

# Genetic Analysis of Japanese Patients With Persistent Hyperinsulinemic Hypoglycemia of Infancy

## Nucleotide-Binding Fold-2 Mutation Impairs Cooperative Binding of Adenine Nucleotides to Sulfonylurea Receptor 1

Yukio Tanizawa, Kazuyuki Matsuda, Michinori Matsuo, Yasuharu Ohta, Nobuhiko Ochi, Masanori Adachi, Mayumi Koga, Seiji Mizuno, Mitsuharu Kajita, Yukichi Tanaka, Katsuhiko Tachibana, Hiroshi Inoue, Susumu Furukawa, Teruo Amachi, Kazumitsu Ueda, and Yoshitomo Oka

To elucidate the genetic etiology of persistent hyperinsulinemic hypoglycemia of infancy (PHHI) in the Japanese population, we conducted a polymerase chain reaction–single-strand conformation polymorphism analysis of the sulfonylurea receptor 1 (SUR1) and Kir6.2 genes in 17 Japanese PHHI patients, including a pair of siblings from a consanguineous family. We also analyzed the glutamate dehydrogenase gene for the exons encoding an allosteric regulatory domain of the enzyme. In the SUR1 gene, we identified one frameshift (I446fsdelT) and two missense (R1420C, R1436Q) mutations. None of these mutations were found in control Japanese subjects. Siblings homozygous for the R1420C mutation had a mild form, whereas two patients heterozygous for the I446fsdelT and R1436Q mutations, respectively, exhibited a severe form of PHHI. Functional consequences of these mutations on  $K_{ATP}$  function were evaluated using  $^{86}Rb^+$  efflux studies in COS-7 cells. SUR1-446fsdelT and SUR1-1436Q did not form a functional  $K_{ATP}$ . Western blot analysis after transient expression in COS-7 cells revealed the expression of SUR1-1436Q protein to be markedly reduced, suggesting SUR1-1436Q to be unstable in these cells.  $K_{ATP}$ (SUR1-1420C) showed reduced responses to metabolic inhibition by oligomycin and 2-deoxyglucose.  $K_{ATP}$  channels are under complex regulation by intracellular ATP and ADP. ATP

both inhibits and activates these channels. The inhibition is probably mediated through direct ATP interaction with a pore-forming subunit Kir6.2, whereas the activation is likely to be through a regulatory subunit SUR1. There is a cooperative regulation of ATP and ADP binding to SUR1, and this cooperativity may be involved in regulating the  $K_{ATP}$  channel. In SUR1-1420C, high-affinity binding of ATP to the nucleotide-binding fold (NBF)-1 was indistinguishable from that of wild-type SUR1. However, stabilization of ATP binding to NBF-1 by MgATP or MgADP was impaired, suggesting that this defect may account for impaired  $K_{ATP}$ (SUR1-1420C) function. This is the first direct biochemical evidence that the cooperativity of nucleotide binding to SUR1 is impaired in a SUR1 mutant causing PHHI. No mutations were identified in the Kir6.2 and glutamate dehydrogenase genes. The genetic etiology of PHHI appears to be heterogeneous. SUR1 mutations may account for no more than 20% of PHHI cases in Japanese patients. Mutations of Kir6.2 and glutamate dehydrogenase genes are likely to be even less common. Diabetes 49:114–120, 2000

From the Third Department of Internal Medicine (Y.Tani., K.M., Y.Oh., H.I., Y.Ok.) and the Department of Pediatrics (M.Ko., S.F.), Yamaguchi University School of Medicine, Yamaguchi; the Laboratory of Biochemistry (M.M., T.A., K.U.), Division of Applied Life Sciences, Kyoto University Graduate School of Agriculture, Kyoto; Aichi Prefectural Hospital and Habilitation Center for Disabled Children (N.O.), Dai-ni Aoitori Gakuen; the Department of Endocrinology and Metabolism (M.A., K.T.) and the Division of Pathology (Y.Tana.), Kanagawa Children's Medical Center, Kanagawa; the Department of Pediatrics (S.M.), Tokai Municipal Hospital, Tokai; and the Department of Pediatrics (M.Ka.), Nagoya University School of Medicine, Nagoya, Japan.

Address correspondence and reprint requests to Yukio Tanizawa, MD, PhD, Third Department of Internal Medicine, Yamaguchi University School of Medicine, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan. E-mail: tanizawa@po.cc.yamaguchi-u.ac.jp.

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NBF, nucleotide-binding fold; PCR, polymerase chain reaction; PHHI, persistent hyperinsulinemic hypoglycemia of infancy; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism; SUR1, sulfonylurea receptor 1.

