Insulin Resistance and Diabetes Due to Different Mutations in the Tyrosine Kinase Domain of Both Insulin Receptor Gene Alleles*

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Mutations in the insulin receptor gene can lead to in vivo and in vitro insulin resistance and can be the cause of diabetes mellitus in selected patients. We have studied a 22-year-old diabetic woman with Type A insulin resistance and acanthosis nigricans. Insulin binding to the patient’s erythrocytes, monocytes, adipocytes, fibroblasts, and transformed lymphocytes was decreased. Receptor autophosphorylation and tyrosine kinase activity toward an exogenous substrate were reduced in partially purified insulin receptors from the proband’s transformed lymphocytes. Determination of the nucleotide sequence of the patient’s insulin receptor cDNA revealed that the subject was a compound heterozygote who inherited two different mutant insulin receptor gene alleles. The paternal allele contains a missense mutation encoding the substitution of glycine for arginine at position 981 in the tyrosine kinase domain of the receptor. The maternal allele contains a nonsense mutation causing premature termination after amino acid 988 in the β-subunit, thereby deleting most of the kinase domain. The mRNA encoded by the allele with the premature stop codon is likely to be unstable, since mRNA transcripts from this allele were decreased markedly compared with the other allele. The mother, who is heterozygous for the nonsense mutation, exhibited only mild insulin resistance, whereas the proband was severely insulin-resistant; this indicates that the missense mutation is biologically significant. In summary, (1) we have identified a patient and her family with a genetic form of insulin resistance and diabetes due to a defect at the level of the insulin receptor; (2) the proband is a compound heterozygote displaying a missense mutation (position 981) in one allele and a nonsense mutation (position 988) in the other insulin receptor gene allele; (3) the missense mutation is in the kinase domain and encodes a receptor with impaired in vitro kinase activity; and (4) based on the in vitro and in vivo phenotype, the kinase domain mutation at position 981 is biologically significant leading to insulin resistance.

The insulin receptor is a heterotetrameric protein consisting of two extracellular α-subunits and two membrane-spanning β-subunits assembled in an α3β2 structure (1). Insulin binds to the α-subunits stimulating autophosphorylation and tyrosine kinase activity of the β-subunits (2–4). This is associated with a conformation change in the β-subunit (5). Following these events a variety of putative cellular protein substrates are phosphorylated and numerous biologic events are initiated (4, 6–8). Insulin receptor autophosphorylation and/or kinase activation appear necessary for many, if not all, of insulin’s biologic effects, and this subject recently has been reviewed extensively (9–14). Defects in insulin receptor autophosphorylation/kinase activity have been associated with a number of insulin-resistant states both in animals and in man, including noninsulin-dependent diabetes mellitus and obesity (15–18). In addition, several different types of mutations in the insulin receptor gene have been identified in patients with inherited syndromes of extreme insulin resistance and diabetes (19–27). In some patients mutations have been described that impair proreceptor processing and transport or receptor turnover, or alter the affinity with which the receptor binds insulin (20–22, 24). Other mutations have been described in the kinase region of the insulin receptor which presumably interfere with transmembrane signaling by compromising the receptor tyrosine kinase function (25–28).

In an earlier study, we described a female diabetic patient who had acanthosis nigricans and severe insulin resistance (29). Two siblings were also hyperinsulinemic and insulin-resistant as was one parent (29). The patient’s in vivo dose-response curve for insulin-stimulated glucose disposal and in vitro dose-response curve for insulin-stimulated glucose transport in isolated adipocytes were shifted to the right and showed marked decreases in the maximal response. Insulin binding to the patient’s erythrocytes, monocytes, adipocytes, and cultured fibroblasts was decreased markedly. Less severe insulin binding defects were observed in the other family members. Taken together, these observations demonstrated that this family manifested an inherited form of insulin resistance and diabetes which involved abnormalities at the level of the insulin receptor (29). In the current study, we report functional studies of insulin receptors from the patient as well as the primary sequence of the insulin receptor gene in the patient and the other family members. Our results demonstrate that the proband is a compound heterozygote for two mutations in the insulin receptor β-subunit and that both abnormal alleles are inherited independently in the other family members.

MATERIALS AND METHODS

Clinical Characterization of the Subject—The proband is a 22-year-old woman with an inherited form of severe insulin resistance and diabetes. She was diagnosed at 14 years of age. Details of her clinical condition were reported previously (29). In brief, she is severely
insulin-resistant with acanthosis nigricans and mild hirsutism. She requires insulin therapy to maintain her fasting glucose level of approximately 250 mg/dl, but no ketonemia is present. She is not a product of a consanguinous marriage.

