In beta cells from the pancreas, ATP-sensitive potassium channels, or K<sub>ATP</sub> channels, are composed of two subunits, SUR1 and K<sub>IR</sub>6.2, assembled in a (SUR1/K<sub>IR</sub>6.2)<sub>4</sub> stoichiometry. The correct stoichiometry of this form of hyperinsulinism is a failure of K<sub>ATP</sub> channels to exit the ER/cis-Golgi compartments. In addition to these retrograde signals, we show that the C terminus of SUR1 has an anterograde signal, composed in part of a dileucine motif and downstream phenylalanine, which is required for K<sub>ATP</sub> channels to exit the ER/cis-Golgi compartments and transit to the cell surface. Deletion of as few as seven amino acids, including the phenylalanine, from SUR1 markedly reduces surface expression of K<sub>ATP</sub> channels. Mutations leading to truncation of the C terminus of SUR1 are one cause of a severe, recessive form of persistent hyperinsulinemic hypoglycemia of infancy. We propose that the complete loss of beta cell K<sub>ATP</sub> channel activity seen in this form of hyperinsulinism is a failure of K<sub>ATP</sub> channels to traffic to the plasma membrane.

In pancreatic beta cells, the high affinity sulfonylurea receptor, SUR1<sup>1</sup>, and the potassium inward rectifier, K<sub>IR</sub>6.2, combine to form octameric ATP-sensitive potassium channels, K<sub>ATP</sub> channels, that link glucose metabolism to membrane potential (1–5). These channels play a key role in the regulation of insulin secretion, and loss of K<sub>ATP</sub> channel activity has been shown to cause a severe, recessive form of congenital or neonatal hyperinsulinism, designated HI-SUR1 or HI-KIR6.2, depending on which subunit harbors the mutation (5). K<sub>IR</sub>6.2 forms the channel pore that is regulated by SUR1, and both subunits are required to form a fully functional channel sensitive to ATP, MgADP, sulfonylureas, and potassium channel openers (for reviews, see Refs. 4–7). C-terminal truncated K<sub>IR</sub>6.2 subunits generate homomeric K<sup>+</sup> channels, (K<sub>IR</sub>6.2)<sub>4</sub>, in the absence of SUR1 that have the correct conductance and are weakly inhibited by ATP but have altered kinetics and lack the other properties of wild-type K<sub>ATP</sub> channels (8–10). Zerangue et al. (11) have identified a novel endoplasmic reticulum retention (ER) or retrograde signal in the C terminus of K<sub>IR</sub>6.2 and in K<sub>IR</sub>6.2. The same motif is found in SUR1 and SUR2 on the N-terminal side of NBF1. Deletion or mutation of the K<sub>IR</sub> signal permits surface expression of K<sub>IR</sub> subunits without SUR1, whereas mutation of the SUR1 signal gives surface expression without a K<sub>IR</sub>. These signals are proposed to serve as a quality control mechanism that ensures only the surface expression of properly assembled octameric channels (11). We show here that there is an additional level of regulation of trafficking; the C terminus of SUR1 has an anterograde signal that is required for surface expression of K<sub>ATP</sub> channels. The deletion of this anterograde signal can account for the loss of channel activity in some HI-SUR1 mutations.

**EXPERIMENTAL PROCEDURES**

The hamster SUR1 cDNA (1), encoding 1582 amino acids, in the pECE vector (12) was truncated by introducing stop codons at positions 1581 (SUR1<sup>ΔC2</sup>), 1579 (SUR1<sup>ΔC4</sup>), 1576(SUR1<sup>ΔC7</sup>), 1570(SUR1<sup>ΔC13</sup>), 1561(SUR1<sup>ΔC22</sup>), and 1534(SUR1<sup>ΔC49</sup>) using conventional polymerase chain reaction. The amino acid substitutions, V1578A, F1574A, K1580A, and V1576A, were introduced into the SUR1<sup>ΔC49</sup> mutant by overlapping mutagenesis. SUR1<sup>ΔC49</sup> was generated by using two-overlap polymerase chain reaction using overlapping mutagenic primers. PECE<sub>570</sub>-SUR1 and pECE<sub>570</sub> the kind gift of Dr. Susumu Seino (Chiba University, Chiba Japan), was subcloned into the pECE vector before use. Mouse K<sub>IR</sub>6.2 (2), the kind gift of Dr. Susumu Seino (Chiba University, Chiba Japan), was subcloned into the pECE vector before use. The resulting constructs were sequenced to confirm the introduction of the desired mutation.

