Sequence Variants in the Pancreatic Islet β-Cell Inwardly Rectifying K^+ Channel Kir6.2 (Bir) Gene

Identification and Lack of Role in Caucasian Patients with NIDDM

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Signals derived from the metabolism of glucose in pancreatic β-cells lead to insulin secretion via the closure of ATP-sensitive K^+ channels (K_{ATP}). The cloning of the gene encoding the β-cell inward rectifier Kir6.2 (Bir), a subunit of the β-cell K_{ATP} channel, provided the opportunity to look for mutations in this gene that might contribute to the impaired insulin secretion of NIDDM. By single-strand conformational polymorphism (SSCP) analysis on 35 Northern-European Caucasian patients with NIDDM, six sequence variants were detected: $Glu^{10gag \rightarrow}Lys^{10aag}$ (E10K), $Glu^{23gag \rightarrow}Lys^{23aag}$ (E23K), $Leu^{270ctg \rightarrow}Val^{270gtg}$ (L270V), $Ile^{337atc \rightarrow}Val^{337gtc}$ (I337V), and two silent mutations. Allelic frequencies for the missense variants were compared between the NIDDM group (n = 306) and nondiabetic control subjects (n = 175) and did not differ between the two groups. Pairwise allelic associations indicated significant linkage disequilibrium between the variants in Kir6.2 and between them and a nearby pancreatic β-cell sulfonylurea receptor (SUR1) missense variant (S1370A), but these linkage disequilibria did not differ between the NIDDM and control groups. The results of these studies thus revealed that mutations in the coding region of Kir6.2 *1*) were not responsible for the previously noted association of the SUR1 variants with NIDDM (Inoue H et al., *Diabetes* 45:825–831, 1996) and *2*) did not contribute to the impaired insulin secretion characteristic of NIDDM in Caucasian patients. *Diabetes* 46:502–507, 1997

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PCR, polymerase chain reaction; SSCP, single-strand conformational polymorphism; SUR, sulfonylurea receptor.

Although numerous studies have shown that insulin resistance and hyperinsulinism are prominent features in the early stages of NIDDM (2–5), others have noted that symptomatic diabetes requires pancreatic β-cell failure (6–9). We have sought to ascertain whether at least part of the genetic predisposition to NIDDM resides in genes encoding insulin production, as have others (10). It has been known for some time that signals derived from the metabolism of glucose in pancreatic β-cells ultimately result in insulin secretion via the closure of ATP-sensitive K^+ (K_{ATP}) channels (11,12). Sulfonylurea compounds are also potent inhibitors of islet K_{ATP} channels (12).

Recently, the pancreatic β-cell K_{ATP} channel was shown to be a heteromultimeric structure composed of at least two essential subunits, the inward rectifier, Kir6.2 (Bir), and the sulfonylurea receptor (SUR1) (13). The importance of I_{KATP} in regulating insulin secretion was demonstrated when mutations in the SUR1 gene were found in patients with familial hyperinsulinism (14,15). More recently, we have discovered a child with this disorder who is homozygous by descent for a premature stop mutation in Kir6.2, resulting in uncontrolled hyperinsulinism requiring 95% pancreatectomy for treatment (A. Nestorowicz and M.A.P., unpublished observations).

The islet K_{ATP} channel is thus a known key regulator of insulin secretion (16). Consequently, mutations in the K_{ATP} genes might result in the alteration of the glucose-insulin secretion coupling in NIDDM patients, and previous studies have addressed this question by linkage analysis in NIDDM sib pairs (17,18). The Kir6.2 gene resides within 5 kb of the SUR1 gene on chromosome 11p15.1 (13), and the allele sharing at nearby linkage markers D11S902 and D11S921 did not differ from what was expected in Mexican-American (17) and Japanese sibs with NIDDM (18). A more recent analysis of this linkage data, however, disclosed that this locus could not be ruled out as a minor susceptibility locus or even a major one in a subset of NIDDM patients (19).

