Restriction-Fragment–Length Polymorphism in Insulin-Receptor Gene and Insulin Resistance in NIDDM

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Restriction-enzyme analysis of genomic DNA from 52 White and Hispanic nondiabetic subjects and 51 subjects with non-insulin-dependent diabetes (NIDDM) was carried out with insulin-receptor cDNA probes. A polymorphic 5.8-kilobase *Sst*I fragment was found in 12 (23.5%) of 51 NIDDM subjects but only in 4 (7.7%) of 52 nondiabetic control subjects. This association is significant by χ^2-analysis ($P < .05$). Furthermore, the nondiabetic subjects with the polymorphism were found to have hyperinsulinemia and/or nondiagnostic glucose tolerance. The polymorphism is a genetic marker for a phenotype that is neither necessary nor, by itself, sufficient for NIDDM. Nevertheless, it may indicate that insulin resistance functionally related to an insulin-receptor gene polymorphism is the proximal cause of NIDDM in at least one subset of the population. *Diabetes* 37:1071–75, 1988

Non-insulin-dependent diabetes mellitus (NIDDM) is a complex disease syndrome with an underlying genetic predisposition (1–4). However, the trait has highly variable penetrance, and the pattern of inheritance is poorly defined. Reasons for this are that NIDDM is probably a heterogeneous disease with multiple etiologies, and no biochemical or genetic markers for NIDDM have been identified. Insulin resistance is a characteristic feature of NIDDM and plays an important role in the pathophysiology of this syndrome. The etiology of this insulin resistance lies largely in abnormalities of the insulin-action sequence distal to the binding of insulin to its receptor (1–4). The cellular mechanisms underlying this postbinding defect are largely unknown, although recent advances in understanding the biochemistry of the insulin receptor have provided important clues.

The insulin receptor is a transmembrane protein consisting of two α- and two β-chains (5,6; Fig. 1). The α-chain is extracellular. The β-chain spans the membrane, and the molecule's tyrosine kinase domain is found in its cytoplasmic region (7). This region shows sequence homology with other hormone receptors and certain oncogene products, all of which also display tyrosine-specific protein kinase activity (8,9). Many of the details of the structure of the insulin receptor are based on information gained from structural characterization of the insulin-receptor cDNA (8,9).

With this new knowledge of the biochemistry and molecular biology of the insulin receptor, it is possible to test the hypothesis that the pathogenesis of NIDDM may be at least partly due to abnormal functioning of the insulin receptor. It has recently been found that the insulin-receptor tyrosine kinase activity in NIDDM patients was decreased compared with the activity of the enzyme in normal control subjects (10). The reported defects in the insulin receptor could represent posttranslational modifications of the receptor secondary to the diabetic state. Alternatively, the decreased kinase activity could be due to inherited structural alterations in the insulin receptor. To address these issues, we analyzed the insulin-receptor gene of normal and diabetic individuals with cloned cDNA probes (8).

MATERIALS AND METHODS

Subjects. Characteristics of the study group are summarized in Table 1. All were White (n = 97) or Hispanic (n = 6). NIDDM subjects (n = 51) were diagnosed by accepted criteria (11). At the time of the study, ~40% (n = 20) of the NIDDM subjects were being treated with insulin. Because NIDDM can be a silent disease that expresses itself only when the affected individual is obese, aged, or stressed, normal subjects were screened to decrease the possibility of including nondiabetic subjects destined later in life to develop NIDDM. Nondiabetic subjects (n = 52) averaged 54 yr of age, had at least two measurements of fasting glu-

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TABLE 1
Summary of study groups

Subjects	*n*	Age (yr)	Body mass index (kg/m²)	Fasting glucose (mg/dl)	Fasting insulin (μU/ml)	2-h OGTT (mg/dl)
Nondiabetic	52	57.5 ± 2.82	27.6 ± 3.8	95 ± 7.2	15.4 ± 2.6	121 ± 5
NIDDM	51	52.7 ± 2.22	36.2 ± 1.7	202 ± 16.0	31.2 ± 4.4	308 ± 19

Glucose measured 2 h after 75-g load of glucose. Values are means ± SE. OGTT, oral glucose tolerance test.

cose levels, all of which were <110 mg/dl, and had oral glucose tolerance tests (OGTT) that were either normal (n = 41) or indeterminate (n = 11) according to the criteria of the National Diabetes Data Group (11). Half (n = 24) were obese [body mass index (BMI) >25], which further decreased the probability that potential future diabetic patients might be included in the control group.

