Membrane Targeting of ATP-sensitive Potassium Channel

EFFECTS OF GLYCOSYLATION ON SURFACE EXPRESSION*

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Oligosaccharides play significant roles in trafficking, folding, and sorting of membrane proteins. Sulfonylurea receptors (SURx), members of the ATP binding cassette family of proteins, associate with the inward rectifier Kir6.x to form ATP-sensitive potassium channels (K_ATP). These channels are found on the plasma membrane in many tissues and play a pivotal role in synchronizing electrical excitability with cell metabolic state. Trafficking defects resulting from three independent SUR1 mutations involved in the disease persistent hyperinsulinemic hypoglycemia of infancy have been described. Two of these mutations displayed notable decreases in glycosylation. Here we have investigated the relationship between the two N-linked glycosylation sites (Asn10 and Asn1050) and SUR1 trafficking. Using patch clamp analysis, surface biotinylation, and immunofluorescence microscopy, we demonstrate a significant decrease in surface expression of SUR1 single or double glycosylation site mutants (N10Q,N1050Q) when co-expressed with Kir6.2. Additionally, we show prominent retention within the ER of the SUR1 double glycosylation mutant under the same conditions. Further investigation revealed that mutation of the ER retention signal was able to partially restore surface expression of the SUR1 double glycosylation mutant. These studies suggest that SUR1 glycosylation is a key element for the proper trafficking and surface expression of K_ATP channels.

N-Linked oligosaccharides have been shown to play a significant role in trafficking, folding, and sorting of many membrane proteins (for review, see Ref. 1). In the endoplasmic reticulum (ER),1 oligosaccharides are added as “core glycans” during protein synthesis (for review, see Ref. 2). Initially, 14 sugar moieties are added. Following export to the Golgi, the sugar moieties are trimmed back, and additional glycan moieties are then added. This “mature” protein is then shuttled to the plasma membrane (2). The specific role of glycosylation in protein trafficking, stability, and function has been investigated in many systems (3–7). The effect that glycosylation has on the trafficking of the sulfonylurea receptor (SURx) is noteworthy in light of recent evidence that shows a defect in SUR1 trafficking can lead to the severe disease, persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (8–10). This study addresses the influence of glycosylation in the trafficking of the sulfonylurea receptor, SUR1. SURx, a member of the ATP binding cassette family of proteins, associates with the inward rectifier Kir6.x to form ATP-sensitive potassium channels (K_ATP) (11–15). K_ATP channels are found on the plasma membrane in many tissues and play a pivotal role in synchronizing electrical excitability of the cell with its metabolic state (16). These subunits together coordinate ATP and ADP binding with channel opening and closing. SURx regulates the flow of potassium ions through Kir6.x. Different isoforms of SURx and Kir6.x contribute to distinct nucleotide binding affinities and orchestrate tissue-specific electrical responses to metabolism (17). While K_ATP channels have widespread distribution, including expression in the brain and heart, the role of K_ATP channels has been best characterized in pancreatic β cells where it is a key mediator of insulin secretion (18, 19). Increases in blood glucose cause closure of pancreatic K_ATP channels, triggered by an increase in the cellular ATP to ADP ratio. This results in membrane depolarization, Ca2+ influx and insulin release (for review, see Refs. 20–22). Furthermore, mutations within the SUR1 and Kir6.2 genes have been shown to result in PHHI (23). To date, three of the mutations in SUR1 that result in PHHI have been demonstrated to affect protein trafficking specifically (8–10). While the SUR1 ΔF1388 and SUR1 L1544P mutations result in impaired function as well as retention in the ER, the SUR1 R1394H mutation results in retention within the Golgi network (8–10).

