of the receptor for insulin or a slightly decreased kinase activity; in these cases, environmental factors such as obesity may trigger the onset of diabetes.

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27. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln, R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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Human Diabetes Associated with a Mutation in the Tyrosine Kinase Domain of the Insulin Receptor

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Insulin receptor complementary DNA has been cloned from an insulin-resistant individual whose receptors have impaired tyrosine protein kinase activity. One of this individual's alleles has a mutation in which valine is substituted for Gly^{996}, the third glycine in the conserved Gly-X-Gly-X-X-Gly motif in the putative binding site for adenosine triphosphate. Expression of the mutant receptor by transfection into Chinese hamster ovary cells confirmed that the mutation impairs tyrosine kinase activity.

MOST INDIVIDUALS WITH NONINsulin-dependent diabetes mellitus (NIDDM) are resistant to the biological actions of insulin, and this is thought to be one of the primary factors giving rise to the disease (*1*). The binding of insulin to its receptor is the first step in insulin action, and defects in insulin receptor function may explain the insulin resistance in diabetic patients. When insulin binds to the extracellular domain of the receptor, the receptor undergoes autophosphorylation (*2*) and the tyrosine kinase associated with the intracellular domain (*3–5*) of the receptor is activated, thereby triggering the biological response within the target cell (*6, 7*). We have now identified a mutation in the insulin receptor gene of an individual with a form of NIDDM associated with severe insulin resistance.

The proband, a young Japanese male with the syndrome of insulin resistance and acanthosis nigricans (*8*), had a normal plasma glucose concentration when fasted but had the glucose tolerance typical of a diabetic. When the proband was fasted, his insulin level was high (150 μU/ml) and rose to >1000 μU/ml during an oral glucose tolerance test. Insulin binding to his circulating mononuclear cells was decreased, possibly due to down-regulation of insulin receptors. Although the number of insulin receptors on Epstein-Barr virus (EBV)–transformed lymphoblasts derived from the proband was at the lower limit of normal, there was a 50 to 80% decrease in receptor tyrosine kinase activity as measured with both solubilized insulin receptors and intact lymphoblasts (*8*).

To identify the molecular basis of the defect in the insulin receptor–associated tyrosine kinase activity, we cloned the cDNA encoding the proband's insulin receptor (Fig. 1). We determined the nucleotide sequence of four clones encoding the adenosine triphosphate (ATP) binding site of the

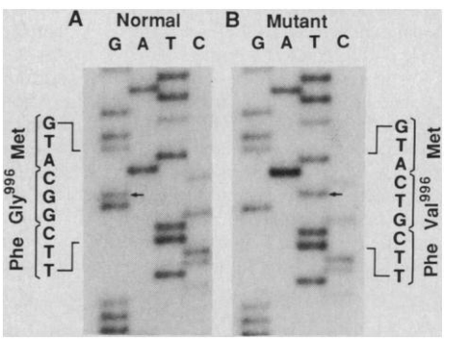

Fig. 1. Partial nucleotide sequence of two alleles of the proband's insulin receptor gene. A cDNA library was constructed in λgt10 from polyadenylated RNA that had been isolated from the proband's EBV-transformed lymphoblasts (*12, 18*). Approximately 2×10^6 recombinant bacteriophage were screened with an insulin receptor cDNA (*4*), and 44 positive clones were obtained. Fourteen clones encoding portions of the β subunit were recloned into pUC13, and both strands were sequenced by the dideoxy chain termination method with T7 DNA polymerase (Sequenase, United States Biochemical Corp.). (**A**) Clone 29 (nucleotides 2415 to 3705) had the same sequence as described (*5*) except at codon 1046 where an alternative histidine codon was present (CAT instead of CAC). Codon 996 (GGC) encoded glycine as in the normal sequence (*4, 5, 12*). The sequence of clone 29 was confirmed in another clone (clone 27, nucleotides 2963 to 3337). (**B**) Clone 8 (nucleotides 2743 to 4394) had the same sequence as described (*5*) except at codon 996, which was GTC instead of GGC, resulting in the substitution of valine for glycine at position 996. The sequence of clone 8 was confirmed in another clone (clone 25, nucleotides 3070 to 4405).