**P**ersistent hyperinsulinemic hypoglycemia of infancy (PHHI) (Online Mendelian Inheritance in Man [OMIM]: 256450) is characterized by inappropriately elevated insulin secretion in the presence of hypoglycemia. Mutations have been identified in the genes encoding subunits of the ATP-sensitive potassium channel ( $K_{ATP}$ ), sulfonylurea receptor 1 (SUR1), and Kir6.2 genes in recessive and sporadic forms of PHHI (1–8). Mutations were also identified in the glucokinase gene (9) and in the glutamate dehydrogenase gene in patients with hyperinsulinemic hypoglycemia and hyperammonemia (10). However, the genetic etiology of PHHI in Japanese patients is unknown.

The  $K_{ATP}$  channel, a heterooctamer composed of two essential subunits, SUR1 and Kir6.2 (BIR) (11), plays a central role in glucose-induced insulin secretion in pancreatic  $\beta$ -cells (12–15). Kir6.2, a pore-forming subunit and a member of the weak inward rectifier family (11), has been suggested

to be a primary site of ATP inhibition of  $K_{ATP}$  (16). SUR1 (17), a member of the ATP-binding cassette superfamily containing two highly conserved nucleotide-binding folds (NBF-1 and NBF-2), allows MgADP to exert a stimulatory effect on  $K_{ATP}$  (2). However, the regulation of  $K_{ATP}$  by adenine nucleotides is complex. Recently, we demonstrated cooperative regulation of adenine nucleotides binding to SUR1, which may be involved in the regulation of  $K_{ATP}$  activity (18,19).

To date, many mutations have been identified in the SUR1 gene in PPHI patients. Some are nonsense or frameshift mutations, and they apparently result in the expression of a truncated SUR1 protein. Others are missense mutations in which a single amino acid has been substituted. Many of these missense mutations were identified in two NBFs of SUR1, especially in NBF-2 (5,20). Because these domains are the primary sites of nucleotide interactions with SUR1, we speculate that these mutations impair  $K_{ATP}$  activity, at least in part, by disrupting nucleotide-SUR1 interactions. However, details of the molecular events involved are not clear. Studying the natural mutants identified in PPHI patients is one approach to elucidating the structure-function relationship and molecular mechanisms underlying regulation of SUR1 by adenine nucleotides, and thus  $K_{ATP}$  function.

Herein, we studied the genetic etiology of PPHI in Japanese patients. We identified three SUR1 mutations. Functional studies of these SUR1 mutations revealed that one of the missense mutations disrupts cooperative binding of adenine nucleotides to SUR1, thereby impairing  $K_{ATP}$  function.

## RESEARCH DESIGN AND METHODS

**Subjects.** We recruited 17 Japanese patients with PPHI from 16 families. Of the families, 15 had only one affected child, and no consanguinity was documented. Two patients were siblings from a consanguineous family. The diagnosis of PPHI was made based on the criteria of Antunes et al. (21). Genomic DNA was extracted from peripheral blood (22).

**Single-strand conformation polymorphism analysis.** In 16 PPHI patients, including a pair of siblings from the consanguineous family, all 39 exons of the SUR1 gene, a coding exon of the Kir6.2 gene, and exons encoding an allosteric regulatory domain of the glutamate dehydrogenase gene (exons 11 and 12) were analyzed. For one patient, only exons of the SUR1 gene encoding NBF-1 (exons 17–24) and NBF-2 (exons 33–38) were analyzed because DNA samples were limited. Exons of the human SUR1 gene and Kir6.2 gene were amplified from peripheral blood leukocyte DNA by polymerase chain reaction (PCR), as described previously (23,24). The exons of the glutamate dehydrogenase gene were also amplified, as described by Stanley et al. (10), from peripheral blood leukocyte DNA. Single-strand conformation polymorphism (SSCP) analysis was performed using a CleanGel DNA analysis kit and Multiphor II electrophoresis system (Amersham Pharmacia Biotech, Tokyo). We mixed 10  $\mu$ l of the PCR products with 30  $\mu$ l of 95% formamide, 20 mmol/l EDTA, 0.05% bromophenol blue, and xylene cyanol. Immediately before electrophoresis, samples were heat-denatured at 95°C for 5 min. Aliquots of the samples (7  $\mu$ l) were electrophoresed at 3 W for 10 min and then at 10 W for 30 min at 15°C. After electrophoresis, DNA bands were visualized by silver staining using a PlusOne DNA silver staining kit (Amersham Pharmacia Biotech).

