Identification of a Familial Hyperinsulinism-causing Mutation in the Sulfonylurea Receptor 1 That Prevents Normal Trafficking and Function of \( K_{\text{ATP}} \) Channels*

Received for publication, January 12, 2002, and in revised form, February 15, 2002
Published, JBC Papers in Press, February 26, 2002, DOI 10.1074/jbc.M200363200

Grit Taschenberger‡, Adam Mougey‡, Shu Shen‡, Linda B. Lester§, Stephen LaFranchi¶, and Show-Ling Shyng§

From the ‡Center for Research on Occupational and Environmental Toxicology, the §Department of Medicine, and the ¶Department of Pediatrics, Oregon Health and Science University, Portland, Oregon 97220

Mutations in the pancreatic ATP-sensitive potassium (\( K_{\text{ATP}} \)) channel subunits sulfonylurea receptor 1 (\( \text{SUR1} \)) and the inwardly rectifying potassium channel \( \text{Kir6.2} \) cause persistent hyperinsulinemic hypoglycemia of infancy. We have identified a \( \text{SUR1} \) mutation, L1544P, in a patient with the disease. Channels formed by co-transfection of \( \text{Kir6.2} \) and the mutant \( \text{SUR1} \) in COS cells have reduced response to MgADP (~10% that of the wild-type channels) and reduced surface expression (~19% that of the wild-type channels). However, the steady-state level of the \( \text{SUR1} \) protein is unaffected. Treating cells with lysosomal or proteasomal inhibitors did not improve surface expression of the mutant channels, suggesting that increased degradation of mutant channels by either pathway is unlikely to account for the reduced surface expression. Removal of the RKR endoplasmic reticulum retention/retrieval trafficking motif in either \( \text{SUR1} \) or \( \text{Kir6.2} \) increased the surface expression of the mutant channel by ~35 and ~20%, respectively. The simultaneous removal of the RKR motif in both channel subunits restored surface expression of the mutant channel to the wild-type channel levels. Thus, the L1544P mutation may interfere with normal trafficking of \( K_{\text{ATP}} \) channels by causing improper shielding of the RKR endoplasmic reticulum retention/retrieval trafficking signals in the two channel subunits.

In pancreatic \( \beta \)-cells, ATP-sensitive potassium (\( K_{\text{ATP}} \)) channels couple metabolic signals to cell excitability and regulate insulin secretion (for review see Refs. 1–4). \( K_{\text{ATP}} \) channels are inhibited by intracellular ATP but stimulated by ADP in the presence of Mg\(^{2+}\) (1–4). When the ATP/ADP ratio increases in response to elevated blood glucose levels, \( K_{\text{ATP}} \) channels close leading to membrane depolarization, activation of voltage-gated Ca\(^{2+}\) channels, and insulin release. In contrast, when blood glucose levels are low, the ATP/ADP ratio decreases, \( K_{\text{ATP}} \) channels open, and insulin secretion ceases. Failure of \( K_{\text{ATP}} \) channels to open in response to glucose deprivation is a major cause of the disease persistent hyperinsulinemia hypoglycemia of infancy (PHHI) (5–8). PHHI is characterized by inappropriate insulin hypersecretion in infants despite low glucose levels (9, 10). In severe cases, subtotal pancreactectomy is required to avoid hypoglycemia-induced brain damage.

The pancreatic \( K_{\text{ATP}} \) channel complex is formed by the sulfonylurea receptor 1 (\( \text{SUR1} \)) and the inward rectifier potassium channel \( \text{Kir6.2} \) in a 4:4 stoichiometry (11–14). The \( \text{Kir6.2} \) subunit forms the potassium-conducting pore. Although homomeric \( \text{Kir6.2} \) channels are sensitive to ATP inhibition, the ATP sensitivity is greatly enhanced by interaction with the \( \text{SUR1} \) subunit, which also confers channel sensitivity to MgADP, sulfonylureas, and diazoxide (1–4). \( \text{SUR1} \), a member of the ATP-binding cassette transporter family, contains two intracellular nucleotide-binding folds (NBFs) (11). Both NBFs have been demonstrated to bind and hydrolyze ATP; the ATP\( \text{ase} \) activity of NBF2 is much higher than that of NBF1 (15–17). It is proposed that the hydrolysis of MgATP at NBF2 stabilizes the binding of ATP at NBF1, which results in functional coupling among \( \text{SUR1} \) and \( \text{Kir6.2} \) and channel activation (16). An increase in the intracellular concentration of ADP following glucose starvation stimulates \( K_{\text{ATP}} \) channel activity by slowing the rate of ATP hydrolysis at NBF2, locking NBF2 in a post-hydrolytic state (18). Sulfonylureas inhibit channel activity by disrupting the cooperative nucleotide binding between the two NBFs (16). On the other hand, diazoxide promotes channel activity by stabilizing \( \text{SUR1} \) in the post-hydrolytic conformation (18).

