

A New ER Trafficking Signal Regulates the Subunit Stoichiometry of Plasma Membrane K_{ATP} Channels

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Summary

Proper ion channel function often requires specific combinations of pore-forming α and regulatory β subunits, but little is known about the mechanisms that regulate the surface expression of different channel combinations. Our studies of ATP-sensitive K^+ channel (K_{ATP}) trafficking reveal an essential quality control function for a trafficking motif present in each of the α (Kir6.1/2) and β (SUR1) subunits of the K_{ATP} complex. We show that this novel motif for endoplasmic reticulum (ER) retention/retrieval is required at multiple stages of K_{ATP} assembly to restrict surface expression to fully assembled and correctly regulated octameric channels. We conclude that exposure of a three amino acid motif (RKR) can explain how assembly of an ion channel complex is coupled to intracellular trafficking.

Introduction

Ion channels form the basis of electrical excitability, contribute to ionic homeostasis, and control neurotransmission and hormone secretion (Hille, 1992). Most ion channels are multimeric protein complexes containing one or more pore-forming α subunits and, in many cases, additional regulatory β subunits. Coassembly among different α subunits and further interaction with β subunits generate a large number of heteromultimeric ion channel combinations, often with dramatically different channel properties (Scott et al., 1994; Rhodes et al., 1997). However, biochemical and electrophysiological data imply that native channels on the plasma membrane often have a specific stoichiometry, and many possible combinations of subunits expressed in the same cell are not observed on the cell surface (Isom et al., 1994; Sheng et al., 1994; Wang et al., 1994; Rhodes et al., 1997). Additionally, there is a growing list of α subunits that require a specific β subunit to facilitate surface expression (Fink et al., 1996; Shi et al., 1996; Trimmer, 1998; Wilson et al., 1998). Similarly, some G protein receptors and transporters also require additional subunits for surface expression (Geering, 1990; McLatchie et al., 1998; White et al., 1998).

While it is clear that only some combinations of ion channel subunits are present on the cell surface, the

regulatory mechanisms that ensure plasma membrane targeting of only physiologically appropriate channel complexes remain to be elucidated. Studies of nicotinic receptor assembly suggest that exit from the endoplasmic reticulum (ER) is likely to be an important checkpoint for controlling ion channel surface stoichiometry (Blount et al., 1990). How could assembly between different subunits promote ER exit of specific channel combinations? One possibility is that only some combinations of assembled subunits can achieve a correctly folded state, and unassembled subunits and partially or incorrectly assembled complexes are retained in the ER by chaperones that recognize unfolded proteins (Kowalski et al., 1998). Additionally, recognition of unfolded proteins can be coupled to degradative pathways (Kopito, 1997), creating another level of quality control. For example, many immune receptor subunits contain strong degradation signals that are masked by subunit assembly (Bonifacino et al., 1990). Another possibility is that ion channel subunits could contain discrete ER retention signals that are hidden or overcome by forward trafficking signals in correctly assembled channels. Cytoplasmic ER retention signals such as the C-terminal KKXX are present in several immune receptor subunits (Letourneur and Klausner, 1992; Letourneur et al., 1995; Teasdale and Jackson, 1996). Correct immune receptor assembly not only prevents rapid degradation, but may also mask the ER retention signal (Klausner et al., 1990).

To address these issues of quality control during ion channel assembly, we have studied the assembly-dependent trafficking of ATP-sensitive K^+ channels (K_{ATP}). K_{ATP} channels respond to intracellular ATP and ADP levels and couple the metabolic state of the cell to membrane excitability (Dunne and Petersen, 1986). K_{ATP} channels are important in many tissues and regulate insulin secretion in the pancreas, control vascular tone, protect neurons and muscles from ischemia, and are responsive to leptin (Ashcroft and Ashcroft, 1990; Nichols and Lederer, 1991; Terzic et al., 1995; Harvey et al., 1997). K_{ATP} channels have an unusual octameric stoichiometry consisting of four pore-lining inward rectifier α subunits (Kir6.1/2), like other K^+ channels, but also contain four regulatory sulphonylurea-binding β subunits (SUR1/2A/2B) that belong to the ATP-binding cassette family (Clement et al., 1997; Inagaki et al., 1997; Shyng and Nichols, 1997; Babenko et al., 1998).

We find that assembly and trafficking of K_{ATP} channels are intricately linked processes. Since only octameric K_{ATP} channel complexes are capable of expressing on the cell surface, quality control mechanisms must exist to prevent monomers and partial complexes from expressing on the cell surface. Surprisingly, we find that the rate-limiting quality control mechanism during K_{ATP} assembly does not involve degradation or ER folding-chaperones but rather the exposure of a novel ER retention/retrieval signal present in each subunit. Mutating the retention sequences allows surface expression of monomers and partially assembled complexes, including improperly gated channel combinations that open under normal metabolic conditions. We further show

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that the new trafficking sequence functions as an ER retention/retrieval signal in a variety of eukaryotic cells, including yeast, mammalian cells, and *Xenopus* oocytes. We conclude that quality control during K_{ATP} assembly is mediated by a short trafficking signal whose exposure reflects the assembly state of the channel.

Results

Kir6.1/2 and SUR1 Require Coexpression for Plasma Membrane Expression

To study how assembly affects the surface targeting of K_{ATP} subunits, we measured plasma membrane protein levels. To do this, we inserted hemagglutinin (HA) epitopes into extracellular loops of Kir6.1, Kir6.2, and SUR1. We also introduced extra residues into the region of Kir6.2 between M1 and the HA tag (6.2-11HA) to ensure that the epitope was accessible when assembled with SUR1. When expressed in *Xenopus* oocytes after metabolic inhibition, HA-tagged subunits exhibited K^+ -selective currents similar to those observed for wild-type channel subunits (data not shown). Because most ion channels are expressed at relatively low levels on the cell surface, we developed an assay to measure surface protein that combines enzyme amplification with the sensitivity and linearity of analytical luminometry. Exposed HA epitopes on the surface of intact oocytes were labeled with a monoclonal antibody to HA, then with a horseradish peroxidase- (HRP-) conjugated secondary antibody. Antibody bound to the cell surface of intact oocytes was quantitated by luminometer measurement of single oocyte chemiluminescence.