Nucleotide changes corresponding to each SSCP were determined by direct sequencing of the PCR products using a model 373A automated sequencer and dye terminator sequencing kit (Applied Biosystems, Norwalk, CT). Amino acid numbers for human SUR1 were assigned based on the cDNA sequences deposited to the public database (accession numbers L78207 and U63421). In those sequences, a serine, which was found in an alternatively spliced form (25) between Ser<sup>742</sup> and Leu<sup>743</sup> according to the current numbering, is excluded. Restriction fragment length polymorphism analysis of the SUR1 gene mutations. The SUR1 gene mutations were screened in Japanese control subjects by PCR–restriction fragment length polymorphism (RFLP) analysis. The I446fsdelT and R1420C mutations created an NlaIII restriction site (CATG). The R1436Q mutation created a BsrI restriction site (CCAGTG). After PCR amplification of exon 9 (I446fsdelT) or exon 35 (R1420C and R1436Q), the products were digested with the appropriate restriction enzymes and analyzed by electrophoresis on 2% agarose gel.

**In vitro mutagenesis and functional analysis of mutant  $K_{ATP}$ .** Expression plasmids for mouse SUR1 (pcDNA-mSUR1a) and mouse Kir6.2 (pcDNA-mBIR) were described previously (23). Site-directed mutagenesis was performed using mutagenesis primers (for I446fsdelT: 5'-acg ctc cgt tca tgc ctc tcc atc atc c-3'; for R1420C: 5'-gcc agt aca gat cat gtg ggc gtg atc ctc c-3'; and for R1436Q: 5'-ttc agc ggc acc atc caa ttc aac ctg gac cc-3') and a GeneEditor in vitro Site-Directed Mutagenesis System (Promega, Madison, WI). Transfection of expression plasmids and Rb-efflux study was performed as described previously (23). Adenine nucleotide binding to SUR1 was studied essentially as described previously (18,19). Membrane proteins from COS-7 cells expressing mouse SUR1 were prepared (18), and aliquots, which contained the same amount of SUR1 protein as determined by Western blot analysis, were incubated with 10  $\mu$ mol/l 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$  and 2 mmol/l ouabain in MgTE buffer (40 mmol/l Tris-Cl [pH 7.5], 3 mmol/l MgSO<sub>4</sub>, 0.1 mmol/l EGTA) for 3 min at 37°C. The reaction was stopped by adding ice-cold MgTE buffer, unbound 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$  was removed by centrifugation at 15,000g for 5 min at 2°C, and the pellets were washed again with ice-cold MgTE buffer. Photoaffinity labeling was performed on ice, using UV irradiation (254 nm, 5.5 mW/cm<sup>2</sup>), immediately after resuspending the pellets in MgTE or after further incubating in MgTE with or without 0.5 mmol/l ATP or ADP for 15 min at 37°C. After the photoaffinity labeling, membrane proteins were separated by 7% SDS-PAGE, and labeled SUR1 was quantitated using a BAS2000 PhosphorImager (Fuji Film, Tokyo). Western blot analysis was performed using anti-rat SUR1 antibody (provided by Dr. S. Seino, Chiba University, Chiba, Japan).

Statistical analysis. The statistical significance of differences in quantitative variables between groups was analyzed by unpaired (two-tailed) Student's *t* tests. *P* values <0.05 were considered significant.

## RESULTS

Mutations of SUR1, Kir6.2, and glutamate dehydrogenase genes in Japanese PPHI patients. The sensitivity of the SSCP analysis used in this study was assessed by testing the detection of known polymorphisms identified by the conventional method using large sequencing gels and electrophoresis under two different conditions (23). With the CleanGel DNA analysis kit and the Multiphor II electrophoresis system, all known polymorphisms were detectable, confirming this system to be as sensitive as the conventional method (data not shown).

SSCP analysis of the human SUR1 gene revealed 15 sequence variants in Japanese PPHI patients (Table 1). We identified three potential disease-causing mutations (I446fsdelT, R1420C, and R1436Q). A nucleotide change (G  $\rightarrow$  A) in intron 8 identified in this study appeared to be a polymorphism because it was not present in the splicing consensus sequences. All other nucleotide changes were also polymorphisms because they have been observed previously in other studies (5,23). Besides these polymorphisms, there were several other sequence discrepancies between the human SUR1 gene sequences among the public databases. All of the Japanese PPHI patients analyzed herein were homozygous for one of the two sequences deposited in these public databases (Table 2).

