Normal Insulin-Receptor cDNA Sequence in Pima Indians With NIDDM

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Pima Indians have served as a model of non-insulin-dependent diabetes mellitus (NIDDM). Within this population, inherited insulin resistance is a primary determinant of abnormal glucose metabolism. The insulin receptor is regarded as a "candidate gene" that could potentially be defective in Pima Indians or other populations with NIDDM. To directly address the question of potential insulin-receptor genetic defects in Pima Indians, we isolated and sequenced insulin-receptor cDNA from two Pima Indians with NIDDM. Small amounts of lymphoblast RNA were used to generate first-strand cDNA, which was then amplified via the polymerase chain reaction (PCR). In this way, seven overlapping segments of insulin- receptor cDNA were obtained. With the exception of the alternatively spliced 36–base pair exon 11, which is not expressed in lymphoblasts, the complete coding region of the mature proreceptor was examined with a combination of direct sequencing and sequencing of subcloned PCR segments. The nucleotide sequence in both subjects was identical to previously published insulin-receptor cDNA sequences obtained from healthy subjects. These data indicate that abnormalities of insulin binding and receptor function that have been previously observed in vitro with fresh and cultured cells from Pima Indians may be consequences of the diabetic milieu and/or genetic abnormalities in molecules that interact with the insulin receptor. *Diabetes* 38:1496–1500, 1989

Non-insulin-dependent diabetes mellitus (NIDDM) is a common disorder resulting from impaired insulin action and a relative deficiency of insulin. Although the pathogenesis of NIDDM is probably heterogenous within the general population, this disorder has been studied in various inbred ethnic groups such as the Pima Indians, in whom NIDDM is extremely prevalent (1). Within this group, the development of impaired glucose tolerance is most closely linked to increasing insulin resistance (2), a physiological parameter that is influenced by genetic factors in the Pima population (3,4). The molecular basis of inherited insulin resistance in Pima Indians is unknown. However, among the proteins potentially responsible for this state, the insulin receptor has received the greatest attention. Abnormalities of insulin binding and insulin-receptor function have been demonstrated in several tissues removed from non-Pima subjects with NIDDM (5–8) and in two studies of Pima Indians (9,10), one of which also used cultured cells (10).

Insulin-receptor mutations have recently been described in several patients with rare syndromes of severe insulin resistance (11–17). The relevance of these severe cases to more usual forms of NIDDM derives from the observation that one defective insulin-receptor allele was capable of conferring milder degrees of insulin resistance in family members of two severely affected patients, in whom both alleles were altered (12,15). Indeed, with the technique of restriction-fragment–length polymorphism (RFLP) there is some evidence to suggest that genetic variation at the insulin-receptor locus may contribute to the development of NIDDM in several patient groups (18,19).

In a direct approach to this question, we isolated and sequenced insulin-receptor cDNA from two Pima Indians with NIDDM. With the polymerase chain reaction (PCR), several overlapping segments of insulin-receptor cDNA encompassing the complete coding region were obtained. Through a combination of direct sequencing and sequencing of subcloned insulin-receptor PCR segments, we determined that the cDNA sequence in both patients is identical to the normal published sequence (20–22).

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Received for publication 1 August 1989 and accepted in revised form 23 August 1989.

RESEARCH DESIGN AND METHODS

Lymphoblast cell lines established from two Pima Indians, 2417 and 2575, were obtained from the National Institute of General Medical Studies Human Genetic Mutant Cell Repository (Camden, NJ). Patient 2417 is a woman with onset of NIDDM at age 21 yr; she has two siblings, a sister with NIDDM and a brother whose status is unknown. Patient 2575 is a Pima-Papago Indian with onset of NIDDM at age 18 yr; he has a sister with NIDDM, and both parents have NIDDM. To our knowledge, these two patients are not related. Total cellular RNA was prepared by the method of Chirgwin et al. (23).