Using this assay, we first tested whether coexpression alters surface protein levels. In the absence of SUR1, the surface labeling for Kir6.1HA or Kir6.2-11HA was not significantly different from that of uninjected oocytes (Figure 1A). However, when Kir6.1HA or Kir6.2-11HA was coexpressed with SUR1, surface signals were increased at least 500-fold (Figure 1A). Not only did surface expression of Kir6.1 or Kir6.2 require the presence of SUR1, surface expression of SUR1HA required coexpression of Kir6.1 or Kir6.2 (Figure 1B). Western blot analysis indicated that coexpression did not cause as dramatic a difference in protein levels as was observed for surface labeling (Figures 1A and 1B). Additionally, we observed a higher molecular weight band only when SUR1 was coexpressed with Kir6.1 or Kir6.2 (Figure 1B). This result is consistent with a previous report that SUR1 does not acquire mature complex glycosylation unless assembled with Kir6.1 or Kir6.2 (Clement et al., 1997).

Short Sequence Determinants in Kir6.1 and Kir6.2 Prevent Surface Expression of Individual α Subunits

Our studies revealed that K_{ATP} subunits did not reach the cell surface when expressed alone. Since it has been reported that removing 26 or 36 residues from the C terminus of Kir6.2 results in ATP-sensitive ion channels in the absence of SUR (Tucker et al., 1997), truncation must increase surface expression of Kir6.2 channels. Indeed, surface labeling of Kir6.2HA Δ 36 was 900-fold higher than full-length Kir6.2HA or uninjected oocytes (Figure 1C). As previously reported (Tucker et al., 1997),

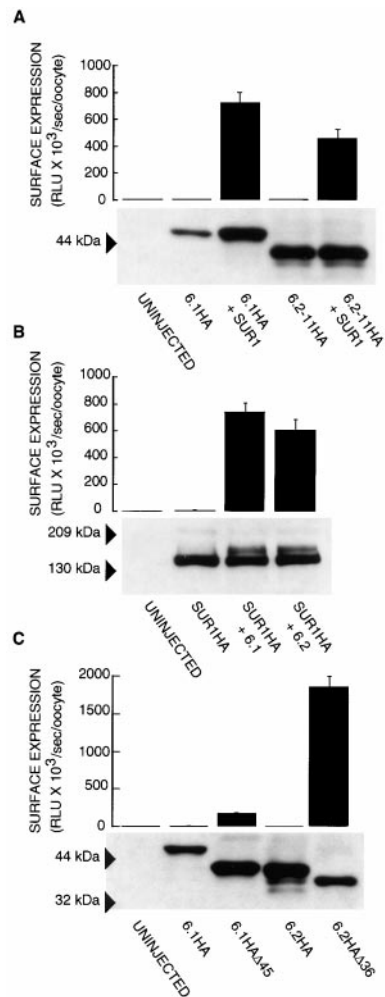


Figure 1. Coexpression of α (Kir6.1/2) and β (SUR1) Subunits or Truncation of α Subunits Is Required for Cell Surface Expression

Extracellular HA epitopes were introduced into K_{ATP} subunits, and levels of tagged protein on the surface of nonpermeabilized oocytes were assessed by labeling with an anti-HA epitope antibody and an HRP-conjugated secondary antibody. Single oocyte chemiluminescence was detected in an analytical luminometer and is reported in relative light units. Error bars represent standard deviations for five to ten oocytes (pertains to all subsequent figures). Western blots of total HA-tagged protein in oocyte homogenates is shown beneath plots.

(A) Kir6.1HA or Kir6.2-11HA was not detectable on the cell surface unless coexpressed with SUR1. Coexpression of SUR1 lacking the HA tag increases Kir6.1HA protein severalfold but does not alter Kir6.2HA protein levels.

(B) Coexpression of an α subunit is required for surface expression of SUR1HA but does not alter total SUR1HA protein (140 kDa band) levels. The mature, higher molecular weight SUR1HA band is only present when an α subunit is coexpressed.

(C) Kir6.1HA and Kir6.2HA subunits lacking 45 or 36 amino acids, respectively, from the C terminus were expressed on the cell surface, but total HA-tagged protein was not greatly affected by truncation.

Kir6.2HA Δ 36 exhibited K^+ -selective inwardly rectifying current that responded weakly to metabolic inhibition by azide treatment and was not blocked by glibenclamide, a compound known to bind SUR (data not shown).

Similar to Kir6.2HA, truncating the corresponding region in Kir6.1HA also resulted in surface expression (Figure 1C), but no azide-stimulated K⁺-selective currents were observed. Western blot analysis indicated that total protein levels for Kir6.2HA were not increased by truncation. Kir6.1HA levels were increased by truncation, although the change in protein levels was much smaller than the 100- to 200-fold increases in Kir6.1HA surface protein (Figure 1C).

The truncation results indicated that both Kir6.1 and Kir6.2 contain C-terminal elements that prevent surface expression. To identify the sequence determinants, we made a series of C-terminal truncations in Kir6.2HA. Removing 20 but not 18 residues resulted in channels that exhibited azide-induced currents and surface labeling (Figure 2B [b]). Like wild type, all Kir6.2 C-terminal mutants that did not express on the surface were detectable by Western analysis and were functional when coexpressed with SUR1 (data not shown). Comparing the sequences of Kir6.1 and Kir6.2 indicates that the last 40 amino acids of the C terminus are poorly conserved, except for a cluster of four amino acids (LRKR) (Figure 2A). This cluster was partially deleted in Kir6.2HAΔ20 but not in Kir6.2HAΔ18. Indeed, replacing LRKR with alanines allowed the channel to reach the surface (Figure 2B [c]). Deleting the ten amino acids separating LRKR from highly conserved upstream sequences also resulted in surface expression (Figure 2B [c]). However, when these residues were replaced with alanines, the channel did not appear on the plasma membrane, perhaps indicating that a flexible linker is needed to expose the LRKR sequence. Next, we tested the effect of single or double alanine mutations in LRKR. Alanine substitution at any of the three basic residues resulted in surface expression (Figure 2B [c]). Substituting arginines with lysines resulted in surface expression, but the reverse substitution had no effect (Figure 2B [d]). When different amino acids (alanine, histidine, asparagine, glutamine, arginine, tryptophan, isoleucine, and glutamic acid) were used to replace the middle lysine, only alanine, asparagine, and glutamate substitution resulted in surface expression (Figure 2B [d]). We conclude that the minimal sequence required for preventing surface expression is RKR, although the middle position prefers a large neutral or positively charged amino acid.