We recently reported that two polymorphic variants in the SUR1 gene were strongly associated with NIDDM in Northern-European Caucasians, suggesting that genetic defects at the SUR1/Kir6.2 locus may account for a component of the inherited basis of NIDDM in this racial group (1). Knowing the

TABLE 1
List of primers used for SSCP

Fragment no.	Forward primers (5′→3′)	Reverse primers (5′→3′)	Annealing temperature (°C)	PCR product size (bp)
1	CCGAGAGGACTCTGCAGTGA	CACCAGCGTGGTGAACACGT	55	278
2	GAAAGGCAACTGCAACGTGG	TAGTCACTTGGACCTCAATG	58	278
3	CTGTGTCACCAGCATCCACT	TGATGATCATGCTCTTGCGG	58	308
4	TCAGCAAGCATGCGGTGATC	ACGCCTTCCAGGATGACGAT	58	322
5	CTACCATGTCATTGATGCCA	GCACTTTGATGGTGTTGCCA	58	248
6	CGTTACTCTGTGGACTACTC	TGGGCTACATACCACATGGT	58	263

central role of Kir6.2 in regulating insulin secretion, we scanned the gene for mutations in patients with NIDDM.

RESEARCH DESIGN AND METHODS

Subjects. Informed consent was obtained for all subjects before testing. The studies were approved by the University of Utah Institutional Review Board, the Central Oxford Research Ethics Committee, and the Washington University Human Studies Committee. All of the patients and controls subjects had been previously studied for sequence variants at the SUR1 locus (1).

Utah. The affected probands for single-strand conformational polymorphism (SSCP) screening were obtained from 35 families with a minimum of two NIDDM siblings with a diagnosis before age 65, as recently described (20). To study the prevalence of the KIR6.2 sequence variants, 134 unrelated NIDDM patients and 79 nondiabetic unrelated control subjects of Northern European descent were recruited at the outpatient clinics of the Veteran's Affairs Medical Center and University of Utah Clinics (mean age, 64 and 55 years, respectively).

United Kingdom. 172 unrelated Caucasian NIDDM patients (Oxford Clinic of the U.K. Prospective Diabetes Study) and 96 unrelated nondiabetic control subjects from the same geographical area were recruited (20).

SSCP analysis. Primers used for polymerase chain reaction (PCR) amplification and the product size are shown in Table 1. Since the coding sequence of the human Kir6.2 gene was encoded by one exon (1173 bp), primers were designed to amplify this region as six overlapping fragments (Fig. 1). For SSCP analysis, ^{32}P-radiolabeled PCR products were heat-denatured and loaded onto a 5% polyacrylamide gel and subjected to electrophoresis at 20W for 2–4 h (25°C) for gels with 10% glycerol and at 40W for 1–2 h (4°C) for gels without glycerol. Variants detected by SSCP analysis were re-amplified and sequenced with a cycle sequencing kit (Perkin-Elmer/Cetus, Norwalk, CT) directly.

Genotyping for variants in the Kir6.2 gene. For genotyping, PCR products were digested with restriction enzymes shown in Table 2 and analyzed by either acrylamide or agarose-gel electrophoresis. For the L270V variant, hybridization with allele-specific oligonucleotides (ASH) using ^{32}P-radiolabeled oligonucleotides corresponding to either the wild-type sequence (270-L: TACGACCTGGCACCCAG) or the sequence of variant (oligo 270-V: CTGGGTGCCACGTCGTA) was also used as described elsewhere (21).

Statistical analysis. Likelihood ratio tests were used to evaluate the statistical significance of linkage disequilibrium at all marker pairs within a sample (NIDDM, Utah; control, U.K.) and to evaluate the significance of haplotype differences between samples. For each marker pair, we used the computer program ASSOC (22) to partition the total likelihood χ^2 into a component due to linkage disequilibrium and a residual component due to all other causes of phenotypic association. To compare haplotype frequencies between various samples, we used the "myriad haplotype" algorithm of MacLean and Morton (23) to obtain maximum likelihood haplotype frequency estimates. The respective likelihoods for the diabetic, control, and combined (total) sample were denoted by L_D, L_C, and L_T. The quantity $-2[\ln L_T - (\ln L_D + \ln L_C)]$ is asymptotically distributed as χ^2 with degrees of freedom $(D-1)+(C-1)-(T-1)$, where D, C, and T are the number of haplotypes with a nonzero frequency in each sample (24).