Genetic studies have identified over 50 PHHI-associated mutations in the \( K_{\text{ATP}} \) channel genes, most of which are in \( \text{SUR1} \) (6–8). Of the missense \( \text{SUR1} \) point mutations studied, many are clustered in NBF2 and cause the inability of the channel to respond to the stimulating effect of MgADP (19, 20). More recently, incorrect channel trafficking to the plasma membrane has been shown as a mechanism by which certain mutations cause PHHI (21–23). Normal expression of functional \( K_{\text{ATP}} \) channels on the cell surface requires co-assembly of \( \text{SUR1} \) and \( \text{Kir6.2} \) into an octameric complex. This co-assembly is thought to cause shielding of a RKR-tripeptide ER retention/retrieval signal in each of the \( \text{SUR1} \) and \( \text{Kir6.2} \) channel subunit (24). Without proper shielding of the RKR signal, individual subunits and partial channel complexes are retained in the ER (24). Sharma et al. (21) reported a second trafficking signal...
present in the C terminus of SUR1 that is required for forward trafficking of the channel complex. Therefore, they (21) propose that mutations leading to C-terminal truncation of SUR1 may cause PHHI by preventing normal channel trafficking. We recently demonstrated that a PHHI-associated single amino acid deletion mutation in SUR1 (ΔF1388) results in defective trafficking and a loss of cell surface expression of K_ATP channels (22). Here, we report functional and cell biological analyses of a SUR1 mutation, L1544P, that we identified in a patient with PHHI. This mutation reduces surface expression of K_ATP channels; it also reduces response of the channel to MgADP and diazoxide. We show that simultaneous removal of the -KK-RK-ER retention/retrieval signal in both SUR1 and Kir6.2 nearly completely reversed the trafficking defect caused by the L1544P SUR1 mutation.

**EXPERIMENTAL PROCEDURES**

**Genetic Analysis—**Genomic DNA was extracted from peripheral blood using the Puregene purification kit. Individual exons and adjacent intron-exon boundaries of the SUR1 gene (GenBank™ accession numbers L78208 and L78216) were amplified by PCR and screened for mutations by direct nucleotide sequencing.

**Construction of SUR1 and Kir6.2 Plasmids—**FLAG epitope (DYKDDDDK) was inserted at the N terminus of the hamster SUR1 cDNA by sequential overlap extension PCR. Point mutations of SUR1 were introduced into hamster SUR1 cDNA in the pECE plasmid using the QuikChange site-directed mutagenesis kit (Stratagene). Epitope tag and mutations were confirmed by DNA sequencing. All SUR1 and SUR1-Kir6.2 fusion constructs were in pECE vector and mouse Kir6.2 cDNA in pCMV6b vector (22). Mutant clones from multiple PCR reactions were analyzed in all experiments to avoid false results caused by undesired mutations introduced by PCR. In some cases, an additional subcloning step (a restriction fragment of NotI and EcoRI corresponding to nucleotide positions 4135-4585 of the plasmid) was used to limit potential PCR-introduced artifacts.

*86Rb+ Efflux Assay—*Cells were incubated for 24 h in culture medium containing 86RbCl (1 μCi/ml) 2–3 days after transfection. Before measurement of 86Rb efflux, cells were incubated for 30 min at 25°C in Krebs–Ringer phosphate solution with metabolic inhibitors (2.5 μg/ml oligomycin plus 1 μM 2-deoxy-D-glucose). At selected time points, the solution was aspirated from the cells and replaced with fresh solution. The percentage efflux at each time point was calculated as the cumulative counts in the aspirated solution divided by the total counts from the solutions and the cell lysates.