**COSm6 cells** were cultured and transfected as described previously (1, 3). Rubidium efflux assays to measure specific glibenclamide-inhibited efflux were done as described (3, 13).

Membranes were prepared from transfected COSm6 cells grown on 150-mm plates. Cells were washed twice with PBS, pH 7.4, and collected by inverting at 4 °C with PBS supplemented with 2 mM EDTA. The cells were pelleted, resuspended in 200–1000 μl of hypotonic buffer (5 mM Tris-HCl, 2 mM EDTA, 0.1 mM NaCl, pH 7.4) and allowed to swell for 40 min on ice. Cells were homogenized, centrifuged at 1000 × g for 10 min at 4 °C. For further purification, the supernatant was collected and centrifuged at 40,000 × g for 60 min. The pelleted membranes were resuspended in 300 μl of membrane buffer supplemented with protease inhibitors (Complete<sup>™</sup>, Roche Molecular Biochemicals) (50 mM Tris-HCl, 5 mM EDTA; pH 7.4) and stored at −80 °C. The protein concentrations varied from 2–5 mg/ml. Membranes were photolabeled with 125<sup>I</sup>-iodoazidoglibenclamide, kindly provided by Professor Uwe Panten (University of Braunschweig, Braunschweig, Germany), as described (3, 14).

The appearance of SUR1 at the cell surface was quantified using a luminometer-based assay to measure SUR1<sub>c</sub>-myc. Transfected COSm6 cells were gently washed in Kreb’s Ringer PBS and incubated for 1 h at 25 °C with the mouse monoclonal IgG<sub>c</sub>-myc antibody (9E10, Santa Cruz Biotechnology) diluted in Dulbecco’s modified Eagles’ medium.
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**RESULTS**

Deletion of the C terminus of SUR1 affects $K_{ATP}$ channel activity measured here by a $^{86}$Rb$^+$ efflux assay. The SUR1AC2 and ΔC4/KIR6.2 channels show glibenclamide-inhibited $^{86}$Rb$^+$ efflux attributable to $K_{ATP}$ channels which is comparable with wild-type channels (Fig. 1A); the SUR1AC7/KIR6.2 channels show approximately a 50% decrease in activity, while a complete loss of activity is observed for the SUR1AC13, ΔC22, and ΔC49/KIR6.2 channels. The results suggest that the last 5 to 13 amino acids of the C terminus of SUR1 are essential for obtaining channel activity. To determine whether the SUR1 C terminus was masking the ER retention signal on KIR6.2 (11), we analyzed the K ATP channel activity as shown in Fig. 1B; the SUR1AC7/KIR6.2 versus SUR1AC/KIR6.2 versus SUR1AC/KIR6.2 channel (Fig. 1A), indicating that the loss of channel activity resulting from deletion of the C terminus of SUR1 does not depend on the presence of the ER retention signal on KIR6.2.

SUR1 is differentially glycosylated, exhibiting a mature or complex glycosylated form (150–170 kDa) and an immature or core glycosylated form (140 kDa) (3, 16). Mature SUR1 is present only when the receptor is co-expressed with KIR6.1 or KIR6.2 and has been shown to assemble with KIR6.2 to form active $K_{ATP}$ channels in the plasma membrane (3). The mature receptor is resistant to Endo H, whereas the immature, core glycosylated species is deglycosylated to a 137-kDa species (data not shown). Endo H removes high mannosyl oligosaccharide chains that are added in the ER, thus the appearance of an Endo H-resistant form indicates processing of the oligosaccharides beyond the cis-Golgi. Co-expression of SUR1C2 or ΔC4 with KIR6.2 yields both the immature and mature glycosylated forms of SUR1 as shown in Fig. 1B using $^{125}$I-iodoazidoglibenclamide to identify the receptors. SUR1AC7, ΔC13, ΔC22, and ΔC49 show essentially a complete lack of complex glycosylation. The results are consistent with either a defect in the processing of oligosaccharides on the C-terminal truncated receptors, with a failure of the receptors to traffic to the medial Golgi apparatus and thus the cell surface, or with a failure of subunits to co-assemble.

