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Mutations in the sulfonylurea receptor gene are associated with familial hyperinsulinism in Ashkenazi Jews

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Familial hyperinsulinism (HI) is a disorder of pancreatic β-cell function characterized by persistent hyperinsulinism despite severe hypoglycemia. To define the molecular genetic basis of HI in Ashkenazi Jews, 25 probands were screened for mutations in the sulfonylurea receptor (SUR1) gene by single-strand conformation polymorphism (SSCP) analysis of genomic DNA and subsequent nucleotide sequence analyses. Two common mutations were identified: (i) a novel in-frame deletion of three nucleotides (nt) in exon 34, resulting in deletion of the codon for F1388 (ΔF1388) and (ii) a previously described g→a transition at position –9 of the 3′ splice site of intron 32 (designated 3992–9g→a). Together, these mutations are associated with 88% of the HI chromosomes of the patients studied. $^{86}Rb^{+}$ efflux measurements of COSm6 cells co-expressing Kir6.2 and either wild-type or ΔF1388 SUR1 revealed that the F1388 mutation abolished ATP-sensitive potassium channel (K_{ATP}) activity in intact cells. Extended haplotype analyses indicated that the ΔF1388 mutation was associated with a single specific haplotype whereas the 3992–9g→a mutation was primarily associated with a single haplotype but also occurred in the context of several other different haplotypes. These data suggest that HI in Ashkenazi Jews is predominantly associated with mutations in the SUR1 gene and provide evidence for the existence of at least two founder HI chromosomes in this population.

INTRODUCTION

Familial hyperinsulinism (HI; OMIM: 256450) is an autosomal recessive disorder of pancreatic β-cell function and is characterized by inadequate suppression of insulin secretion in the presence of severe, recurrent, fasting hypoglycemia. Clinical manifestations of HI, which occurs predominantly in neonates and infants under 1 year of age, include seizures, coma and large birth weight for gestational age (1–3). In the absence of treatment, HI may be lethal or result in irreversible neurologic sequelae. Estimates for the incidence of this disorder vary from 1/40 000 live births in northern Europe (4) to 1/2675 live births in Saudi Arabia, a country in which 51% of all births are from consanguineous marriages (5).

The HI locus was initially assigned to the region flanked by the microsatellite markers D11S926 and D11S899 on chromosome 11p14–15.1 by genetic linkage analyses and homozygosity mapping (6,7). Subsequent linkage and haplotype studies using additional genetic markers refined the position of the HI locus to the D11S419–D11S1310 interval (8). Recently, the gene for the sulfonylurea receptor (SUR1), a modulator of insulin secretion, was localized to chromosome 11p15.1 by fluorescence *in situ* hybridization (FISH) and two separate point mutations were identified in probands with HI by nucleotide sequence analyses of SUR1 cDNA and/or genomic DNA (9). Both mutations, a g→a transition in a 5′ splice site which results in exon skipping and a g→a transition in intron 32, postulated to activate cryptic splice sites, are presumed to result in premature truncation of the SUR1 molecule (9).

Characterization of the full-length complementary DNA (cDNA) sequence and genomic structure of the human SUR1 homologue indicates that the SUR1 gene comprises 39 exons and spans >100kb of genomic DNA (G. Gonzalez, L. Aguilar-Bryan and J. Bryan, GSDB Accession #L78208; H. Inoue and M. A.

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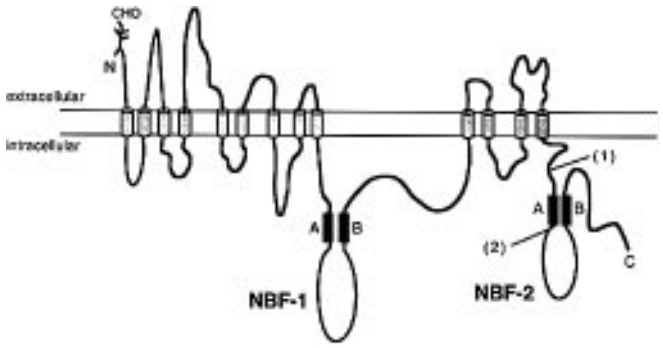

Figure 1. Schematic representation of the predicted membrane topology of the SUR1 protein. The molecule comprises two nucleotide-binding folds (designated NBF-1 and NBF-2), containing the characteristic Walker A and Walker B consensus sequence motifs (denoted by A and B, respectively), and two membrane spanning domains composed of multiple putative transmembrane segments (10–12). Locations of putative disease causing mutations identified in Ashkenazi Jewish probands are denoted as: (1) the position where premature truncation of SUR1 due to the intronic mutation, 3992–9g→a, is postulated to occur (9,10) and (2) ΔF1388. Adapted from Philipson, L. H. and Steiner, D. F. *Science* **268**, 372–373.

Permutt, unpublished data). The human SUR1 cDNA contains a single open reading frame that encodes a protein of 1581 amino acids and an alternative spliced form of 1582 amino acids (MW≈ 177 kDa) (G. Gonzalez, L. Aguilar-Bryan and J. Bryan, GSDB accession #L78208; H. Inoue and M. A. Permutt, unpublished data) (10). Homology analyses of the deduced amino acid sequence revealed that SUR1 is a member of the adenosine triphosphate (ATP)-binding cassette superfamily (11,12). A model for SUR1 predicts that the molecule contains two nucleotide binding domains (NBF-1 and NBF-2) and two membrane spanning domains, each composed of multiple putative transmembrane regions (Fig. 1) (10). Evidence for a functional role of SUR1 in the regulation of ATP-sensitive potassium channels (K_{ATP}) in pancreatic β-cells has recently been provided by the finding that co-expression of SUR1 and a small β-cell inward rectifier potassium channel subunit (Kir 6.2) in COSm6 or COS-1 cells reconstitutes an inwardly rectifying ATP-sensitive potassium current (I_{KATP}) (13). The SUR1 protein is presumed to confer ATP/ADP-sensitivity on I_{KATP} (13). Current models for glucose-regulated insulin secretion propose that closure of such K_{ATP} channels in pancreatic β-cells is required for insulin exocytosis (14). Elevations in blood glucose concentration lead to increased rates of glucose metabolism in β-cells and consequent alterations in the intracellular ratio of ATP/ADP, resulting in inhibition of K_{ATP} channels. The subsequent depolarization of the β-cell plasma membrane activates voltage-sensitive Ca^{2+} channels and the ensuing influx of Ca^{2+} initiates insulin secretion. Absence of K_{ATP} channel activity in HI has been demonstrated by patch-clamp studies on islet β-cells obtained from affected individuals (15,16), a finding consistent with the identification of mutations in the SUR1 gene associated with HI (9,17).