Chemicals—Human insulin and [125I]-insulin, monodiodiated at the A-14 position (300-400 Ci/mg), were kindly provided by Eli Lilly, Inc. Fetal bovine calf serum was from Irvine Scientific. Lymphoprep was from Nyegard & Co. (Oslo, Norway). 32P-labeled ATP (6000 Ci/mg) was from Du Pont-New England Nuclear. Thermus aquaticus (Taq) polymerase was from Perkin-Elmer Cetus. Avian myeloblastosis virus reverse transcriptase, T7 RNA polymerase, T4 polynucleotide kinase, deoxynucleotides, and nick translation kit were from Bethesda Research Laboratories. Nitrocellulose membranes were from Schleicher and Schuell. Poly(Glu-Tyr) and all other reagents were from Sigma.

Transformation of Lymphocytes—Sterile hemiparized blood samples (10 ml) were obtained, and the white blood cell fraction was collected through Lymphoprep. Transformed lymphocytes were generated according to previously reported methods (30).

Insulin Binding Studies—Insulin binding to erythrocytes, adipocytes, and transformed lymphocytes was studied as described previously (30, 31). Insulin binding was measured at 15°C in all studies.

Autophosphorylation and Analysis of [125I]-Labeled Insulin Receptors—(WGA) Purified insulin receptors (WGA) were prepared from transformed lymphocytes (>2 X 10^9) as described previously (32). WGA-purified receptors were incubated with [125I]-B2(2-nitro-4-azidophenylacetyl)-des-PheB' (NAPA) insulin at 4°C for 16 h (33). The autophosphorylation reaction was carried out using 50 μM [γ-32P]ATP by previously described methods (12). After termination of autophosphorylation, photolysis of [125I]-insulin was conducted as described (33).

Labeled insulin receptors were incubated with either anti-insulin receptor antiserum (11), or normal serum and immune complexes were precipitated with protein A. Immunoprecipitates were fractionated on a 7.5% reducing SDS gel. The gel was dried and exposed to −70°C on XAR film using an intensifying screen. Quantification of the intensity of the band on the gel was evaluated by either counting the gel slice directly or by calculating the absorbance of the band on film using a KGB gel scanner system.

Phosphorylation Assays for Exogenous Substrates—WGA-purified receptors (~300 fmol) were preincubated at 22°C for 30 min without or with insulin (500 ng/ml) in a total volume of 80 μl. The substrate poly(Glu-Tyr) (10 mg/ml) and MgCl₂ (10 mM) were added, and the phosphorylation reaction was initiated, as described previously (28). Basal activity, which was less than 10% of total activity, was subtracted to determine the insulin-stimulated kinase activity.

Preparation of Total Cytoplasmic RNA and Genomic DNA—Total cytoplasmic RNA was prepared from transformed lymphocytes (>5 X 10⁹ cells) according to methods described previously (34). Genomic DNA was isolated either from transformed lymphocytes or peripheral blood according to standard procedures (35).

Slot-Blot Autoradiography—Slot-blot analysis of the cytoplasmic RNA was performed according to methods described elsewhere (36). All RNA samples were analyzed at four serial 2-fold dilutions containing 20, 10, 5, and 2.5 μg of total RNA. The autoradiograms were scanned using a KGB gel scanner system. Only values obtained from the linear portion of the concentration curves were used for calculations.

Synthesis of First Strand cDNA from the Patient's Insulin Receptor mRNA and Subsequent Amplification by the Polymerase Chain Reaction—As described earlier (37), five different overlapping sets of single-stranded oligonucleotide DNA primers complementary to the presumed cytoplasmic sequence of the human insulin receptor were prepared. These primers span the region between nucleotide 125 and 4266 (by the numbering method of Ullrich et al. (38)). This includes the entire coding sequence of both α- and β-subunits of the human insulin receptor. A specific first strand cDNA copy of the insulin receptor mRNA was made with the 3'-oligonucleotide in each primer set which served as a primer for reverse transcriptase. Synthesis and subsequent amplification of the cDNA was carried out according to previously described methods (25, 39). To avoid the subcloning step for sequencing of PCR-amplified DNA, the 5'-oligonucleotide of each set of primers contained a T7 phage promoter sequence at its 5'-end. Thus, the 5'-end of the amplified DNA segments contained this phage promoter which was used as a primer for T7 RNA polymerase followed by direct sequencing of the RNA (40).