We used luminometry to quantify the appearance of SUR1 in subunits at the cell surface (Fig. 1C). The results confirm the observation that co-expression of SUR1 and KIR6.2 markedly increases their surface expression (11). Co-expression of KIR6.2

**FIG. 1. Analysis of $K_{ATP}$ channels assembled from KIR6.2 and SUR1AC subunits.** A, relative $K_{ATP}$ channel activity determined by measuring the glibenclamide-inhibited efflux of $^{86}$Rb$^+$ from COSm6 cells transfected either with wild-type KIR6.2 (open bars) or KIR6.2ΔC35 (striped bars) and the indicated SUR1AC constructs. The data are expressed as a percentage of the value obtained with full-length SUR1. B, comparison of the relative amounts of mature and immature receptors produced by expression of the SUR1AC constructs with KIR6.2. The large and small arrows show the positions of the mature and immature forms of the receptors, respectively. The receptors were identified, in isolated membrane preparations, by specific photolabeling with 3 nm $^{125}$I-iodoazidoglibenclamide. The + and – lanes are photolabeling reactions done in the presence and absence of 1 μM unlabeled glibenclamide, respectively. C, surface expression of SUR1, measured in the presence (filled bars) and absence (open bars) of wild-type KIR6.2. The dotted line is an average value for the endogenous peroxidase activity in COSm6 cells which have not been transfected. The measurements were done as described under “Experimental Procedures”; the data are expressed as relative light units (RLU) per μg of protein. The error bars are S.D.; measurements were done in triplicate.

Fig. 2. SUR1AC subunits with KIR6.2. Left panel, full-length SUR1, SUR1AC49, and SUR1AC221 receptors and KIR6.2 were co-photolabeled with $^{125}$I-idoazidoglibenclamide in the presence (+) or absence (−) of 1 μM glibenclamide, respectively. In the ΔC49 + 6.2 lane note the absence of mature receptor and the specific labeling of KIR6.2. Right panel, co-immunoprecipitation, Co-IP, of N-hisSUR1AC49 and KIR6.2 using anti-his tag antibodies. SUR = full-length SUR1; Δ49 = SUR1AC49; Δ221 = SUR1AC221; 6.2 = KIR6.2; cont = SUR1 with no his tag.
with full-length SUR1c-myc produced the greatest differential. The SUR1Dc2-myc and SUR1Dc4-myc constructs give 60% of this increase, whereas further deletions reduced surface expression to background values. The loss of surface expression observed with C-terminal deletion parallels the loss of mature SUR1 (Fig. 1B) and K<sub>ATP</sub> channel activity (Fig. 1A). The results are consistent with a failure of the C-terminal deleted receptors to traffic beyond the cis-Golgi to the plasma membrane.

To determine whether K<sub>IR</sub>6.2 and the SUR1D subunits were co-assembling, we tested whether the inward rectifier would photolabel with 125I-azidoglibenclamide and co-immunoprecipitate when expressed with the truncated receptors. Fig. 2 (left panel) shows that when SUR1A249 or SUR1A221 are co-expressed with K<sub>IR</sub>6.2, both the receptor and inward rectifier are photolabeled consistent with their physical association with SUR1. We have previously shown that the inward rectifier alone does not label (3). Comparison with the wild-type control indicates that the mature form of the receptor is absent in both cases. The right panel in Fig. 2 demonstrates that the truncated receptor and inward rectifier co-assemble. When N-6X-hisSUR1Dc49, tagged with six histidine residues at its N terminus, is co-expressed with K<sub>IR</sub>6.2, the inward rectifier labels with 125I-iodoazidoglibenclamide and can be co-immunoprecipitated using anti-histidine tag antibodies (Fig. 2, Co-IP). The results indicate the C-terminal truncated receptors can assemble with K<sub>IR</sub>6.2 but then fail to traffic into the Golgi and plasma membrane.