One of the patients (patient 1) was heterozygous for I446fsdelT, a novel mutation. This mutation causes a frameshift such that a termination codon appears at codon 461. Thus, the expression of a truncated SUR1 protein is predicted from this mutated allele. Analysis of the proband's parents by PCR-RFLP revealed her unaffected father also to be heterozygous for this mutation. Two other mutations (R1420C and R1436Q) were missense mutations, both in NBF-2, a functionally important domain of SUR1. Although they are outside of the Walker's consensus sequences, known nucleotide-interacting sequences highly conserved among the ABC transporter family (26), the arginine residues at 1420 and 1436 are both conserved among human, hamster, rat, and mouse SUR1 and also in rat SUR2, suggesting the func-

**TABLE 1**  
Mutations and polymorphisms of the SUR1 and Kir6.2 genes in Japanese PHHI patients

Position	Common	Variant	Allelic frequency		
			PHHI	Type 2 diabetes	Control
<b>SUR1 gene</b>					
<b>Mutations</b>					
Exon 9	ATT (Ile <sup>446</sup> )	Deletion of T*	1/34	—	ND
Exon 35	CGC (Arg <sup>1420</sup> )	TGC (Cys <sup>1420</sup> )	4/34	—	0/134
Exon 35	CGA (Arg <sup>1436</sup> )	CAA (Gln <sup>1436</sup> )	1/34	—	0/204
<b>Polymorphisms</b>					
Exon 2	CCT (Pro <sup>69</sup> )	CCC (Pro <sup>69</sup> )	24/32	71/200	ND
Intron 8	G†	A	3/32	—	ND
Exon 12	CAT (His <sup>562</sup> )	CAC (His <sup>562</sup> )	28/32	137/200	95/134
Exon 14	AAG (Lys <sup>649</sup> )	AAA (Lys <sup>649</sup> )	2/32	27/200	ND
Intron 15	C‡	T	22/32	109/200	67/134
Intron 18	C§	T	4/32	—	ND
Intron 18	T	C	4/32	—	ND
Exon 21	CTG (Leu <sup>830</sup> )	TTG (Leu <sup>830</sup> )	1/34	11/200	ND
Exon 31	AGG (Arg <sup>1274</sup> )	AGA (Arg <sup>1274</sup> )	2/32	20/200	ND
Exon 33	TCC (Ser <sup>1370</sup> )	GCC (Ala <sup>1370</sup> )	16/34	74/200	51/134
Intron 33	T¶	C	3/34	24/200	ND
Intron 33	C#	T	2/34	3/200	ND
<b>Kir6.2 gene</b>					
	GAG (Glu <sup>23</sup> )	AAG (Lys <sup>23</sup> )	6/32	ND	ND
	GCT (Ala <sup>190</sup> )	GCC (Ala <sup>190</sup> )	23/32	ND	ND
	ATC (Ile <sup>284</sup> )	ATA (Ile <sup>284</sup> )	2/32	ND	ND
	ATC (Ile <sup>337</sup> )	GTC (Val <sup>337</sup> )	14/32	ND	ND

Amino acid numbers for human SUR1 were assigned based on the cDNA sequences deposited to the public database (accession numbers L78207 and U63421). In those sequences, a serine, which was found in an alternatively spliced form (25) between Ser<sup>742</sup> and Leu<sup>743</sup> according to the current numbering, is excluded. ND, not determined. Type 2 diabetes data come from Ohta et al. (23). Control data come from this study and Ohta et al. (23). \*Frameshift and premature termination at codon 446, †28 bp downstream from exon 8 (exon 8-CAG gtactagatgggctgaggggaagggagg/a), ‡3 bp upstream from exon 16 (c/tag GCC-exon 16), §36 bp upstream from exon 19 (c/tttccghcaccctactccgccccgcttttccag GAA-exon 19), ¶34 bp upstream from exon 19 (t/cccgghcaccctactccgcccgcttttccag GAA-exon 19), ¶25 bp upstream from exon 34 (t/cggccggtgccttctcttccag ATC-exon 34), #16 bp upstream from exon 34 (c/tcttctcttccag ATC-exon 34).

tional importance of these residues. In addition, neither of these two mutations existed in control Japanese subjects when examined by SSCP or PCR-RFLP analyses. Two patients (patients 2 and 3), siblings from a consanguineous family, were homozygous for the R1420C mutation. This mutation was recently reported by Verkarre et al. (20) in a PHHI patient with focal adenomatous hyperplasia of pancreatic islets. R1436Q is a novel missense mutation. One patient (patient 4) was heterozygous for this mutation. These

lines of evidence suggest that the two missense mutations identified here are likely to impair SUR1 function and are thus causally related to the development of PHHI.