We previously demonstrated a founder effect for HI in Ashkenazi Jews by extended haplotype analyses of 21 kindreds (8). In this report, probands from these and an additional four families of Ashkenazi Jewish descent were screened for mutations in the SUR1 gene by single-strand conformation polymorphism (SSCP) analysis of genomic DNA. Two common mutations, deletion of F1388 (ΔF1388) and a g→a transition at position –9 of the 3′ splice site of intron 32, were identified. The effects of the ΔF1388 mutation upon K_{ATP} channel activity were examined by $^{86}RbCl$ efflux measurements in transfected cells expressing Kir6.2/ΔF1388 SUR1 K_{ATP} channels. The data indicate that the ΔF1388 mutation impairs K_{ATP} channel activity, consistent with the HI disease phenotype of probands possessing this mutation. Extended haplotype analyses of kindreds that possess identical mutations suggest that at least two founder mutations for HI may exist within the Ashkenazi Jewish population.

RESULTS

SSCP analyses

To define the molecular basis of HI in Ashkenazi Jews, DNA samples from 25 probands were screened for mutations within all 39 exons of the SUR1 gene and also, within flanking intron–exon boundaries by SSCP analyses. Exons are numbered consecutively from the 5′ end of the gene, with the two putative nucleotide-binding folds, NBF-1 and NBF-2, of the SUR1 protein encoded by exons 15–22 and 33–38, respectively (G. Gonzalez, L. Aguilar-Bryan and J. Bryan, GSDB accession #L78208) (10). Samples demonstrating electrophoretic mobility shifts were detected in some probands and/or control samples for exons 6, 12, 16, 18, 21, 27, 31, 33, 34, 35 and 39 (Figs 2a and 3a; data not shown). Mutations in the SUR1 gene in samples displaying such band shifts were identified by nucleotide sequence analyses of polymerase chain reaction (PCR) products amplified in independent reactions from genomic DNA samples. A total of 14 different mutations were identified, including 12 putative polymorphisms (see below).

Nucleotide sequence analyses of PCR products encompassing exon 34 from DNA samples displaying aberrant SSCP patterns revealed an in-frame deletion of three nucleotides (Fig. 2a and b). Although it is not possible to distinguish by nucleotide sequence analyses whether the nucleotides (nt) 4162–4164 (ttc) or 4163–4165 (tct) are deleted in the mutant allele (Fig. 2b), both alternatives result in deletion of the codon for phenylalanine at amino acid position 1388 (ΔF1388) in NBF-2 and conservation of the adjacent downstream serine amino acid residue (G. Gonzalez, L. Aguilar-Bryan and J. Bryan, GSDB accession #L78208) (10). Amino acid and nucleotide positions for the human SUR1 protein are numbered relative to the alternatively spliced form of the cDNA, encoding a protein of 1582 amino acids (G. Gonzalez, L. Aguilar-Bryan and J. Bryan, GSDB accession #L78208). Deletion of either nt 4162–4164 (ttc) or nt 4163–4165 (tct) creates a unique restriction enzyme recognition sequence for *Bse*RI in exon 34 of the mutant allele, allowing identification of non-carriers and individuals homozygous or heterozygous for the ΔF1388 mutation. A representative mutation analysis profile for one pedigree is shown in Figure 2c. Following digestion of the 274 bp PCR product with *Bse*RI, probands homozygous for the ΔF1388 allele possessed fragments of 144 and 130 bp whereas both heterozygous parents had an undigested 274 bp fragment and two restriction fragments of 144 and 130 bp. Allelic frequencies for the ΔF1388 mutation were determined for HI and normal chromosomes by PCR amplification of exon 34 from all 25 probands and from 89 unaffected unrelated Ashkenazi Jews, followed by digestion of the resultant PCR products with *Bse*RI. The ΔF1388 mutation was observed on nine of 48 (18.8%) HI alleles and was not detected in the 89 control DNA samples (data not shown). Eight probands were heterozygous and one proband, identical by descent for a region (D11S1901–D11S1310) encompassing the SUR1 locus on chromosome 11p15.1, was

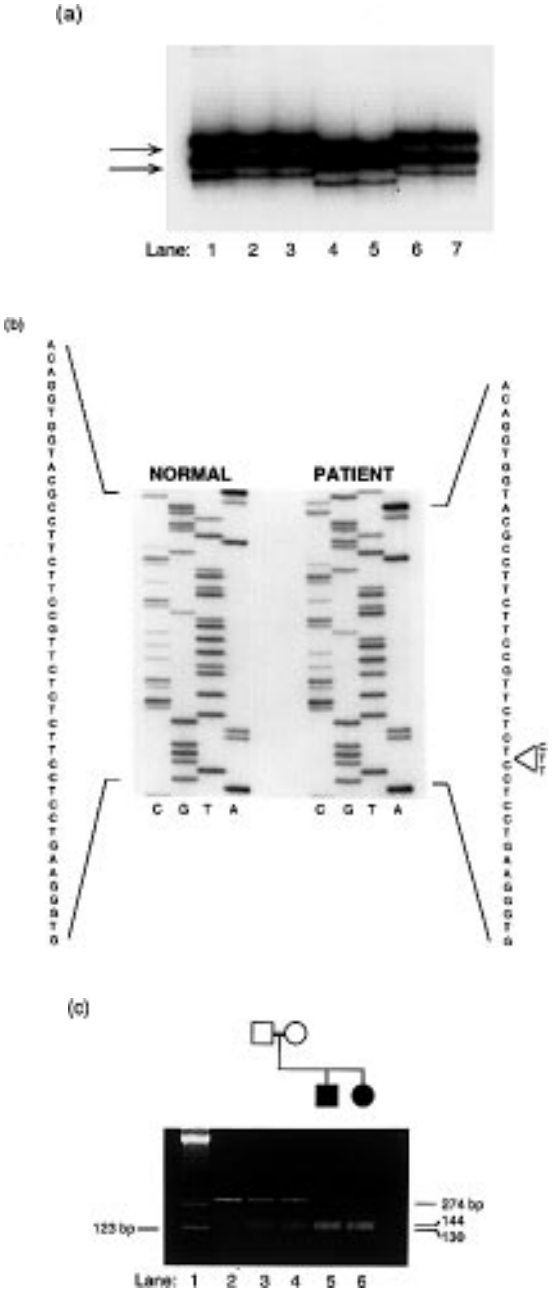

Figure 2. Identification of the exon 34 ΔF1388 mutation. (**a**) SSCP analyses of seven individuals of Ashkenazi Jewish descent. Lanes 1, 2, 3, 5 and 6: unrelated probands; lane 4: affected sib of the proband in lane 5; lane 7: unaffected control individual. Arrows indicate the position of shifted bands in individuals possessing the ΔF1388 mutation. (ii) Nucleotide sequence analyses of exon 34 amplified by PCR from genomic DNA of individuals indicated in lanes 5 (patient) and 7 (normal) in (a). The nucleotide sequence shown is of the positive-sense strand. Although deletions of either nt 4162–4164 (ttc) or 4163–4165 (tct) may have occurred in the mutant allele, only the position of the ttc deletion is indicated for purposes of clarity. (**c**) RFLP analyses of a kindred possessing the ΔF1388 mutation. PCR products encompassing exon 34 were amplified from genomic DNA of all family members and digested with *Bse*RI for 2 h. Restriction digestion products were analyzed on a 3% NuSieve (3:1) agarose gel and visualized by staining with ethidium bromide. Lane 1: 123 bp marker DNA ladder (Gibco); 2: undigested 274 bp exon 34 PCR product; 3 and 4: maternal and paternal PCR products following digestion with *Bse*RI, indicating the presence of the undigested wild-type (274 bp) fragment and mutant allele products (144 bp and 130 bp); 5 and 6: affected sibs homozygous for the ΔF1388 mutation.

homozygous for this mutation (see Fig. 2c). Restriction fragment length polymorphism (RFLP) analyses of all immediate family members of the nine probands possessing the ΔF1388 mutation revealed co-segregation of this mutation with the HI disease phenotype in each kindred (Fig. 2c; data not shown).