**Patch Clamp Recordings—**COSm6 cells were transfected using LipofectAMINE or FuGENE 6 and plated on coverslips. The cDNA for the patient’s SUR1 was co-transfected with SUR1 and Kir6.2 to facilitate the identification of positively transfected cells. Patch clamp recordings were made 36–72 h post-transfection. All experiments were performed at room temperature as described previously. Micro pipettes were pulled from nonheparinized Kimble glass (Fisher Scientific) on a horizontal puller (Sutter Instrument Co., Novato, CA). Electrode resistance was typically 0.5–1 MΩ when filled with K INT solution as noted below. Inside-out patches were voltage-clamped with an Axopatch 1D amplifier (Axon Inc., Foster City, CA). The standard bath (intracellular) and pipette (extracellular) K INT solution had the following composition: 140 mM KCl, 10 mM K-HEPES, 1 mM K-EGTA, pH 7.3. ATP was added as the potassium salt. All currents were measured at a membrane potential of −50 mV (pipette voltage +50 mV). Data were analyzed using pCLAMP software (Axon Instrument). Offline analysis was performed using Microsoft Excel programs. Data were presented as the mean ± S.E.

**Immunofluorescence Staining—**COSm6 cells were plated in 6-well tissue culture plates transfected with 0.5 μg of SUR1 and 0.4 μg of Kir6.2/well using FuGENE 6 (Roche Molecular Biochemicals) according to manufacturer’s directions. Cells were analyzed 48–72 h post-transfection.

**Functional Analysis of the Mutant Channel—**The functional consequences of the L1544P mutation for macroscopic K_ATP channel activity in intact cells were assessed by the 86Rb+ efflux assay. Prior to 86Rb+ efflux measurement, cells were incubated with metabolic inhibitors, which reduce the intracellular ATP/ADP ratio to stimulate channel activity. In cells expressing wild-type K_ATP channels, 84.8 ± 8.0% (n = 8) of intracellular 86Rb+ was released in a 40-min period following metabolic inhibition. In contrast, only 31.5 ± 9.6% (n = 4; compare with 18% in untransfected control cells) of 86Rb+ was released in cells expressing L1544P-SUR1 mutant channels (Fig. 2A). The poor response of the mutant channel to metabolic inhibition predicts that the channel would not function well in vivo in response to glucose deprivation, thereby causing the PHHI phenotype.

A well documented K_ATP channel defect caused by a number of PHHI-SUR1 mutations is the lack of response to MgADP (19, 20). To determine whether a similar defect occurs in the L1544P-SUR1 mutant channel, we examined the channel response to nucleotides by inside-out patch clamp recordings. In membrane patches with wild-type K_ATP channels, MgADP antagonizes the inhibitory action of ATP and stimulated channel activity. This stimulatory effect of MgADP is greatly reduced in L1544P-SUR1 mutant channels (Fig. 2B, upper panel). How-
ever, the sensitivity of the mutant channels to inhibition by ATP remains the same as wild-type channels (Fig. 2B). We also tested mutant channel response to the potassium channel opener diazoxide, an agonist for pancreatic KATP channels that has been successfully used to treat some forms of PHHI (1–4).

The mutation nearly abolished the ability of the channel to be stimulated by diazoxide (Fig. 2B, lower panel), consistent with the unsuccessful treatment of the patient with diazoxide.

The L1544P Mutation in SUR1 Causes Reduced Surface Expression of KATP Channels—Membrane currents in patches with L1544P mutant channels were generally much smaller compared with patches containing wild-type channels. To determine whether this is the result of a decrease in the number of channels expressed on the cell surface, we performed immunofluorescent staining of surface SUR1 by tagging a FLAG epitope at the N terminus of SUR1 (hereafter referred to as fSUR1). Control experiments confirmed that the FLAG epitope does not alter the behavior of the wild-type nor the mutant protein (22) (data not shown). To ensure that only surface protein was labeled, the staining procedures were carried out at 4 °C, a temperature at which no membrane trafficking occurs. Surface staining in cells co-expressing L1544P-fSUR1 and Kir6.2 was markedly reduced compared with cells co-expressing WT-fSUR1 and Kir6.2 (Fig. 3A, upper panel). The weak surface expression of L1544P-fSUR1 did not result from reduced biosynthesis of the mutant protein; the staining of cells fixed and permeabilized with methanol showed comparable expression levels between the mutant and the wild-type protein (Fig. 3A, lower panel). However, a clear difference in the staining pattern was noted where the L1544P-fSUR1 exhibited strong perinuclear distribution, indicative of intracellular accumulation of the protein. These results are extended by biochemical analyses of the mutant protein. Western blots showed that the steady-state level of L1544P-fSUR1 is not significantly different from that of WT-fSUR1 both when expressed alone and when co-expressed with Kir6.2 (Fig. 3B). In the presence of Kir6.2, the WT-fSUR1 was resolved into two bands, an upper band that corresponds to the mature complex glycosylated form and a lower band that corresponds to the immature core glycosylated form (22). The mature complex-glycosylated form was barely detectable in cells co-expressing L1544P-fSUR1 and Kir6.2, consistent with the reduced surface expression observed in immunofluorescent staining experiments.