To determine if the RKR sequence reduces surface protein by targeting the C terminus for ubiquitination, we mutated all four lysines in the last 36 amino acids to remove these potential sites for ubiquitination (Staub et al., 1997; Ciechanover, 1998). Changing these lysines to arginine did not result in surface expression (Figure 2B [e]). Furthermore, phosphorylation of a serine adjacent to the RKR sequence (LRKRS) is not required for the function of the RKR sequence, since replacing the serine with alanine had no effect on surface expression (Figure 2B [c]).

The C terminus also contains a dileucine motif that has been shown to function in endosomal targeting (Trowbridge et al., 1993; Sandoval and Bakke, 1994). Mutating the dileucine sequence did not cause surface expression, but when combined with a mutation in RKR (RAA), it caused a 5-fold increase of surface signal (Figure 2B [f]). This finding explains why truncating 26 residues or mutating LRKR results in only 20%–30% as

much surface expression as observed for Kir6.2HAΔ36, which lacks both the dileucine and the RKR sequences.

SUR1 Also Contains an RKR Sequence that Prevents Its Surface Expression in the Absence of Kir6.1 or Kir6.2

A database search for membrane proteins containing the RKR sequence revealed that SUR1 contains the RKR sequence in a cytoplasmic loop between the putative eleventh transmembrane domain and the first nucleotide binding fold (Figure 3A). Mutating the RKR sequence resulted in a dramatic increase in surface protein in the absence of Kir6.1 or Kir6.2 (Figure 3B). Indeed, the level of surface expression was similar to that observed when SUR1HA was coexpressed with Kir6.1 or Kir6.2 and was also comparable to surface expression levels of Kir6.2HAΔ36. Furthermore, mutating RKR in SUR1HA caused a dramatic shift of protein into a higher molecular weight band, presumably the mature complex glycosylated form observed when wild-type SUR1 is coexpressed with Kir6.1/2 (Figure 1B) (Clement et al., 1997). We conclude that SUR1 does not express on the cell surface, because it contains the same motif as Kir6.1 and Kir6.2, and because the RKR motif can function to prevent surface expression even in a cytoplasmic loop.

The RKR Sequence Functions as an ER Retention/Retrieval Signal

We next addressed whether the RKR sequence can prevent surface expression of proteins that normally traffic to the cell surface. First, we transferred the last 36 amino acids of Kir6.2 to either the N or C terminus of another inwardly rectifying K⁺ channel, Kir2.1 (IRK1), that differs from Kir6.2 in its ability to traffic to the cell surface without β subunits. The addition of these amino acids greatly reduced both surface expression and currents (Figure 4A). Mutating the RKR completely reversed the effect. After 5–6 days, oocytes expressing Kir2.1HA_{C+36} showed small strongly rectifying currents (Figure 4B). The ratio of current to surface protein was similar to wild-type Kir2.1 (data not shown), indicating that the RKR sequence controls surface expression but does not affect channel gating.

To determine whether RKR is recognized by general eukaryotic cellular machinery, we took advantage of the fact that in yeast, Kir2.1 rescues the *trk1trk2* double mutant for growth on low-potassium medium (Tang et al., 1995). No complementation was observed for Kir2.1_{C+36}, but mutating RKR restored complementation (Figure 4C). The RKR sequence also prevented surface expression when transferred to the C terminus of an epitope-tagged β2 adrenergic receptor (β2-AR) as measured by flow cytometric analysis of surface-labeled COS-7 cells (Figure 4D). In control experiments, transfection efficiency was similar for both constructs as determined by immunofluorescence of permeabilized cells (data not shown).

The ability of the RKR sequence to prevent the surface expression of proteins unrelated to potassium channels enabled us to examine whether the motif acts at an early stage in the secretory pathway. For this purpose, we attached the last 36 residues of Kir6.2 to the C terminus of human CD4 (Nilsson et al., 1989). Immunofluorescent

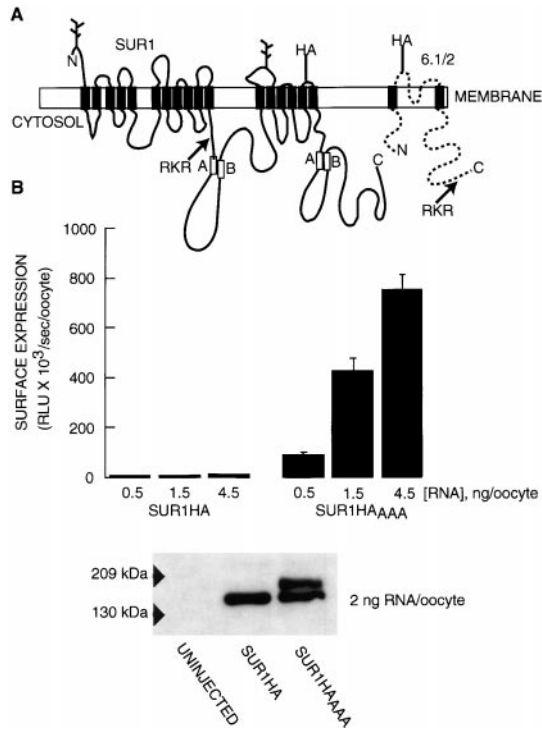


Figure 3. Mutating an RKR Sequence in a Cytoplasmic Loop of SUR1 Allows Surface Expression in the Absence of Kir6.1 or Kir6.2 (A) Location of the RKR sequence in SUR1 based on the topology proposed by Tuszny et al. (1997), adopted from Bryan and Aguilar-Bryan (1997). Kir6.2 with the location of the RKR sequence is shown for reference. (B) SUR1HA with RKR mutated to AAA showed a concentration-dependent increase of surface expression in oocytes. Below, Western analysis of total HA-tagged protein indicated that mutating RKR in SUR1 did not alter protein levels. A higher molecular weight band was only seen for SUR1HA_{AAA}.