Clinical studies. Glucose tolerance was assessed by determining plasma glucose at 30-min intervals for 3 h after an oral glucose load of 75 g. Serum insulin levels after a 12-h fast were measured by radioimmunoassay (12). More than half (n = 56) of the subjects had insulin levels drawn during the OGTT.

Purification of probe DNA fragments. The larger *Eco*RI fragment of the insulin-receptor cDNA [base pairs (bp) 1012–5200; Fig. 1] was subcloned into pUC12, nick-translated, and used as a probe for restriction mapping (8). For higher resolution, a 780-bp *Sfa*NI fragment (bp 2702–3482) was prepared (13).

Genomic restriction analysis. High-molecular-weight DNA was purified from peripheral blood monocytes as described (13). DNA (10 μg) was digested with 40 U of restriction enzyme for 16 h at 37°C in a buffer recommended for the enzyme. The DNA was precipitated in ethanol, fractionated on 1% agarose gels, and blotted onto nitrocellulose. Hybridization probes (sp act ~10^9 cpm/μg) were prepared by nick translation (4100-bp *Eco*RI fragment) or oligonucleotide-primed translation (780-bp *Sfa*NI fragment) (14). Hybridization to Southern blot filters was performed at 10^6 cpm/cm² in 5× saline sodium citrate (SSC) at 68°C for 16 h with washing at 68°C in 0.1× SSC and 0.5% sodium dodecyl sulfate as described (13). Autoradiograms were developed after exposure for 5 days at −70°C with Kodak XAR-5 film and Du Pont intensifying screens.

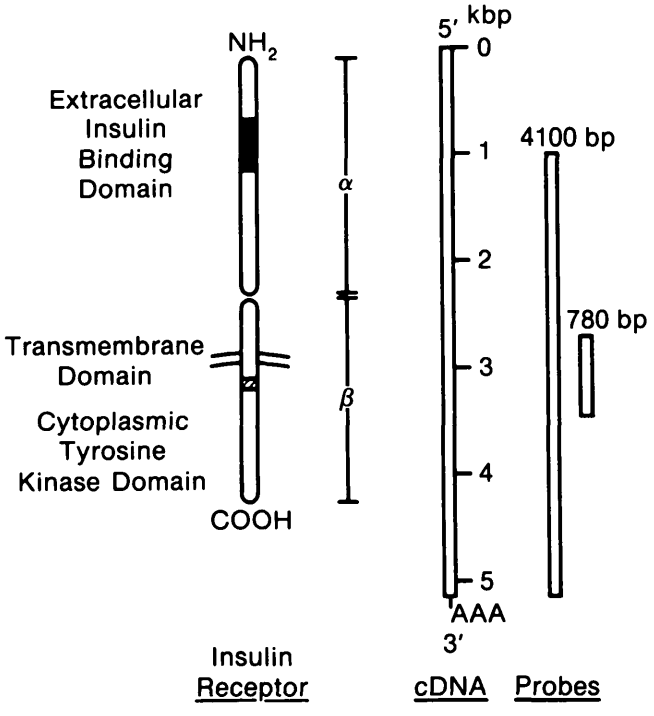

FIG. 1. Schema of insulin-receptor protein, cDNA, and cDNA fragments used as probes. Native protein is $\alpha_2\beta_2$ heterotetramer. α-Chain (135,000 M_r) is extracellular and contains cysteine-rich region (*solid bar*) and hormone binding site; it is linked by disulfide bonds to 95,000-M_r β-chain that spans membrane and contains tyrosine kinase domain. Probable ATP binding site is *hatched*. From cDNA, 2 fragments were generated for use as probes in restriction-map analysis, including 4100–base pair (bp) *Eco*RI fragment and 780-bp fragment generated by *Sfa*NI digestion. The 780-bp fragment includes transmembrane region and putative tyrosine kinase domain. cDNA includes ~800 nontranslated nucleotides.