Surface expression of the mutant channels was further quantified using a chemiluminescence assay similar to that developed by Margeta-Mitrovic et al. (25). Cells were fixed with paraformaldehyde. The surface FLAG tag was labeled with the M2 anti-FLAG antibody followed by secondary antibody conjugated to the horseradish peroxidase. The antibody bound to the cell surface was quantified by measuring the chemilumines-

![Fig. 1. Identification of the L1544P mutation in SUR1. A, a T → C homozygous mutation in exon 39 of SUR1, which results in substitution of leucine at amino acid position 1544 to proline in the protein, was detected in a patient diagnosed with PHHI. B, location of the L1544P mutation in SUR1. Also shown are the locations of the RKR motif in SUR1 and Kir6.2. The topology of SUR1 is based on Conti et al. (26).]
ence released from the horseradish peroxidase substrate. Surface expression of the L1544P-SUR1 mutant channel was only ~20% that of the wild-type channel (Figs. 4-6). As a control, we measured surface expression of cells transfected with fSUR1 alone and found it to be similar to untransfected cells, demonstrating that paraformaldehyde fixation did not lead to labeling of intracellular fSUR1. The results are consistent with observations obtained with immunostaining that the L1544P mutation causes a marked decrease in surface expression of K_{ATP} channels.

Reduced Surface Expression of the L1544P-SUR1 Mutant Channel Is Not because of Increased Protein Degradation

**Fig. 2.** Functional analysis of channels formed by Kir6.2 and L1544P-SUR1. A, macroscopic K_{ATP} channel activity in response to metabolic poisoning assessed by \(^{86}\)Rb\(^+\) efflux assay. Efflux was measured in COSm6 cells coexpressing Kir6.2 and wild-type or mutant SUR1 following incubation with metabolic inhibitors that reduce the ATP/ADP ratio. The representative experiment shows Rb efflux during a 40-min time interval. Although cells expressing wild-type SUR1 channels showed a robust \(^{86}\)Rb\(^+\) release upon metabolic poisoning, \(^{86}\)Rb\(^+\) efflux in cells expressing L1544P mutant channels was only slightly higher than in untransfected controls. B, response of wild-type and mutant channels to ATP, ADP, and diazoxide. K_{ATP} currents were measured in inside-out membrane patches containing wild-type or mutant channels. Patches were exposed to ATP, ADP, and diazoxide at concentrations as indicated. Free Mg\(^{2+}\) in all nucleotide-containing solutions was kept at 1 mM. Holding potential was -50 mV. Representative current traces show that ATP inhibited both wild-type and mutant channels with similar dose response. The application of ADP or diazoxide in the presence of Mg\(^{2+}\) activated wild-type channels but had little effect on L1544P-SUR1 mutant channels.
investigate the mechanism by which the L1544P-SUR1 mutation causes reduced surface expression, we first considered the possibility that the mutant channel might have an increased degradation rate. Although Western blot analysis showed that the steady-state expression levels of total cellular L1544P-fSUR1 and WT-fSUR1 did not differ significantly from each other, this method might not be sensitive enough to detect kinetic differences in the turnover of surface fSUR1. The lysosome is the major pathway by which surface membrane proteins are degraded. The proteasome, although generally thought to be more involved in the degradation of misfolded or excess proteins in the secretory pathway before their exit from the ER, has been shown to be responsible for degradation of certain surface membrane proteins (27). Therefore, we examined the effects of lysosomal and proteasomal inhibitors on surface expression of the mutant channel using the chemiluminescence assay described above. Cells expressing wild-type or mutant channels were treated with the lysosomal inhibitor chloroquine (200 μM) or the proteasomal inhibitor lactacystin (10 μM) for 6 or 12 h, and the surface expression of the channel was quantified. Neither chloroquine nor lactacystin had significant effects on surface expression of the wild-type or the mutant channels (Fig. 4). As a positive control for the effectiveness of the drugs in blocking the lysosomal and proteasomal degradation pathways, we labeled cellular proteins with [35S]methionine for 1 h and chased the labeled proteins in the presence or absence of chloroquine or lactacystin for 6 h. Specific protein bands with increased intensities could be seen in the chloroquine- and lactacystin-treated samples, indicating decreased degradation rates of these proteins (data not shown). Taken