The Function of RKR during K_{ATP} Assembly

Do Kir6.2 subunits interact in the absence of SUR1, and is the trafficking of these Kir6.2 complexes controlled by the RKR sequences? To answer these questions, we injected a constant concentration of Kir6.2HA Δ 36 RNA with various concentrations of Kir6.2 RNA. Coinjecting an equal concentration of Kir6.2 with Kir6.2HA Δ 36 reduced surface expression of Kir6.2HA Δ 36 by over 90%, and coinjecting a 2-fold higher concentration of Kir6.2 blocked 78% of Kir6.2HA Δ 36 surface expression (Figure 6A). The strong dominant-negative effect of Kir6.2 on the surface expression of Kir6.2HA Δ 36 implies that Kir6.2HA Δ 36 was trapped by Kir6.2 in complexes that did not traffic to the cell surface (Figure 6A). The magnitude of the effect was consistent with the theoretical prediction for the effect of a dominant-negative subunit in a tetramer (solid line in Figure 6A). Western blot analysis indicated that Kir6.2 did not cause degradation of Kir6.2HA Δ 36 (data not shown). In contrast to the dramatic effects of coinjecting Kir6.2 with Kir6.2HA Δ 36, coinjecting a 4-fold higher concentration of Kir6.2 Δ 36 or GIRK1 had only a small, presumably nonspecific, effect on surface expression of Kir6.2HA Δ 36 (Figure 6B). Interestingly, full-length Kir6.1 also trapped Kir6.2HA Δ 36, suggesting that the two subunits can form heteromultimers.

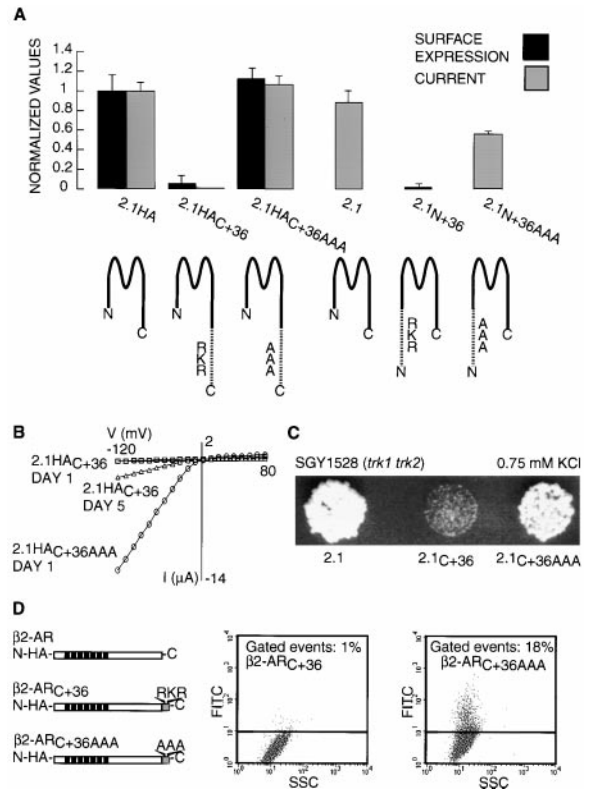


Figure 4. The RKR Sequence Prevents Surface Expression When Transferred to the N or C Terminus Reporter Proteins and Works in Yeast and Mammalian Cells

(A) Transplanting the last 36 amino acids of Kir6.2 to the C or N terminus of Kir2.1 prevented surface expression, but not when RKR was replaced with alanines. (B) I-V relationships in 90 mM extracellular KCl for HA-tagged Kir2.1_{C+36} at day 1 (squares) and day 5 (triangles) after cRNA injection and for HA-tagged Kir2.1_{C+36AAA} 1 day after cRNA injection (circles). (C) Transplanting the last 36 amino acids of Kir6.2 to Kir2.1 interfered with the functional complementation of a *trk1 trk2* double knockout yeast strain, but not when RKR was changed to AAA. Transformed SGY1528 yeast colonies were grown in liquid dropout media containing 100 mM KCl, and serial dilutions of the stationary cultures in 1 M sorbitol were spotted on minimal media containing 0.75 mM KCl. The photograph was taken after 5 days of growth at 30°C. (D) Flow cytometry of β 2-AR_{C+36} and β 2-AR_{C+36AAA} surface expression in COS-7 cells. Only the alanine replacement mutant yielded cells with fluorescent surface signals greater than the signal for untransfected cells. FITC indicates fluorescein fluorescence and SSC, side scatter. The threshold for gated events was determined by measuring the FITC signal of mock-transfected cells labeled with the same antibodies as transfected cells.

We conclude that Kir6.2 channels form tetramers in the absence of SUR and that the presence of the RKR sequence prevents these tetramers from expressing on the cell surface.

Having established that the RKR sequence prevents surface targeting of tetrameric Kir6.2 channels and SUR1 monomers, we next addressed the trafficking of partially assembled K_{ATP} complexes containing both Kir6.2 and SUR1. To create partial K_{ATP} complexes with a defined subunit stoichiometry, we used a previously developed strategy (Clement et al., 1997; Inagaki et al., 1997;

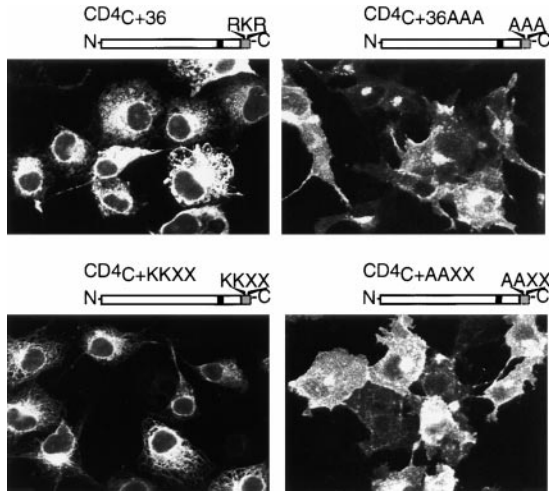


Figure 5. The RKR Sequence Functions to Slow the Progression from ER to *Trans*-Golgi Network

The last 36 amino acids of Kir6.2 or the last 10 amino acids of the KKXX-containing yeast protein Wbp1p were fused to the cytoplasmic C terminus of CD4. Transiently transfected COS-7 cells were immunostained for CD4. A strong perinuclear staining pattern consistent with the accumulation of protein in the ER is observed for both fusion proteins (left). Greatly reduced perinuclear staining was observed when RKR or KKXX was mutated (right).