For two diallelic loci x and y with allele frequencies $p_x/p_{(1-x)}$ and $p_y/p_{(1-y)}$, respectively, there are four haplotypes with estimated frequencies denoted as H_{xy}, $H_{x(1-y)}$, $H_{(1-x)y}$, and $H_{(1-x)(1-y)}$. Linkage disequilibrium (D_{xy}) is defined as the difference between the estimated haplotype frequency and the product of the allele frequencies (i.e., $D_{xy} = H_{xy} - p_x p_y$). For positive values of D_{xy}, the maximum attainable value (D_{max}) is constrained to be the minimum of $p_x(1-p_y)$ or $(1-p_x)p_y$. For negative values of D_{xy}, D_{max} is constrained to be $-p_x p_y$ if $p_x + p_y < 1$ or $-(1-p_x)(1-p_y)$ if $p_x + p_y > 1$. Accordingly, rather than report the numerical value of D_{xy}, which carries little information by itself, we report the observed disequilibrium as a percentage of its maximum possible value (i.e., D_{xy}/D_{max}).

RESULTS

Identification of sequence variants in the Kir6.2 gene. Thirty-five NIDDM probands were screened by SSCP and six variants were detected. When the variants were sequenced, four encoded missense variants (E10K, E23K, L270V, and I337V) and two encoded silent changes (A190A and L267L), as shown in Table 2. In addition to these variants detected by SSCP analysis, sequence analysis of two subjects revealed a possible variant at Ser^{148} (S^{148agc} to I^{148atc}) when compared with the reported sequence. However, when genomic DNA from 79 subjects was screened, all were homozygous I^{148atc}, suggesting that the previously reported S^{148} allele was also rare in Caucasians and Japanese (data not shown; N.I., S.S., unpublished observations).

Frequencies of the Kir6.2 sequence variants in NIDDM patients and control subjects. To evaluate the possible biological consequences of the Kir6.2 variants, genomic DNA from individuals in two Northern-European Caucasian populations (Utah and U.K.) was evaluated for frequencies of the four missense variants (E10K, E23K, L270V, and I337) as shown in Table 3. The E10K missense variant was rare (1/586 chromosomes in NIDDM cases vs. 1/350 in control subjects, NS). While genotypic frequencies for both E23K and I337V variants differed between the NIDDM patients and control subjects, the allelic frequencies did not differ between the groups. Neither genotypic nor allelic frequencies of the L270V variants differed between NIDDM and control groups. The clinical characteristics of NIDDM subjects carrying each mutation were compared with those with homozygous wild-type variant, and no differences were observed for age of diagnosis of diabetes, fasting plasma glucose, HbA_{1c}, BMI, waist-to-hip ratio, plasma insulin, and β-cell function assessed by Homeostasis Model Assessment (25) (data not shown).

Pairwise analysis of variants at Kir6.2 and SUR1. To determine the extent of linkage disequilibrium between the three common missense variants at Kir6.2 and whether this

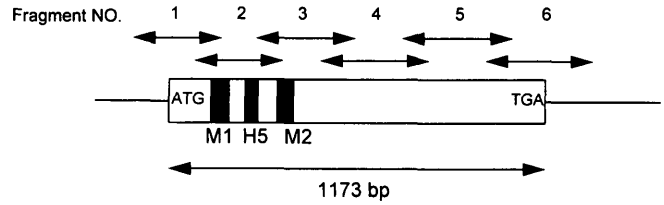

FIG. 1. Strategy for SSCP analysis of the Kir6.2 gene in six overlapping fragments. M1 and M2 refer to the first and second membrane-spanning domains, respectively, and H5 refers to the putative pore region of inwardly rectifying K$^+$ channels. The open box indicates the protein-coding region of the gene.