Sulfonylurea drugs and potassium channel openers bind to SURx directly and have been used extensively to regulate K_ATP channel activity (24, 25). Accordingly, SUR1 is a drug target in the treatment of PHHI, type II diabetes, as well as other conditions involving SUR2 such as hypertension (for review, see Ref. 20). Understanding the mechanism of trafficking and protein maturation of SURx is a fundamental first step in resolving its role in disease. SURx is a multispanning integral membrane protein with a predicted molecular mass of ~170 kDa. Data support a topology model containing 17 transmembrane segments (26–28). Both SURx isoforms contain two N-linked glycosylation acceptor sites, and SURx glycosylation results in core and mature forms of the protein (11, 13, 27–29). SUR1 is glycosylated on the N terminus (Asn19) and the external loop following transmembrane segment 12 (Asn1059) (13, 27). Four subunits of SURx are predicted to surround the pore formed by a Kir6.x tetramer, resulting in a heterooctomer (13). This complicated structure presumably comes together in the ER. Both SURx
and Kir6.1x have been shown to contain ER retention signals (30). It has been suggested that the physical interaction between SURx and Kir6.1x masks the respective ER retention signals allowing export to the Golgi and then the plasma membrane.

Recent studies of voltage-gated potassium channels showed that glycosylation promotes surface expression (5, 31). Another study, however, of the inward rectifier potassium channel Kir3.1 showed no effect of glycosylation (6). While specific correlative studies on SUR1 glycosylation and the surface expression of $K_{\text{ATP}}$ have not been carried out, SUR1 has been shown to have an increase in surface expression as well as glycosylation with mutation of the ER retention signal in the absence of Kir6.2 (30). Furthermore, the SUR1 AF1388 and SUR1 L1544P mutants that lacked surface expression displayed diminished glycosylation (8, 10).

Here we have investigated the relationship between the glycosylation of SUR1 and surface expression of $K_{\text{ATP}}$. We utilized patch clamp analysis, surface immunofluorescence localization, and surface biotinylation techniques to demonstrate a significant decrease in the surface expression of SUR1 in the absence of glycosylation. Additionally, we show prominent retention within the ER of the SUR1 double glycosylation mutant. Furthermore, we characterized the influence of Kir6.2 with regard to SUR1 glycosylation as well as in the facilitation of SUR1 to the plasma membrane.

**MATERIALS AND METHODS**

**Construction of Mutants**

In all constructs, hamster SUR1 was tagged at the C terminus with the V5 epitope by subcloning into the vector pcDNA3.1/V5HisA (Invitrogen). N-Linked glycosylation sites were removed by replacing asparagine 10 and/or asparagine 1050 with glutamines (SUR1 N10Q, SUR1 N1050Q, and SUR1 N10Q,N1050Q) (27). SUR1 AF1388 was introduced into SUR1 with the V5 epitope in pcDNA3.1/V5HisA with ligation (8). In some constructs, denoted SUR1AAA, the endoplasmic reticulum retention signal RKR (amino acids 648–650) was replaced with alanines. Constructs used in immunofluorescence also contain the M2 FLAG tag at external amino acid position 337 (28). All mutations were sequenced (27, 28), and all constructs were confirmed with restriction enzyme digests and/or sequencing.

**Electrophysiology**

COS-1 cells were transiently transfected with constructs encoding SUR1 (0.7 μg), rat Kir6.2 (0.4 μg) (27), and pEGFP (0.1 μg) (CLONTECH), a plasmid encoding enhanced green fluorescent protein (EGFP) transfected with FuGene6 (Roche Molecular Biochemicals), and were plated onto cover-slips. Patch clamp recordings from excised inside-out patches were made 48–72 h following transfection. The standard bath (intracellular) and pipette (extracellular) recording solution (K-INT) contained 140 mM KCl, 20 mM Hepes, pH 7.5, and three 10-min washes with PBSCM followed each antibody incubation. Following fixation with cold methanol (–20 °C, 15 min), cells were washed and mounted onto slides with Pro-Long (Molecular Probes) mounting medium. Cells were visualized with a ×20 objective lens using an Olympus BX60 epifluorescent microscope, and images were recorded with an Optronics CCD-camera (DCC-750).