Three different band shifts were also detected by SSCP analysis of exon 33 in a subset of probands and/or control samples (Fig. 3a; data not shown). Nucleotide sequence analyses of genomic DNA, amplified by PCR from samples displaying mobility shifts, identified two putative polymorphisms (see below) and a previously reported g→a transition at position –9 of the 3′ splice site of intron 32 (designated 3992–9g→a, where nt 3992 is the first nucleotide of exon 33) (Fig. 3b) (9). This mutation has been postulated to activate three cryptic RNA splice sites, resulting in frameshifts and a predicted truncation of the SUR1 molecule (9). A recognition sequence for the restriction endonuclease *Nci*I is abolished by the 3992–9g→a mutation so that mutant alleles retain an intact fragment of 258 bp upon digestion with this enzyme whereas wild-type alleles are digested into 168 and 90 bp fragments (Fig. 3c). Allelic frequencies for the 3992–9g→a mutation, determined by RFLP analyses of 25 probands and 89 normal Ashkenazi individuals, indicated that the 3992–9g→a mutation was the predominant mutation within SUR1 in the probands studied, occurring on 33 of 48 (68.8%) HI chromosomes compared with one of 178 (0.6%) chromosomes from control individuals (data not shown). Of the 23 probands who possessed this mutation, 11 were homozygotes and 12 were heterozygotes. Co-segregation of the 3992–9g→a mutation with the HI disease phenotype was observed for 21 of 23 families by RFLP analyses of individual family members (data not shown). In one family, the proband homozygous for this mutation had two unaffected sibs who were haploidentical for the D11S861–D11S1310 interval encompassing the SUR1 locus (data not shown). Although both sibs were large for gestational age, neither possessed any of the other clinical manifestations of HI (B. Glaser and H. Landau, pers. comm.). In a second family, assuming a recessive mode of inheritance for HI, the proband who was presumed to be a compound heterozygote for the 3992–9g→a mutation and an unidentified mutation also had an unaffected sib who was haploidentical for all microsatellite markers within the D11S861–D11S1310 interval (data not shown). Although the proband fulfilled the diagnostic criteria for HI, the possibility that defect(s) in insulin secretion in this patient are attributable to either (i) mutation(s) at another locus or (ii) a mutation at the SUR1 locus and a second mutation at a different locus cannot be excluded at present.

Together, the 3992–9g→a and ΔF1388 mutations are associated with ≈88% HI alleles in the patient cohort. Mutations were identified on both alleles for 19 of 25 (76.0%) probands, with 11 probands (44.0%) homozygous for the 3992–9g→a mutation, one (4.0%) homozygous for the ΔF1388 mutation, seven (28.0%) were compound heterozygotes possessing both the 3992–9g→a and ΔF1388 mutations and the remaining six (24.0%) probands were presumed to be compound heterozygotes for the 3992–9g→a or ΔF1388 mutations and an unidentified mutation.

$^{86}Rb^{+}$ efflux assays

The functional consequences of the ΔF1388 mutation upon K_{ATP} channel activity were examined by measurement of $^{86}Rb^{+}$ efflux in intact COSm6 cells co-transfected with the murine homologue of the K_{ATP} channel subunit Kir6.2 (13) and either wild-type hamster

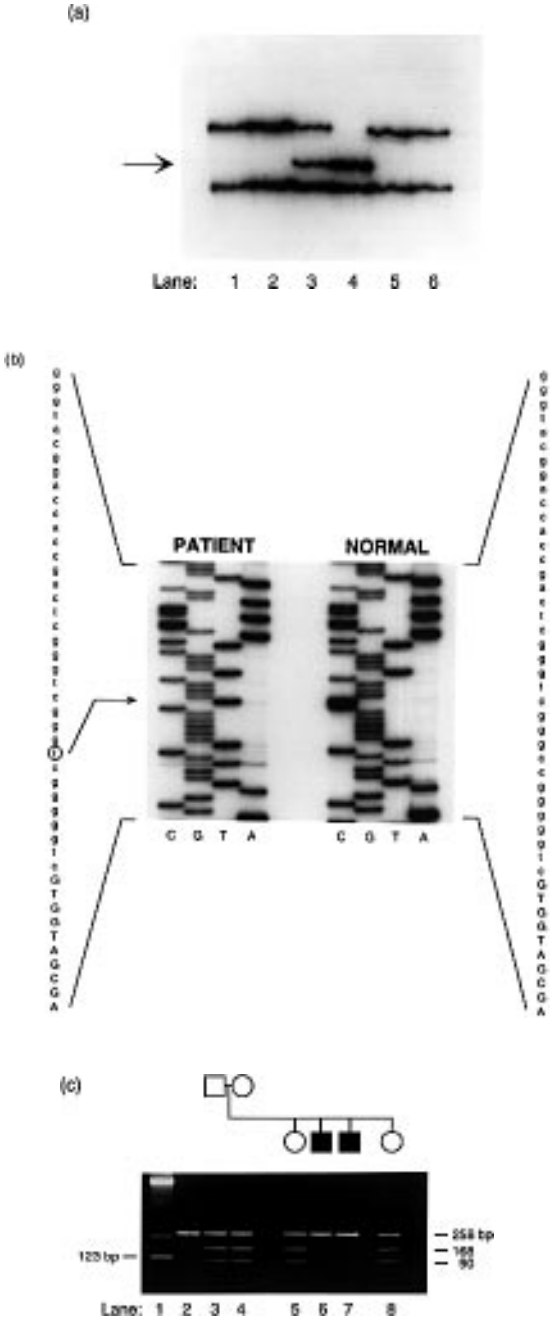

Figure 3. Identification of the 3992–9g→a mutation. (**a**) SSCP analyses of six individuals of Ashkenazi Jewish descent. Lanes 1, 2, 4 and 5: unrelated probands; lane 3: heterozygous parent of the proband (lane 4); lane 6: normal unrelated control. Band shifts in DNA samples possessing the 3992–9g→a mutation are indicated by an arrow. (**b**) Nucleotide sequence analyses of PCR products spanning exon 33 amplified from genomic DNA of individuals indicated in lanes 4 and 6 in (a). The nucleotide sequence shown is of the reverse complement. Exonic and intronic sequences are shown in upper and lower case, respectively. (**c**) RFLP analyses of a kindred possessing the 3992–9g→a mutation. PCR products encompassing exon 33 from all available family members were amplified from genomic DNA and digested with *Nci*I. Restriction digestion products were analyzed on a 3% NuSieve (3:1) agarose gel and visualized by staining with ethidium bromide. Lanes 1: 123 bp marker DNA ladder (Gibco); 2: undigested 258 bp exon 33 PCR product; 3 and 4: PCR products amplified from paternal and maternal DNA follwing digestion with *Nci*I, indicating the presence of the mutant (258 bp) fragment and wild-type allele digested into 168 bp and 90 bp fragments; 5 and 8: unaffected carrier siblings; 6 and 7: affected siblings homozygous for the 3992–9g→a mutation.