**FIG. 3.** Reduced surface expression of the L1544P-SUR1 mutant K<sub>ATP</sub> channel. A, surface staining of COSm6 cells transiently transfected with Kir6.2 and either WT-fSUR1 or L1544P-fSUR1 using the M2 anti-FLAG mouse monoclonal antibodies (upper panels). Staining was performed in living cells at 4 °C. Surface expression of L1544P-fSUR1 mutant channels is clearly reduced compared with that of WT channels. Total cellular expression of FLAG-tagged WT or mutant SUR1 was assayed by immunostaining of cells co-expressing Kir6.2 fixed and permeabilized with methanol (lower panels). WT and mutant fSUR1 showed similar levels of staining. Intense perinuclear staining was more frequently observed in cells expressing the L1544P-fSUR1 mutant channel. B, total fSUR1 protein levels estimated by Western blots. Solid arrowhead indicates core glycosylated SUR1; open arrowhead indicates complex-glycosylated SUR1. Molecular mass markers (kDa) are indicated on the right. The total steady-state protein level of L1544P-fSUR1 is not significantly different from that of WT-fSUR1, both in the presence and the absence of Kir6.2. However, the complex-glycosylated form is barely detectable in cells co-expressing L1544P-fSUR1 and Kir6.2. Note that in the absence of Kir6.2, the expression level of fSUR1 is usually higher. To see the glycosylated band, the blot on the left was exposed longer. Therefore, the intensities of bands between blots cannot be compared.
Neither chloroquine nor lactacystin had significant effect on surface expression of the L1544P-SUR1 mutant channel. COSm6 cells plated in 35-mm dishes were transfected with Kir6.2 and WT-SUR1 or L1544P-SUR1. 2–3 days after transfection, cells were treated with the lysosomal inhibitor chloroquine (200 μM) or the proteasomal inhibitor lactacystin (10 μM) for 6 or 12 h. Surface expression of FLAG-tagged SUR1 was measured by the chemiluminescence assay (see “Experimental Procedures”). Data from the 6 and 12 h treatment were pooled together because no difference was observed between the two time points. In this and subsequent figures, relative luminescence units from mock-transfected cells were subtracted from the relative luminescence units of each dish. Surface expression was normalized to values obtained from cells expressing WT-SUR1 and Kir6.2. Data plotted represent mean ± S.E. of 9–12 dishes from multiple experiments. Neither chloroquine nor lactacystin had significant effect on surface expression of WT or mutant channels.

together, the results suggest that the reduced surface expression of the L1544P-SUR1 mutant channel is unlikely to be the result of increased degradation of the surface channel.

**Surface Expression of the L1544P-SUR1 Mutant Channel Is Restored by Removing the RKR ER Retention/Retrieval Signal in SUR1 and Kir6.2**—Proper trafficking and surface expression of K\textsubscript{ATP} channels require that SUR1 and Kir6.2 assemble into an octameric complex. This assembly process is thought to shield a tripeptide ER retention/retrieval signal, RKR, present in both the SUR1 and the Kir6.2 subunit (exposure of the RKR-trafficking motif prevents individual channel subunits and partially assembled channel complexes from exiting the ER) (24). We next tested whether the RKR signals in the two subunits are not effectively shielded because of the L1544P mutation by inactivating or removing the RKR signals in SUR1 and Kir6.2. The RKR motif in SUR1 is located in a cytoplasmic loop between the putative eleventh transmembrane domain and the first nucleotide-binding fold (Fig. 1B). When we inactivated the RKR signal in L1544P-SUR1 by mutating it to AAA (L1544P\_AAA-SUR1), we observed increased surface expression of the mutant protein both when expressed alone and when co-expressed with Kir6.2 (Fig. 5). Interestingly, in the absence of Kir6.2, the surface expression of L1544P\_AAA-SUR1 was nearly as high as WT\_AAA-SUR1 (Fig. 5A), suggesting that the L1544P mutation per se does not cause significant intracellular retention of the protein. This finding is in contrast to ΔF1388\_AAA-SUR1, whose surface expression is only ∼25% that of WT\_AAA-SUR1 (Fig. 5A). In the presence of Kir6.2, the surface expression of L1544P\_AAA-SUR1 increased by almost 3-fold compared with L1544P-SUR1 (Fig. 5B) but was still lower than that of WT-SUR1 or WT\_AAA-SUR1, indicating that the trafficking defect of the L1544P-SUR1 mutant channel was only partially corrected.