**RESULTS**

**Glycosylation of SUR1 and SUR1 Glycosylation Mutants**—To examine the effect of glycosylation in SUR1 trafficking and surface expression, the two N-linked glycosylation sites on SUR1 were replaced with glutamines to form single glycosylation mutants (SUR1 N10Q, SUR1 N1050Q) and double site glycosylation mutant (SUR1 N10Q,N1050Q). When co-expressed with Kir6.2, wild type (WT) SUR1 and single glycosylation mutants showed two immunoreactive bands corresponding to immature core glycosylated SUR1 and mature complex glycosylated SUR1 as described previously (27) (Fig. 1). The complex glycosylated products showed characteristic shifts in mobility with each single glycosylation mutant displaying a decrease in apparent molecular mass when compared with WT SUR1. While the SUR1 N10Q complex and core glycosylated bands showed distinct separation from one another, the SUR1 N10Q,N1050Q double mutant produced only a single

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**Glycosylation Promotes Surface Expression of SUR1**

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**Tunicamycin Treatment**

COS-1 cells were transiently transfected with SUR1 constructs using FuGENE 6 (Roche Molecular Biochemicals). Twenty-four h following transfection, tunicamycin (1 μg/ml) was added to the medium. Twenty-four h following addition of tunicamycin, cells were harvested by addition of lysis buffer as described above. Proteins were subject to SDS-PAGE analysis, and SUR1 was visualized using enhanced chemiluminescence.
The affect of SUR1 N with WT SUR1 when co-expressed with Kir6.2 (Fig. 2, striking and significant decrease in channel activity compared with WT SUR1. SUR1 N10Q,N1050Q displayed a reduced magnitude of the double mutant SUR1 N10Q,N1050Q was inhibited by the addition of 1 mM ATP.

COS-1 cells. In addition, all currents were completely inhibited for all glycosylation mutants (SUR1 N10Q, SUR1 N1050Q, and SUR1 N10Q,N1050Q) yielded functional channels when co-expressed with Kir6.2 in transiently transfected COS-1 cells. In addition, all currents were completely inhibited by the addition of 1 mM ATP.

To quantitatively examine $K_{\text{ATP}}$ channel expression, the current magnitude of the double mutant SUR1 N10Q,N1050Q was compared with WT SUR1. SUR1 N10Q,N1050Q displayed a striking and significant decrease in channel activity compared with WT SUR1 when co-expressed with Kir6.2 (Fig. 2, A and B). Currents in the double mutant were reduced to only 11% of WT SUR1 levels, and this corresponded to both a decrease in the number of patches expressing current. These data suggest that there is a dramatic reduction in $K_{\text{ATP}}$ channels on the plasma membrane when the SUR1 subunit lacks glycosylation.

Surface Labeling of SUR1 Is Reduced in the Absence of Glycosylation—To determine whether the decrease in current observed in Fig. 2 was a consequence of reduced surface expression, immunofluorescent labeling of surface SUR1 was carried out to assess surface expression. Transiently expressed SUR1 and SUR1 glycosylation mutants containing a FLAG epitope tag inserted at position 337 (at the third external loop) (28) were expressed with and without Kir6.2. Live cell labeling with the anti-FLAG antibody was performed at 4 °C to avoid permeabilization or antibody uptake. Consistent with patch clamp analysis, SUR1 N10Q,N1050Q with Kir6.2 showed reduced labeling when compared with WT SUR1 with Kir6.2 (Fig. 3). As a negative control, the PHHI mutation SUR1 AF1388 demonstrated previously to lack surface labeling when assayed with immunofluorescence, was used in the absence of Kir6.2 (11). As expected, there was no external labeling of SUR1 AF1388 (Fig. 3) nor was there external labeling of SUR1 or the glycosylation mutants in the absence of Kir6.2 (data not shown). The decrease in surface labeling of the double glycosylation mutant in the presence of Kir6.2 was due to an alteration in trafficking, not a difference in protein expression, as all FLAG-tagged constructs showed expression comparable with WT SUR1 with Western blot analysis (data not shown).