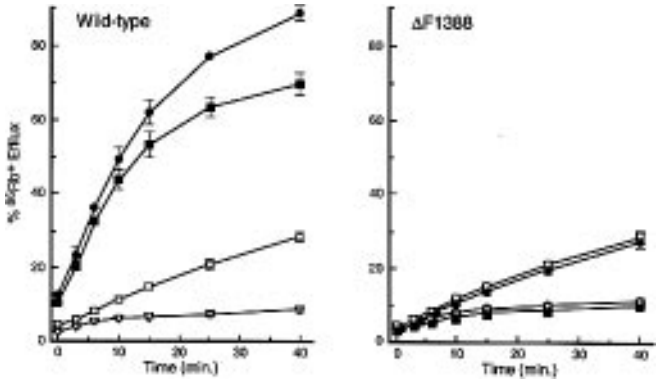

Figure 4. Comparison of $^{86}Rb^+$ efflux through wild-type SUR1 and ΔF1388SUR1 K_{ATP} channels. COSm6 cells were co-transfected with pCMV-Kir6.2 and either pECE–SUR1 (left panel) or pECE–ΔF1388SUR1 (right panel) and assayed for $^{86}Rb^+$ efflux following incubation with either metabolic inhibitors (2-deoxy-D-glucose and oligomycin), which alter the intracellular ATP/ADP ratio, or diazoxide, which activates K_{ATP} channel activity. Open squares indicate averaged basal efflux values from transfected and untransfected cells; solid squares denote efflux in the presence of 2-deoxy-D-glucose (1 mM) and oligomycin (2.5 μg/ml); solid circles represent efflux in the presence of diazoxide (300 μM); open triangles (left panel) indicate efflux in the presence of metabolic inhibitors and glibenclamide (1 μM) and open diamonds (right panel) represent control COSm6 cells incubated in the presence of 2-deoxy-D-glucose and oligomycin. Error bars indicate± standard deviation.

SUR1 or SUR1 containing the ΔF1388 mutation (designated ΔF1388SUR1). $^{86}Rb^+$ efflux was assayed in transfected cells following incubation with (i) metabolic inhibitors (2-deoxy-D-glucose and oligomycin) which reduce intracellular ATP levels and increase ADP levels, resulting in opening of K_{ATP} channels, or (ii) diazoxide, an activator of K_{ATP} channels in islet β-cells or (iii) glibenclamide, an inhibitor of K_{ATP} channel activity. In cells expressing wild-type Kir6.2/SUR1 K_{ATP} channels, $^{86}Rb^+$ efflux was stimulated above basal levels by the presence of either diazoxide or metabolic inhibitors (Fig. 4). The K_{ATP} channel activity elicited by metabolic inhibition in cells expressing wild-type Kir6.2/SUR1 K_{ATP} channels was sensitive to glibenclamide. Untransfected COSm6 cells or cells transfected with a control plasmid expressing β-galactosidase displayed no stimulation of basal $^{86}Rb^+$ efflux levels by diazoxide and inhibition of efflux by metabolic inhibitors (Fig. 4; data not shown). In contrast to cells expressing wild-type K_{ATP} channels, cells expressing Kir6.2/ΔF1388SUR1 channels displayed the same responses as untransfected control cells; no stimulation by diazoxide and reduced efflux in the presence of metabolic inhibitors. Photolabeling of the 140 kDa SUR1 receptor in transfected cells, using ^{125}I-glibenclamide, indicated that similar levels of SUR1 were expressed in cells co-transfected with Kir6.2/ΔF1388SUR1 or wild-type Kir6.2/SUR1 K_{ATP} channels (data not shown). Together, these data suggest that ΔF1388SUR1 is expressed in transfected cells but is unable to form a functional K_{ATP} channel with Kir6.2.

Haplotype analyses

To identify possible founder haplotypes associated with the ΔF1388 or 3992–9g→a mutations, extended haplotypes were derived for HI and normal chromosomes in parents of the 25 Ashkenazi probands by typing all available individuals within each family for four microsatellite markers (D11S1397, D11S902, D11S921, D11S1890), which are linked to the HI locus (8,18,19). The relative

Table 1. Association between haplotypes on HI chromosomes and SUR1 mutations

HAPLOTYPE		D11S1397	D11S902	D11S921	D11S1890	No.(%) HI CHROMOSOMES n=47			No. (%)NORMAL CHROMOSOMES n=47
GROUP	SUBGROUP					3992-9g→a	ΔF1388	Mutation unknown	
A		3	10	3	3	-	-	1(2.1%)	-
B		1	4	4	3	-	-	1(2.1%)	3(6.4%)
C		3	7	5	3	-	-	1(2.1%)	1(2.1%)
D		1	8	3	3	-	-	1(2.1%)	1(2.1%)
E		3	8	4	2	-	-	1(2.1%)	1(2.1%)
F	I	3	4	3	2	17(36.2%)	-	-	-
	II	2	4	3	2	2(4.3%)	-	-	-
	III	3	5	3	2	1(2.1%)	-	-	1(2.1%)
	IV	3	3	3	2	4(8.5%)	-	-	-
	V	3	4	3	3	1(2.1%)	-	-	2(4.3%)
G		3	3	3	4	1(2.1%)	-	-	-
H		2	4	1	3	5(10.6%)	-	-	1(2.1%)
I		1	4	3	2	1(2.1%)	-	-	1(2.1%)
J		3	3	3	1	1(2.1%)	-	-	1(2.1%)
K	I	3	4	5	2	-	8(17.0%)	-	-
	II	2	4	5	2	-	1(2.1%)	-	-
	III	3	4	5	3	-	-	-	3(6.4%)
	IV	2	4	5	2	-	-	-	1(2.1%)
	V	3	4	4	2	-	-	-	1(2.1%)
-	+24 OTHERS					-	-	-	30(63.8%)