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tyrosine kinase domain. On the basis of their nucleotide sequences, the clones could be assigned to two groups, corresponding to the individual's two alleles of the insulin receptor gene. In the first group, which consisted of two clones, G was substituted for T at position 3116, thereby converting codon 996 from GGC (glycine) to GTC (valine) (Fig. 1) (*4*). This mutation was confirmed by amplification of a 144-bp region of genomic DNA (nucleotides 3067 to 3210) by the polymerase chain reaction and subsequent differential hybridization of amplified DNA with allele-specific oligonucleotide probes (Fig. 2). In the second group, which also consisted of two clones, C was substituted for T at position 3267, thereby converting codon 1046 from CAC to CAT, both of which encode histidine.

Gly^{996} is the third glycine in the conserved Gly-X-Gly-X-X-Gly (where X represents any amino acid) motif in the ATP binding site of protein kinases (*9, 10*). Most of what is known about this conserved sequence in protein kinases is inferred by analogy to the structures of other nucleotide-binding proteins such as glyceraldehyde-3-phosphate dehydrogenase, *p*-hydroxybenzoate hydroxylase, and lactate dehydrogenase. It has been suggested that the first and third glycines permit the polypeptide chain to make a sharp turn between β strand and α helix (*10*). In addition, the absence of side chains at the positions of the first and second glycines may allow for close approach of the ribose and pyrophosphate moieties of the ATP, respectively (*10*). In all tyrosine kinases and most serine kinases whose sequences are known, this third glycine is conserved. In a few serine kinases, either alanine or serine has been substituted for the third glycine (*9*).

To assess the significance of the Val^{996} mutation, we expressed both wild-type and mutant insulin receptors by transfection of cDNA into Chinese hamster ovary (CHO) cells. The Val^{996} mutation in the intracellular domain of the receptor did not affect the binding of ^{125}I-labeled insulin to the extracellular domain of the receptor (*11*). Autophosphorylation activity of insulin receptors from the transfected cell lines was assayed after their solubilization and partial purification by chromatography over wheat germ agglutinin-agarose. Autophosphorylation activity of the solubilized mutant receptor was reduced by >90% (Fig. 3). Apparently, the large side chain of valine at position 996 disrupts the three-dimensional structure of the protein. This result demonstrates the importance of the Gly-X-Gly-X-X-Gly motif for protein kinase activity.

Thus, the proband appears to be heterozygous for a mutation that substitutes valine for Gly^{996}, the third essential glycine in the Gly-X-Gly-X-X-Gly motif. This mutation impairs the tyrosine kinase activity of the insulin receptor, probably by disrupting the ATP binding site. Furthermore, because tyrosine kinase activity is thought to be necessary for the insulin receptor to mediate insulin action (*6, 7*), it seems likely that this mutation contributes to the cause of the proband's insulin-resistant diabetes mellitus.