Enzymatic Amplification of Insulin Receptor Exons 11 and 17—By using sets of primers as described by Seino et al. (41), the regions representing exons 11 and 17 were amplified using standard conditions (42). Each reaction was performed in a volume of 25 μl containing 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1 mM MgCl₂, 100 μg/ml gelatin; 200 μM each of dATP, dCTP, dTTP, and dGTP; 1 μM each of oligonucleotide primer; 0.25 μg of HindIII digested genomic DNA; and 1.25 units of Taq DNA polymerase. After initial denaturation at 94°C for 10 min, the samples were subjected to 40 cycles of amplification with an Eppendorf automated thermal cycler; annealing at 50°C for 2 min, extension at 72°C for 3 min, and denaturation at 94°C for 1 min. The upstream oligonucleotide of each set of primers contained a T7 phage promoter sequence at its 5'-end for direct sequence analysis (40).

Allele-specific Oligonucleotide Hybridization—Exon 17 of the insulin receptor gene was amplified by the method described above. One fifth of the amplified double-stranded DNA (~200 ng of DNA) was analyzed by ethidium bromide through a 1% agarose gel and transferred to nitrocellulose membranes. The DNA blots were hybridized in buffer containing 5 x SSPE (0.9 M NaCl, 50 mM sodium phosphate, 5 mM EDTA (pH 7.5), 5 X Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.1% SDS, and 100 μg/ml of heat-denatured salmon sperm DNA for 16 h at 37°C with 32P-labeled synthetic oligonucleotides (1-2 x 10⁶ cpm/ml). The blots were then washed three times: first, with 2 x SSPE containing 0.1% SDS at room temperature for 30 min; second, with 0.1 x SSPE containing 0.1% SDS at room temperature for 30 min; and finally, with 0.1 x SSPE containing 0.1% SDS at 42°C for 10 min.

RESULTS

Insulin Binding to Receptors—Insulin binding to the patient's freshly isolated erythrocytes and adipocytes is shown in Fig. 1. As can be seen, insulin binding is reduced markedly in both cell types, more so in erythrocytes than in the isolated adipocytes. Eight years ago, we published similar studies on this same patient (29), and these earlier results are superimposed on the current figures for comparison. As can be seen, the insulin binding results are essentially identical to those we obtained on this same patient previously. Freshly isolated cells are exposed to a variety of in vivo influences, and consequently insulin binding to these cells may reflect a complex interplay of primary and secondary events. Previously, we reported that insulin binding to cultured fibroblasts was decreased markedly in this patient and in other family members, indicating a primary or genetic origin of this abnormality (29). In the current studies we have prepared Epstein-Barr virus-transformed cells from the patient's circulating lymphocytes, and the results of binding studies to these transformed cells are shown in Fig. 2A. The binding capacity varied substantially across different clonal isolates. Of the four lymphocyte clones prepared, two showed insulin binding capacity ~50% below the normal range, and the other two demonstrated insulin binding at the lower limit of normal.

The reasons for this variation in receptor expression are unknown, but it may be due to differences in the level of insulin receptor gene expression in the transformed lymphocytes. To assess this possibility we measured insulin receptor mRNA levels by slot-blot analysis in the patient-derived and normal cell lines. As can be seen in Fig. 2B, a strong direct correlation was present between the mRNA level and the magnitude of insulin binding.

Autophosphorylation/Kinase Activity—Insulin receptors were prepared by lectin affinity chromatography from the patient-derived and control lymphocytes, and in vitro autophosphorylation was measured (Fig. 3A). Receptors were incubated with or without insulin in the presence of [32P]ATP and analyzed on SDS-PAGE. In controls, a dose-responsive
Mutant Insulin Receptor in Insulin-resistant Diabetes

A 25r,
1 10 100 1000
Total Insulin Concentration (ng/ml)

B 4r
1 10 100 1000
Total Insulin Concentration (ng/ml)

FIG. 1. Insulin binding to erythrocytes (A) and adipocytes (B) from the control subjects (●—●) and a diabetic patient with the Type A syndrome of insulin resistance and acanthosis nigricans (○—○). The previously published insulin binding data from 1982 (—–––) are included for comparison. Results are expressed as percent 

~20-fold stimulation of β-subunit autophosphorylation was observed, with little stimulation with the patient’s receptors. Identical results were obtained from patient-derived lymphocytes expressing decreased receptor number (P2) and those with binding capacity in the lower range of normal (P1) (see Fig. 2). To assess autophosphorylation by a different method, equal amounts of patient and control WGA-purified receptors were photoaffinity-labeled with [125I]-NAPA-insulin. The receptors were then exposed to [32P]ATP and analyzed on SDS-PAGE. As seen in Fig. 3B, equal α-subunit labeling was observed, confirming that comparable amounts of receptors were used. In contrast, β-subunit autophosphorylation was reduced by ~85% for the patient’s receptors compared to controls. Interestingly, the mobility of the α- and β-subunits from the patient’s insulin receptors was normal.