order of these markers with respect to the SUR1 locus has been previously established as 11pter– D11S1397–(D11S902– SUR1–D11S921–D11S1890)–11pcen by genetic linkage (18,19), radiation hybrid (20) and physical (21) mapping. For 21 of these families, the D11S902–D11S921 haplotypes have been described previously (8). Complete haplotypes were constructed for 47 of 48 HI chromosomes and for 47 of 50 normal chromosomes. Subgroups were determined by combining haplotypes that differ by ≤2 bp at a single locus on the assumption that such differences are more likely to represent CA repeat slippage than different mutation events (22). No recombination was observed between the HI locus and any of the four microsatellite marker loci (data not shown). Table 1 shows the haplotypes present on Ashkenazi HI chromosomes and their corresponding frequencies on normal chromosomes. Mutations within SUR1 identified on HI chromosomes are also indicated for each haplotype. A total of 35 different haplotypes were detected, with 11 present on HI chromosomes (Table 1). A single haplotype, F, was predominant on HI chromosomes, accounting for 25 of 47 (53.2%) disease chromosomes and three of 47 (6.4%) normal chromosomes. The three most frequent F subgroups, F.I, F.II and F.IV, which together account for 48.9% HI chromosomes, were not detected on non-HI chromosomes. Two other frequent haplotypes, H and K, were detected on 10.6% and 19.1% of HI chromosomes, respectively, with corresponding frequencies on normal chromosomes of 2.1% and 10.6%. Together, haplotypes F, H and K account for 83.0% of the HI chromosomes and 19.1% normal chromosomes of the parents studied. All other haplotypes were rare, occurring on one of 47 (2.1%) HI chromosomes. Of the 34 HI chromosomes bearing the 3992–9g→a mutation, the majority were represented by the F (73.5%) haplotype and less frequently, by haplotypes H (14.7%), G (2.9%), I (2.9%) and J (2.9%). In contrast, the ΔF1388 mutation was associated exclusively with haplotype K (sub-haplotypes K.I, K.II). Neither of these mutations were detected on normal chromosomes bearing the F, H, I, J or K haplotypes. These data are consistent with the presence of at least two founder chromosomes for HI in Ashkenazi Jews.

Polymorphisms within the SUR1 gene

In addition to the SSCP variants described above for exons 33 and 34, band shifts were also detected for some probands and/or control samples on SSCP for exons 6, 12, 16, 18, 21, 27, 31, 33, 34, 35 and 39 (data not shown). Nucleotide sequence analyses revealed a total of 12 different point mutations within the SUR1 gene which were classified as polymorphisms on the basis that they: (i) do not modify the amino acid sequence of the protein; or (ii) result in conservative amino acid substitutions in apparently non-highly conserved residues of the protein; or (iii) are present on normal chromosomes from the control Ashkenazi population (Table 2). To determine whether these polymorphisms created potential novel 5′ or 3′ splice sites, Shapiro/Senapathy consensus values (23) were calculated for positions –3 to +6 for possible 5′ splice site sequences (containing GT) and for positions –14 to +1 for potential 3′ splice site sequences (containing AG). Consensus values were calculated for all subsequences encompassing the

Table 2. Polymorphisms within the SUR1 gene

REGION	NUCLEOTIDE SUBSTITUTION[a]	CODON	AMINO ACID SUBSTITUTION	DOMAIN	REASON FOR INCLUSION
exon 6	nt 945 c→t	315	None	Tm[b]	silent
exon 12	nt 1686 t→c	562	None	-	silent
intron 15	nt 2117-3 c→t	-	-	-	normal chromosomes[c]
exon 18	nt 2280 c→t	760	None	NBF-1	silent
exon 21	nt 2488 c→t	830	None	NBF-1	silent
intron 26	nt 3332 +6 c→t	-	-	-	normal chromosomes
exon 31	nt 3822 g→a	1274	None	Tm	silent
exon 33	nt 4082 t→g	1361	Val→Gly	NBF-2	normal chromosomes
exon 33	nt 4108 t→g	1370	Ser→Ala	NBF-2	normal chromosomes
intron 33	nt 4123 -25c→t	-	-	-	intronic
intron 35	nt 4310 +62g→a	-	-	-	intronic
exon 39	nt 4717 g→a	1573	Val→Ile	-	conservative

[a]Nucleotide and codon positions are according to the full-length human SUR1 cDNA sequence incorporating the alternative spliced form of exon 17 (GenBank accession no L78208; L78224).
[b]Tm designates putative transmembrane domain.
[c]Mutation was detected on HI and/or normal chromosomes.

putative polymorphism and varied from a minimum value of 0.214 (nt 4717g→a) to a maximum of 0.731 (nt 1686t→c) for possible 5′ splice sites, and from 0.302 (nt 4123–25c→t) to 0.829 (nt 3822g→a) for 3′ splice site consensus values. However, in each case the consensus values for the mutated sites were lower than values calculated for the normal site (data not shown), suggesting that novel 5′ or 3′ splice sites are unlikely to be created by the polymorphisms identified here. Nonetheless, the possibility that some of the identified polymorphisms either augment or ameliorate the deleterious effects of familial hyperinsulinism mutations upon K_{ATP} channel activity and insulin secretion cannot be excluded at present.

DISCUSSION

The sulfonylurea receptor (SUR1) is involved in nucleotide regulation of K_{ATP} channel activity and is, therefore, a central component in the mechanism of glucose-regulated insulin secretion in pancreatic islet β-cells (13,14). In this study, 25 HI patients of Ashkenazi Jewish descent were screened for mutations in the SUR1 gene and specific haplotypes associated with identified mutations were defined. Two common mutations associated with HI in this population were identified, a novel deletion of F1388 in NBF-2 and a previously described point mutation at position –9 of the 3′ splice site of intron 32 (9). The 3992–9g→a mutation, which is the most prevalent mutation in the SUR1 gene in Ashkenazis with HI, has also been previously detected in one proband of Saudi Arabian origin (9). In contrast, the ΔF1388 mutation, which accounted for 19% HI alleles, was not observed in RFLP analyses of 50 non-Ashkenazi probands of various ethnic origins and may, therefore, be associated predominantly with HI in Ashkenazi Jews (A. Nestorowicz, unpublished data). To date, only four mutations in the SUR1 gene have been reported. In addition to the 3992–9g→a and ΔF1388 mutations described here, we previously decribed a missense mutation (G1479R) in exon 37 in a proband of Sephardic Jewish origin (17), and a g→a transition at position –1 of the 5′ splice site of intron 35 was reported for seven probands of Saudi Arabian origin and one proband of German descent (9). However, neither of these additional mutations were detected, either by RFLP or SSCP analyses of exons 35 and 37, in the 25 Ashkenazi Jewish families studied here (data not shown). The allelic homogeneity described here for HI in Ashkenazi Jews presumably reflects the relative isolation of this population (24) and has also been observed for several other autosomal recessive disorders in Ashkenazis, including Canavan disease (25,26), Gaucher disease (27), Fanconi anemia (28) and glycogenesis VII (29).