Oligonucleotide primers and insulin-receptor cDNA synthesis. Seven sets of two 25–base pair (bp) oligonucleotide primers were synthesized with an Applied Biosystems 381A DNA synthesizer. These seven primer sets flank 672- to 1396-bp overlapping regions of insulin-receptor mRNA/cDNA, encompassing the entire coding region (Fig. 1, *A* and *B*). Each primer was modified at the 5′ end to create a restriction enzyme recognition site that could be used for subsequent subcloning if necessary (13). Specific first-strand cDNA was synthesized from 5–10 μg of total RNA with the 3′ flanking oligonucleotide in each primer set and Moloney murine leukemia virus reverse transcriptase (Bethesda Research, Gaithersburg, MD) as previously described (13,24).

PCR amplification of insulin-receptor cDNA. For each PCR primer set, the cDNA reaction mixture was subjected to 40 cycles of PCR amplification with *Thermus aquaticus* (*Taq*) DNA polymerase in an automated DNA thermal cycler (Perkin-Elmer/Cetus, Norwalk, CT) as described (13,24). After PCR amplification, the reaction mixture was digested with ribonuclease A (Sigma, St. Louis, MO) at 0.1 mg/ml at 37°C for 15 min. The amplified material was electrophoresed in 1.5% agarose gels. Bands containing amplified insulin-receptor cDNA were excised and purified with Gene Clean (Bio 101, San Diego, CA).

Direct sequencing of insulin-receptor cDNA. Purified PCR-product cDNA fragments were sequenced directly by the dideoxy-chain–termination method (25) with modified T7 DNA polymerase and Sequenase reagents (U.S. Biochemical, Cleveland, OH). One hundred to 200 ng of PCR-product cDNA was denatured by alkaline treatment, neutralized, precipitated with ethanol, and then annealed to 1 pmol of ^{32}P end-labeled (with T4 polynucleotide kinase) sequencing primer (either 1 of the 2 primers used for PCR amplification or an internal primer) in a 10-μl reaction volume at 37°C for 15 min. Extension/termination reactions were performed according to the manufacturers' instructions (Sequenase), except that the labeling step was omitted.

M13 subcloning and sequencing of insulin-receptor cDNA. With certain PCR primer sets, the quantity of the PCR-product cDNA was inadequate for direct sequencing. In these cases, purified PCR-product cDNA was digested with the appropriate restriction enzymes and subcloned into M13 (13). Single-stranded M13 DNA template derived from individual clones or pooled clones was sequenced with ^{35}S-labeled dATP by the dideoxy-chain–termination method (25). The sequencing reactions from both direct sequencing and M13 sequencing were electrophoresed in 6% polyacrylam-