RESULTS

Initial investigation of genomic DNA digested with several enzymes was performed with a 4.1-kb cDNA probe that includes coding sequences for most of the α-subunit and all of the β-subunit of the insulin receptor (8; Fig. 1). Analysis of *Sst*I-digested DNA revealed restriction-fragment–length polymorphisms. However, because of the complexity of the Southern blots revealed by this combination of restriction enzyme and probe, we chose to use a smaller cDNA probe for further analysis. This probe is a 780-bp *Sfa*NI fragment of the receptor cDNA and spans nucleotides 2702–3482 (according to the numbering of Ullrich et al., 8). It includes the transmembrane region of the β-chain as well as areas of high homology with the human epidermal growth factor receptor that presumably encodes the tyrosine-kinase domain (8,9).

As shown in Fig. 2 and Table 2, most subjects (78 of 103) had DNA fragments of 9.6 and 5.3 kb that were recognized by the 780-bp probe (Fig. 2, *lane a*). One polymorphism is expressed as the absence of the 9.6-kb band and the presence of a new band at 7.2 kb (Fig. 2, *lane b*). Six subjects (5.6%) had both the 9.6- and 7.2-kb bands (Fig. 2, *lane c*). Three subjects (3%) were homozygous for the 7.2-kb fragment (Fig. 2, *lane b*). A 2.4-kb band was sometimes visible when the 7.2-kb band was present, but the presence of the 2.4-kb band was not reproducible, possibly due to our relatively stringent hybridization conditions. A second polymorphism was also revealed when the 780-bp probe was used. Most subjects [87 (81.3%) of 103] had only the smaller 5.3-kb band (Fig. 2, *lanes a–c, f*), whereas 16 (18.7%) of 103 had an additional band at 5.8 kb (Fig. 2, *lane d*). That these two bands represent allelic forms of the polymorphism was suggested by the decreased relative intensity of the

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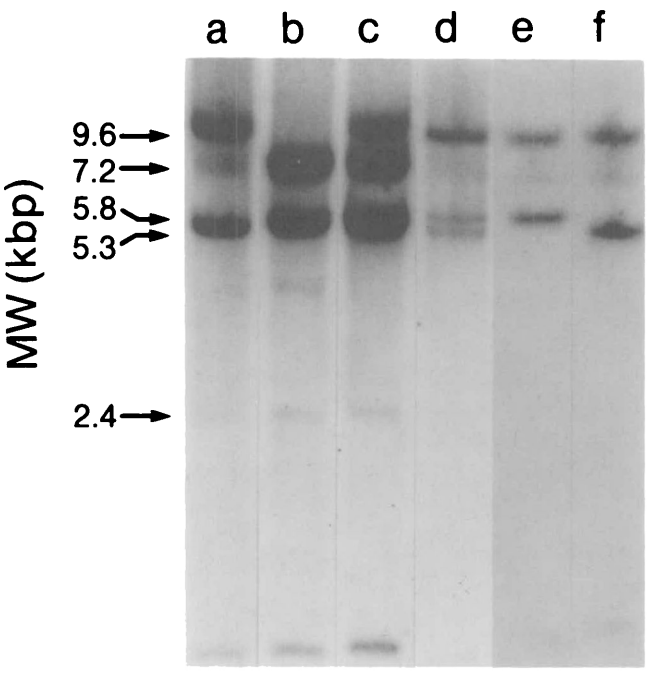

FIG. 2. Restriction fragments generated by *Sst*I digestion of genomic DNA probed with 780-bp fragment. *Lane a*: majority pattern with prominent bands at 9.6 and 5.3 kb. *Lane b*: loss of band at 9.6 kb with new bands at 7.2 and 2.4 kb. *Lane c*: presumed heterozygote of *a* and *b*. *Lane d*: presence of new band at 5.8 kb. *Lane e*: presence of band at 5.8 kb with loss of band at 5.3 kb. *Lane f*: majority pattern.