To determine which amino acids in the C terminus of SUR are important, we compared the distal 25 amino acids of SUR1, SUR2A, and SUR2B (Fig. 3A). The amino acids 24, 22, 17, 9, and 5 residues from the C-terminal were conserved, whereas amino acid 16 was one of a dileucine pair. We engineered alanine substitutions at positions Val-1578, Phe-1574, Leu-1567, Leu-1566, and Asp-1561 in SUR1c-myc, co-expressed them with K<sub>IR</sub>6.2, and analyzed their channel activity and surface expression. As shown in Fig. 3, B and C, surface expression of the myc-tag in the presence (filled bars) and absence (open bars) of wild-type K<sub>IR</sub>6.2 for the same constructs. The data are expressed as relative light units (RLU) per μg of protein. The error bars are S.D.; measurements were done in triplicate.

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FIG. 3. Alanine substitutions in the C terminus of SUR1 affect the surface expression of K<sub>ATP</sub> channels. A, alignment of the C-terminal 25 amino acids of hamster and human SUR1 with mouse and human SUR2A and -2B; the conserved residues and dileucine pair are in bold face. B, relative K<sub>ATP</sub> channel activity determined by measuring the glibenclamide-inhibited efflux of 86Rb<sup>+</sup> from COSm6 cells transfected with wild-type K<sub>IR</sub>6.2 and the indicated myc-tagged SUR1 mutant constructs. C, surface expression of the myc-tag in the presence (filled bars) and absence (open bars) of wild-type K<sub>IR</sub>6.2 for the same constructs. The data are expressed as relative light units (RLU) per μg of protein. The error bars are S.D.; measurements were done in triplicate.

FIG. 4. Analysis of currents through wild-type and SUR1F1574A/K<sub>IR</sub>6.2 channels. Potassium currents through excised patches from COSm6 cells transfected with K<sub>IR</sub>6.2 plus wild-type SUR1, top trace, or SUR1F1574A, bottom trace, were recorded as described (15). The quasi-steady state sensitivity to inhibitory ATP was estimated as described (15). The relative N<sub>Po</sub> values versus [ATP] for the wild-type channels (filled circles) and SUR1F1574A/K<sub>IR</sub>6.2 channels were fit to a pseudo-Hill equation. The values obtained were: IC<sub>50</sub>(ATP) = 6.9 ± 0.3 (h = 1.05) versus 9.1 ± 0.3 (h = 1.1) for the wild-type versus SUR1F1574A/K<sub>IR</sub>6.2 channels, respectively.

Single channel recordings of the SUR1F1574A/K<sub>IR</sub>6.2 channels (Fig. 4) shows that their ATP sensitivity is similar to wild-type channels (IC<sub>50</sub>(ATP) = 8.9 ± 0.3 μM versus 6.9 ± 0.3 μM for wild type (WT)), but their surface density (N) is ~5–6 times lower than that of the wild-type, consistent with the 86Rb<sup>+</sup> efflux and surface expression results. We conclude that amino acids Phe-1574 and Leu-1566 are a part of an anterograde trafficking
signal that is required for the surface expression of $K_{\text{ATP}}$ channels.

**DISCUSSION**

These results identify an export or anterograde signal on the C terminus of the sulfonylurea receptor, SUR1, which is required for surface expression of ATP-sensitive potassium channels. The anterograde signal and the recently described ‘–RKR–’ signal in SUR and in the C termini of KIR6.1 and KIR6.2 are summarized schematically in Fig. 5. These signals act as a quality control mechanism to ensure that only fully assembled, detergent-resistant octameric ATP-sensitive potassium channels reach the plasma membrane.