Surface biotinylation was utilized to quantitate relative amounts of total SUR1 protein present on the plasma membrane and thus accessible to external labeling. The cysteine-modifying reagent, biotin maleimide, was used to label endogenous external cysteines of transiently transfected COS-1 cells
expressing SUR1. In a previous study of the transmembrane topology of SUR1, we showed that this assay labels endogenous cysteine residues on the external side of SUR1 residing on the plasma membrane, and the reagent does not permeate the cysteine residues on the external side of SUR1 residing on the top of SUR1, we showed that this assay labels endogenous expressing SUR1. In a previous study of the transmembrane age of the total SUR1 and represents a lower limit for the amount of SUR1 on the plasma membrane. Multiple experiments were analyzed and plotted (Fig. 4B). At least 5% of WT SUR1 was labeled on the plasma membrane of COS-1 cells. The double glycosylation mutant displayed only 1.3% surface labeling of SUR1. This reduction corresponds to a decrease to 26% in the surface labeling of mutant compared with WT SUR1 when accounting for the total amount of SUR1 protein expressed. The single glycosylation mutants (SUR1 N10Q, and SUR1 N1050Q) were also investigated and similarly showed a significant decrease in surface expression (Fig. 4B). Accordingly, biotin maleimide labeling for all of the glycosylation mutants was similar or slightly greater than the negative control SUR1 ΔF1388.

Additionally, the apparent molecular mass of the surface-labeled, pulled down SUR1 could be used to infer the glycosylated state of SUR1 on the plasma membrane. While both the immature and mature forms of WT SUR1 were present in whole cell lysates, only the higher apparent molecular mass, mature SUR1 was surface-labeled and pulled down (Fig. 4A). This indicates that only mature glycosylated SUR1 was on the plasma membrane when the native glycosylation sites were available. This trend also was observed for both single glycosylation mutants, SUR1 N10Q and SUR1 N1050Q. An additional upper band also was observed for WT SUR1, most likely a result of aggregation that occurs at the high temperature required to elute biotinylated SUR1 from the NeutrAvidin beads (28). Thus, glycosylation promotes surface expression of SUR1 and is clearly indicative of this surface expression.

Subcellular Localization of SUR1 Double Glycosylation Mutant Reveals Prominent Retention within the ER—The bulk of the quality control aspects of trafficking have been attributed to the ER. While many protein mutants that display defects in trafficking are retained within the ER, one SUR1 mutation shows retention within the Golgi (9). To determine subcellular distribution of SUR1 or SUR1 N10Q,N1050Q, immunofluorescent labeling of permeabilized COS-1 cells expressing SUR1 constructs, in the presence of Kir6.2, was investigated. Previous studies with SUR1 ΔF1388 revealed an ER retention-like pattern by immunofluorescence microscopy (8). COS-1 cells were co-transfected with the plasmids encoding enhanced cyan fluorescent protein fused to a sequence targeted to the endoplasmic reticulum or the Golgi (pECFP-ER or pECFP-Golgi). This enabled simultaneous fluorescence visualization of SUR1 with either the ER or the Golgi. WT SUR1 + Kir6.2 showed distinct plasma membrane staining, which was only rarely observed with SUR1 N10Q,N1050Q + Kir6.2, and almost never with SUR1 ΔF1388 (Fig. 5, A and B, arrowheads). In addition, labeling of SUR1 + Kir6.2, SUR1 ΔF1388, and SUR1 N10Q,N1050Q + Kir6.2 all displayed prominent ER co-localization with pECFP-ER (Fig. 5A).

Retention of SUR1 within the Golgi network was described recently for the PHHI mutant SUR1 R1394H in the presence of Kir6.2 (9). Examination of the SUR1 labeling pattern with all constructs in the presence of Kir6.2 indicated that labeling was never restricted to the Golgi (Fig. 5B). While WT SUR1 + Kir6.2 tended to overlap with the Golgi at low intensity, SUR1 ΔF1388 or SUR1 N10Q,N1050Q + Kir6.2 did not generally overlap with the pECFP-Golgi marker. However, individual cells that showed high levels of SUR1 expression sometimes appeared co-localized with the Golgi network, possibly due to actual Golgi localization or nearby ER localization surrounding the Golgi. These data suggest that WT SUR1 + Kir6.2 was present in the plasma membrane as well as Golgi and ER compartments, whereas the double glycosylation mutant was mainly retained in the ER as a result of ER quality control.