TABLE 2
Sequence variations in the human Kir6.2 (Bir) gene

Amino acid	Nucleotide change	Assay	Restriction enzyme	Effect
E10K	GAG to AAG	PCR-RFLP	*Ava* I	Loss of *Ava* I site
E23K	GAG to AAG	PCR-RFLP	*Ban* II	Loss of *Ban* II site
A190A	GCT to GCC	—	—	—
L267L	CTC to CTG	—	—	—
L270V	CTG to GTG	PCR-RFLP, ASH	*Mae* II	Gain of *Mae* II site
I337V	ATC to GTC	PCR-RFLP	*Msl* I	Loss of *Msl* I site

value differed between NIDDM and control groups, allelic associations were calculated for all pairs as shown in Table 4. The *P* values for allelic association are shown above the diagonal, and the percent of maximum disequilibrium below. Significant linkage disequilibrium was evident between each pair of Kir6.2 missense variants for both NIDDM and control groups.

Three variants at SUR1 had been previously evaluated in these same two populations of NIDDM patients and control subjects (1). The SUR1 exon 24 and exon 22 variants were shown to be significantly associated with NIDDM, while the exon 7 variant was present at the same frequency in both groups. The extent of linkage disequilibrium was thus determined between pairs of variants at SUR1 and Kir6.2 (Table 4). The results of these analyses show significant allelic association between exon 24 and exon 22 variants for the NIDDM group only. While the SUR1 exon 7 variant was associated

TABLE 3
Genotypic and allelic frequencies of variants between NIDDM patients and normal subjects

	U.K. population		Utah population		U.K. and Utah combined	
	NIDDM patients	Control subjects	NIDDM patients	Control subjects	NIDDM patients	Control subjects
E10K						
Genotype						
G/G	172 (1.00)	96 (1.00)	120 (0.992)	78 (0.987)	292 (0.997)	174 (0.994)
G/A	0 (0.00)	0 (0.00)	1 (0.008)	1 (0.013)	1 (0.003)	1 (0.006)
A/A	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Allele						
G	344 (1.00)	192 (1.00)	241 (0.996)	157 (0.994)	585 (0.998)	349 (0.997)
A	0 (0.00)	0 (0.00)	1 (0.004)	1 (0.006)	1 (0.002)	1 (0.003)
E23K						
Genotype						
G/G	72 (0.42)	38 (0.40)	52 (0.44)	21 (0.31)	124 (0.43)*	59 (0.36)*
G/A	78 (0.45)	52 (0.54)	55 (0.46)	44 (0.65)	133 (0.46)*	96 (0.59)*
A/A	22 (0.13)	6 (0.06)	12 (0.10)	3 (0.04)	34 (0.12)*	9 (0.05)*
Allele						
G	222 (0.65)	128 (0.67)	159 (0.67)	86 (0.63)	381 (0.65)*	214 (0.65)*
A	122 (0.35)	64 (0.33)	79 (0.33)	50 (0.37)	201 (0.35)*	114 (0.35)*
L270V						
Genotype						
C/C	160 (0.93)	88 (0.92)	128 (0.96)	64 (0.86)	288 (0.94)	152 (0.89)
C/G	12 (0.07)	8 (0.08)	6 (0.04)	10 (0.14)	18 (0.06)	18 (0.11)
G/G	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Allele						
C	332 (0.97)	184 (0.96)	262 (0.98)	138 (0.93)	594 (0.971)	322 (0.947)
G	12 (0.03)	8 (0.04)	6 (0.02)	10 (0.07)	18 (0.029)	18 (0.053)
I337V						
Genotype						
A/A	62 (0.36)	25 (0.26)	12 (0.39)	ND	74 (0.36)†	25 (0.26)†
A/G	85 (0.49)	64 (0.67)	17 (0.55)	ND	102 (0.50)†	64 (0.67)†
G/G	25 (0.15)	7 (0.07)	2 (0.06)	ND	27 (0.13)†	7 (0.07)†
Allele						
A	209 (0.61)	114 (0.59)	41 (0.66)	ND	250 (0.62)†	114 (0.59)†
G	135 (0.39)	78 (0.41)	21 (0.34)	ND	156 (0.38)†	78 (0.41)†