In the Kir6.2 gene, no significant mutation but two missense variants and two silent changes were identified (Table 1). Both missense variants and one of the silent changes were the same as those previously identified in Caucasian subjects with type 2 diabetes and in control subjects (24). In the exons encoding the allosteric regulatory domain of the glutamate

**TABLE 2**  
Sequence discrepancy among public databases

Position	Sequence in databases and accession numbers				Japanese PHHI patients	
	L78207	U63421	U63429	L78214	Sequence observed	Frequency
Exon 1	GTC (Val <sup>30</sup> )	GCG (Ala <sup>30</sup> )	—	—	GCG (Ala <sup>30</sup> )	32/32
Exon 4	GCG (Ala <sup>163</sup> )	GGC (Gly <sup>163</sup> )	—	—	GCG (Ala <sup>163</sup> )	32/32
Exon 4	GTA (Val <sup>167</sup> )	CTA (Leu <sup>167</sup> )	—	—	GTA (Val <sup>167</sup> )	32/32
Exon 9	ACG (Thr <sup>487</sup> )	AGC (Ser <sup>487</sup> )	—	—	ACG (Thr <sup>487</sup> )	32/32
Intron 8	—	—	Exon 8- CAGgtactagatggg	Exon 8- CAG gtggg	Exon 8- CAGgtactagatggg	32/32

TABLE 3  
Profiles of patients with SUR1 gene mutations

Patient	Mutation	Onset (day)	Birth weight (g)	Treatment/outcome
1	I446fsdelT	0	5,014	Partial pancreatectomy
2	R1420C	0	5,254	Remission
3	R1420C	0	5,080	Remission
4	R1436Q	0	3,410	Partial pancreatectomy

dehydrogenase gene (exons 11 and 12), no sequence variants were identified.

Clinical characteristics of PHHI patients with SUR1 gene mutations. Clinical characteristics of PHHI patients with SUR1 gene mutations are summarized in Table 3. Two patients (patients 1 and 4) were born at full term and developed severe hypoglycemia several hours after birth. Despite medical treatment, including diazoxide administration, hypo-

glycemia persisted and required partial pancreatectomy at 4 months 21 days and 3 months 10 days, respectively. A sibling pair (patients 2 and 3), who were homozygous for the R1420C mutation, had a milder form of PHHI. They were also born at full term and developed hypoglycemia several hours after birth. Although both required vigorous intravenous glucose infusion and/or glucagon administration to maintain a normal plasma glucose level, the glucagon requirement was reduced after 3 months of age, and eventually hypoglycemia occurred only occasionally after prolonged fasting. Three patients were large for their gestational ages. The mother of patient 1 had gestational diabetes, which was managed with diet therapy. Her unaffected brother was 4,000 g at birth, whereas patient 1 weighed 5,014 g (Table 3). Functional studies of mutant  $K_{ATP}$  channel. Functional consequences of SUR1 gene mutations identified in this study were examined by reconstituting the  $K_{ATP}$  channel in COS-7 cells. I<sup>446</sup>, R<sup>1420</sup>, and R<sup>1436</sup> were all found to be conserved between human and mouse SUR1. Therefore, we introduced I446fsdelT, R1420C, and R1436Q mutations by site-directed mutagenesis to the corresponding positions of mouse SUR1 cDNA, and mutant SUR1 and mouse Kir6.2 were transiently coexpressed in COS-7 cells to reconstitute the mutant  $K_{ATP}$  channel. When intracellular ATP was depleted by metabolic inhibition with 2-deoxyglucose (1 mmol/l) and oligomycin (2.5  $\mu$ g/ml), significant  $Rb^+$  efflux was observed from the cells expressing wild-type  $K_{ATP}$  (Fig. 1A). However,  $^{86}Rb^+$  efflux from the cells expressing  $K_{ATP}$  (SUR1-446fsdelT) and  $K_{ATP}$  (SUR1-1436Q) was indistinguishable from that from control COS-7 cells expressing Kir6.2 and bacterial  $\beta$ -galactosidase (lacZ) (Fig. 1). These results suggest that SUR1-446fsdelT and SUR1-1436Q are unable to form functional  $K_{ATP}$  channels detectable under this experimental condition. Because the R1436Q mutation is a missense mutation, the protein expression level of SUR1-1436Q was assessed by Western blot analysis. Expression of SUR1-1436Q protein was reduced to less than one-tenth that of wild-type SUR1 (Fig. 2).  $^{86}Rb^+$  efflux from COS-7 cells expressing  $K_{ATP}$  with SUR1-1420C was reduced by 50% when examined under the metabolic inhibition and compared with that from wild-type  $K_{ATP}$  (Fig. 1). The efflux was completely inhibited by glibenclamide (1  $\mu$ mol/l). Activation of the channel by diazoxide also appeared to be impaired in the  $K_{ATP}$ (SUR1-1420C) mutant,