Shyng and Nichols, 1997). First, we created a tandem triple fusion protein in which SUR1 was linked to two Kir6.2 subunits and contained an extracellular HA epitope to monitor cell surface expression as illustrated in Figure 6C. Similar to previous reports (Clement et al.,

1997; Inagaki et al., 1997), we observed no K_{ATP} currents for the triple fusion. Furthermore, surface signal for oocytes injected with the triple fusion was not significantly different than that of uninjected oocytes (Figure 6C). Coexpressing free, untagged SUR1 with the triple fusion protein was required for surface expression (Figure 6C), indicating that partial complexes containing two SUR subunits per Kir6.2 tetramer are incapable of expressing on the cell surface.

We next tested whether the failure of partial K_{ATP} complexes to traffic to the cell surface was due to the RKR sequence. It has been previously shown that a tandem double fusion between SUR1 and Kir6.2 readily forms functional K_{ATP} channels unlike the tandem triple fusion (Clement et al., 1997; Shyng and Nichols, 1997). Functional channels that arise from the expression of double fusion constructs have an octameric stoichiometry (Clement et al., 1997; Shyng and Nichols, 1997). Coexpression of unfused Kir6.2 subunits with the double fusion protein results in nonfunctional partial complexes containing both double fusion subunits and free Kir6.2 subunits (Shyng and Nichols, 1997). These partial complexes containing fewer than four SUR1 subunits are rescued by expressing additional free SUR1 subunits, thereby demonstrating that an octameric stoichiometry is necessary for K_{ATP} function (Shyng and Nichols, 1997). We took advantage of this method of generating partial K_{ATP} complexes to test whether RKR prevents partial complexes from expressing on the cell surface. To do this, we created similar SUR1-Kir6.2 fusions (R-R) and added an extracellular HA epitope to the 6.2 portion of the fusion. We then mutated the RKR motif in either the Kir6.2 subunit (R-A) or SUR1 (A-R), or both (A-A), as shown in

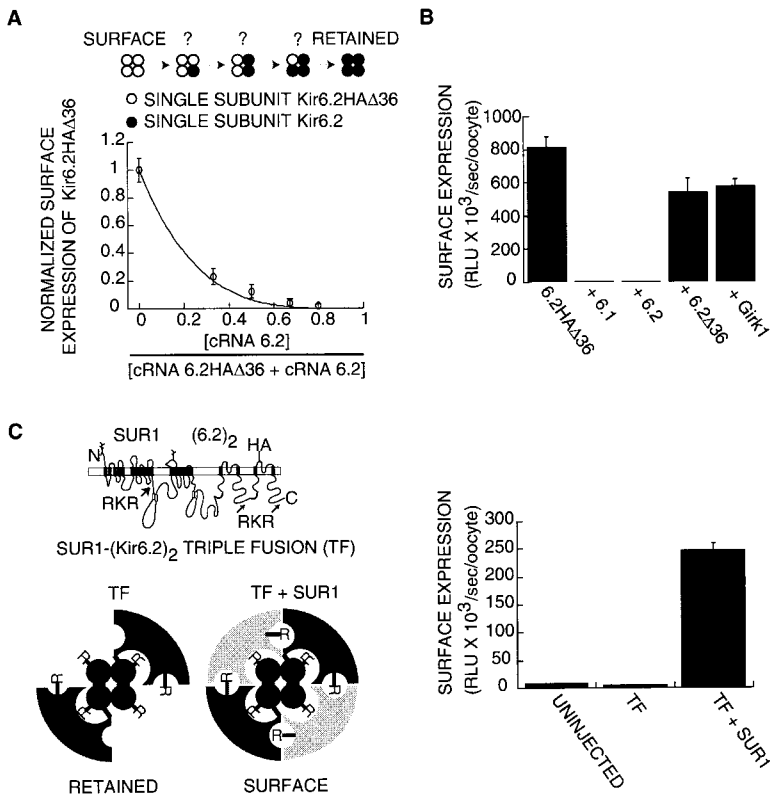


Figure 6. Trafficking of Kir6.2 Tetramers Is Controlled by the RKR Motif, and a 1:1 Stoichiometry of SUR1 and Kir6.2 Is Required for Surface Expression

(A) Kir6.2 Δ 36 containing an extracellular HA tag (Kir6.2HA Δ 36) was coexpressed with different concentrations of untagged full-length Kir6.2. Surface levels of Kir6.2HA Δ 36 are plotted as a function of the fraction of full-length Kir6.2 RNA in the total amount of RNA injected. The solid line represents the theoretical prediction for the effect of a dominant-negative subunit in a tetramer.

(B) Coinjection of an equal RNA concentration of Kir6.2HA Δ 36 and various untagged channel subunits.

(C) A triple tandem fusion containing one SUR1 subunit and two Kir6.2 subunits with an extracellular HA tag is expected to assemble into complexes with a fixed stoichiometry of two SUR1 subunits per Kir6.2 tetramer as illustrated (left). Triple fusion constructs are not present on the cell surface unless free SUR1 is coexpressed (right).