Next, we examined the effects of removing the RKR ER retention/retrieval signal in Kir6.2 on surface expression of the mutant channel. Previous studies have shown that deletion of the last 25 amino acids from the C terminus (Kir6.2ΔC25), which removes the RKR signal, allows the subunit to form functional channels on the cell surface without co-expression with SUR1 (28, 29). We co-expressed Kir6.2ΔC25 with L1544P-SUR1 and found that the surface expression of L1544P-SUR1 increased by 2-fold (Fig. 5B). The result indicates that improper shielding of the RKR signal in Kir6.2 after channel assembly also contributes to the reduced surface expression of the L1544P-SUR1 mutant channel.

The above observations suggest that improper shielding of the RKR motif in SUR1 and in Kir6.2 both play a role in preventing normal surface expression of the L1544P mutant channel. This revelation led us to determine whether simultaneous removal of the RKR signal in both SUR1 and Kir6.2 subunit is sufficient to completely restore surface expression of the mutant channel. Fig. 5B shows that the level of surface FLAG tag signal in cells co-expressing L1544P\_AAA-SUR1 and Kir6.2C25 is equivalent to that in cells co-expressing WT-SUR1 and Kir6.2. Therefore, simultaneous removal of the RKR motif in SUR1 and Kir6.2 abolished the trafficking defect caused by the L1544P mutation. Cells co-expressing L1544P\_AAA-SUR1 and Kir6.2C25 also showed a concomitant increase in K\textsubscript{ATP} currents in inside-out patches (∼5-fold that of cells co-expressing L1544P-SUR1 and Kir6.2, data not shown). This result confirms that the increased surface L1544P\_AAA-SUR1 detected in cells co-expressing L1544P\_AAA-SUR1 and Kir6.2C25 is actually within functional channel complexes.

Taken together, the results suggest that the L1544P mutation causes reduced surface expression of K\textsubscript{ATP} channels primarily by preventing proper shielding of the ER retention/retrieval signals in both SUR1 and Kir6.2, leading to reduced forward trafficking of channel complexes.

**K\textsubscript{ATP} Channel Drugs Have No Effect on the Trafficking of the L1544P-SUR1 Mutant Channel**—The trafficking defects of

---

**Fig. 4.** Lysosomal or proteasomal inhibitors have no effect on surface expression of the L1544P-SUR1 mutant channel. COSm6 cells plated in 35-mm dishes were transfected with Kir6.2 and WT-SUR1 or L1544P-SUR1. 2–3 days after transfection, cells were treated with the lysosomal inhibitor chloroquine (200 μM) or the proteasomal inhibitor lactacystin (10 μM) for 6 or 12 h. Surface expression of FLAG-tagged SUR1 was measured by the chemiluminescence assay (see “Experimental Procedures”). Data from the 6 and 12 h treatment were pooled together because no difference was observed between the two time points. In this and subsequent figures, relative luminescence units from mock-transfected cells were subtracted from the relative luminescence units of each dish. Surface expression was normalized to values obtained from cells expressing WT-SUR1 and Kir6.2. Data plotted represent mean ± S.E. of 9–12 dishes from multiple experiments. Neither chloroquine nor lactacystin had significant effect on surface expression of WT or mutant channels.

**Fig. 5.** Inactivation or removal of the RKR ER retention/retrieval signal in SUR1 and Kir6.2 rescue the trafficking defect of the L1544P-SUR1 mutant channel. A, mutation of the RKR sequence to AAA in wild-type (WT\_AAA)-SUR1 and L1544P (L1544P\_AAA)-SUR1 allows surface expression of both proteins in the absence of Kir6.2. Note that surface expression was normalized to measurements obtained from cells expressing WT\_AAA-SUR1 alone. Each data point represents the mean ± S.E. of 9–12 dishes from multiple experiments. B, inactivation of the RKR signal in L1544P-SUR1 as well as removal of the signal in Kir6.2 increased surface expression of the mutant channel (from 18.9 ± 1.1% to 54.5 ± 2.4% and 46.3 ± 6.2%, respectively, n = 12–40). Simultaneous disruption of the RKR signal in both SUR1 and Kir6.2 restored surface expression of mutant channels to levels equivalent to the wild-type channel (normalized surface expression = 106.5 ± 9.2%, n = 18).
respectively, using the chemiluminescence assay. Neither glibenclamide (5 μM) nor diazoxide (100 μM) had significant effects on surface expression of the L1544P-SUR1 mutant channel after 24–48 h of treatment (Fig. 6), although glibenclamide consistently increased while diazoxide decreased surface expression of WT-fSUR1 channels by ~30 and ~20%, respectively.