To assess receptor kinase activity directed toward an exogenous substrate, equal amounts of patient and control WGA-purified receptors were allowed to phosphorylate an artificial substrate (Glu-Tyr, 4:1). In parallel to the decreased autophosphorylation, Glu-Tyr phosphorylation was reduced by ~90% (Fig. 3C).

Insulin Receptor Sequence—To determine whether the decreased receptor expression and impaired autophosphorylation/kinase activity could be due to a structural alteration of the patient’s insulin receptors, we analyzed the insulin receptor mRNA sequence obtained from the patient’s transformed lymphocytes. As described previously (37), five sets of primers were used to amplify 1107-, 1160-, 759-, 673-, and 773-base pair overlapping segments that include the entire coding sequence for both α- and β-subunits. The sequence was then determined from RNA transcripts of the amplified DNA segments (39). One small region of the subject’s insulin recep-
purified insulin receptors were obtained from Epstein-Barr virus-transformed lymphocytes as described under "Materials and Methods." Based on insulin binding data, the number of insulin receptors used was the same in each lane. The preparations were incubated without insulin (lanes 1 and 4), with 10 ng/ml insulin (lanes 2 and 5), or with 1000 ng/ml insulin (lanes 3 and 6). Receptor phosphorylation was measured as described previously (12). The receptors were immunoprecipitated with antireceptor antibody (11) and subjected to receptor autophosphorylation of purified insulin receptors from a normal individual (lanes 1 and 4), with 10 ng/ml insulin (lanes 2 and 5), or with 1000 ng/ml insulin (lanes 3 and 6). Receptor phosphorylation was measured as described previously (12). The receptors were immunoprecipitated with either anti-insulin receptor antibody (11) and subjected to electrophoresis on SDS-PAGE under reducing conditions. B, double labeling of purified insulin receptors from a normal individual (lanes 1, 2, 5, and 6) and the proband (lanes 3 and 4). The receptor preparations were incubated with 10 ng/ml of \(^{125}\)I-NAPA-insulin as described under "Materials and Methods." Receptor autophosphorylation was measured as in A above. The receptors were immunoprecipitated with either anti-insulin receptor antibody (lanes 2, 4, and 6) or normal serum (lanes 1, 3, and 5), and immunoprecipitates were fractionated on a 7.5% reducing SDS-PAGE gel. C, tyrosine kinase activity of purified insulin receptors from normal subjects (N, n = 3) and the proband (P). Lectin-purified insulin receptors were obtained from Epstein-Barr virus-transformed lymphocytes as described. The insulin receptor content in the preparations was equalized based on insulin binding data. The preparations were assayed with a synthetic copolymer containing glutamate and tyrosine (4:1) either in the absence or presence of 500 ng/ml insulin as described (28). The incorporation of \(^{32}\)P into the polypeptide was measured in a liquid scintillation counter. The results are expressed as percent over basal stimulation and represent the mean ± S.E. (n = 3). Basal tyrosine kinase activities for the control and the proband are 4.4 ± 0.9 and 1.9 ± 0.3 (mean ± S.E., pmol ATP/mg substrate/10 min/100 fmol insulin receptor), respectively.

**Table I**

Comparison of nucleotide and amino acid sequences of the insulin receptor in healthy subjects and the patient.

| Amino acid Nucleotide | Ullrich et al. (38) | Ebina et al. (41) | Seino et al. (43) | Patient |
|-----------------------|--------------------|------------------|------------------|---------|
| 144                   | 559                | TAC              | Tyr CAC          |         |
| 276                   | 957                | CAG              | Gin CAA          |         |
| 421                   | 1391               | ATC              | Ile ACC          |         |
| 465                   | 1522               | CAG              | Gin AAG          |         |
| 519                   | 1686               | GAC              | Asp GAT          |         |
| 523                   | 1698               | GCA              | Ala GCC          |         |
| 861                   | 2711               | GAC              | Asp GTC          |         |
| 981                   | 3071               | CGA              | Arg CCA          |         |
| 1239                  | 3846               | AAC              | Asn AAG          |         |

| 1                           | 3                  | TACG             | Arg Glu          | G-3'    |
| 2                           | 1                  | TACG             | Arg Glu          | G-3'    |
| Mutant allele A: 5'-T       | CAGA               | A                | Arg TGA          | G-3'    |
| Mutant allele C: 5'-T       | CAGA               | A                | Arg TGA          | G-3'    |