Data are number of subjects with each genotype or number of alleles (% of each group). *Combined NIDDM vs. control genotypes, $\chi^2 = 8.89$, $P = 0.012$, allelic frequencies, Fisher's exact $P = 0.94$; †combined NIDDM vs. control genotypes, $\chi^2 = 7.37$, $P = 0.025$, allelic frequencies, Fisher's exact $P = 0.65$. ND, not done.

TABLE 4
Allelic associations calculated for all pairs of variants to determine the extent of linkage disequilibrium between the three common missense variants at Kir6.2 and SUR1 and whether this value differed between the NIDDM and control groups

Percentage maximum disequilibrium	*P* values for allelic association					
	Exon 24	Exon 22	Exon 7	E23K	L270V	I337V*
NIDDM patients (U.K. and Utah combined)						
Exon 24	—	$<10^{-4}$	0.79	0.12	0.89	0.94
Exon 22	89.1	—	0.81	0.19	0.69	0.86
Exon 7	1.7	6.5	—	$<10^{-6}$	0.11	$<10^{-6}$
E23K	10.6	19.7	77.5	—	0.009	$<10^{-6}$
L270V	4.3	49.0	40.0	62.6	—	0.0005
I337V*	0.9	6.2	94.9	98.6	100.0	—
Control subjects (U.K. and Utah combined)						
Exon 24	—	0.57	0.46	0.25	0.49	0.046
Exon 22	31.8	—	0.66	0.92	0.95	0.30
Exon 7	9.2	14.0	—	$<10^{-6}$	0.0009	$<10^{-6}$
E23K	16.0	9.5	86.3	—	0.003	$<10^{-6}$
L270V	28.7	13.8	75.7	84.4	—	0.02
I337V*	42.8	100.0	84.9	97.4	100.0	—

The *P* values for allelic association are shown above the diagonal, and the percent of maximum disequilibrium below. *I337V only typed in the U.K. sample.

with Kir6.2 variants, this was observed for both the NIDDM and control groups. Thus, the previously noted association of SUR1 variants with NIDDM (1) was not due to linkage disequilibrium with variants at the adjacent Kir6.2 locus.

Further evidence of the lack of a significant role for the SUR1 exon 7 and Kir6.2 variants in the etiology of NIDDM was obtained when extended haplotypes were compared between NIDDM patients and control subjects. Table 5 reports the extended haplotype array for the Utah samples and the U.K. samples (which were typed for the additional Kir6.2 I337V variant). The likelihood ratio test for the Utah samples (χ^2 = 11.5, df = 5) is nominally significant at P = 0.04, due largely to the increased frequency of the 2,1,2 haplotype in diabetic patients. A modest Bonferroni correction to account for multiple tests renders this comparison nonsignificant. The likelihood ratio test for the U.K. samples (χ^2 = 7.7, df = 6) indicates no difference between NIDDM patients and control subjects.

DISCUSSION

In the current study, four missense variants were uncovered by systematic screening of Kir6.2 in 35 Caucasian probands (70 chromosomes) with NIDDM. All four of the variants detected occur in amino acids that are conserved between mouse and human Kir6.2. The location of the variants represent changes in the cytoplasmic NH_2-terminus, E10K and E23K, and COOH-terminus, L270V and I337V, regions of unknown function. The frequencies of the variants were assessed in two independent Northern-European Caucasian populations (Utah and U.K.). Analysis of the total sample for each of the four variants revealed no differences in allele frequencies between NIDDM patients and control subjects (Table 3), strongly suggesting that Kir6.2 variants in the coding region of the gene contribute little to the enhanced genetic risk for NIDDM in Caucasians. Other racial groups were not evaluated at this locus. Furthermore, regulatory regions were not assessed, and defects in expression of the gene (e.g., promoter or intronic mutations) would not be detected by these methods. Interestingly, decreased expression of the Kir6.2 gene was recently noted in the Zucker fatty rat, a model for NIDDM (26).