**DISCUSSION**

K<sub>ATP</sub> channels regulate the resting membrane potential of pancreatic β-cells, the failure of K<sub>ATP</sub> channels to open in response to glucose deprivation results in constant membrane depolarization and persistent insulin secretion (1–4). The dysfunction of K<sub>ATP</sub> channels attributed to genetic mutations is now recognized as the major cause of the insulin secretion dysfunction (6–8). PhHII-associated SUR1 mutations (9–12). The clinical observation of the patient not only causes defects in channel function but also patient not only causes defects in channel function but also patient not only causes defects in channel function but also

Figure 6. K<sub>ATP</sub> channel drugs glibenclamide and diazoxide do not improve surface expression of the L1544P-SUR1 mutant channel. Cells expressing wild-type or mutant channels were treated with 5 μM glibenclamide or 100 μM diazoxide for 24–48 h, and surface expression of channels were measured by the chemiluminescence assay. No significant difference in expression of the mutant channels was observed between control and drug-treated cells. However, glibenclamide increased surface expression of the wild-type channel, whereas diazoxide decreased surface expression (126.6 ± 11.6% and 80.3 ± 9.9%, respectively, n = 9–12).

Some membrane proteins such as human P-glycoprotein and human Ether-a-go-go channels can be corrected by treating cells with protein-specific agonists or antagonists (30, 31). Recently, Partridge et al. (23) reported that the K<sub>ATP</sub> channel agonist diazoxide reversed the channel-trafficking defect caused by a PHHI-associated SUR1 mutation R1394H. We determined whether the trafficking defect caused by the L1544P mutation can also be corrected by drug treatment using the chemiluminescence assay. Neither glibenclamide (5 μM) nor diazoxide (100 μM) had significant effects on surface expression of the L1544P-SUR1 mutant channel after 24–48 h of treatment (Fig. 6), although glibenclamide consistently increased while diazoxide decreased surface expression of WT-fSUR1 channels by ~30 and ~20%, respectively.

The L1544P Mutant Channel Phenotype Is Consistent with Clinical Observations of the Patient—Reconstitution of L1544P-SUR1 mutant K<sub>ATP</sub> channels in COS cells shows that the mutant channel is expressed at only ~20% of the wild-type channel. Moreover, the response of the mutant channel to stimulation by MgADP or diazoxide is greatly reduced. As a result, channel activity in intact cells under metabolic inhibition is markedly compromised. The functional phenotype of the L1544P-SUR1 mutant channels that we observed from these in vitro studies is consistent with the clinical phenotype. The patient did not respond well to diazoxide treatment and therefore underwent subtotal pancreatectomy at 8 weeks of age. Although the L1544P mutant channels have some residual response to diazoxide (Fig. 2B) and might be expected to respond to high dosage of diazoxide treatment, the reduced cell surface expression of the channel is likely to exacerbate the problem and render any diazoxide effect negligible. Our findings underscore the importance of normal K<sub>ATP</sub> channel expression and channel response to MgADP in proper function of pancreatic β-cells.

**Functional Defect Caused by the L1544P-SUR1 Mutation**—The L1544P mutation greatly reduces the response of the resulting channel to stimulation by MgADP, a functional defect shared by a number of other PHHI-SUR1 mutations (19, 20, 23). Most of these other mutations are located within the NBF2, which binds and hydrolyzes nucleotides to promote K<sub>ATP</sub> channel activity (16, 18). Therefore, a loss of channel response to MgADP may result from the effects of the mutations on nucleotide binding and/or hydrolysis (16, 17, 35). Leucine 1544 is located near the C terminus of SUR1 just downstream of the predicted NBF2; it is not expected to participate directly in nucleotide binding and hydrolysis. Another mutation, F591L, which is also outside of the two NBFs, has similar detrimental effects on channel response to MgADP and diazoxide (20). We speculate that these two mutations may reside in regions involved in the functional coupling between SUR1 and Kir6.2 following nucleotide binding and hydrolysis at the NBFs.