At present, it is unclear whether the 3992–9g→a mutation has a causal role in the disease process or is a polymorphism highly associated with a disease causing mutation located elsewhere, either within the SUR1 gene or a nearby locus. The effect(s) of the 3992–9g→a mutation upon RNA splicing have been examined by transfection of a human glioblastoma cell line (SNB19) with mini-gene constructs containing the exon 33 and flanking exons of SUR1 inserted downstream from a RSV promoter (9). Comparison of cDNA products from wild-type and mutant constructs revealed that activation of three separate 3′ cryptic splice sites occurred in constructs containing the 3992–9g→a mutation, resulting in a 7 bp addition or deletions of 20 or 30 bp. Consequent frameshifts associated with these additions/deletions are predicted to result in deletion of the entire NBF-2 domain (Fig. 1) (9). Although specific role(s) of NBF-2 remain to be elucidated, site-directed mutagenesis studies of NBF-2 in which G1479 was replaced with R have shown that MgADP stimulation of K_{ATP} channel activity occurs through binding in this domain (17). Deletion of NBF-2 in 3992–9g→a alleles may, therefore, not only prevent binding of ATP to NBF-2 but may also abolish responsiveness of SUR1 to MgADP. Unresponsiveness to MgADP is predicted to result in a net reduction of channel activity at any ADP/ATP ratio, a hypothesis consistent with the loss of regulation of glucose-induced insulin secretion observed in the HI disease phenotype (17). Although cryptic spice site activation was demonstrated in transfected glioblastoma (SNB19) cells (9) it is unknown whether such aberrant RNA splicing of 3992–9g→a mutant alleles occurs in islet β-cells *in vivo* in the HI patients studied here. Formal proof of cryptic splice site utilization will require isolation and nucleotide sequence analyses of SUR1 cDNA from affected probands possessing the 3992–9g→a allele.

Intrafamilial variation in disease phenotype was observed for one family in which the proband, who was homozygous for the 3992–9g→a mutation, had two unaffected haploidentical sibs (data not shown). However, both unaffected haploidentical sibs were large for gestational age (H. Landau and B. Glaser, unpublished data) and it is possible that these children exhibit(ed) subtle defects in regulation of glucose-induced insulin secretion. Such variation in phenotypic expression has also been described for a number of other autosomal recessive disorders including Gaucher disease (27). The mechanism(s) underlying this variable phenotypic expression of HI is unknown. Clinical heterogeneity may be due to the effects of modifier genes (genetic background), metabolic interferences and/or exogenous factors that modulate the expression of the HI phenotype. Alternately, if cryptic splice

site utilization does occur in 3992–9g→a mutant alleles in islet β-cells *in vivo*, it is feasible that some individuals may express variable proportions of wild-type transcript sufficient to maintain phenotypically normal regulation of insulin secretion.

A second common mutation identified in the Ashkenazi probands was deletion of the codon for F1388. $^{86}Rb^{+}$ efflux measurements on intact cells expressing either Kir6.2/wild-type SUR1 or Kir6.2/ΔF1388SUR1 K_{ATP} channels revealed that channel activity was impaired by the ΔF1388 mutation (Fig. 4). Both metabolic inhibition and addition of diazoxide increased efflux in COSm6 cells expressing wild-type K_{ATP} channels whereas cells expressing Kir6.2/ΔF1388SUR1 channels were unresponsive to diazoxide and, similar to untransfected COSm6 control cells, showed reduced efflux in the presence of metabolic inhibitors. These data were obtained for intact cells and thus, are predicted to be representative of the activity of the mutant ΔF1388 K_{ATP} channel in pancreatic β-cells. However, we have not yet resolved whether the ΔF1388 K_{ATP} channel is completely inactive or is unable to respond to changes in MgADP, as demonstrated for other mutations in NBF-2 (17). Amino acid alignments of SUR1 NBF-2 with other members of the ABC-superfamily indicates that residue 1388 is usually non-polar in nature but that the primary structure is not strictly conserved, with some members of this protein family containing L (cystic fibrosis transmembrane conductance regulator; CFTR), T (P-glycoprotein multidrug resistance protein family; MDR) or V(MDR) at the homologous position (30). Amino acid residue 1388 is located two residues downstream from the Walker A consensus sequence in NBF-2 and resides in a flexible cytoplasmic loop predicted to protrude from the core-nucleotide binding fold (Fig. 1) (10–12,30). A conserved sequence (linker) motif located within this loop has been proposed for other members of the ABC protein family to couple, presumably via a conformational change(s), the energy of ATP hydrolysis to transport or activity of the protein (30). Three-dimensional modeling of a number of ABC proteins has shown that the core nucleotide-binding fold composed of Walker A and B motifs has a strict requirement for a particular conformation rather than primary structure to maintain binding and hydrolysis of ATP (11,31). It is possible that deletion of F1388 disrupts the requisite tertiary structure of the nucleotide-binding pocket, perhaps preventing binding of ATP or ADP and/or alters the conformation and function(s) associated with the conserved cytoplasmic linker motif in SUR1. Patch-clamp experiments on cells co-transfected with Kir6.2 and ΔF1388SUR1 cDNA constructs may provide further information regarding the effect(s) of the ΔF1388 mutation upon the properties of the K_{ATP} channel. Further studies are also needed to determine whether the ΔF1388 mutation disrupts post-translational processing of the SUR1 protein, as has been described for deletions of certain amino acids within the cytoplasmic loop of NBF-1 in CFTR (32,33).

Extended haplotype analyses revealed associations between the ΔF1388 and 3992–9g→a mutations in SUR1 and specific haplotypes (Table 1). Although only a small number of probands with the ΔF1388 mutation were identified, the data suggest that this mutation may be associated exclusively with the group K haplotype, a finding consistent with a single origin of this mutation. In contrast to the ΔF1388 mutation, the 3992–9g→a mutation was observed in the context of five (F, G, H, I, J) different (i.e. haplotypes that differ by >2 bp at one or more markers) haplotypes, with the majority of alleles carrying this mutation represented by haplotypes F and H. It is possible that these haplotypes represent independent origins of the 3992–9g→a mutation and/or that this mutation is ancient, so that multiple recombination events with normal haplotypes may have resulted in variant haplotypes. As 12 polymorphisms were detected within the SUR1 gene, construction of haplotypes using these intragenic RFLP markers as well as intragenic microsatellite markers, may provide further insight into the origins of these chromosomes in Ashkenazi Jews. An additional five distinct haplotypes (A–E) were observed on single HI chromosomes for which no mutations were defined within the 39 exons of the SUR1 gene. These haplotypes may represent separate mutational events or rearrangements of more common haplotypes by an ancestral recombination event. Assuming that 2 bp variation are more likely to represent CA repeat slippage than different mutation events (22), we estimate that there may be in the order of five additional mutations in this Ashkenazi patient cohort that remain to be identified. These mutations may be present within the exon sequences or flanking intron–exon boundaries screened here but were undetected by SSCP analyses or may be located either within intronic sequences and/or the 5′ or 3′ untranslated regions of the SUR1 gene which have not yet been analyzed.