Kir6.2 Promotes Surface Expression of SUR1—Previous ex-
experiments have suggested that Kir6.2 association is necessary for the proper trafficking of SUR1 (30). Another study, however, suggested that SUR1 could traffic to the plasma membrane alone (32). To examine in more detail the role of Kir6.2 in the facilitation of SUR1 surface expression, surface biotinylation was carried out in the presence or absence of Kir6.2 (Fig. 6). As expected, a significant decrease in the surface expression of SUR1 was noted in the absence of Kir6.2. However, the presence of some labeling suggested that some SUR1 was capable of trafficking to the plasma membrane without Kir6.2. These data were contrasted with immunofluorescence surface labeling in which signal was not detected in the absence of Kir6.2. However, the presence of some labeling suggested that some SUR1 was capable of trafficking to the plasma membrane without Kir6.2. These data were contrasted with immunofluorescence surface labeling in which signal was not detected in the absence of Kir6.2 (data not shown). This may be explained by differences in assay sensitivity. Biotinylation of the glycosylation mutants in the absence of Kir6.2 showed further reduction in surface biotinylation, comparable with the SUR1 ΔF1388 mutant (Fig. 6), and also lacked positive labeling with surface immunofluorescence data (data not shown). These data suggest that both glycosylation and association with Kir6.2 are determinants of surface expression.

**Mutation of the ER Retention Signal Partially Restores Surface Expression**—To investigate the role of the ER retention signal with regard to glycosylation in SUR1 surface expression, surface biotinylation was carried out in SUR1 constructs with the ER retention motif RKR mutated to AAA (SUR1AAA). Previous studies have shown that SUR1AAA has increased surface expression in the absence of Kir6.2 (30) and is able to partially restore surface expression in the SUR1AAA ΔF1388 mutant (8). In agreement with this expectation, surface biotinylation indicated a significant increase in the surface expression of SUR1AAA with or without Kir6.2 compared with WT SUR1 with Kir6.2 (Fig. 7). Additionally, mutation of the ER retention signal sustained partial recovery of SUR1AAA N10Q,N1050Q surface expression in the presence or absence of Kir6.2 (Fig. 7). The ability of the AAA mutation to restore surface expression was clearly very low. Glycosylation still played the major role in surface expression in the absence of the RKR endoplasmic reticulum retention signal, as seen by the greatly reduced surface expression that was present for all of the glycosylation mutants.

**Kir6.2 Influences Glycosylation State of SUR1**—The influence of Kir6.2 in promoting the complex glycosylation of SUR1 has been documented previously (13). Presumably, this glycosylation is facilitated by promoting the ER export of SUR1. The ability of SUR1AAA to traffic to the plasma membrane without Kir6.2 allows for insight into the influences of Kir6.2 on the glycosylation of SUR1. SUR1AAA displays prominent glycosylation in the absence of Kir6.2 (30). Close inspection of SUR1 bands on Western blots shows a significant and consistent increase in the apparent molecular mass of the glycosylated band of SUR1AAA in the absence of Kir6.2 compared with WT SUR1 in the presence of Kir6.2 as well as SUR1AAA when co-expressed with Kir6.2 (Fig. 7, arrowheads). The increase in apparent molecular mass of SUR1AAA and SUR1AAA glycosylation mutants (SUR1AAA N10Q, and SUR1AAA N1050Q) was demonstrated to be a result of oligosaccharide modification. Incubation of transfected cells with tunicamycin, an antibiotic that prevents N-linked glycosylation, eliminated the upper band (Fig. 8). Both SUR1AAA N10Q and SUR1AAA N1050Q show increased apparent molecular mass in the absence of Kir6.2 (Fig. 7, Input, right panel), which decreased toward the size of SUR1 N10Q and SUR1 N1050Q, respectively, in the presence of Kir6.2 (Fig. 7, Input, left panel). This suggests that the interaction with Kir6.2 influences the degree of glycosylation. Thus, both Asn and Asn sites appear to have an increase in the degree of glycosylation of SUR1AAA in the absence of Kir6.2.