FIG. 3. A, autophosphorylation of purified insulin receptors from a normal individual (lanes 1-3) and the proband (lanes 4-6). WGA-purified insulin receptors were obtained from Epstein-Barr virus-transformed lymphocytes as described under "Materials and Methods." Based on insulin binding data, the number of insulin receptors used was the same in each lane. The preparations were incubated without insulin (lanes 1 and 4), with 10 ng/ml insulin (lanes 2 and 5), or with 1000 ng/ml insulin (lanes 3 and 6). Receptor phosphorylation was measured as described previously (12). The receptors were immunoprecipitated with antireceptor antibody (11) and subjected to electrophoresis on SDS-PAGE under reducing conditions. B, double labeling of purified insulin receptors from a normal individual (lanes 1, 2, 5, and 6) and the proband (lanes 3 and 4). The receptor preparations were incubated with 10 ng/ml of \(^{125}\)I-NAPA-insulin as described under "Materials and Methods." Receptor autophosphorylation was measured as in A above. The receptors were immunoprecipitated with either anti-insulin receptor antibody (lanes 2, 4, and 6) or normal serum (lanes 1, 3, and 5), and immunoprecipitates were fractionated on a 7.5% reducing SDS-PAGE gel. C, tyrosine kinase activity of purified insulin receptors from normal subjects (N, n = 3) and the proband (P). Lectin-purified insulin receptors were obtained from Epstein-Barr virus-transformed lymphocytes as described. The insulin receptor content in the preparations was equalized based on insulin binding data. The preparations were assayed with a synthetic copolymer containing glutamate and tyrosine (4:1) either in the absence or presence of 500 ng/ml insulin as described (28). The incorporation of \(^{32}\)P into the polypeptide was measured in a liquid scintillation counter. The results are expressed as percent over basal stimulation and represent the mean ± S.E. (n = 3). Basal tyrosine kinase activities for the control and the proband are 4.4 ± 0.9 and 1.9 ± 0.3 (mean ± S.E., pmol ATP/mg substrate/10 min/100 fmol insulin receptor), respectively.

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| 465                   | 1522               | CAG              | Gin AAG          |         |
| 519                   | 1686               | GAC              | Asp GAT          |         |
| 523                   | 1698               | GCA              | Ala GCC          |         |
| 861                   | 2711               | GAC              | Asp GTC          |         |
| 981                   | 3071               | CGA              | Arg CCA          |         |
| 1239                  | 3846               | AAC              | Asn AAG          |         |

| 1                           | 3                  | TACG             | Arg Glu          | G-3'    |
| 2                           | 1                  | TACG             | Arg Glu          | G-3'    |
| Mutant allele A: 5'-T       | CAGA               | A                | Arg TGA          | G-3'    |
| Mutant allele C: 5'-T       | CAGA               | A                | Arg TGA          | G-3'    |

FIG. 4. A, partial nucleotide sequence of the patient's insulin receptor cDNA containing two novel point mutations. At nucleotide position 3071 (according to the numbering system of Ullrich et al. (38)), there are two bands (G and A, indicated by arrow) instead of one for G as reported for the normal human insulin receptor cDNA (38, 48). There are also two bands at position 3091 (C and T) instead of one for C only. Substitutions of G by A and C by T in the cDNA CAGA result in a missense and a nonsense mutation, respectively, in one of the two alleles of the subject's insulin receptor gene. B, partial nucleotide sequence of the sense strand of insulin receptor exon 17 from a control subject (1) and the proband (2). By using a set of primers as described by Seino et al. (41), a 341-base pair region of lymphocyte genomic DNA corresponding to exon 17 and adjacent intron regions of the insulin receptor gene was amplified by PCR, and the sequence was analyzed directly.

differences since no alterations in biologic behavior are noted when normal receptors are expressed in transfected cells (45-47). Nine single nucleotide differences exist between the two initially reported receptor cDNAs (38, 48) at nucleotide positions 559, 957, 1391, 1522, 1686, 1698, 2711, 2713, and 3846 (according to the numbering system of Ullrich et al. (38)). At five of these positions (559, 957, 1391, 1522, and 1686) the sequence from our patient was identical to that reported by Ullrich et al. (38). At four positions (1698, 2711, 2713, and 3846) the sequence corresponded to that of Ebina et al. (48). At seven of these nine positions our sequence was the same as the genomic sequence of Seino et al. (43). The sequence of exon 11 (amino acid positions 718-729 (41)) of the subject's insulin receptor gene was identical to the reported normal sequences (41, 48).