In summary, we have established that HI in Ashkenazi Jews is associated with two frequent mutations within the SUR1 gene. Identification of additional mutations in HI and characterization of their effects upon the structure and properties of the islet β-cell K_{ATP} channel may provide further insight into the structure–function relationship of the SUR1 protein, the mechanism of insulin exocytosis in islet β-cells and the molecular and cellular mechanisms underlying familial hyperinsulinism.

MATERIALS AND METHODS

Families

A total of 25 Ashkenazi Jewish families were ascertained from Israel and the United States. Twenty-one of these families have been described previously (8) and an additional four families were recruited from Israel for this study. Consanguinity was present in three families on the panel in which the parents within each family were related to a common distant ancestor (8). Clinical diagnosis of HI was based on the following criteria (2,34,35): hypoglycemia with increased glucose utilization in infants that are large for gestational age and the combination of inappropriately elevated serum insulin levels, low plasma ketones and brisk response to glucagon injection. For this study, probands were classified as Ashkenazi Jewish when both parents were descended from eastern European ancestors (Germany, Czechoslovakia, Poland, Romania, Hungary, Ukraine, Russia or the Baltic States). Genomic DNA from probands and their families was isolated from Epstein–Barr virus (EBV)-transformed lymphoblasts or from peripheral blood lymphocytes using standard procedures. Genomic DNA from 89 unrelated, normal Ashkenazi Jewish individuals was kindly supplied by Dr D. Abliovitz (Department of Genetics, Hadassah Hospital, Jerusalem).

SSCP analysis

For SSCP analysis, individual exons and adjacent intron–exon boundaries were amplified by the polymerase chain reaction (PCR). Oligonucleotide sequences for SSCP were complementary

Table 3. Oligonucleotide sequences, annealing temperatures and PCR product sizes for SSCP analysis of SUR1

REGION	PRIMER SEQUENCE		ANNEALING TEMP.(°C)	FRAGMENT SIZE (bp)
	FORWARD	REVERSE		
Exon 1	5'-GCGGAGCCAGCGGAGCCAG-3'	5'-CCCCTCCTCCGCGGCTCG-3'	65	232
Exon 2	5'-CATGCACCCTCTTCCTCCTC-3'	5'-CCTGGAGCAGATTCACTTTC-3'	62	248
Exon 3	5'-CCCCATCCTGTCTGTTTCTC-3'	5'-GGACTGCCCCTCCGTCCTA-3'	65	186
Exon 4	5'-GTACACACATGATGCACACACG-3'	5'-AGGACAGAGCCAGAGCCTCT-3'	62	275
Exon 5	5'-GAGAAGTTGACTTACCCATCC-3'	5'-AGTGAATAGATGGTGTGCTGT-3'	65	318
Exon 6	5'-GTGAATTAGCCCTCAGGCAC-3'	5'-CCATCTAGAGGGTGCCTTAC-3'	60	279
Exon 7	5'-TCATTTTGCTGGTCAATGAC-3'	5'-GGTTCTTATGGCAAAGTGAA-3'	58	253
Exon 8	5'-TGGTGATGATGATGAGAATG-3'	5'-TTTGGTTGTGAGTCCTGCTG-3'	58	263
Exon 9	5'-AACAGGGATTTTGCCAGCAC-3'	5'-GGGGAAGAGGGACAAAACAC-3'	65	252
Exon 10	5'-AGAAGCCTCTCCCCTCTGAC-3'	5'-CTCTCCTTGCATGTACGCAG-3'	65	252
Exon 11	5'-ACGCTCCTCCCACTAAAC-3'	5'-AAATCTGGGCAGCCTGTC-3'	65	159
Exon 12	5'-CACCCTGGCCTCACTTGTGC-3'	5'-CATCACTCGAGCAAGCCTTG-3'	60	253
Exon 13	5'-AGGGGTGTCTCTGTGCTTCC-3'	5'-TGCTGGGAGTAGCAAGGGGA-3'	60	207
Exon 14	5'-TCCTCACCTCCAAACCACCC-3'	5'-GAGTAGGTGCTCAATAAATGC-3'	60	184
Exon 15	5'-GGGAACTGGCTGCCTTTCTG-3'	5'-GAGTAGGTGCTCAATAAATGC-3'	58	228
Exon 16	5'-CCCGGCCCCACTCACATCTG-3'	5'-GGAGGATGGTTAAAAGGAGATT-3'	60	202
Exon 17	5'-GGGGTGGGAACTAACTGGTG-3'	5-CTTCTGACCCCAGTCCCAAG-3'	58	110
Exon 18	5'-AGAGCTCAGTATGCCTTTCC-3'	5'-GGTGATGTGGCTCCCTTGG-3'	58	145
Exon 19	5'-GAGAACAAGCCCTGAGAATGC-3'	5'-AGTTCCTCCCCTCCACATCC-3'	60	200
Exon 20	5'-TGCTCGGGCCAACTCTGACC-3'	5'-TTGGGGGTCCTGGCTTTGAA-3'	60	190
Exon 21	5'-GGCATGATGTGTTCCTCCTCT-3'	5'-GGGAGGCAGCCAGAGACCAG-3'	58	156
Exon 22	5-CCTACCCTCTACTCTCTTCC-3'	5'-CTGCCCCCTATAGCCTGACC-3'	60	211
Exon 23	5'-ATTTGAGTGAGGAAAGGGATGT-3'	5'-CTTGGCCATCCCTGGATAT-3'	60	256
Exon 24	5-CCATATATCCTAAGACATTGCC-3'	5'-AGGCCATAATTTCACTCCCA-3'	60	170
Exon 25	5'-TGATTCACCCTCAGAGGGAT-3'	5'-ATTTGGAGTTCCAGGGTGC-3'	60	366
Exon 26	5'-CTGGAACTCCAAATCACTCTC-3'	5'-GAGAACAGTGGACTTGCTCAC-3'	62	250
Exon 27	5'-TGAATGACTCCAGAGACACTTA-3'	5'-GCCCCCAGGGGTCCGAGG-3'	60	239
Exon 28	5'-AAAATTCACCTCACTCTCTCC-3'	5'-CTAGCAATGTACACCAAACTGC-3'	55	258
Exon 29	5'-ATTCACCCACACAGCGGTA-3'	5'-GAGAGAGCAACGTGTCCTTG-3'	65	305
Exon 30	5'-CTATGTGGACCAACACTGTTC-3'	5'-ATCCTATCCTCTCTTTCATCC-3'	50	245
Exon 31	5'-GTAGAACAGGGTCCTGTGGC-3'	5'-TGTCTCCAGTGACGAAGGTG-3'	65	249
Exon 32	5'-GTGGAACAGGGTCCTGTGGC-3'	5'-CTTGGACTCTTCCCCACC-3'	62	210
Exon 33	5'-AGTCCAAGGAGGAGTGTGTC-3'	5'-AGCATTGGGTTGGGCCCGT-3'	65	258
Exon 34	5'-CCCTGTGACCTCCCACACCT-3'	5'-ACTGCTGGGCCTCCTGTCAT-3'	65	274
Exon 35	5'-GGTCAGACATGACAGGAGG-3'	5'-GATCTGATGGAACTGAGCC-3'	62	235
Exon 36	5'-ACCCTGCTCCCTCCTACTG-3'	5'GTCCTTGAGTGCCCAACC-3'	60	192
Exon 37	5'-ATCCCATCTGCTCCACTCAC-3'	5'-ATCCCACTAAACCCTTTCCAG-3'	65	254
Exon 38	5'-CCAGGCCAGACCCCATCCAC-3'	5'-CCAGGCCAGACCCCATCCAC-3'	60	153
Exon 39	5'-GGGTGGTATTCCCACCATC-3'	5'-GTATGGGCAGGGTCCGAAT-3'	55	230

