95 and 99.9% confidence limits for this estimate of the frequency of the Val^{382} mutation in the normal population are 1.9 and 4.2% respectively.

to both oligonucleotides, consistent with the previous conclusion that all six of these unaffected family members are heterozygous carriers of the mutation.

To rule out the possibility that the mutation is a common sequence polymorphism found in the general population, we analyzed DNA from 80 normal individuals. The Val^{382} mutation was not detected in any of the 160 normal chromosomes (Figure 3).The rarity of the substitution also strengthens the genetic linkage analysis above. The frequency of the complete haplotype carried by the affected sisters is likely to be <1% (Table I), implying a LOD score of 2.25 in favor of linkage (Figure 2).

Val^{382} mutation impairs post-translational processing and transport of receptors expressed in transfected NIH3T3 cells

To evaluate the significance of the substitution of valine for phenylalanine at position 382, we expressed the mutant form of the insulin receptor cDNA in cultured cells. The transfection experiments described below demonstrate that this mutation impairs post-translational processing of the receptor precursor and thereby decreases the number of receptors on the cell surface.

Table III. Effect of Val^{382} mutation upon receptor biosynthesis and transport

	Wild-type receptor		Val^{382} insulin receptor	
	WT-2	WT-8	V382-1	V382-2
Insulin receptor mRNA (molecules/cell)	2000	1800	2000	20 000
Proreceptor biosynthesis (arbitrary units)	4	8	14	32
Cell surface receptors (binding sites/cell)	500 000	650 000	78 000	540 000

NIH3T3 cells were transfected with an expression vector containing human insulin receptor cDNA encoding either wild-type or Val^{382} receptors. Levels of human insulin receptor mRNA were assayed with an RNase A protection assay. The rate of proreceptor biosynthesis was measured as outlined in Materials and methods with the exception that the chase period was omitted. The number of insulin receptors on the cell surface was estimated from the Scatchard plots shown in Figure 5. Untransfected NIH3T3 cells have ~1000 receptors/cell.

When levels of mRNA and receptor biosynthesis are held constant, does the Val^{382} mutation reproduce the patients' phenotype of a decrease in the number of receptors on the cell surface? To answer this question and facilitate studies of the intracellular transport of receptors, one would ideally compare cell lines that expressed equal quantities of mRNA encoding either wild-type or mutant insulin receptor. However, transfected cell lines usually vary with respect to the level of expression of the transfected cDNA. To allow us to take this variation into account, we measured the content of human insulin receptor mRNA in the transfected cells (Table III). The two cell lines transfected with wild-type receptor (WT-2 and WT-8) have similar levels of insulin receptor mRNA (~2000 molecules/cell). In contrast, the three cell lines expressing mutant cDNA had a wide range in the level of insulin receptor mRNA: ~500, 2000 or 20 000 molecules of insulin receptor mRNA/cell in V382-3, V382-1 and V382-2 cells respectively.

We measured the rate of biosynthesis of the 190-kd insulin receptor precursor by incubating cells for 3 h in the presence of [^{35}S]methionine (Table III). One of the cell lines transfected with mutant cDNA (V382-2, the cell line with the highest level of insulin receptor mRNA) synthesized the insulin receptor precursor 4–8-fold more rapidly than was observed with the two cell lines transfected with wild-type insulin receptor cDNA (WT-2 and WT-8). The other cell line transfected with mutant cDNA (V382-1) synthesized insulin receptors at a rate intermediate between that observed in V382-2 and the cells expressing wild-type insulin receptor cDNA. Because of the relatively short labeling period, the major form of the receptor was the 190 kd precursor (data not shown).

To evaluate post-translational processing of the precursor to the mature receptor, more detailed experiments were carried out in which cells were pulse-labeled with [^{35}S]methionine for 3 h followed by a chase period of 1.5 or 4 h (Figure 4). These pulse-chase studies provided the clearest evidence for a defect in the post-translational processing of the mutant receptor. In the cells transfected