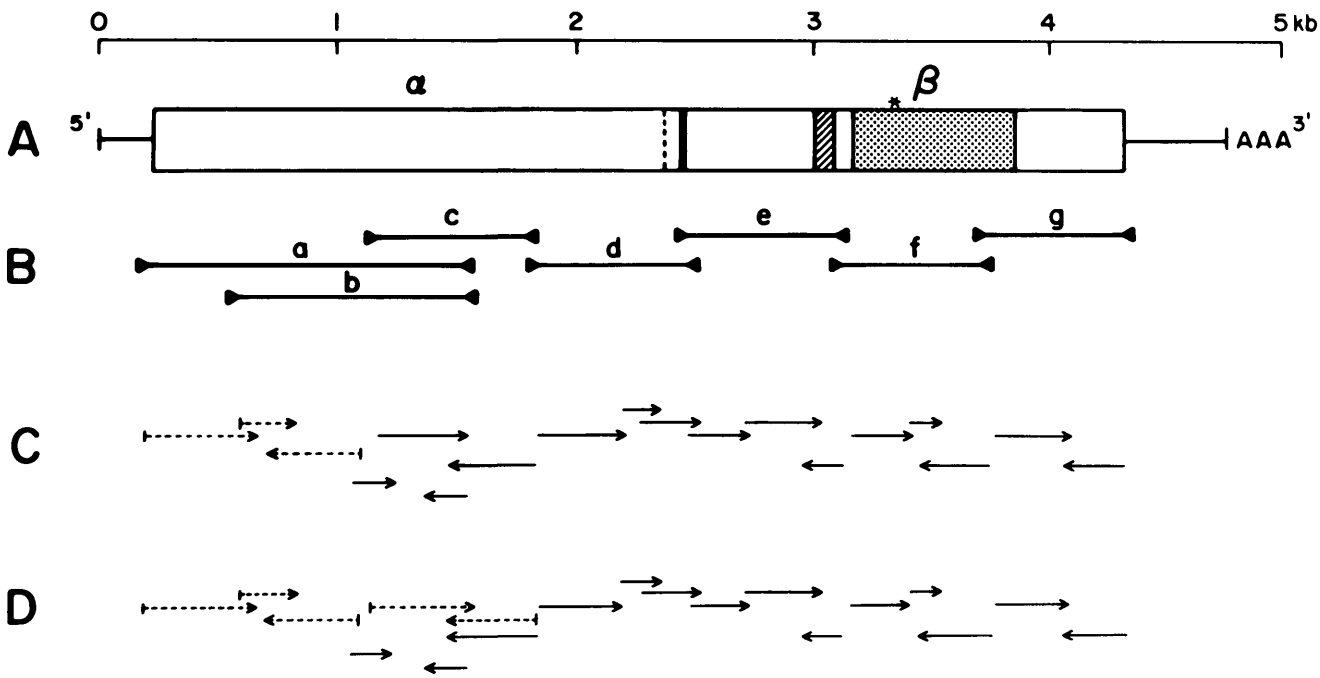

FIG. 1. *A*: schema of insulin-receptor cDNA that encodes single α/β-proreceptor protein (1355 amino acids). The α/cleavage site (*dark vertical bar*), transmembrane region (*hatched area*), and tyrosine kinase domain (*stippled area*) are shown. *Vertical broken line* indicates position of 36–base pair segment encoded by exon 11, which is alternatively spliced (and is absent in lymphoblast-derived cDNA). *Position of ATP-binding site within tyrosine kinase domain. *B*: location of 7 overlapping polymerase chain reaction (PCR) primer sets (*triangles*) that were used to amplify 7 segments of insulin-receptor cDNA (*a–g*) encompassing complete coding region. *C*: patient 2417. *Broken arrows* indicate direction and length of sequencing reactions performed with several (minimum of 6) clones of insulin-receptor cDNA after subcloning of PCR-product cDNA segments *a* and *b* (*B*) into M13 as described in RESEARCH DESIGN AND RESULTS. *Solid arrows* indicate length and direction of direct dideoxy-sequencing reactions performed with PCR-product cDNA segments *b–g*. *D*: patient 2575. Same as *C*, except that sequencing of M13 clones was also performed with PCR-product cDNA segment c.

ide-urea gels, which were then dried and exposed to Kodak XAR-5 film.

RESULTS

Seven sets of oligonucleotide primers were used to amplify insulin-receptor cDNA with the PCR method after synthesis of first-strand cDNA. In this way, seven double-stranded insulin-receptor cDNA fragments comprising the entire coding region were obtained from each subject (Fig. 1). The 36 bp encoded by exon 11 that are alternatively spliced were not included in the amplified cDNA because lymphoblasts, used as the source of RNA in these cases, and leukocytes do not express the exon 11–containing splice variant (22,24). In addition, only the last six codons of the 21–amino acid signal peptide were included in the amplified cDNA due to primer location (Fig. 1).