5.3-kb band when the 5.8-kb band was present and by the finding of two subjects who had only the 5.8 kb band (Fig. 2, *lane e*). The latter subjects were presumably homozygous for this polymorphism. These polymorphisms have been previously described and characterized by a similar cDNA probe (15,16).

A summary of the frequencies of the polymorphisms revealed by hybridizing the 780-bp probe to the *Sst*I digests is given in Table 2. The polymorphism defined by the 7.2-kb band was seen in a total of nine subjects, four diabetic and five nondiabetic. The homozygotes for the 7.2-kb band included two nondiabetic subjects and one with NIDDM. Thus, this polymorphism did not associate with the diabetic phenotype. In contrast, the presence of the 5.8-kb band appears to be associated with diabetes. It was seen in only 4 (7.7%) of 52 nondiabetic subjects but in 12 (23.5%) of 51 NIDDM subjects (Table 3). By χ^2-analysis, the association of the 5.8-kb band with diabetes was significant ($\chi^2 = 4.92$, $P < .05$). The six Hispanic subjects were distributed equally among the groups: two nondiabetic and four with NIDDM, one of whom had the 5.8-kb band. If they are excluded from the χ^2-analysis, the association remains significant ($\chi^2 = 4.40$, $P < .05$).

TABLE 2
Frequency of *Sst*I polymorphisms in normal and NIDDM subjects

Subjects	*n*	-9.6- -5.3-	-9.6- -7.2- -5.3-	-7.2- -5.3-	-9.6- -5.8- -5.3-	-9.6- -5.8-
Nondiabetic	52	43	3	2	3	1
NIDDM	51	35	3	1	11	1

Size of polymorphic fragments designated in kilobases.

TABLE 3
Frequency of 5.8-kb *Sst*I fragment in normal and NIDDM subjects

Subjects	Present	Absent
NIDDM (*n* = 51)	12*	39
Nondiabetic (*n* = 52)	4	48

*$\chi^2 = 4.92$, $P = .037$. When the 6 Hispanics are excluded from analysis, $\chi^2 = 4.40$, $P = .044$.

The association of the 5.8-kb polymorphism with NIDDM was clear, yet some nondiabetic subjects also expressed the polymorphism. Because the genetic predisposition to diabetes is not always expressed as disease and the phenotype of NIDDM is complex, we reexamined the subgroup of subjects expressing the 5.8-kb polymorphism. All (*n* = 11) NIDDM subjects heterozygous for the 5.8-kb marker were obese at diabetes onset; two have been able to discontinue sulfonylurea therapy with weight loss, although they remain glucose intolerant and hyperinsulinemic (fasting insulin levels 33 and 22 μU/ml, respectively; normal 9 ± 1). The single subject homozygous for the 5.8-kb polymorphism presented with diabetes at a relatively early age (44 yr), was only moderately overweight (BMI = 30.6), and required 150 U/day of insulin for control of his diabetes. Off insulin therapy, endogenous fasting insulin levels were 74 ± 3 μU/ml. Only one of the nondiabetic subjects with the 5.8-kb band was mildly obese (Table 4). The others were not obese, yet all exhibited some abnormality of glucose homeostasis. Although none met the established criteria for impaired glucose tolerance, subjects 2–4 had nondiagnostic OGTTs. Subject 3, a homozygote for the 5.8-kb band, had a fasting glucose of 109 mg/dl and a 2-h value of 201 mg/dl. All four subjects had hyperinsulinemia during the OGTT, although in subjects 1 and 2 this was not out of the range of the hyperinsulinemia seen with aging (17). Subject 3 had fasting hyperinsulinemia beyond that normally seen in association with aging (17 vs. 13 ± 1 μU/ml for elderly control subjects), and subject 4 had profound hyperinsulinemia both before and during the OGTT.