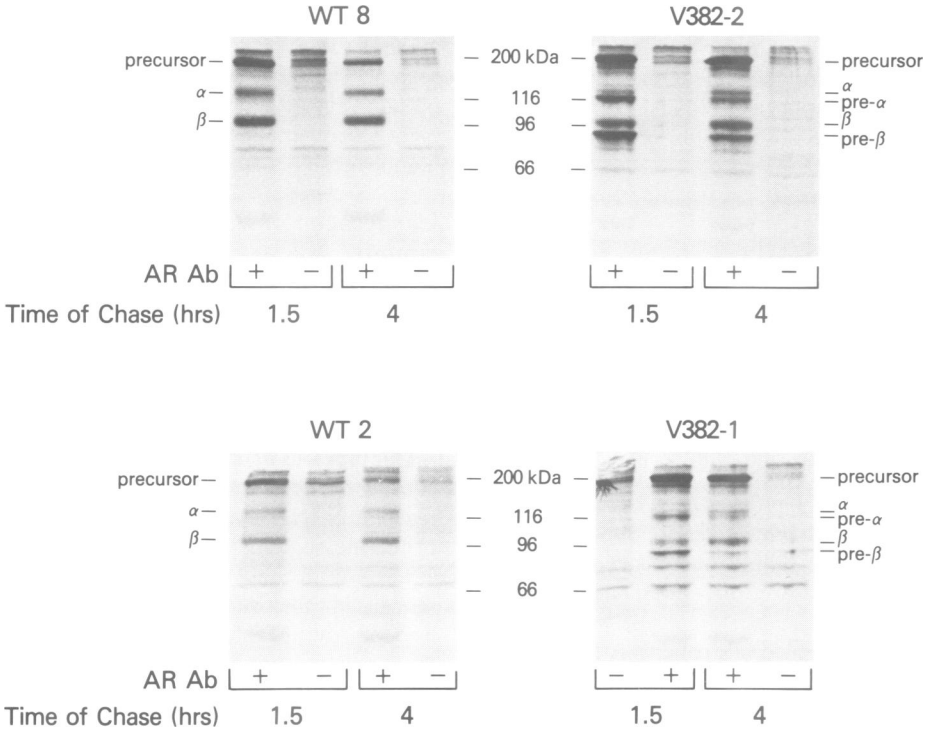

Fig. 4. Pulse-chase labeling of insulin receptor with [^{35}S]methionine. NIH3T3 cells transfected with wild-type (WT-2 and WT-8) or with mutant insulin receptor cDNA (V382-1, V382-2 and V-382-3) were pulse-labeled with [^{35}S]methionine for 3 h followed by a chase with unlabeled methionine for either 1.5 or 4 h. The cells were solubilized, the receptors were immunoprecipitated and the immunoprecipitates were analyzed by SDS–PAGE followed by fluorography.

with wild-type cDNA, two bands (M_r = 130 and 100 kd) were seen in the regions of the α- and β-subunits. In the cells transfected with mutant cDNA, two bands (M_r = 120 and 130 kd) were seen in the region of the α-subunit and two bands (M_r = 90 and 100 kd) in the region of the β-subunit. Previous studies had suggested that proteolytic cleavage of the receptor precedes maturation of the N-linked carbohydrate moieties (Hedo *et al.*, 1983; Ronnett *et al.*, 1984). Therefore, it seemed likely that the low mol. wt forms of the α- and β-subunits might represent precursors in which the N-linked oligosaccharides had not undergone final processing in the Golgi. As has been suggested previously (Hedo *et al.*, 1983), the kinetics of labeling are consistent with the 90 and 120 kd bands being precursors of the 100 and 130 kd bands respectively. Specifically, the ratio of the mature α- and β-subunits (M_r = 130 and 100 kd) to the lower mol. wt precursor forms (M_r = 120 and 90 kd) increases as a function of time during the chase period. Thus, the post-translational processing of the 190 kd proreceptor is less efficient in the cells transfected with mutant cDNA. Despite comparable rates of proreceptor biosynthesis in cell lines WT-8 and V382-1, a larger number of mature receptors are synthesized in the cells transfected with wild-type cDNA (WT-8, Figure 4). Furthermore, there are comparable levels of mature receptors in cell lines V382-2 and WT-8; however, the cell line (V382-2) transfected with mutant cDNA requires ~4-fold more precursor to achieve the same level of mature receptor biosynthesis (Table III, Figure 4).

Studies of [^{125}I]iodoinsulin binding to the surface of the cells (Table III, Figure 5) are consistent with the conclusions of the pulse-chase studies. Thus, the cells transfected with wild-type cDNA bound 22% (WT-2 cells) to 31% (WT-8 cells) of the [^{125}I]iodoinsulin in the medium. In contrast,