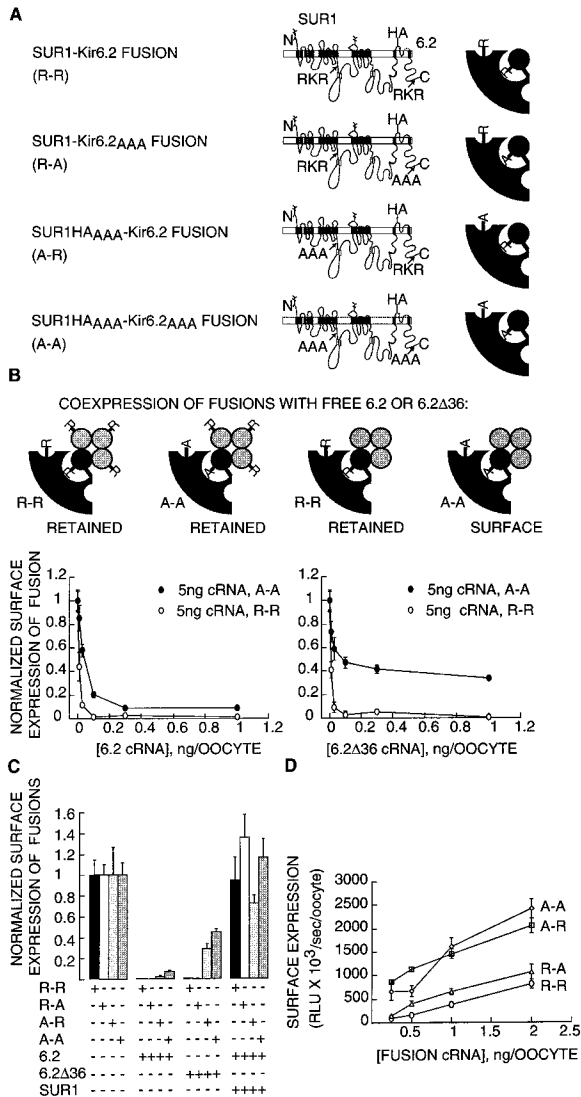


Figure 7. Partially Assembled K_{ATP} Complexes Are Prevented from Expressing on the Cell Surface by RKR Sequences

(A) Fusion proteins were created by linking the C terminus of SUR1 to the N terminus of Kir6.2 with a flexible alanine-glycine linker (R-R). The Kir6.2 subunit contains an extracellular HA epitope. The RKR motif was mutated to alanine in either Kir6.2 (R-A) or SUR1 (A-R), or both (A-A).

(B) Coinjection of different concentrations of Kir6.2 or Kir6.2Δ36 RNA with either fusion protein R-R or A-A (5 ng). Levels of the fusion protein were normalized to levels in the absence of untagged free subunits.

(C) Surface expression of fusion proteins (5 ng) when coexpressed with 1 ng free Kir6.2 or Kir6.2Δ36. All data were normalized to the surface levels of fusion protein expressed alone and are labeled as in (A). Coexpression of 5 ng untagged wild-type SUR1 reversed the effect of free α subunits.

(D) RNA dose responses of fusion proteins alone, labeled as in (A).

Figure 7A. By coinjecting various concentrations of unfused Kir6.2 or Kir6.2Δ36 RNA with a constant concentration of RNA for the fusion protein, we created a continuum of partially assembled complexes, and by injecting different combinations of fusion and free subunits, we

systematically varied the number and position of RKR motifs in the partial complexes.

Coexpression of either Kir6.2 or Kir6.2Δ36 with the R-R or R-A fusion protein completely blocks surface expression of the fusion protein in a dose-dependent manner, and the effect can be fully reversed by expressing additional free SUR1 (Figures 7B and 7C). This result indicates that partial complexes do not traffic to the cell surface, as we found for the triple fusion (Figure 6C). In contrast, when the A-A fusion is coexpressed with Kir6.2Δ36, the complexes are on the cell surface at levels that are 200-fold higher than the background signal or the surface signal for R-R coexpressed with Kir6.2 (Figures 7B and 7C). This result indicates that removing the RKR sequence from both SUR1 and Kir6.2 allows partial complexes to express on the plasma membrane.

How might the RKR motifs prevent the surface expression of partial complexes? Since Kir6.2Δ36 blocked surface expression of R-R or R-A, and Kir6.2 blocked surface expression of A-A (Figures 7B and 7C), it is unlikely that mutating the RKR sequences impairs the assembly of fusion and free subunits. Furthermore, these results suggest that the RKR sequence on either SUR1 or Kir6.2 can independently block surface expression of partial complexes, most likely by retaining or retrieving the partial complexes early in the secretory pathway. It also follows that the RKR motif in SUR1 cannot be hidden by interactions with Kir6.2, since coexpression of Kir6.2Δ36 completely blocks surface expression of the R-A fusion, while A-A coexpressed with Kir6.2Δ36 is present on the plasma membrane (Figure 7C). Low levels of surface expression (5% of control) were detectable for A-A coexpressed with Kir6.2, but surface expression was never observed for R-R with coexpressed Kir6.2Δ36, indicating that the RKR motif in SUR1 may affect the trafficking of partial complexes more strongly than the RKR motif in Kir6.2. It should be noted that Kir6.2Δ36 reduced surface expression of the A-A fusion by 50% (Figures 7B and 7C). This effect could arise from nonspecific effects of coexpression or may indicate that other sequences exposed in partial complexes slow their traffic to the cell surface or enhance their targeting to intracellular compartments such as the endosomes. We conclude from these experiments that partial complexes do not express on the plasma membrane due to the presence of exposed RKR motifs.

Are the RKR sequences fully masked in the octameric channel? RNA concentration responses for the different fusion combinations indicated that, in contrast to the strong effects of RKR in retaining Kir6.2 tetramers, mutating the RKR in Kir6.2 did not greatly alter surface expression of the octameric channel formed by the SUR1-Kir6.2 fusion protein (Figure 7D). However, mutating RKR in SUR1 increases surface expression levels, especially at lower RNA concentrations (Figure 7D). These results suggest that the RKR motif in SUR1 but not in Kir6.2 is partially exposed in the fully assembled octamer.