**Cell Biological Defects Caused by the L1544P Mutation**—The L1544P-SUR1 mutant channels exhibit reduced surface expression compared with wild-type channels. Our results suggest that increased degradation of the mutant channel on the cell surface is unlikely to be a major cause of reduced surface expression. First, the steady-state level of total cellular L1544P-SUR1 was not significantly different from that of wild-type SUR1. Second, the inhibition of the lysosomal or the proteasomal degradation pathway did not improve surface expression of the L1544P-SUR1 mutant channels. Rather, the reduced surface expression is primarily because of improper shielding of the tripeptide -RKR- ER retention/retrieval signal in the channel subunits. The inactivation or removal of the signal in either SUR1 or Kir6.2 partially corrected the trafficking defect of the mutant channels, and simultaneous inactivation of the signal in both channel subunits completely reversed the trafficking defect. The observation that in the absence of Kir6.2 the surface expression of L1544P-AAA-fSUR1 is as efficient as WT-AAA-fSUR1 (Fig. 5A) indicates that the L1544P mutation itself does not impair the ability of the protein to traffic to the cell surface. Thus, the trafficking defect caused by the L1544P-SUR1 mutation is Kir6.2-dependent, consistent with the idea that the mutation prevents normal interaction between the SUR1 and Kir6.2 subunits such that the ER retention signals are not effectively shielded. Because L1544P-AAA-SUR1 is capable of trafficking to the cell surface in the absence of Kir6.2, one might argue that the L1544P-AAA-fSUR1 detected on the surface of cells co-transfected with ΔC25Kir6.2 is not incorporated into the channel complex. However, this argument is counteracted by the observation that surface expression of L1544P-AAA-SUR1 in cells co-transfected with wild-type Kir6.2 is much lower than in cells co-transfected with ΔC25Kir6.2 (Fig. 5B). Furthermore, the increased surface expression of L1544P-AAA-SUR1 in cells co-transfected with wild-type Kir6.2 suggests that the L1544P mutation causes a cell-surface expression defect that is due to changes in the L1544P mutant channel.
expression of L1544P_{AA4}-SUR1 in cells co-transfected with ΔC25Kir6.2 is accompanied by a concomitant increase in $K_{ATP}$ currents that cannot be accounted for by the expression of ΔC25Kir6.2 homomeric channels alone.

An anterograde channel trafficking signal at the C terminus of SUR1 has been shown as necessary for efficient surface expression of $K_{ATP}$ channels (21). Leucine 1544 is located near the C terminus of SUR1 (human SUR1 has 1582 amino acids). We cannot rule out the possibility that L1544 is part of an anterograde trafficking signal. However, whatever negative effect the L1544P mutation has on the anterograde trafficking signal, it is overcome by eliminating the ER retention signal.

The fact that ~20% L1544P-SUR1 mutant channels do reach the cell surface indicates that the mutant SUR1 is able to assemble with Kir6.2 to form functional channels. Nevertheless, it is possible that the L1544P mutation could interfere with the ability of SUR1 to physically associate with Kir6.2, thereby reducing the number of properly assembled channel complexes. To address this issue, we examined surface expression of a SUR1-Kir6.2 dimer containing the L1544P mutation. This dimer construct links the C terminus of SUR1 to the N terminus of Kir6.2 and forces physical association between the two subunits. The wild-type dimer construct has been previously shown to form functional channels with proper surface expression (12, 14, 24). We found that the L1544P-SUR1-Kir6.2 dimer did not correct the trafficking defect caused by the mutation (data not shown), arguing that the mutation is unlikely to reduce channel expression simply by preventing SUR1 from associating with Kir6.2.

**Comparison between the L1544P-SUR1 Mutation and Other SUR1 Mutations That Cause Defective $K_{ATP}$ Channel Trafficking**—In addition to L1544P, two other PHHI-SUR1 mutations, ΔF1388 and R1394H, have been reported to cause defective $K_{ATP}$ channel trafficking (22, 23). The ΔF1388 mutation differs from the L1544P mutation in several respects. First, the ΔF1388 mutation completely abolishes surface expression of $K_{ATP}$ channels. Second, the inactivation of the RKR ER retention signal in ΔF1388-SUR1 only led to minimal surface expression of the protein both in the presence and absence of Kir6.2 (22) (Fig. 5A). Third, the co-expression with Kir6.2ΔC25 did not increase surface expression of ΔF1388-SUR1.2 These results show that the ΔF1388 mutation causes retention of SUR1 even after the removal of the RKR signal possibly by causing protein misfolding. In contrast, L1544P does not hinder the trafficking of SUR1 as long as the retention signal in SUR1 is inactivated, suggesting that the mutation is unlikely to cause major misfolding of the protein.

The R1394H mutation has recently been reported to cause channel retention in the Golgi compartment when expressed in HEK293 cells. Furthermore, the trafficking defect of R1394H-SUR1 could be corrected by diazoxide treatment, which was blocked by glibenclamide (23). We did not observe significant effects of either diazoxide or glibenclamide on surface expression of the L1544P-SUR1 mutant channels. Therefore, the potential therapeutic value of $K_{ATP}$ channel drugs in correcting trafficking defects is limited to specific mutations.

In summary, we have identified a novel SUR1 mutation, L1544P, in a patient with the severe form of PHHI. The result-