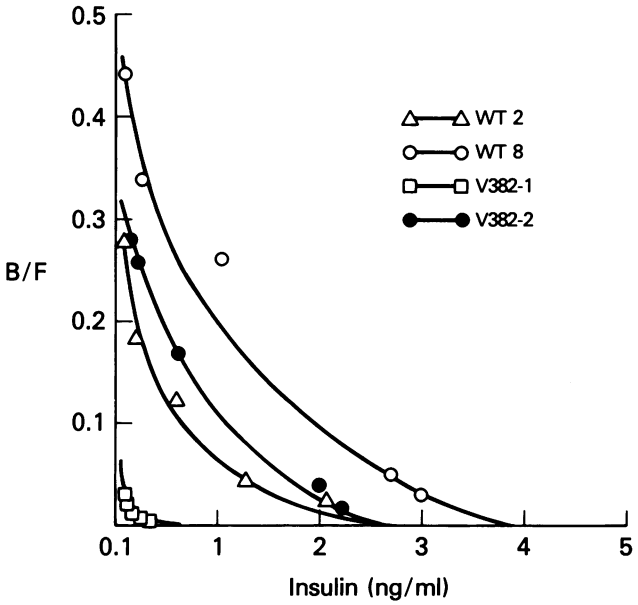

Fig. 5. [^{125}I]Iodoinsulin binding to surface of transfected cells. In the same experiment presented in Table III [^{125}I]iodoinsulin binding to confluent monolayers of NIH3T3 cells transfected with human insulin receptor cDNA is shown. The number of insulin receptors per cell was estimated from the *x*-intercept of the Scatchard plot (Table III).

V382-1 cells bound considerably less [^{125}I]iodoinsulin (3.5%) despite having similar amounts of insulin receptor mRNA. V382-2 cells, which had 10-fold higher levels of insulin receptor mRNA than the cells transfected with wild-type cDNA, bound comparable amounts of [^{125}I]iodoinsulin (22%). Thus, because transport of receptors to the plasma

membrane is retarded rather than totally blocked, the 10-fold greater over-expression of the insulin receptor gene in V382-2 cells compensated for the defect in intracellular transport by restoring the number of cell surface receptors to a level comparable to that observed in WT-2 cells.

Discussion

Mutations in receptor genes

Mutations in the genes encoding cell surface receptors have been observed in patients with familial hypercholesterolemia (Brown and Goldstein, 1986; Yamamoto *et al.*, 1986; Hobbs *et al.*, 1987; Lehrman *et al.*, 1987; Esser and Russell, 1988) and genetic forms of insulin resistance (Taylor, 1987; Kadowaki *et al.*, 1988; Moller and Flier, 1988; Yoshimasa *et al.*, 1988; Odawara *et al.*, 1989). In addition, mutations have been described that convert cell surface receptors into transforming proteins such as the proteins encoded by v-*erbB*, v-*fms* and the *neu* oncogene (Downward *et al.*, 1984; Hampe *et al.*, 1984; Bargmann *et al.*, 1986; Coussens *et al.*, 1986; Roussel *et al.*, 1988; Woolford *et al.*, 1988; Yarden and Ullrich, 1988).

Previously, we had demonstrated that patients A-5 and A-8 had a mutation that was associated with a decrease in the number of insulin receptors on the surface of their cells (Hedo *et al.*, 1985), despite the fact that the levels of insulin receptor mRNA were normal (Ojamaa *et al.*, 1988) as was the rate of proreceptor biosynthesis (Hedo *et al.*, 1985). Thus, these patients were predicted to have a defect in transport of receptors to the cell surface. This prediction has been confirmed in the present study. Several lines of evidence support the conclusion that the disease is caused by the mutation encoding the substitution of valine for phenylalanine at position 382 of the α-subunit of the insulin receptor.

Genetic linkage to the insulin receptor gene

In light of the phenotype of insulin resistance in association with a decrease in the number of insulin receptors on the cell surface (Hedo *et al.*, 1985), the insulin receptor gene seemed a likely candidate for the locus of the mutation causing the disease in these patients. In fact, the pattern of inheritance of RFLPs in the pedigree is consistent with the hypothesis that the mutation is genetically linked to the insulin receptor gene (Figures 1 and 2). The LOD score in favor of linkage ranges from 1.10 according to standard linkage analysis to 2.25 according to the homozygosity mapping method (Lander and Botstein, 1987). A LOD score of 2.25 is sufficiently high to support the hypothesis that the disease-causing mutation is linked to the insulin receptor locus (For details of the calculation, see Materials and methods.)