Within portions of the proximal α-subunit, the PCR-product cDNA was subcloned into M13 before sequencing (Fig. 1). To maximize the likelihood that both alleles of each subject were analyzed, we devised a novel method of pooling six M13 clones that were then sequenced together. The validity of this method was confirmed by pooling six clones where one of the six was known to contain a single-base substitution; both sequences could be unambiguously seen on the sequencing-gel autoradiogram (data not shown). By sequencing at least six clones, the probability of including only one of two alleles would be 0.5^6 or 1.56% assuming that both alleles were equally expressed as mRNA and equally amplified. With this method, three separate single-base substitutions (none duplicated in either or both patients) were detected. These base changes were present in only one of a minimum of nine clones when we separately sequenced individual M13 clones. We conclude that all three of these are PCR-misincorporation errors, which occur randomly with low frequency due to incomplete fidelity of DNA copying by *Taq* polymerase (26). A base change present in one clone of a sample of ≥nine clones is statistically different ($P \leq .039$ by the 2-tailed sign test; 27) from a population where that same base change is truly present in 50% of the population. Thus, it is extremely unlikely that skewed sampling of two equally expressed alleles, where one contains a heterozygous mutation, could account for the occurance of the nucleotide changes we observed. Furthermore, the frequency of PCR-misincorporation errors that we observed in these two patients is ~1 in 4000 nucleotides of M13 sequence, which is consistent with our experience and that of others (26).

Direct sequencing without prior subcloning was performed with most of the seven insulin-receptor cDNA fragments (Fig. 1). As has been previously reported, this method allows for the simultaneous sequencing of both alleles and eliminates the problem of PCR-misincorporation errors, because the average population of amplified DNA is sequenced rather than individual clones (26,28). In our hands, the fidelity of direct sequencing is high, and direct sequencing can detect both alleles of a known heterozygous insulin-receptor point mutation (13; data not shown).

The insulin-receptor nucleotide cDNA sequence obtained from our subjects was compared to the previously published normal receptor sequence (20–22) and was found to be identical to the initially reported normal cDNA sequence of Ebina et al. (20) or Ullrich et al. (21) for both subjects. Of the nine single-nucleotide differences between the two initially reported receptor cDNAs, five corresponded to the sequence of Ullrich et al. (21), and three corresponded to the sequence of Ebina et al. (20) for both subjects, similar to the genomic sequence of Seino et al. (22; Table 1). At amino acid 523 (Ala), one subject had only the Ebina codon (GCG), and the other subject had only the Ullrich codon (GCA); thus, this site is likely to be a true sequence polymorphism rather than a cloning artifact.

DISCUSSION

NIDDM is a genetically determined disorder, and the identification of responsible genes is a major goal of diabetes research (29). The Pima Indians serve as an informative model of NIDDM in which studies have been carried out on epidemiological, physiological, and cellular levels. Insulin resistance is the primary determinant of impaired glucose tolerance within the Pima population (2). Measurements of insulin sensitivity show a strong familial aggregation independent of other effects such as age or obesity (3), and the pattern of insulin resistance within studied groups suggests a single-gene, codominant mode of inheritance for this parameter (4).

The insulin receptor is an important candidate gene in the search for molecular defects that underlie the pathogenesis of insulin resistance. Studies that used RFLPs in an attempt to find an association between certain RFLPs and NIDDM susceptibility at the insulin-receptor locus have in some cases shown positive results (18,19). One study with a very small sample number failed to find linkage between NIDDM and the insulin receptor in Pima Indians (30). RFLP studies may fail to prove linkage disequilibrium even when the candidate gene causes the disease if sites of high-frequency recombination occur in or near the gene or if the genetic mutation is old enough to have allowed crossing over to reach equilibrium with RFLPs (31). Moreover, in a population such as the Pima Indians, insulin-receptor defect(s) might exist as a prevalent susceptibility trait with other gene(s) determining the onset of NIDDM.