to flanking intronic sequences (G. Gonzalez, L. Aguilar-Bryan and J. Bryan, GSDB accession nos L78208– L78222, L78224–L78243, L78254, L78255) and were designed to generate PCR products of 110–366 bp. Primer sequences and annealing temperatures for amplification of each exon are listed in Table 3. Exons are numbered in consecutive order from the 5′ end of the gene. For

exon 25, the 366 bp PCR product was digested with the restriction endonuclease *Rsa*I prior to electrophoresis through non-denaturing gels. Genomic DNA (50 ng) was amplified by PCR in a 10 µl reaction containing: 10 pmol each primer, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.0 U *Taq* DNA polymerase (Gibco) and 1 µCi [α-^{32}P]-dCTP. For amplification of exon 1, the reaction mix included 5% formamide. Amplification conditions were an initial denaturation at 94°C (4 min); followed by 30 cycles of 94°C (1 min), optimal annealing temperature (1 min) and 72°C (1 min). Amplified samples were diluted two-fold with formamide buffer (95% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol), denatured at 94°C for 5 min and chilled on ice prior to electrophoresis. PCR products were analyzed on gels containing a 1/4 dilution of GeneAmp gel matrix (Perkin-Elmer) and 0.5×Tris–borate–EDTA (TBE), with or without 10% glycerol. Electrophoresis was done at 10–30 W for 5–12 h, at 4°C or room temperature (RT) and gels were exposed to Kodak X-OMAT film for autoradiography.

Identification of mutations

Nucleotide sequence analyses to identify mutations were done on patient samples exhibiting shifted bands relative to control samples on SSCP. Genomic DNA was amplified by PCR with primers used for SSCP analysis (Table 3). The resultant PCR products were excised from 2% low melting temperature (LMP) agarose gels, purified and directly sequenced by double-stranded DNA cycle sequencing (Perkin-Elmer) according to the manufacturer's instructions. Both strands of the PCR product were sequenced using PCR amplification primers end-labeled with ^{32}P.

For mutations that created or abolished restriction endonuclease sites, the presence of the mutation was verified by restriction endonuclease digestion of PCR products with appropriate enzymes. To confirm the presence of the 3992–9g→a and ΔF1388 mutations, genomic DNA was amplified using SSCP primers (Table 3) and the resultant PCR products were digested for 2 h with *Nci*I or *Bse*RI, respectively. Restriction digestion products were analyzed by electrophoresis on 3% NuSieve (3:1) (FMC BioProducts) agarose gels and were visualized by staining with ethidium bromide.

Haplotype analysis

All available individuals within each family were genotyped with the four di- and tetranucleotide repeat polymorphic markers D11S1397, D11S902, D11S921 and D11S1890. D11S1397 is distal to the SUR1 locus but the relative order of D11S902–SUR1–D11S921–D11S1890 is unknown (18–21). Haplotypes for 21 of these families at the D11S902 and D11S921 loci have been described previously (8). Oligonucleotide sequences and PCR amplification conditions for all microsatellite markers were obtained from Genome Data Base. Haplotypes were derived for parental chromosomes by inferring phase from their genotypes and those of their children: in 22 families there were at least two children, providing unequivocal haplotypes and in three families only one child was available for haplotype analyses. All haplotypes were constructed to minimize recombinants. For consanguineous marriages where the parental HI chromosomes were identical by descent (IBD), only one parental disease chromosome was included in the analyses. Control allele distributions were drawn from the non-carrier chromosomes within the same families.

Plasmid constructions

Construction of the plasmids (i) pCMV-Kir6.2, expressing full-length murine Kir6.2, generously provided by Dr Susumu Seino (Chiba University, Japan) and (ii) pECE-SUR1 expressing full-length hamster SUR1 cDNA, have been described in detail elsewhere (10,13). F1388 was deleted from wild-type hamster SUR1 using an overlap PCR strategy. Briefly, complementary mutant oligonucleotides missing the phenylalanine codon (F) codon and two flanking primers were synthesized. The outside primers flanked unique restriction sites in the pECE-SUR1 plasmid. Two PCR products with a 20 bp overlap spanning the desired deletion were amplified using (i) the forward mutant primer (5′-AGTCCTCCTCCCTGGCCTTTTTCCG-3′) with the downstream outside reverse primer (5′-CGTAGAGGATCCAGACATGA-3′) and (ii) the forward outside primer (5′-GTCATCTCCTATGTCACACC-3′) and the reverse mutant primer (5′-AAAGGCCAGGGAGGAGGACTTCCCG-3′), respectively. Amplification was done with *Taq* DNA polymerase (Perkin-Elmer) for 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The two resultant PCR products were purified by agarose gel electrophoresis, mixed and re-amplified using only the forward and reverse outside primers. The PCR product was digested with the restriction enzymes *Eco*RI and *Not*I, purified by agarose gel electrophoresis and subcloned using standard methodology into pECE–SUR1 (10) following digestion with *Eco*RI and *Not*I. The region amplified by PCR was sequenced to ensure no additional mutations were introduced during amplification. The resulting plasmid is designated pECE–ΔF1388SUR1.

Cell culture, $^{86}Rb^+$ efflux and ^{125}I-glibenclamide photolabeling

COSm6 cells were maintained in Dulbecco's modified Eagle's medium with high glucose (DMEM-HG) and supplemented with 10% fetal calf serum (FCS). Cells were co-transfected with pCMV-Kir6.2 (5 µg) and either pECE–SUR1 (5 µg) or pECE–ΔF1388SUR1 (5 µg) using DEAE-dextran as described previously (13). Co-transfected cells were incubated for 12–24 h with ^{86}RbCl and assayed for efflux in the presence or absence of diazoxide, metabolic inhibitors (oligomycin and 2-deoxy-D-glucose) and glibenclamide as described in detail elsewhere (10,13). Co-transfected cells were photolabeled with ^{125}I-glibenclamide as described previously (10,13).

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