In genetic mapping, a systematic effort is made to detect linkage to any region of the human genome. Since many regions are analyzed, some spurious correlations will arise by chance. For example, if 50 well-spaced RFLPs are tested for linkage, one is likely to be significant at the 2% level by random chance. In order to guard against false positives, a LOD score threshold of 3.0 is required when the entire genome is examined for linkage (Morton, 1955). However, when only a single pre-chosen candidate locus is tested, a lower threshold of ~ 1.5 is sufficient to ensure a comparable chance of false positives. Since the insulin receptor was a candidate locus chosen for precise biological reasons, the observed LOD score of $2.0-2.3$ is thus highly significant—corresponding approximately to the significance level that a LOD score of 3.5 would have in a complete genomic search. The fact that we were able to demonstrate linkage by analyzing RFLPs in a single pedigree containing only eight members demonstrates the statistical power of homozygosity mapping (Lander and Botstein, 1987).

Identification of Val^{382} mutation as the cause of the disease

The genetic linkage data strongly supported the hypothesis that the insulin receptor gene was the most likely candidate for the mutation causing the disease in these patients. The mutation appears to be recessive in that the parents and the four siblings—all of whom are heterozygous carriers of the mutation—appear not to be affected with the disease.

Our previous data suggested that there would be a mutation altering the coding sequence of the gene inasmuch as insulin receptor mRNA levels were normal as was the translatability of the message *in vivo* (Hedo *et al.*, 1985; Ojamaa *et al.*, 1988). With one exception, the predicted amino acid sequence of the patients' insulin receptor cDNA was normal. The sequence of the patient's insulin receptors differed from the sequences predicted by Ullrich *et al.* (1985) and Ebina *et al.* (1985) by virtue of a substitution of valine for phenylalanine at position 382 in the α-subunit. In the normal insulin receptor, there is a pair of phenylalanines at positions 381 and 382. Interestingly, paired phenylalanines are found elsewhere in the insulin receptor, and also at positions 24 and 25 of the insulin B-chain. In fact, the first two mutations described in the insulin gene were substitutions of serine and leucine for Phe-B24 and Phe-B25 respectively. Both substitutions decreased the biological activity of the insulin molecule by >90% (Tager *et al.*, 1979). Similarly, site-directed mutants of the insulin receptor in which leucine was substituted for Phe^{88} or Phe^{89} markedly decreased the affinity with which the mutant receptor bound insulin (Whittaker *et al.*, 1988). These observations suggest that protein structure can be disrupted by substitution of a branched chain amino acid for one of the phenylalanine residues in the sequence. On the other hand, it is noteworthy that the homologous region of the human IGF-1 receptor contains the sequence Phe-Leu rather than Phe-Phe at positions $371-372$ (Ullrich *et al.*, 1986). Because of the close structural homology between the insulin receptor and the IGF-1 receptor, the presence of a branched chain amino acid at position 372 in the IGF-1 receptor raised the possibility that substitution of a branched chain amino acid at position 382 in the insulin receptor might not disrupt the receptor structure.

Transfection of mutant insulin receptor cDNA into NIH3T3 cells

Therefore, we transfected mutant cDNA in NIH3T3 cells to determine the effect of substituting valine for phenylalanine at position 382 in the α-subunit of the insulin receptor. This point mutation alters the three-dimensional structure of the receptor in such a way as to interfere with post-translational processing. The defect in post-translational processing is associated with impaired transport of the receptor to the plasma membrane. However, it is hard to be certain which is cause and which is effect. Is the defect in post-translational processing caused by a failure of the receptor to be transported to the region of the Golgi where the relevant

processing enzymes are located? Does the defect in processing abolish a signal that contains the information for targeting the receptor as a molecule that is destined for transport to the plasma membrane? There are some data that support the latter possibility. For example, synthesis of functional receptors is inhibited by drugs such as tunicamycin that inhibit the enzymes involved in synthesis of the carbohydrate moiety of the receptor (Rosen *et al.*, 1979; Ronnett *et al.*, 1984). Furthermore, insulin binding has been reported to be abnormal in variants of CHO cells that are defective in the pathways of protein glycosylation (Podskalny *et al.*, 1984).

There are precedents for structural gene mutations that impair the transport of proteins to the plasma membrane. This type of mutation has been described in the low density lipoprotein receptor gene of the Watanabe heritable hyperlipidemic rabbit (Yamamoto *et al.*, 1986) and also in patients with familial hypercholesterolemia (Lehrman *et al.*, 1987; Esser and Russell, 1988). In addition, the mutations that transform the CSF-1 receptor into the v-*fms* oncogene have an effect that closely resembles the effect of the Val^{382} substitution upon the insulin receptor. For example, substitution of serine for leucine at position 301 in the extracellular domain of the CSF-1 receptor partially inhibits glycosylation processing and retards transport of the mutant receptor to the plasma membrane (Roussel *et al.*, 1988; Woolford *et al.*, 1988). Mutations impairing transport to the plasma membrane have also been described in viral proteins, e.g. influenza hemagglutinin molecules, in which case the impaired intracellular transport was also associated with a defect in protein glycosylation (Gething *et al.*, 1986). In several of these examples, there has been evidence that BiP, an immunoglobulin binding protein originally described in B-lymphocytes (Haas and Wabl, 1983; Munro and Pelham, 1986), binds to the defective protein. It is possible that a similar mechanism may play a role in the defective processing of the mutant insulin receptors in our patients' cells.