Our previous analysis of these same individuals from the Utah and U.K. groups revealed significant association of variants at the SUR1 locus with NIDDM (1). While Kir6.2 variants were not associated with NIDDM, the Kir6.2 gene resides within 5 kb of the SUR1 gene on chromosome 11p15.1 (13). In the SUR1 study, three variants were assessed, an exon 24 –3 c to t and an exon 22 c to t silent change (both associated with NIDDM) and an exon 7 missense variant. The relative order of the variants studied at SUR1/Kir6.2 are 5′-SUR1: exon 24-exon 22-exon 7-Kir6.2: E10K-E23K-L270V-I337V-3′. The results of the combined analysis of SUR1 and Kir6.2 indicate that the exon 22 and 24 variants are in linkage disequilibrium with a putative mutation in or near the SUR1 locus that contributes to diabetes. As these SUR1 variants are not in linkage disequilibrium with variants in the Kir6.2 gene and were presumably independently inherited, the Kir6.2 variants provide no information as to the site of the putative SUR1-related mutations.

How does this report, which finds no significant mutations in the coding region of Kir6.2, contribute to the results from previous linkage studies (17–19) and to our understanding of the genetic basis for NIDDM? The ability to detect linkage in a complex disorder such as NIDDM is predominantly determined by the risk contributed by a particular locus and the number of sib pairs tested (27). Given the number of families studied, the previous linkage studies would be unlikely to detect genes in a heterogeneous disease where more than five genes are contributing. Thus, limiting the search for diabetogenic genes to linkage studies in families could result in failure to detect genes that might encompass as much as 5–10% of the genetic risk. An alternative approach is the direct screening at the single nucleotide level for variants in the coding region of a candidate gene, followed by assessment of the frequency of the variant in patients and control subjects (28). Candidate genes have been shown to be involved in

TABLE 5
Maximum likelihood estimate of haplotype frequency

	Exon 7	E23K	L270V	I337V	NIDDM patients	Control subjects
Utah						
	1	1	1	—	0.5477	0.5934
	1	1	2	—	—	—
	1	2	1	—	0.0158	0.0273
	1	2	2	—	0.0053	—
	2	1	1	—	0.1128	0.0321
	2	1	2	—	—	0.0039
	2	2	1	—	0.3053	0.2955
	2	2	2	—	0.0131	0.0479
U.K.						
	1	1	1	1	0.5984	0.5762
	1	1	1	2	0.0350	0.0677
	1	1	2	1	—	—
	1	1	2	2	0.0029	—
	1	2	1	1	—	—
	1	2	1	2	0.0556	0.0799
	1	2	2	1	—	—
	1	2	2	2	0.0059	0.0053
	2	1	1	1	0.0062	0.0176
	2	1	1	2	—	—
	2	1	2	1	—	—
	2	1	2	2	0.0029	0.0052
	2	2	1	1	0.0030	—
	2	2	1	2	0.2670	0.2170
	2	2	2	1	—	—
	2	2	2	2	0.0232	0.0311

Exon 7: 1 = Ser^{1370}, 2 = Ala^{1370}; E23K: 1 = Glu^{23}, 2 = Lys^{23}; L270V: 1 = Lev^{270}, 2 = Val^{270}; 1 = Ile^{337}, 2 = Val^{337}.

other complex diseases by this type of analysis (29–31). The results of our Kir6.2 study in NIDDM patients will thus provide important direction to the search for genes that increase the risk for NIDDM. Having eliminated this important candidate gene in this racial group, future efforts might now be directed to the evaluation of Kir6.2 in other racial groups, to the assessment of regulatory regions of the gene for possible defects in expression, or to other loci.

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