DISCUSSION

We have described two restriction-fragment–length polymorphisms within the human insulin-receptor gene, one of which appears to be related to the NIDDM phenotype. The polymorphisms were revealed by analyzing *Sst*I-digested genomic DNA of diabetic and nondiabetic control subjects with a radiolabeled 780-bp receptor cDNA probe. The form of the polymorphism associated with NIDDM involves an increase in the size of the restriction fragment from 5.3 to 5.8-kb and was seen in 4 (7.7%) of 52 normal and 12 (23.5%) of 51 diabetic subjects. This polymorphism has been determined to result from an insertion (15).

The 5.8-kb restriction-fragment polymorphism was described previously and reported not to be associated with NIDDM (16). Subsequently, an association study of the polymorphism was undertaken in a large population of Black subjects with NIDDM (18). In that study, the presence of the 5.8-kb polymorphism was found, in contradistinction to the

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TABLE 4
Clinical characteristics and results of oral glucose tolerance test in nondiabetic subjects expressing 5.8-kilobase marker

			Plasma glucose (mg/dl)						Serum insulin (μU/ml)					
Subject	Age (yr)	BMI	Fasting	30 min	60 min	90 min	120 min	180 min	Fasting	30 min	60 min	90 min	120 min	180 min
1	60	26.8	93	134	157	138	138	85	9	58	102	93	119	27
2	70	24.7	90	128	155	157	156	74	14	60	72	88	90	28
3*	71	21.7	109	147	123	174	201	70	17	51	44	48	81	30
4	88	22.1	101	150	203	155	93	87	39	66	265	405	123	31

Glucose was measured after 75-g carbohydrate load. Normal fasting insulin levels are 9 ± 1 μU/ml for subjects <60 yr, 13 ± 1 for subjects >60 yr. Normal peak insulin values during an OGTT are 85 ± 10 (15). BMI, body mass index; expressed in kg/m².
*Subject homozygous for 5.8-kilobase marker.

current results, to be significantly but negatively correlated with NIDDM. We are not able to reconcile these two disparate findings except to suggest that NIDDM is a heterogenous disease and that the form of the disease seen in Blacks may have different genetic determinants in that the 5.8-kb polymorphism denotes a subset of Whites with NIDDM. Interestingly, both studies may suggest a relationship between the insulin receptor and NIDDM but possibly through different mechanisms. Recently, a family study was undertaken in an attempt to demonstrate formal genetic linkage between the insulin-receptor gene and maturity-onset diabetes in youth (15). In that study, the 5.8-kb polymorphism, referred to as S_1 (16), cosegregated with diabetes in two pedigrees but not in two other pedigrees. Interestingly, however, in one of the pedigrees in which S_1 (or the 5.8-kb fragment) segregated with diabetes, the affected family members were hyperinsulinemic. However, the small size of the pedigrees precluded statistical proof of linkage.

This study relies on the association between a genotype and a phenotype in a population; therefore, no formal genetic linkage can be inferred. Note, however, that the polymorphism is found near sequences that code for the β-subunit transmembrane region of the receptor as well as for a region of extensive homology with oncogenes of the tyrosine kinase family (8,9), which includes the ATP binding site of the insulin receptor (19). In this regard, the decreased kinase activity of insulin receptors in NIDDM may be related to this polymorphism (10,20,21).

Although the polymorphism is associated with NIDDM, the presence of the 5.8-kb band is itself neither necessary nor sufficient for NIDDM. Only 23.5% of the diabetic subjects in this study had the diabetes-associated polymorphism. This is probably related to the heterogeneous etiology of NIDDM. There could be several distinct structural abnormalities in the insulin receptor or other proteins critical for glucose homeostasis that alone or in combination may result in diabetes. Further structural analysis of the insulin-receptor gene in NIDDM should help clarify these issues. If there are several distinct biochemical abnormalities responsible for the broad spectrum of NIDDM, then the one associated with the 5.8-kb polymorphism is relatively mild. Two of the subjects with that pattern were able to normalize fasting glucose with weight loss, although they remain glucose intolerant and hyperinsulinemic. It is interesting that the single diabetic subject homozygous for the 5.8-kb fragment had onset of the disease at a relatively early age (44 yr), was only moderately overweight, and required high doses of insulin (150 U/day) despite a weight-maintenance diet.