The results are consistent with our previous observations on glycosylation of the receptor. The mature, complex glycosylated receptor is Endo H-resistant, whereas the immature, core-glycosylated receptor is Endo H-sensitive. The Endo H resistance of mature SUR1 indicates it has been transported from the ER and cis-Golgi to the medial and trans-Golgi where further processing takes place. The mature receptor has been shown to be associated with KIR6.2 as a large octameric complex consistent with its being in active KATP channels on the cell surface (3). Their Endo H sensitivity indicates the immature, core-glycosylated receptors are retained in the ER and cis-Golgi and do not undergo further processing. Expression of SUR1 without KIR6.1 or KIR6.2 produces the immature form of the receptor (3). This is consistent with the observed lack of surface expression of SUR1 in Xenopus oocytes (11) and in COSm6 cells (Fig. 1C) unless KIR6.1 or KIR6.2 are present and indicates the retrograde signals must be masked before the assembled channels can exit the ER and cis-Golgi. Deletion of as few as seven residues from the C terminus of SUR1 reduces the amount of mature receptor, the level of surface expression, and the density of $K_{\text{ATP}}$ channels when the receptor is expressed with KIR6.2. Co-photolabeling of SUR1 and KIR6.2 with $^{125}$I-iodoazidoglibenclamide and co-immunoprecipitation of the two subunits indicates they can assemble, which suggests the C terminus of SUR is not required for subunit association. Analysis of the currents from SUR1Phe-1574/KIR6.2 channels indicates they retain normal sensitivity to ATP, but the density of channels is reduced. Expression of KIR6.2ΔC subunits with SUR1AC subunits does not lead to surface expression of channels, indicating that the C terminus of SUR does not mask the –RKR– signal in KIR, and our preliminary data indicate it does not mask the –RKR– signal in SUR1. Previous work indicates that SUR1 increases the surface expression of KIR6.2ΔC subunits approximately 8-fold, consistent with the involvement of the anterograde signal in a process that facilitates trafficking to the cell surface (10).

Substitution of alanines at positions Phe-1574 and Leu-1566 indicate the importance of these residues for anterograde transit of KATP channels. Dileucine motifs have been shown to be important in protein trafficking from the trans-Golgi to a late endosomal/lysosomal compartment (17–20) and for internalization of receptors following protein kinase activation and phosphorylation (21–23). An acidic residue 4–5 residues on the N-terminal side of the dileucine motif can also be important for sorting (24, 25), and a C-terminal glutamate/dileucine motif has been shown to be important for surface expression of the vasopressin V$_2$ receptor (26). Interestingly, mutation of the first leucine of the vasopressin V$_2$ receptor has a much larger inhibitory effect than substitution of the second, the same pattern as we observe for SUR1. However, mutation of Asp-1561, five residues upstream of the dileucine motif in SUR1 had no significant effect, whereas an E→Q substitution in the vasopressin V$_2$ receptor largely eliminates surface expression. The vasopressin V$_2$ receptor has no downstream phenylalanine corresponding to SUR1Phe-1574. The cellular receptors for these signaling motifs have not been identified, and it is unclear whether the C terminus of SUR interacts with COP1 or COP2 proteins or with COP-associated adaptor proteins (for reviews, see Refs. 27–32).

These observations give a molecular insight into the lack of KATP channel activity observed in pancreatic beta cells from patients with persistent hyperinsulinemic hypoglycemia of infancy. Mutations in both SUR1, HI-SUR1, and KIR6.2, HI-KIR6.2, are the cause of a recessive form of this disorder which is characterized by an inappropriate secretion of insulin despite hypoglycemia (5, 6, 33). Nonsense and splice site mutations in SUR1 produce C-terminal deletions (5) that have been shown to result in a complete loss of KATP channel activity (34). Although many of the truncated receptors may be incapable of producing functional channels for other reasons, our results indicate that even if channels do assemble they will not reach the cell surface and that the “primary” defect associated with mutations in SUR1 that truncate the receptor will be a failure to traffic correctly as a...
result of deleting the anterograde signal.

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