To address the role of the insulin-receptor gene more directly, we isolated and sequenced the insulin-receptor cDNA from two Pima Indians with NIDDM. The finding of a normal

TABLE 1
Comparison of insulin-receptor nucleotide differences in healthy subjects and Pima Indian subjects 2417 and 2575

Amino acid codon no.	Ref. 20	Ref. 21	Ref. 22	Subject 2417	Subject 2575
144	His (CAC)	Tyr (TAC)	U	U	U
276	Gln (CAA)	Gln (CAG)	E	U	U
421	Thr (ACC)	Ile (ATC)	U	U	U
465	Lys (AAG)	Gln (CAG)	U	U	U
519	Asp (GAT)	Asp (GAC)	E	U	U
523	Ala (GCG)	Ala (GCA)	E	E	U
873	Val (GTC)	Asp (GAC)	E	E	E
874	Ser (TCC)	Thr (ACC)	E	E	E
1251	Lys (AAG)	Asn (AAC)	E	E	E

U, identical to nucleotide sequence of Ullrich et al. (ref. 21). E, identical to nucleotide sequence of Ebina et al. (ref. 20).

nucleotide sequence in the complete coding region of the mature receptor from both subjects suggests that primary structural changes in the insulin receptor are not responsible for the genetically mediated insulin resistance seen in this population. It is possible that a defect might lie in the small region of exon 11 or that the mRNA derived from a single defective allele, if expressed in low abundance relative to the normal allele, might have been missed by the methods employed; however, we consider these possibilities extremely unlikely. Recent preliminary data on the sequence of the intracellular portion of the β-subunit of an insulin-resistant but nondiabetic Pima Indian are consistent with our findings (32).

Several studies noted abnormal insulin binding and insulin-receptor tyrosine kinase function in freshly isolated cells from patients with NIDDM (5–8). It is not clear which (if any) of these defects might be due to mutations of the insulin-receptor gene versus acquired consequences of the diabetic milieu. Recent data suggests that some of these findings (in particular altered tyrosine kinase function) may be reversible with treatment of diabetes (33). Our data suggest that the abnormal insulin-receptor autophosphorylation seen in freshly isolated adipocytes from Pima Indians is not due to a primary change in the insulin-receptor sequence (9). An observed decrease in affinity of insulin binding to cultured fibroblasts from obese Pima Indians has raised the possibility of genetic differences at the insulin-receptor locus (10). If this earlier observation is correct, our data lead us to conclude that it is more likely that the genes responsible for insulin resistance encodes a protein that is capable of initiating events that modify insulin-receptor function. Although our knowledge of such molecules is incomplete, protein kinase C or a phosphoprotein phosphatase could play such a role (34).

Insulin-receptor mutations have been identified in several patients with severe forms of insulin resistance (11–17). It is possible that insulin-receptor genetic defects are also present in some fraction of patients with NIDDM. This study is the first report of insulin-receptor cDNA sequence obtained from subjects with NIDDM; the prevalence of potential receptor defects in more heterogenous populations with NIDDM is not addressed by this study. The PCR method and subsequent DNA-sequencing methods (in particular, direct sequencing of the PCR product) as described here can be more efficiently applied to larger numbers of patients than traditional cloning strategies. The direct sequencing of PCR-product DNA can also now be automated (35). Further application of these techniques to studies of the insulin-receptor and other candidate genes in patients with NIDDM will help clarify the molecular mechanisms responsible for this heterogenous disorder.

ACKNOWLEDGMENTS

We thank Drs. Matthew Warmon (Dept. of Genetics, Children's Hospital, Boston, MA) and Bernard J. Ransil (Beth Israel Hospital) for helpful discussions. We appreciate the clinical information on patients 2417 and 2575 provided by Dr. Clifton Bogardus (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health [NIH], Phoenix, AZ).

This work was supported by NIH Grant NIDDK-28082-07 and a grant from the Juvenile Diabetes Foundation (J.S.F.). D.E.M. is the recipient of a Physician Scientist Award from the National Institute on Aging.

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