Finally, we examined the functional properties of channels that arise from coexpressing α and β subunits that both lacked the ER retention/retrieval signal. Similar to

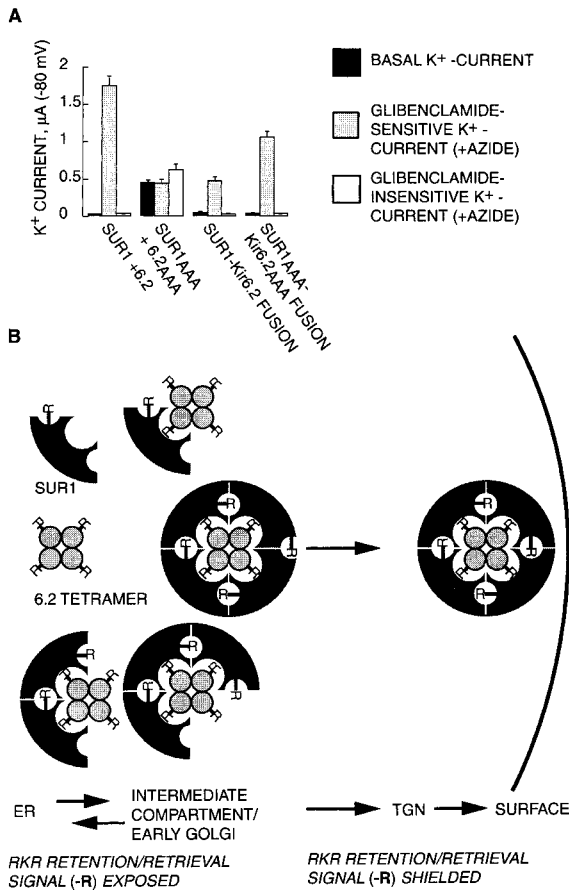


Figure 8. The RKR Sequence Is Required for Preventing Surface Expression of Improperly Gated Channel Combinations

(A) Currents were measured by two electrode voltage-clamp. Oocytes coinjected with wild-type subunits did not exhibit basal K⁺ currents, but following treatment with azide (3 mM for 4 min), large K⁺ currents were induced that could be blocked with glibenclamide (10 µM). Coexpression of subunits lacking the RKR sequence resulted in basal K⁺ currents and azide-stimulated currents that were only partially blocked by glibenclamide. Expression of R-R and A-A gave rise to currents that resembled coinjection of unfused wild-type subunits but not unfused subunits lacking RKR.

(B) A model of quality control during K_{ATP} assembly based on data in Figures 1–3, 6, and 7.

previous studies (Inagaki et al., 1995; Gribble et al., 1997), coexpressing wild-type K_{ATP} subunits resulted in native-like K_{ATP} channels that were not open under normal cellular conditions but could be activated by azide-induced metabolic inhibition and inhibited by glibenclamide. These properties are the hallmark of native octameric K_{ATP} complexes. In contrast, coexpressing Kir6.2_{AAA} and SUR1H_{AAA} resulted in large basal K⁺ currents in the absence of metabolic inhibition, and, following azide treatment, substantial glibenclamide-insensitive currents were apparent (Figure 8A). However, R-R and A-A fusion proteins exhibited similar azide-stimulated currents that were fully blocked by glibenclamide. Therefore, a gating effect arising from mutating RKR cannot account for the atypical currents observed when Kir6.2_{AAA} and SUR1H_{AAA} are coexpressed. Rather, the RKR motifs function to prevent the surface expression

of channel stoichiometries that are active under normal metabolic conditions and do not respond to K_{ATP} channel blockers. These probably include tetramers of Kir6.2_{AAA}. Indeed, expression of Kir6.2_{AAA} alone results in glibenclamide-insensitive basal K⁺ currents (data not shown). Considering the results in Figure 7, it is also possible that partial complexes of SUR1_{AAA} and Kir6.2_{AAA} are on the surface and are partially responsible for the atypical currents.

Discussion

Cell Surface Expression of K_{ATP} Subunits Is Regulated by Intracellular Trafficking Signals

Previous studies provide convincing evidence that the coexpression of both α and β subunits of the K_{ATP} complex is required for the formation of functional channels (Inagaki et al., 1995; Sakura et al., 1995). However, the effect of coexpression on the subcellular distribution and plasma membrane targeting of individual subunits is less well-understood (John et al., 1998; Lorenz et al., 1998; Makhina and Nichols, 1998). Our results provide direct evidence that coassembly of α and β subunits results in a profound enhancement of surface expression compared with each subunit expressed alone (Figures 1A and 1B).

We show that a simple three amino acid trafficking sequence (RKR) is responsible for preventing the surface expression of Kir6.2 tetramers and SUR1 monomers. This finding explains why C-terminal truncations of Kir6.2 lead to the observation of SUR-independent currents (Tucker et al., 1997). It has been reported that overexpression of Kir6.2 in HEK 293 cells results in low levels of channels with properties similar to truncated Kir6.2 in the absence of SUR (John et al., 1998). Taken together with our finding that truncating Kir6.2 dramatically enhances surface expression, we consider it unlikely that truncation profoundly alters channel gating.

The RKR sequence we have identified not only prevents the surface targeting of SUR1 and Kir6.2 subunits expressed alone but also functions at later stages of assembly to retain partial complexes containing both SUR1 and Kir6.2 (Figure 7). Systematically removing RKR motifs from each of the subunits in the partial complexes indicates that the RKR in SUR1 is most important for the trafficking of partial complexes, although RKR in Kir6.2 also contributes to preventing partial complexes from reaching the cell surface (Figure 7). Because partial complexes containing RKR in SUR1 but not Kir6.2 did not express on the cell surface (Figures 7B and 7C), it follows that the RKR in SUR1 is not shielded by assembly with Kir6.2 but probably is hidden by interactions with adjacent SUR1 subunits. The results are schematically summarized in Figure 8B.

Previous studies (Clement et al., 1997; Inagaki et al., 1997; Shyng and Nichols, 1997) have shown that an octameric K_{ATP} stoichiometry is necessary for K_{ATP} function. Our measurements of the surface targeting of partial complexes (Figures 6C and 7) indicate that the necessity for an octameric stoichiometry can be explained by channel trafficking. Since only octameric channels

are present on the cell surface, it remains an open question whether an octameric stoichiometry is necessary for K_{ATP} channel gating.