At nucleotide positions 3071 and 3091, the subject was heterozygous for two novel point mutations (Table I and Fig. 4A) corresponding to amino acid positions 981 and 988 of the subject's insulin receptor. An arginine codon (CGA) at 981 was converted to CAA encoding glutamine, resulting in a missense mutation. Additionally, an amber chain termination codon (TGA) was substituted for the arginine codon 988 (CGA) producing a nonsense mutation. The patient was heterozygous for both of these mutations as judged by the fact that bands corresponding to both G and A (CGA (Arg) → CAA (Gln)) and C and T (CGA (Arg) → TGA (stop)) could be detected in the sense strand at nucleotides 3071 and 3091 in the sequencing ladder (Fig. 4A). These mutations were confirmed in two additional independent experiments. The T signal at position 3091 converting the Arg codon (CGA) to a stop codon (TGA) is less intense than the corresponding C signal at the same position. This indicates that mRNA transcripts arising from the insulin receptor gene allele containing the stop codon mutation are less abundant than mRNA transcripts from the other allele. In contrast, at position 3071, the A signal converting the normal Arg codon (CGA) to Gln (CAA) is more intense than the normal G signal. This indicates that transcripts arising from the insulin receptor gene allele with the CAA mutation are more abundant than the
mRNA transcripts from the allele with the normal sequence at this position. These results indicate that the patient is a compound heterozygote with one allele containing a premature stop signal (TGA) at codon 988 with a normal sequence at codon 981 and that the content of mRNA transcripts from this allele is decreased. The other allele contains a normal sequence at codon 988 with CAA (Gln) instead of CGA (Arg) at position 981, and the mRNA transcripts from this allele are more abundant than those from the allele with TGA at position 988.

Both of these mutations are contained within the kinase domain encoded by exon 17. To verify these genetic abnormalities further, we used PCR to amplify exon 17 from the patient’s genomic DNA. Sequence analysis (Fig. 4B) of the amplified DNA confirmed our observations derived from mRNA based sequencing. Thus, the subject displayed two separate mutations, one at nucleotide position 3071 and the other at 3091. The double signals (G and A at 3071 and C and T at 3091) confirm that the subject was heterozygous for both of these mutations. Comparable intensities of both nucleotide signals at these two positions indicate that amplified DNA from both alleles was represented equally in the reactions. When exon 17 was amplified and sequenced from a normal individual, only G and C were detected at nucleotide positions 3071 and 3091, respectively (Fig. 4B).

These results were elucidated further by use of allele-specific probes. Thus, oligonucleotides specific for either the wild type sequence or the respective mutant sequences were prepared and hybridized to DNA corresponding to exon 17. As seen in Fig. 5, the oligonucleotide probe specific for either the missense (981) or nonsense (988) mutation hybridized to DNA from the patient (right lanes PT, Fig. 5) but did not hybridize to the DNA from a control subject (right lanes C, Fig. 5). In contrast, the oligonucleotide specific for the wild type sequences hybridized to the DNA from both the control subject (left lanes C, Fig. 5) and the patient (left lanes PT, Fig. 5). Thus, normal genomic DNA does not contain either mutant sequence, whereas the patient’s genomic DNA contains both mutant sequences as well as both wild type sequences. These results support the conclusion that the subject is a compound heterozygote for both the missense and the nonsense mutation.