**DISCUSSION**

Mutagenesis studies have demonstrated the presence of two N-linked glycosylation sites on SUR1 at positions Asn and...
Glycosylation promotes surface expression of SUR1 and SUR1 AAA glycosylation constructs shows that mutation of the ER retention signal partially rescues the surface expression of SUR1 in the presence or absence of Kir6.2. Surface biotinylation of endogenous external cysteines was performed as described in the legend to Fig. 3. Top panels, input of total cell lysate (5% of the total volume used for the pull down with NeutrAvidin beads), visualized following SDS-PAGE analysis with anti-V5 antibody. Bottom panels, biotinylated surface proteins pulled down with NeutrAvidin beads, visualized identical to input. Molecular mass markers are shown to the left.

Glycosylation of SUR1 Accompanies Surface Expression—Previous studies have suggested that core glycosylation is associated with ER pools of protein, while more complexly glycosylated products can be correlated with Golgi and plasma membrane pools of protein (for review, see Ref. 2). The position of immunoreactive bands pulled down from biotinylated cell surface proteins suggests that the SUR1 present on the plasma membrane is composed of the mature, complex glycosylated form (Figs. 4 and 7). Moreover, when just one glycosylation acceptor site was available (Asn<sup>10</sup> or Asn<sup>1050</sup>), still the mature form of SUR1, exclusively, was present on the plasma membrane. Only when glycosylation was completely prevented with the double glycosylation mutant did a small amount of the SUR1 nonglycosylated form traffic to the plasma membrane (Fig. 4). We believe these data reflect the character of SUR1 in vivo, as earlier studies that investigated the subcellular localization of SUR1 from insulin secreting cells also suggested association of mature glycosylated SUR1 with plasma membranes (33).

Glycosylation Promotes Surface Expression of K<sub>AATP</sub> Channels—To investigate the influence of SUR1 glycosylation on K<sub>AATP</sub> surface expression, channel activity was examined with patch clamp analysis. Prevention of glycosylation at both Asn<sup>10</sup> and Asn<sup>1050</sup> sites showed a dramatic decrease in K<sub>AATP</sub> channel activity (Fig. 2). Surface immunofluorescent labeling (Fig. 3) as well as biotinylation data (Fig. 4) demonstrated that the absence of a glycosylation acceptor site reduced surface expression of SUR1. Quantitation of surface biotinylation data demonstrated a significant decrease in surface expression when either site was not glycosylated (Fig. 4). These data indicate that both glycosylation sites are important in promoting the surface expression of SUR1. Qualitatively, surface immunolabeling as well as surface biotinylation data suggest that glycosylation at position Asn<sup>10</sup> has a greater impact on surface expression of SUR1 (Figs. 4 and 7). Sequence comparison with SUR2A and SUR2B reveals preservation of this glycosylation site at the N terminus, while the second glycosylation acceptor site is variable in position (residue Asn<sup>1050</sup> in SUR1 and predicted at residue Asn<sup>298</sup> in SUR2). The similarity in sequence and conservation of the Asn<sup>10</sup> glycosylation site suggests it is likely that SUR2A and SUR2B would be influenced by glycosylation in a similar manner to SUR1.

Glycosylation Mutants Result in Endoplasmic Reticulum Retention—The endoplasmic reticulum has been implicated as a checkpoint for protein quality control (for review, see Ref. 34). Localization of the double glycosylation mutant in the ER (Fig. 5) suggests that unglycosylated SUR1 does not meet proper ER criteria for further transit to the Golgi. Previous studies have identified an RKR motif as an ER retention signal in both SUR1 and Kir6.2. In these studies, it was shown that mutation of the RKR motif to alanines negated the requirement of SUR1 co-assembly with Kir6.2 (30). These data led to the suggestion that SUR and Kir6.2 physically mask their respective ER retention signals. Interestingly, our studies indicate that mutation of the ER retention signal only partially overrides the retention of the SUR1 glycosylation mutants (Fig. 7). This suggests that the mechanism involved in retention of the double glycosylation mutant is independent of the RKR retention signal and thus independent of association with Kir6.2. Indeed, association with Kir6.2, as assayed by co-immunoprecipitation, seemed unaffected by the absence of both glycosylation sites (data not shown). Decreased protein stability of other membrane proteins has been attributed to a lack of glycosylation (5, 7, 35), raising the possibility that the residing immature glycosylated SUR1 may be destined for degradation. While overall protein levels of the glycosylation mutants were comparable with that of WT SUR1, the rate of protein turnover was not investigated specifically.