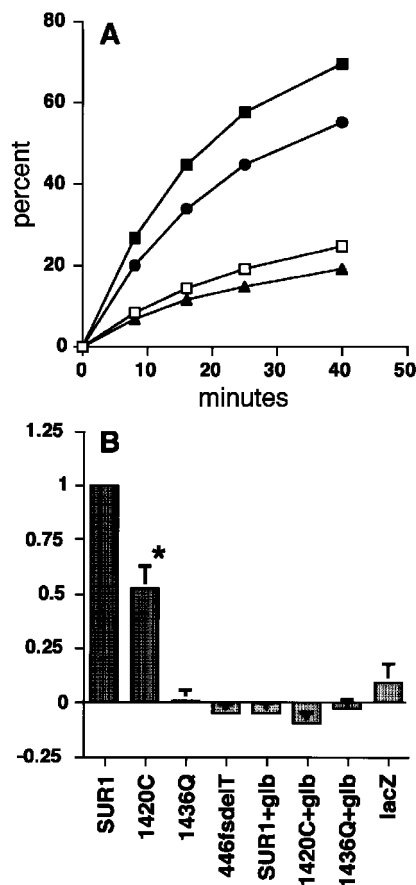


FIG. 1.  $Rb^+$  efflux studies. A: Time courses of  $Rb^+$  efflux from COS-7 cells expressing wild-type or mutant  $K_{ATP}$  in the presence of oligomycin (2.5  $\mu$ g/ml) and 2-deoxyglucose (1 mmol/l). Means of measurements made in triplicate for the representative experiment are plotted. ■, SUR1 (wild-type); ●, SUR1-R1420C; ▲, SUR1-446delT; □, LacZ. B:  $Rb^+$  efflux during a 40-min incubation in the presence or absence of glibenclamide (1  $\mu$ mol/l) under metabolic inhibition. Net  $Rb^+$  efflux through  $K_{ATP}$  (values obtained from the cells expressing  $K_{ATP}$  minus values obtained from control cells expressing bacterial  $\beta$ -galactosidase [lacZ] and Kir6.2) is presented as a ratio relative to the efflux from COS-7 cells expressing wild-type  $K_{ATP}$ . Bars represent the means of two to five experiments. Error bars indicate SE. \* $P = 0.0154$ .

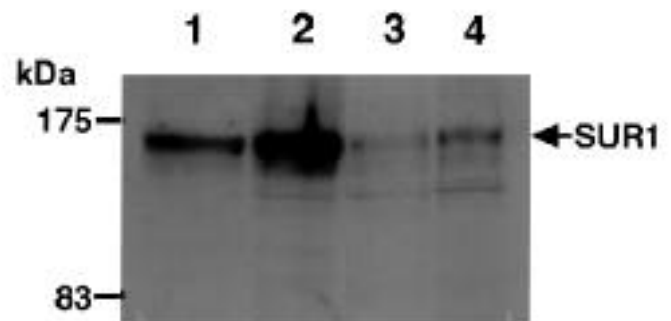


FIG. 2. Western blot analysis. Expressions of wild-type SUR1 and SUR1-1436Q in the crude membrane fractions of COS-7 cells were determined by Western blot analysis. Crude membrane fractions from the cells expressing wild-type SUR1 (lanes 1 and 2; 10 or 20  $\mu$ g protein, respectively) and those from the cells expressing SUR1-1436Q (lanes 3 and 4; 20 or 40  $\mu$ g protein, respectively) were electrophoresed, blotted, and probed with anti-SUR1 antibody.