Family Studies of the Mutant Alleles—The patient’s mother and two sisters have variable degrees of glucose intolerance, insulin resistance, and hyperinsulinemia (29). In addition, measurements of insulin binding to receptors from these family members show 40–70% reductions in binding capacity using circulating erythrocytes and cultured fibroblasts. Therefore, it was of obvious interest to track the mutated insulin receptor alleles in the family. To accomplish this, genomic DNA was obtained from circulating mononuclear leukocytes from each family member, and exon 17 was amplified by PCR and sequenced. The exon 17 sequence results from all family members are shown in Fig. 6. The patient’s mother is heterozygous for the arginine to stop nonsense mutation in codon 988, while the father is heterozygous for the arginine to glutamine missense mutation in codon 981. One sister is heterozygous for the 988 nonsense mutation, whereas the other sister has the same genotype as the patient; i.e. she is a compound heterozygote for both mutations. This latter sister is also insulin-resistant, extremely hyperinsulinemic, and diabetic (29). The segregation of the mutant alleles, one to the father and one to the mother, confirms our conclusions based on the sequencing data in the patient that each of the patient’s insulin receptor gene alleles contains one mutation.
The cellular mechanisms of insulin action involve a number of sequential steps, some of which are poorly understood. Theoretically a defect at any point in the insulin action cascade could lead to insulin resistance (49). The insulin receptor represents the first target protein in the insulin signalling sequence, and abnormal expression or function of the insulin receptor has been implicated in numerous insulin-resistant states in man (16–18, 28, 50), animals (51), and cell lines (50, 52–54). This is particularly true for the unusual genetic forms of severe insulin resistance and diabetes in man (19–27). Subsequent to the cloning of the insulin receptor cDNA (38, 48) and gene (43), it has become possible to analyze the molecular genetics of insulin resistance/diabetes in such patients. Indeed, several patients have been described with mutations in the insulin receptor gene which impair receptor expression or function. In the current studies we have investigated a patient and family members who display an inherited syndrome of severe insulin resistance and diabetes. Our results show that the proband displays decreased numbers of insulin receptors on several freshly isolated or cultured cell types and that the expressed receptors display impaired autophosphorylation/kinase activity. Molecular studies revealed that the patient is a compound heterozygote for a missense mutation (Arg → Glu) at position 981 and a nonsense (pre-mature chain termination signal) mutation at position 988, both in the kinase domain. Analysis of the insulin receptor gene sequence in all family members confirmed the conclusion that each receptor allele contained one mutation and that one or both mutated alleles were present in each family member.

In 1982 we reported insulin binding and in vivo data on the proband and her family and concluded that the insulin resistance was an inherited syndrome due to a defect at the level of the insulin receptor (29). Interestingly, the patient’s in vivo phenotype is essentially the same as it was 8 years ago, and the results of glucose clamp studies and insulin binding studies to freshly isolated erythrocytes and adipocytes are almost superimposable on the earlier results. Our current functional and molecular studies appear to explain adequately the insulin resistance/diabetes phenotype in the family. Thus, the sequence of the patient’s insulin receptor showed two deviations from normal: (a) substitution of glutamine for arginine at position 981 in the kinase domain, and (b) a premature amber stop codon for arginine at position 988. Sequence analysis and allele-specific hybridization of the patient’s PCR-amplified genomic DNA (exon 17) further demonstrated that the patient was heterozygous for each individual mutation.

One of the patient’s alleles contained a stop codon (nonsense mutation) causing premature chain termination at position 988, thereby deleting most of the kinase and all of the C-terminal domain of the β-subunit. The sequencing data on DNA amplified from the patient’s mRNA revealed double nucleotide signals at positions 981 and 988. The mutant signal (T) at position 988 and the wild type signal (G) at position 981 both are reduced markedly (by ~90%) compared with the mutant signal at position 981 (A) and the wild type signal at position 988 (C). This indicates that mRNA transcripts generated from the insulin receptor allele containing the 988 stop codon mutation are reduced markedly in comparison to mRNA transcripts from the allele containing the 981 mutation. It is now well established that nonsense mutations can be associated with decreased mRNA levels (55–58). The exact mechanisms by which premature termination mutations lead to decreased mRNA levels are unclear, but may involve altered intranuclear stability, abnormal nuclear to cytoplasmic mRNA transport, or decreased stability of the mRNA (59, 60). Recently, Urlaub et al. (58) showed that RNA processing was affected by a nonsense mutation resulting in a low level of mature mRNA. Whatever the mechanism, however, the reduced mRNA expression from this allele is sufficient to explain the decreased insulin binding capacity in the patient’s transformed lymphocytes. It is likely that the further reduction in insulin binding capacity to the patient’s freshly isolated erythrocytes, adipocytes, and monocytes can be explained by in vivo down-regulation (61, 62). Thus, due to her insulin resistance, the patient displayed hyperinsulinemia which would lead to increased degradation of the insulin receptors encoded by the insulin receptor allele that did not contain the premature stop codon. It is of interest to note that insulin binding was reduced by more than 50% in the patient’s cultured fibroblasts. In a simplistic sense, one might expect normal expression of insulin receptors from the allele containing the 981 missense mutation resulting in only an ~50% decrease in binding capacity. The reasons for this more marked decrease in insulin binding to fibroblasts is unknown and may suggest that additional mechanisms are operative in this cell type.