Materials and methods

Patients

Patients A-5 and A-8 are two sisters with type A extreme insulin resistance (Barnes *et al.*, 1974). The syndrome of type A insulin resistance affects young women and is characterized by extreme insulin resistance in association with two additional characteristic features: (i) a hyperkeratotic, hyperpigmented skin lesion called acanthosis nigricans, and (ii) masculinization caused by overproduction of androgenic steroids by the ovary (Kahn *et al.*, 1976). As reported elsewhere, patients A-5 and A-8 both have an 80–90% decrease in the number of insulin receptors on the surface of their circulating monocytes and EBV-transformed lymphoblasts (Hedo *et al.*, 1985). Neither the parents nor the four unaffected children are diabetic. The pedigree of this Caucasian family from Venezuela is presented in Figure 1. Note that the parents are first cousins so that the children are products of a consanguineous marriage. DNA samples from 20 normal Venezuelan subjects and 60 additional Caucasians (from the panel of the Centre d'Etudes du Polymorphisme Humaine) were kindly provided by Drs James Gusella and Nicholas Dracopoli respectively.

Southern blots

Genomic DNA (10 μg) was isolated from EBV-transformed lymphoblasts according to standard techniques (Maniatis *et al.*, 1982). Thereafter the DNA (10 μg) was digested with restriction endonuclease, electrophoresed on 0.8% agarose gels and transferred to nylon membranes (Schleicher and Schuell, Keene, NH) for Southern blotting according to standard methods (Maniatis *et al.*, 1982).

Measurement of mRNA levels in NIH3T3 cells by RNase A protection assay

An antisense RNA probe was obtained by *in vitro* transcription catalyzed by T7 RNA polymerase in the presence of [α-^{32}P]CTP with a template comprised of a fragment of human insulin receptor cDNA (nucleotides 2013–2854) ligated into the *Eco*RI site of plasmid pGEM-4Z (Melton *et al.*, 1984). Prior to transcription of the template, the plasmid was linearized by digestion with *Stu*I. Total cellular RNA (20 μg) was hybridized for 12 h at 45°C with 50 000 c.p.m. of ^{32}P-labeled antisense RNA probe (0.18 pmol) complementary to nucleotides 2398–2854 of human insulin receptor cDNA in 30 μl of solution containing 80% formamide, 40 mM Pipes (pH 6.7), 400 mM NaCl and 1 mM EDTA. After hybridization, the RNA–RNA duplexes were digested for 30 min at 30°C in 0.3 ml of solution containing 300 mM NaCl, 20 mM Tris–HCl (pH 7.5), 5 mM EDTA and 40 μg/ml of RNase A (Sigma Corp., St Louis, MO). Thereafter, proteinase K (0.1 mg/ml) and SDS (0.5%) were added and the incubation was continued for an additional 30 min at 37°C to digest the RNase A. The samples were extracted with phenol–chloroform and precipitated with ethanol. After being dissolved in 5 μl of 97% formamide containing bromphenol blue (0.04%), xylene cyanol (0.04%), plus Tris (5 mM, pH 7.5), the RNA was analyzed by gel electrophoresis through a denaturing 6% polyacrylamide/8 M urea gel for 2 h at 60 W (Myers *et al.*, 1985). Scanning densitometry was performed on the autoradiographs of the gels to quantitate the radioactivity in the bands (Table III).

In some experiments, RNA samples were treated with 0.3 N NaOH for 30 min at 37°C. Thereafter, the samples were neutralized with 0.1 M Tris (pH 7.5), 10 mM EDTA, plus 50 mM HCl for 10 min at 4°C and desalted by gel filtration through a Sephadex G-50 spin column prior to annealing with the ^{32}P-labeled antisense RNA probe. This alkaline treatment destroyed the RNA and also eliminated the ability of the samples to protect the labeled probe from digestion with RNase A. Thus, there was no major contaminant with insulin receptor DNA that might have interfered with the quantitation of insulin receptor mRNA (Kim and Wold, 1985). Furthermore, when tRNA (20 μg) was substituted for total cellular RNA (20 μg) in the hybridization with antisense RNA probe, the probe was completely digested by RNase A (data not shown).