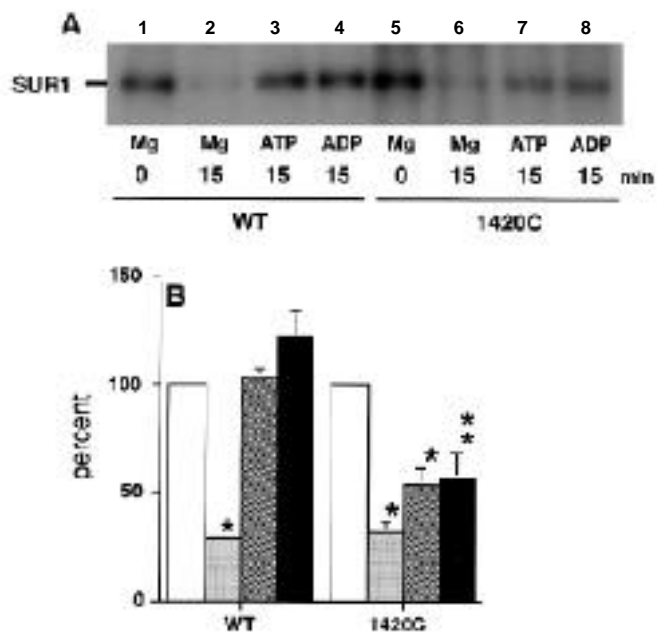


FIG. 3. Cooperative binding of MgADP and 8-azido-[ $\alpha$ - $^{32}$ P]ATP. Membrane proteins from COS-7 cells expressing wild-type SUR1 (WT) or SUR1-1420C (1420C) were preincubated with 10  $\mu$ mol/l 8-azido-[ $\alpha$ - $^{32}$ P]ATP. A: After removal of free 8-azido-[ $\alpha$ - $^{32}$ P]ATP, the proteins were photoaffinity-labeled immediately (0) or after a 15-min incubation (15) in MgTE (Mg) with or without ATP or ADP. Samples were electrophoresed and autoradiographed. B: Photoaffinity labeling is expressed as a percentage of the control immediate photoaffinity-labeling value performed in a parallel experiment. Experiments were performed in triplicate. □, control; ■, Mg; ▨, ATP; ■, ADP. \* $P < 0.01$ , \*\* $P < 0.0248$ .

although the difference was not statistically significant (data not shown).

Effect of R1420C mutation on cooperative binding of adenine nucleotides in SUR1. There is a cooperative regulation of ATP and ADP binding to SUR1, and this cooperativity may be involved in MgATP and MgADP regulation of the  $K_{ATP}$  channel (18,19). ATP binds strongly to SUR1 at NBF-1 and MgADP binds at NBF-2. Prebound MgADP at NBF-2 inhibits ATP binding to NBF-1 (18), whereas prebound ATP at NBF-1 is stabilized by MgADP or MgATP (19). Effects of the R1420C mutation in NBF-2 on cooperative binding of ATP and ADP were assessed. As shown in Fig. 3, SUR1-1420C bound 8-azido-[ $\alpha$ - $^{32}$ P]ATP as effectively as wild-type SUR1 (lanes 1 and 5). The 8-azido-[ $\alpha$ - $^{32}$ P]ATP was dissociated equally from SUR1-1420C and from wild-type SUR1 in the absence of MgATP or MgADP in 15 min at 37°C (lanes 2 and 6). The binding was almost completely stabilized in the presence of MgATP or MgADP for wild-type SUR1 (lanes 3 and 4), while the effect was reduced for SUR1-1420C by 50% (lanes 7 and 8).

## DISCUSSION

PHHI is a heterogeneous disorder. In patients with a recessive form of the disease, as well as in some of the sporadic cases, mutations have been identified in genes encoding the  $K_{ATP}$  channel, the SUR1 and Kir6.2 genes. An autosomal dominant form is also known (27,28), and mutations have been identified in the glutamate dehydrogenase gene (10) and the glucokinase gene (9) in some patients with dominantly inherited disease.

We identified three mutations in four patients from a cohort of Japanese PHHI patients. Two patients (patients 1 and 4) with apparently sporadic PHHI were heterozygous for a mutation of the SUR1 gene. Mutations in the  $K_{ATP}$  channel genes appear to be recessive (3), and, as such, mutations are required in both alleles for PHHI to manifest. In some reported cases, however, mutations were found in only one allele when peripheral leukocyte DNA was examined (3,5,6,20). Histopathologically, pancreatic lesions in PHHI patients can be classified into two forms: 1) a focal form with adenomatous hyperplasia of islet cells, and 2) a diffuse form (29). Recent reports have demonstrated that in focal hypertrophic lesions of pancreatic islets, the maternal allele of chromosome region 11p15, including SUR1 and Kir6.2 genes, was specifically lost (20,30). In addition, paternally inherited mutations of the SUR1 gene were found in these patients (20). Therefore, these patients are heterozygous for the SUR1 gene mutations, based on examination of their leukocyte DNA, but hemizygous or homozygous for SUR1 gene mutations in hypertrophic islets. In one of our patients, patient 1, a I446delT mutation was paternally inherited, and pathological examination of the resected pancreas revealed hypertrophic islets distributed throughout the organ forming adenomatous lesions mostly in the pancreatic head (data not shown). Although we could not test this possibility directly, it is likely that loss of heterozygosity occurred in this patient's islets during islet development and caused her disease. This may also be the case for patient 4, who was heterozygous for a germline R1436Q mutation.

SUR1 mutations in Japanese PHHI patients are heterogeneous. No common mutation has been found, such that no founder effect exists. This is in contrast to Ashkenazi Jews (3) and Finns (6). In these populations, a single or a few mutations explain the majority of PHHI cases.