The patient’s other insulin receptor allele contains a missense mutation at position 981 (Arg → Gln). It seems highly likely that this substitution is of biologic significance. This mutation is positioned within the tyrosine kinase domain of the β-subunit, and there is substantial evidence that receptor autophosphorylation/kinase activity is essential for many of insulin’s actions (9–14). Direct measurements of receptor autophosphorylation and kinase activity showed markedly reduced activity for the patient’s receptors. Since the mRNA levels from the 988 (stop codon) allele are strikingly reduced, and since any truncated receptor protein encoded by these few transcripts is likely to be unstable (49), the 981 (missense) allele encodes essentially all of the insulin receptors expressed in this patient’s cells. It follows then that the reduced autophosphorylation/kinase activity we measured reflects the functional properties of receptors encoded by the 981 allele. Furthermore, the patient and one sister express the same compound heterozygote genotype and both are far more insulin-resistant than the sister who is heterozygous for only the nonsense mutation. Thus, it seems likely that substitution of an amino acid with an uncharged side chain (glutamine) for a basic amino acid (arginine) at position 981 alters the structure of the insulin receptor compromising kinase activity and biologic signaling. It is interesting to note that position 981 lies near the GXGXXG (where X represents any amino acid) motif of the insulin receptor ATP binding site (26). To elucidate all of the biologic sequelae of the 981 mutation definitively, however, studies of cells transfected with cDNA containing this mutation will be necessary.

Both of the mutations in this patient’s insulin receptor gene alleles are at arginine positions. The CGA codon (arginine) has been identified previously as a hot spot for mutations (63), presumably because the CpG sequence is a substrate for methylation and the presence of 5-methylcytosine predisposes to errors in DNA replication converting the CG base pair to a TA base pair (63, 64). Along these lines, it is interesting to note that the 981 mutation we observed is thus far unique, whereas the stop codon mutation at position 988 has been observed recently in another patient with a genetic form of severe insulin resistance (65). This is the first instance in which identical mutations in an insulin receptor allele have been reported, raising the possibility that a stop codon mutation at position 988 may occur more frequently. When occurring in isolation, this mutation would inactivate functionally one allele which would only reduce insulin receptor
expression by ~50%. Due to the normal presence of spare receptors, an isolated mutation of this sort would only lead to a mild state of insulin resistance (such as in this patient’s mother and sister 1). By itself, this phenotype is unremarkable, and probably only leads to overt diabetes if other diabetogenic environmental or genetic factors coexist. Based on this, it is interesting to speculate that the 988 stop codon mutation may appear more frequently in populations predisposed toward insulin resistance or diabetes.

To investigate the mode of inheritance of these mutations and to establish the relationship between the genotype and phenotype in this family, we determined the sequence of exon 17 of the insulin receptor gene from the patient’s two sisters and parents. The sequence results revealed that the mother and younger sister (sister 1) are both heterozygous for the 988 nonsense mutation with normal sequences at position 981. The father is a heterozygote for the 981 missense mutation with a normal sequence in both alleles at position 988. Finally, the patient’s older sister (sister 2) is a compound heterozygote for both mutations, thus sharing the same genotype as the proband and sister 2 have inherited both abnormal alleles.

In this family, the diabetic phenotype is only expressed in those with fasting insulin levels >100 microunits and postprandial glucose intolerance. The father is more insulin-resistant but does not have overt diabetes. Sister 2 is also a compound heterozygous sharing the exact same genotype as the proband, and this sister also demonstrates extreme insulin resistance. However, recent studies show that this sister is much less hyperglycemic (having only mild diabetes) than the proband. This sister is also far more hyperinsulinemic than the proband with fasting insulin levels >100 microunits and postprandial levels >2000 microunits/ml. Perhaps the enormous capacity of this sister’s β cells to secrete insulin prevents decompen-

In summary, we have studied a patient with a genetic form of severe insulin resistance and diabetes. Our results demonstrate that this patient is a compound heterozygote for two mutations in both insulin receptor alleles. One mutation leads to a premature chain termination signal, whereas the other leads to a glutamine for arginine mutation at position 981 in the tyrosine kinase domain. It is likely that these insulin receptor gene mutations explain the phenotype in this patient as well as in affected family members.

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