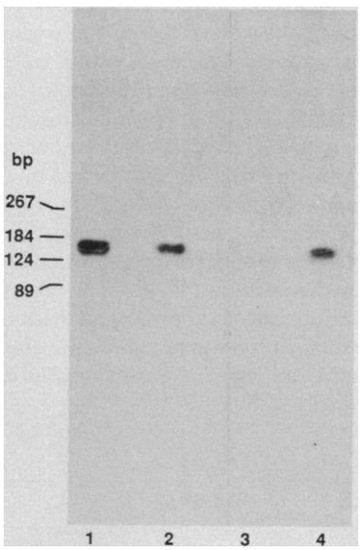

Fig. 2. Hybridization of enzymatically amplified genomic DNA with allele-specific oligonucleotide probes. Genomic DNA (1 μg) was prepared from EBV-transformed lymphoblasts of the proband (lanes 2 and 4) and a normal subject (lanes 1 and 3). The region of interest was amplified by performing 35 cycles of the chain reaction catalyzed by Taq DNA polymerase (*19*) (Perkin-Elmer–Cetus) and primed with synthetic oligonucleotides (nucleotides 3067 to 3086, sense strand; nucleotides 3191 to 3210, antisense strand). The amplified DNA (1 μg per lane) was analyzed by electrophoresis through a 1.2% agarose gel and transferred to nitrocellulose membranes. The DNA blots were hybridized in buffer containing 20% formamide, 5× Denhardt's solution, 5× SSPE [0.9 *M* NaCl, 50 m*M* sodium phosphate, and 5 m*M* EDTA (*p*H 7.7)], 0.5% SDS, and denatured salmon sperm DNA (0.1 mg/ml) for 16 hours at 37°C with ^{32}P-labeled synthetic oligonucleotides (1×10^5 to 2×10^5 cpm/ml) corresponding to either the normal (GC TCC TTC GGC ATG GTG TA, lanes 1 and 2) or mutant (GC TCC TTC GTC ATG GTG TA, lanes 3 and 4) sequences (nucleotides 3107 to 3125). The blots were then washed three times: first, with 3× standard saline citrate (SSC) containing 0.1% SDS at room temperature for 30 min; second, with 0.1× SSC containing 0.1% SDS at room temperature for 30 min; and finally, with 0.1× SSC containing 0.1% SDS at 37°C for 30 min. Autoradiography of the blots was performed overnight at room temperature. The amplified normal DNA hybridized only with the oligonucleotide corresponding to the normal nucleotide sequence. The amplified DNA from the proband hybridized with both normal and mutant sequences, confirming that the proband is heterozygous for both sequences at codon 996.

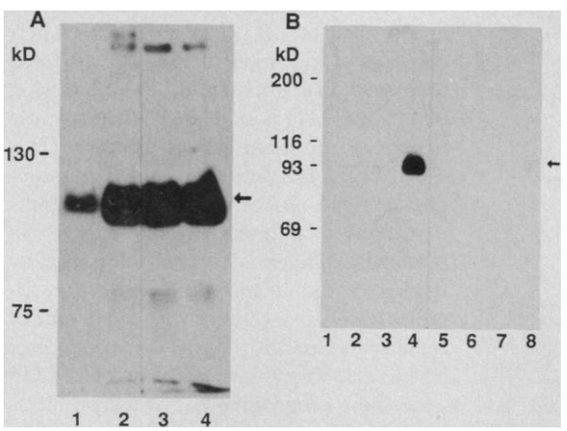

Fig. 3. Expression of the normal and mutant human insulin receptor cDNA in CHO cells. Normal human insulin receptor cDNA was used to construct the expression vector. Because this cDNA clone lacked the consensus initiation sequence, this sequence was added by ligation of an appropriate oligonucleotide (*20*). In addition, Hind III linkers were ligated onto the ends, and the cDNA was ligated into the Hind III site of pSV2*neo* to give the expression vector pSV2hIR. The mutant (GV-996) expression vector was constructed by substituting a 1267-bp Xho I–Spe I fragment (base pairs 3068 to 4334) of insulin receptor cDNA derived from clone 8. CHO-K1 cells were transfected with 10 μg of expression vector (either normal or GV-996) and 1 μg of pSV2*neo* by the calcium phosphate precipitation technique. Stable transfectants were selected by cultivation in the presence of the neomycin analog G418. (**A**) Protein blotting of partially purified insulin receptors. A site-specific antibody (Ab-IRC) directed against the COOH-terminus of the human insulin receptor β subunit was used to quantitate the number of insulin receptors in the cells as described previously (*7, 21*). (Lane 1) Nontransfected cells; (lane 2) cells transfected with normal human insulin receptor cDNA (Gly^{996}); (lanes 3 and 4) two cell lines transfected with mutant insulin receptor cDNA (Val^{996}). Based on insulin binding studies, we estimate that the transfected cell lines have 30,000 to 50,000 receptors per cell. In contrast, the nontransfected cells have approximately 1500 receptors per cell. (**B**) Autophosphorylation of partially purified insulin receptors from nontransfected cells (lanes 1 and 2), cells transfected with normal human insulin receptor cDNA (Gly^{996}) (lanes 3 and 4), or cells transfected with Val^{996} mutant insulin receptor cDNA (lanes 5 to 8). CHO cells were solubilized in the presence of Triton X-100, and the receptors were partially purified on wheat germ agglutinin–agarose (*22*). Receptors were incubated in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 10^{-7} *M* insulin and then were phosphorylated in the presence of 50 μ*M* [γ-^{32}P]ATP and 4 m*M* $MnCl_2$ as described previously (*2*). Receptors were immunoprecipitated with Ab-IRC and analyzed by SDS–polyacrylamide gel electrophoresis (10 μg per lane) and autoradiography (*2, 7*). In both (A) and (B) the arrow points to the β-subunit of the insulin receptor.