To assess the causal relationship of mutations to PHHI more directly, we conducted functional studies. As expected, SUR1-446fsdelT could not reconstitute functional  $K_{ATP}$  when it was coexpressed with Kir6.2 in COS-7 cells and studied by  $Rb^+$  efflux assays (Fig. 1). Neither did SUR1-1436Q form functional  $K_{ATP}$  (Fig. 1). When SUR1-1436Q alone was expressed in COS-7 cells, the expression level of SUR1 protein in the crude membrane fraction was less than one-tenth that of wild-type SUR1 (Fig. 2). This result suggests instability of the protein in the cell, or defective transport to the membrane fraction, and may account for the absence of functional  $K_{ATP}$  in cells coexpressing SUR1-1436Q and Kir6.2. Single channel function of  $K_{ATP}$  constituted with this mutant SUR1 needs to be further investigated by electrophysiological studies using the patch-clamp technique.

Impairment of  $K_{ATP}$  channel activity with SUR1-1420C was modest but significant when assayed by  $^{86}Rb^+$  efflux studies under metabolic inhibition by oligomycin and 2-deoxyglucose. Inhibition of the channel by glibenclamide (1  $\mu$ mol/l) was complete (Fig. 1). When cooperative binding of ATP and MgADP or MgATP was assessed, stabilization of 8-azido-[ $\alpha$ - $^{32}$ P]ATP binding to NBF-1 by MgADP or MgATP was impaired in SUR1-1420C (Fig. 3). This may explain impaired  $K_{ATP}$  (SUR1-1420C) activation in COS-7 cells by metabolic inhibition.  $K_{ATP}$  channels are under complex regulation by intracellular ATP and ADP. Inhibition of the  $K_{ATP}$  channel by ATP appears to be mediated by direct interaction of ATP with the pore-forming subunit Kir6.2 (16,31,32), whereas acti-

vation is conferred by the regulatory subunit, SUR1 (2,33). According to our proposed model (19),  $K_{ATP}$  channel activation is induced when SUR1 binds ATP in NBF-1 and MgADP in NBF-2 cooperatively (19). At high intracellular MgADP concentrations, most SUR1 is in this condition. When the intracellular MgADP concentration decreases, MgADP dissociates from NBF-2, leading to closure of the  $K_{ATP}$  channel. MgADP dissociation from NBF-2 further leads to instability of ATP binding at NBF-1, allowing the release of ATP. ATP binding at NBF-1 is required for the channel-activating effect of SUR1.

Failure of SUR1-1420C to stabilize ATP binding at NBF-1 by MgADP could be due to impaired MgADP binding to NBF-2. Or alternatively, it may be due to impaired cooperative interaction between two NBFs (possibly conformational change of SUR1). Glibenclamide was as effective on SUR1-1420C as on wild-type SUR1 (Fig. 1). Because this drug is proposed to work by releasing ATP from NBF-1 in cooperation with MgADP at NBF2 (19), MgADP binding to NBF-2 might be normal, but the cooperative interaction between two NBFs is impaired in SUR1-1420C. However, it is to be determined directly whether this mutation affects MgADP binding to NBF-2.

Most of the SUR1 mutants identified in PHHI patients have exhibited a reduced response to MgADP stimulation, when reconstituted channel activities were measurable (2,34). This may also be the case for the R1420C mutation, although we did not test this possibility directly. Direct interaction of mutant SUR1 and adenine nucleotides has not previously been assessed. The data presented herein constitute the first direct biochemical evidence of defective nucleotide interaction with SUR1 linked to impaired  $K_{ATP}$  channel activity.

In our  $^{86}\text{Rb}^+$  efflux studies, impairment of  $K_{ATP}$  channel function was modest with the R1420C mutation, while no channel activities were observed for the other two mutations, R1436C and I446fsdelT. This parallels clinical disease severities of these patients: patients with the R1420C mutation achieved seemingly spontaneous remission after a few months of medical therapy, whereas patients who had R1436C or I446fsdelT mutations required partial pancreatectomy. This may be a coincidence, however, since it has also been reported that in vitro functional results did not explain clinical severity (5,34), and the number of patients in our study was small.

In conclusion, we identified three SUR1 mutations in four patients from a cohort of 17 Japanese PHHI subjects, including a pair of siblings. No mutation was identified in the Kir6.2 gene or in exons of the glutamate dehydrogenase gene encoding an allosteric regulatory domain. The genetic etiology of PHHI in the Japanese population appears to be heterogeneous. SUR1 mutations account for only ~20% of PHHI cases in Japan. Mutations of Kir6.2 and glutamate dehydrogenase genes are even more rare. We also present herein the first direct biochemical evidence that the SUR1 mutant identified in PHHI patients impairs cooperative adenine nucleotide binding, thereby leading to dysfunction of  $K_{ATP}$  channel activity and dysregulation of insulin secretion.

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