The existence of the 5.8-kb fragment in four nondiabetic subjects in the original study group reveals that the polymorphism is a marker for an abnormality that by itself is also not sufficient for NIDDM. However, all subjects in this category had hyperinsulinemia, nonnormal glucose tolerance, or both. In the most striking case, subject 4 had marked fasting hyperinsulinemia and, by raising his serum insulin levels severalfold higher than normal during an OGTT, was still only able to maintain indeterminate, but not normal, glucose tolerance (Table 4). Just one of the normal subjects with the 5.8-kb fragment was obese but only minimally (BMI 26.8). These features raise the possibility that the polymorphism is a marker for insulin resistance and that another physiologic abnormality, e.g., insufficient insulin secretion or obesity, needs to be present before NIDDM is expressed. These data are clearly speculative and require further study.

To reveal differences between NIDDM and normal subjects, we biased our selection of normal subjects toward older obese individuals because NIDDM has its onset in middle age or later. Younger people with a genetic predisposition can have normal glucose tolerance, only to develop NIDDM in later years. Thus, in a group of younger subjects, individuals with the diabetes-related polymorphism who had not yet developed clinically detectable diabetes could be found. The existence of the polymorphism in two diabetic subjects who were able to normalize their fasting glucose with strict dieting emphasizes this point. Had these two been selected at an earlier age or only by virtue of fasting glucose levels after weight loss, they would have been incorrectly identified as nondiabetic. Because of our selection bias, we are not yet able to specify the predictive value of finding the 5.8-kb polymorphism in a given person. The data would suggest an approximately threefold relative risk (23.5 vs. 7.7%) for NIDDM when the 5.8-kb band is present in Whites. Assuming 5–10% of the population >50 yr old has NIDDM (22) and that 23.5% of these people have the 5.8-kb marker, whereas 7.7% of all nondiabetic individuals have this band, then ~67% of all individuals with the 5.8-kb restriction fragment will be nondiabetic. Approximately 2–3% of the total population >50 yr old (25% of everyone with the polymorphism) will have both the polymorphism and NIDDM. The polymorphism may be more predictive for insulin resistance, however, as suggested by the OGTT results of the nondiabetic subjects with the 5.8-kb marker.

Further family studies should help clarify the relationship of the polymorphism to disease. However, even these studies may not be completely unambiguous for several reasons.

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The low penetrance of the genetic predisposition will lead to false positives (people with markers but without disease); however, such occurrences are expected and can be dealt with statistically. Furthermore, physiologic testing (e.g., OGTT and insulin levels) can be used to narrow the definition of the nondiseased population. The high prevalence of the disease and its possibly multiple etiologies is more confounding. If 10% of the population >50 yr old had NIDDM (21), it is likely that in some families more than one "diabetes gene" will coexist. This will lead to diseased subjects within a family that do not segregate to a single marker (false negatives).

In summary, using insulin-receptor cDNA to probe the genomic DNA of a well-defined population of normal and NIDDM subjects, we have analyzed several restriction-fragment–length polymorphisms in the insulin-receptor gene, one of which appears to be significantly associated with the NIDDM phenotype. The presence of the polymorphism is neither sufficient nor necessary for diabetes. Nevertheless, these results raise the possibility that the polymorphism may be a marker for certain forms of insulin resistance and may denote a subgroup of subjects who can develop NIDDM. Further studies on the structure of the insulin receptor in NIDDM and on the detailed physiologic abnormalities present in subjects with the polymorphism should clarify the significance of this finding.

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