We have not determined the complete nucleotide sequence of this individual's other allele of the insulin receptor gene and do not know whether it is normal. The proband may be a compound heterozygote for two different mutations or, alternatively, the Val^{996} mutation may be dominant (*12*). Previous studies have shown the participation of acquired defects in insulin receptor tyrosine kinase activity in the pathogenesis of NIDDM (*13*). Furthermore, insulin-resistant patients have been described who may have primary genetic defects in their insulin receptor kinase activity (*14, 15*).

In conclusion, our data suggest that a defect in the insulin receptor tyrosine kinase can interfere with insulin action in vivo, thus supporting the hypothesis that mutations in the insulin receptor gene may be the cause of NIDDM, at least in a subpopulation of individuals (*12, 15–17*).

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Histone H5 in the Control of DNA Synthesis and Cell Proliferation

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The linker histones (H1, H5, H1°) are involved in the condensation of chromatin into the 30-nanometer fiber. This supranucleosome organization correlates with the resting state of chromatin, and it is therefore possible that the linker histones play an active role in the control of chromatin activity. The effect of H5 has been directly determined by expression of an inducible transfected H5 gene in rat sarcoma cells, which do not produce H5. Transfection resulted in the reversible inhibition of DNA replication and arrest of cells in G_1, at which time H5 concentrations approached that of terminally differentiated avian erythrocytes. The arrest of proliferation was accompanied by specific changes in gene expression probably related to the cell cycle block. The selectivity of these effects suggest that H5 plays an active role in the control of DNA replication and cell proliferation.

THE DIFFERENT LINKER HISTONE subtypes share similar structural features and bind, albeit with different affinity, to seemingly equivalent chromatin sites (*1*). The homologous variants H5 and H1° are synthesized in the maturing avian erythrocyte [H5 (*2, 3*)] or during the differentiation of mammalian cells [H1° (*4*)] and may have analogous functions. Expression of H5 and H1° is regulated independently of that of the main cellular histones [H1, H2A, H2B, H3, and H4] and is not coupled to DNA replication (*3, 5*). This results in increased amounts of H5 or H1° in terminally differentiated cells. H5, an early marker of the avian erythroid lineage, increases in relative content with the degree of cellular maturity as a direct consequence of an increase in transcription rate of the H5 gene and a progressive loss of the proliferation potential of the cells (*3*). The increase in linker histone content (H1 + H5) accompanies a progressive condensation of chromatin and the inactivation of most cellular genes (*2, 3*). Despite these correlations, the extent to which H5 contributes to the differentiation and loss of self-renewal capacity of the erythroid cells is still a matter of speculation.

We have directly determined the effect of H5 by transfection of a glucocorticoid-inducible H5 gene (pMSH5) (*6*) into rat sarcoma XC cells (*7*). This transformed cell line expresses relatively high levels of the glucocorticoid receptor (GR), and its growth characteristics are not noticeably affected by dexamethasone (Dex). In addition, XC cells do not differentiate, a trait important for distinguishing possible effects of H5 on cell proliferation from indirect effects due to cell differentiation.

The steroid hormone–responsive H5 gene and a selectable marker gene (*pRSVneo*) (*8*) were cotransfected (*9*) into XC cells, and G418-resistant colonies were isolated in which expression of H5 was hormone-dependent. An electrophoretic analysis of the basic nuclear proteins (*10*) from one such clone, XC10, grown in the presence and absence of 0.5 μM Dex, is shown in Fig. 1. The hormone induced the expression of a protein with the mobility of H5, not induced in the control XC8 cells, and its identity as H5 was confirmed by immunoblotting (*11*).

After hormone treatment, the amount of H5 in XC10 cells progressively increased and reached a maximum after 2 days (Fig. 1B) comparable to that found in mature erythrocytes (Fig. 1A) and, at the same time, the amounts of H1 decreased (Fig. 1B). The H5 content of XC10 cells began

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