This assay, which requires near-perfect base pairing between the probe and the mRNA, is specific for human insulin receptor mRNA but does not detect murine insulin receptor mRNA in untransfected NIH3T3 cells.

cDNA cloning

A random-primed cDNA library was constructed in λgt10 from poly(A)$^+$ RNA from EBV lymphocytes of patient A-8 (Gubler and Hoffman, 1983; Bevins *et al.*, 1988). When 1×10^6 independent clones were screened with a 5167 bp human insulin receptor cDNA, 24 positive clones were identified. The cDNA inserts were excised from 15 selected bacteriophage clones, recloned into either the *Eco*RI site in plasmid pGEM-4Z or the *Not*I site of plasmid pGEM-5Z (Promega Biotec, Madison, WI) and sequenced by the dideoxynucleotide method. In some reactions, dITP and ddITP were substituted for dGTP and ddGTP respectively, to resolve ambiguities in the sequence. The clones derived from the λgt10 library covered 4380 bp of sequence (extending from the nucleotide 535 bp upstream of the ATG codon marking the start site of translation, to nucleotide 3846 in the sequence of Ullrich *et al.* (1985).

To obtain the remainder of the coding sequence (nucleotides 3846–4185),

Table IV. Sequences of oligonucleotides

1. 5′-CCTTAAGATGAACAGAAATGTATA-3′ (nt 4309–4286)
2. 5′-TCCAGAGAGAGTCACTGACCTCAT-3′ (nt 3796–3818)
3. 5′-tcaggaattcGCGCATGTGCTGGCAATTCA-3′ (nt 3819–3838)
4. 5′-gcaggaattcTGCCGCCCCCGCCACGGTAG-3′ (nt 4188–4167)
5. 5′-ACAATCTGGCAGCTGAGCTA-3′ (nt 1172–1191)
6. 5′-AGGTCTCTCCTCGAATCAGA-3′ (nt 1287–1306)
7. 5′-CTTTCCTTCTTCCGGAAG-3′ (nt 1264–1281, wt)
8. 5′-CTTTCCTTCGTCCGGAAG-3′ (nt 1264–1281, M)
9. 5′-TACGCTCTGGTGTCACTT-3′ (nt 1249–1266, C)

The oligonucleotides were synthesized with phosphoramidite chemistry using a DuPont Coder 300 (Wilmington, DE). The oligonucleotides were purified by reverse-phase chromatography on a NEN-Sorb Prep column (DuPont-NEN, Wilmington, DE). Upper-case letters depict nucleotides derived from the normal insulin receptor sequence. Lower-case letters depict nucleotides appended to the 5′ termini to provide *Eco*RI linkers to facilitate ligation of amplified DNA into a plasmid. The boldface **G** in oligonucleotide 8 corresponds to the mutation identified in patients A-5 and A-8. Oligonucleotides 1 and 4 correspond to the antisense strand of human insulin receptor cDNA; the remainder of the oligonucleotides correspond to the sense strand.

we used the polymerase chain reaction catalyzed by *Taq* DNA polymerase (Simpson *et al.*, 1988). First-strand cDNA synthesis was catalyzed by reverse transcriptase with random hexanucleotide primers as in the construction of the λgt10 library. The mRNA template was hydrolyzed by incubation in the presence of NaOH (0.2 N) for 60 min at 60°C. The region of interest was amplified sequentially using two nested sets of primers (oligonucleotides 1–4 in Table IV). In the first amplification, 1 ng of single-stranded cDNA was incubated with 0.6 mg of oligonucleotides 1 and 2 in the presence of *Taq* DNA polymerase (2.5 units; Perkin Elmer-Cetus, Emeryville, CA) in a total volume of 0.1 ml. The amplification was carried out for 30 cycles with each cycle consisting of incubations of 90 s at 92°C for denaturation, 90 s at 55°C for annealing, 90 s at 72°C for primer extension. At the beginning of the first cycle, the denaturation period was lengthened to 5 min; at the end of the last cycle, the extension was lengthened to 3 min. Thereafter, one-tenth of the product of the first amplification was amplified in the presence of oligonucleotides 3 and 4 for an additional 35 cycles. The amplified fragment of DNA was digested with *Eco*RI and ligated into the plasmid pGEM-4Z to allow for nucleotide sequencing by the dideoxy technique (Sanger *et al.*, 1977).