Drugs or substrates have been shown to increase the stability of various proteins during assembly, resulting in higher
expression on the cell surface (36–38). Indeed, diazoxide was shown recently to facilitate the surface expression of the SUR1 R1394H trafficking mutant associated with PHHI (9). To address whether drugs could increase the stability of SUR1 N10Q,105Q and consequently increase surface expression, surface biotinylation was carried out following 24-h incubation with sulfonylureas or potassium channel openers. The addition of diazoxide or glibenclamide had no apparent impact on the surface expression of SUR1 N10Q,N105QQ (data not shown).

Kir6.2 Influences Surface Expression and Glycosylation of SUR1—The role that Kir6.2 plays in the facilitation of SUR1 to the plasma membrane has been well documented (30). Previous studies, however, have suggested that SUR1 also can traffic to the plasma membrane in the absence of Kir6.2 (32). Here we used immunofluorescence and surface biotinylation assays to ascertain the importance of Kir6.2 for surface expression of SUR1. Our studies suggest that co-expression of Kir6.2 has a significant impact on the surface expression of SUR1. While no surface labeling was observed with immunofluorescence in the absence of Kir6.2, weak surface labeling was observed with surface biotinylation. This discrepancy may be due to differences in assay sensitivity. Additionally, the presence of SUR1 at low levels on the plasma membrane in the absence of Kir6.2 may be a result of overexpression in the COS-1 expression system.

The influence of Kir6.2 on glycosylation of SUR1 was also investigated. Initial assessment of the SUR1 single glycosylation mutants co-expressed with Kir6.2 showed a difference in apparent molecular mass of the complex glycoproduct expressed with each mutant. This suggests that there is a qualitative difference in the sugar moieties at each N-linked glycosylation site. The greater apparent molecular mass of the SUR1 N10Q upper band compared with the SUR1 N105Q upper band suggests that the Asn1050 site is glycosylated more extensively. Furthermore, an increase in the apparent molecular mass of the SUR1AA upper complex glycosylated band when compared with WT SUR1 co-assembled with Kir6.2 suggests that SUR1AA is glycosylated to a greater extent than WT SUR1 co-assembled with Kir6.2. This influence of Kir6.2 on the glycosylation state of SUR1AA was surprising. A decrease in the apparent molecular mass of the upper SUR1 band for each glycosylation site in the presence of Kir6.2 suggests some form of interaction with Kir6.2. A physical interaction may directly alter accessibility to glycosylating enzymes by physically blocking the site or causing a conformational change that alters the magnitude of glycosylation. Alternatively, interaction may change the residence time in the Golgi, resulting in a modification of synthesis or trimming of sugar moieties. Further experiments are required to assess the nature of interaction.

Appropriate protein trafficking of K<sub>ATP</sub> channels is essential for accurate insulin secretion. Recently, three mutations of SUR1, resulting in the disease PHHI, have been attributed to trafficking defects (8–10). SUR1 A1388 mutation, suggested to result in ER retention, displayed diminished glycosylation in the presence of Kir6.2 as did the SUR1 L1544P mutant (8, 10). Elucidating the dependence of K<sub>ATP</sub> surface expression with SUR1 glycosylation and Kir6.2 association is important in creating treatments targeted at overcoming trafficking defects. The significance of SUR1 glycosylation to proper K<sub>ATP</sub> protein trafficking is necessary to understand its role in disease.

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