Amplification of genomic DNA

The 134 bp region of genomic DNA corresponding to nucleotides 1172–1306 of insulin receptor cDNA was amplified with *Taq* DNA polymerase (Saiki *et al.*, 1988). Genomic DNA (1 μg) was digested with *Rsa*I, extracted with phenol–chloroform, precipitated with ethanol, and then incubated with 0.6 mg of oligonucleotides 5 and 6 (Table IV) in the presence of *Taq* DNA polymerase (2.5 units; Perkin Elmer-Cetus) in a total volume of 0.1 ml. The amplification was carried out for 35 cycles according to the protocol described above.

Allele-specific oligonucleotide hybridization

100 ng of amplified genomic DNA corresponding to bp 1172–1306 was heat-denatured and applied to a nitrocellulose membrane using a Minifold apparatus (Schleicher and Schuell). The blots were hybridized to ^{32}P-labeled oligonucleotides 7, 8 or 9 (Table IV) (10^6 c.p.m./ml) in a solution containing 5 × SSPE, 5 × Denhardt's solution and 0.1% SDS. The filters were washed twice at room temperature in 2 × SSPE + 0.1% SDS, followed by 10 min wash at 55°C in 5 × SSPE + 0.1% SDS.

Transfection of insulin receptor cDNA into NIH3T3 cells

The expression vector used for these studies was based on a plasmid described earlier (Riedel *et al.*, 1986). Transcription of insulin receptor cDNA is driven by the SV40 early promoter. In addition, the plasmid contains both dihydrofolate reductase and neomycin resistance genes as selectable markers. To construct a plasmid for expression of the mutant cDNA, a 249 bp fragment (*Aat*II/*Mst*II) of wild-type insulin receptor cDNA was replaced with the comparable sequence derived from one of the patient's insulin receptor cDNA clones (A-8-11). As described previously, NIH3T3 cells transfected with wild-type insulin receptor cDNA possess ~100-fold more insulin receptors than do the non-transfected NIH3T3 cells (Kadowaki *et al.*, 1988).

The expression vector (0.1 μg) was transfected into NIH3T3 cells by the calcium phosphate procedure. Two days after transfection, the neomycin analog G-418 was added to the culture medium to select for resistant clones. Thereafter, cells were grown in the presence of increasing concentrations of methotrexate (50–200 nM) for stepwise amplification of cDNA expression. Stable transfectants expressing insulin receptors were identified by assaying for the ability to synthesize insulin receptors. To assay for insulin receptor biosynthesis, cells were incubated overnight in the presence of [^{35}S]methionine (Kris *et al.*, 1985), solubilized in detergent and insulin receptors were immunoprecipitated with a polyclonal antibody raised against the C-terminal pentadecapeptide of the insulin receptor kindly provided by Dr J.Ramachandran.

Biosynthetic labeling with [^{35}S]methionine

Confluent monolayers of transfected NIH3T3 cells were incubated for 1 h at 37°C in methionine-free DMEM. Thereafter, cells were pulse-labeled for 3 h with DMEM containing 0.1 mCi/ml of [^{35}S]methionine (1111 Ci/mmol; Dupont-NEN, Wilmington, DE) prior to the addition of DMEM containing unlabeled methionine for 1.5, 4 or 8 h of chase. At each time point, cells were washed with ice-cold PBS and solubilized in 1 ml of solution containing Triton X-100 (1%), Hepes (50 mM, pH 7.6), NaCl (150 mM), bacitracin (10 μg/ml), leupeptin (10 μg/ml) and phenylmethanesulfonyl fluoride (0.2 mM) for 1 h at 4°C. After ultracentrifugation at 50 000 r.p.m. for 30 min at 4°C, the solubilized material was preadsorbed with protein A–agarose (20% suspension) for 30 min at 4°C, and 0.5-ml aliquots were immunoprecipitated for 2 h at 4°C with either anti-receptor antibody Ab-50 (Cama *et al.*, 1988) or normal rabbit serum at a dilution of 1:100. The immune complexes were precipitated by incubation with 10% Pansorbin (Calbiochem, La Jolla, CA), and washed three times with a solution containing Triton X-100 (0.1%), NaCl (150 mM) and Hepes (50 mM, pH 7.6). The immunoprecipitates were analyzed by SDS–PAGE followed by fluorography (Hedo *et al.*, 1985).

Insulin binding

Transfected NIH3T3 cells were grown to confluency in 6-well dishes. On the day of the experiment, cells were washed with assay buffer containing NaCl (120 mM), $MgSO_4$ (1.2 mM), KCl (2.5 mM), Na acetate (15 mM), glucose (10 mM), EDTA (1 mM), Hepes (50 mM, pH 7.8) and bovine serum albumin (10 mg/ml). The cells were incubated overnight at 4°C in the same medium with ~0.1 ng/ml of [^{125}I]iodoinsulin (receptor grade, 360 Ci/g; Dupont-NEN, Wilmington, DE) in the presence of varying concentrations of unlabeled insulin (1 ng/ml–50 μg/ml). Thereafter, the cells were washed twice with ice-cold PBS to remove unbound insulin, the cells were solubilized in 1 N NaOH for 2 h and the cell-associated radioactivity was counted for 10 min in a gamma counter. All binding experiments were performed in duplicate.

Calculation of LOD score

The odds ratio is defined as the ratio of the probability of the observations assuming that two loci are genetically linked divided by the probability of the same observations assuming that the loci are unlinked. The LOD score is defined as the $\log_{10}$ of the odds ratio.

Standard linkage analysis. We observed that the two affected sisters have the same genotype at the insulin receptor locus while the four unaffected sibs have a different genotype. This is the only inheritance pattern consistent with the hypothesis that the disease-causing locus is linked to the insulin receptor locus assuming that there was no crossing over in the region of the insulin receptor locus on chromosome 19 during the meioses producing the children. Thus, the probability of this inheritance pattern is 100% if the insulin receptor locus is tightly linked to the disease-causing locus. However, if these two loci were unlinked, then the probability is 1/4 that the two affected sisters would have the same genotype and $(3/4)^4$ that the four unaffected sibs would have a different genotype. Thus, if the two loci are unlinked, the probability of the inheritance pattern is 8% [$= (1/4)(3/4)^4$]. In this case, the LOD score is $\log_{10}$ (1.0/0.08) = 1.10.

Homozygosity mapping. As emphasized by Lander and Botstein (1987), the consanguinity of the parents provides additional linkage information. The previous probabilities may each be multiplied by the probabilities (assuming either tight linkage or non-linkage) that the affected sibs would be homozygous for haplotype A. Let q be the population frequency of haplotype A. Assuming tight linkage, the probability that the affected sibs are homozygous for haplotype A is q. Assuming non-linkage, the probability is $(1/16)q + (15/16)q^2$, where the first term arises due to actual homozygosity by descent in a first cousin marriage, and the second term arises from chance meeting in the population of two chromosomes with haplotype A. Thus the additional contribution to the LOD score due to consanguinity is $\log_{10}[16/(1+15q)]$. As shown in Figure 3, the LOD score for the family would range from 1.10 if the haplotype is extremely common ($q \approx 1$) to 2.30 if the haplotype is extremely rare ($q \approx 0$). [For more details, see Lander and Botstein (1987).]

This derivation may be simplified by considering the special case where haplotype A is so uncommon in the population ($q \approx 0$) that one can be certain that homozygosity for the A allele is due to homozygosity by descent. Under the assumption that the disease-causing locus is tightly linked to the insulin receptor locus, one would predict that the two parents should have inherited allele A by virtue of descent from a common ancestor. (The probability of observing this by random chance in the absence of genetic linkage is 1/4.) Furthermore, the two affected children should be homozygous for allele A. [The probability of observing this by random chance in the absence of genetic linkage is $(1/4)^2$.] Finally, the four unaffected children should not be homozygous for allele A. [The probability of observing this by random chance in the absence of genetic linkage is $(3/4)^4$.] Thus the odds of observing this kindred if the mutation were not linked to the insulin receptor locus can be calculated as the product of the individual probabilities [$(1/4)^3(3/4)^4 = 0.005$]. Therefore,

$$\text{LOD} = \log_{10} \frac{1}{(1/4)^3(3/4)^4} = -\log_{10}(0.005) = 2.3$$

Based on the frequencies of the seven individual RFLPs and the fact that they appear to be in linkage equilibrium (Table I), we estimate the frequency (q) of haplotype A at ~2%. Based on this estimate, the LOD score is ~2.20

(Figure 3). Even if q were as high as 10%, the LOD score would be 1.90. If the Val^{382} mutation, which was shown in Figure 5 to be rare in the population, is considered in addition to the seven RFLPs, then the haplotype at the insulin receptor locus found in the affected sisters appears to have a population frequency $q < 1\%$ (Table I). This corresponds to a LOD score of ~ 2.25—close to the maximum of 2.30 obtainable by homozygosity mapping in this family (Figure 3). These LOD scores (1.9–2.25) exceed the threshold for declaring linkage when studying a single candidate locus (see Discussion).

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