RKR Is a Novel Cytoplasmic ER Retention/Retrieval Sequence

The best characterized ER retention signals are the luminal KDEL and cytoplasmic KKXX sequences (Teasdale and Jackson, 1996). The KDEL signal is found at the luminal C terminus of soluble and type II transmembrane proteins, whereas the KKXX sequence is found at the cytoplasmic C terminus of some transmembrane proteins. Proteins containing these sequences are retained in the ER or retrieved from the Golgi compartment back to the ER (Teasdale and Jackson, 1996). Like KDEL and KKXX, the RKR sequence also causes ER retention/retrieval when fused to proteins normally localized to the plasma membrane (Figures 4 and 5). Furthermore, our finding that the RKR sequence functions in yeast, *Xenopus* oocytes, and mammalian cells suggests that the RKR motif is recognized by general eukaryotic trafficking machinery.

Although the ER retention/retrieval sequence we have identified is composed of basic residues, it clearly differs from KKXX in both specific amino acid requirements and its lack of dependence on proximity to the C terminus. It has been reported that alternatively spliced transcripts of major histocompatibility complex (MHC) class II invariant (Ii) chain and syntaxin 5 contain an arginine-based sequence in the extended cytoplasmic N terminus that causes ER retention (Schutze et al., 1994; Hui et al., 1997). Both proteins contain RRR or RKR, similar to the minimal ER retention sequence we have identified. However, in contrast to Schutze et al. (1994), we found that the middle lysine residue did not tolerate mutation to certain amino acids. Two important properties of the RKR signal we have characterized is that it did not require proximity to either the N or C terminus, and it functioned in a cytoplasmic loop of SUR1. These properties distinguish it from previously described ER retention/retrieval signals, and along with the minimal sequence requirement, they greatly increase the number of membrane proteins in which RKR could function.

Physiological Implications of Trafficking Signals in K_{ATP} Subunits

From a physiological perspective, there are important consequences of having retention/retrieval signals on the α and β subunits of K_{ATP} . Unlike wild-type K_{ATP} channels, which do not open under typical metabolic conditions, coexpression of SUR1 and Kir6.2 subunits lacking ER retention signals allows surface expression of improperly regulated K^+ channels that open under typical metabolic conditions (Figure 8A). These aberrant channels probably reflect tetramers of Kir6.2_{AAA} but may also include partial complexes with fewer than four SUR1 subunits. If such channels were expressed on the plasma membrane, they could have harmful effects. For example, pancreatic β cells have a high input resistance (Rorsman and Trube, 1985), and the presence of these improperly regulated channels on the cell surface would hyperpolarize the cell and impair insulin secretion.

An important but poorly understood aspect of ion

channel regulation is the control of the number of channels on the cell surface. This is especially critical for pancreatic β cells whose high input resistance makes them very sensitive to changes in only a few channels. The fact that the RKR motif in SUR1 influences the number of fully assembled octameric channels on the cell surface (Figure 7D) as well as the identification of a dileucine motif in Kir6.2 raises the possibility that these motifs regulate the supply of K_{ATP} channels to or from the cell surface. Perhaps slow trafficking rates create an intracellular reservoir of K_{ATP} channels. Such a reservoir could help buffer channel number on the cell surface from large fluctuations in subunit translation rates due to the stochastic variation of RNA number. In addition, the dileucine motif is known to mediate endosomal trafficking and targeting to the basolateral surface of epithelial cells (Trowbridge et al., 1993; Matter et al., 1994). Since it has been reported that K_{ATP} is preferentially located on the basolateral surface of some epithelial tissues (Tsuchiya et al., 1992), the dileucine signal could also be involved in the polarized trafficking of K_{ATP} . Further experiments will be needed to determine whether these trafficking signals have additional roles in the regulation of K_{ATP} function.

One of the most surprising findings in our study is that individual K_{ATP} subunits were not sufficiently unfolded to cause strong retention by ER resident chaperones. Removal of a short, discrete trafficking signal allowed surface expression of individual subunits at levels exceeding that which was observed for coassembled α and β subunits and allowed surface expression of partial complexes containing both SUR1 and Kir6.2. Therefore, at multiple stages of K_{ATP} assembly, ER retention by trafficking signals must be rate limiting for surface expression of SUR1 monomers, Kir6.2 tetramers, and partial complexes with both subunits. We conclude that trafficking sequences play an essential role in quality control during K_{ATP} assembly.

Experimental Procedures

Molecular Biology

General protocols were from Ausubel et al. (1997). HA epitopes were introduced into rat Kir6.1 and mouse Kir6.2 cDNAs by sequential overlap extension polymerase chain reactions (PCR). The epitope (YPYDVPDYA) was inserted at position 114 for Kir6.1 and position 102 for Kir6.2. Construct Kir6.2-11HA has 11 amino acids inserted into Kir6.2: the sequence before the HA epitope reads ⁹⁸GDLAYME-KGIT⁹⁹DL. For insertion of the HA epitope into the loop between the putative sixteenth and seventeenth transmembrane domains of SUR1 (Tusnady et al., 1997; Kast and Gros, 1998), complementary oligonucleotides with the appropriate overhangs were ligated into an SfiI site endogenous to the hamster SUR1 cDNA. Some residues were duplicated to position the epitope in the extracellular loop. The amino acid sequence of the HA-tagged SUR1 reads ¹²⁷²LHREL-SAGLVYPYDVPDYAHRELSAGLVGLG¹²⁸⁴ at the site of the epitope insertion. Truncations $\Delta 45$ in Kir6.1 and $\Delta 3$, $\Delta 10$, $\Delta 20$, $\Delta 26$, and $\Delta 36$ in Kir6.2 (Figure 3B [b]) as well as constructs Kir6.2 LRKR368/369/370/371AAAA (Figure 3B [c]; Kir6.2_{AAA}), Kir6.2 KKKK370/377/379/381RRRR (Figure 3B [e]), and SUR1HA RKR648/649/650AAA (SUR1-HA_{AAA}) were constructed by PCR as well. For further mutagenesis, using a cassette strategy, artificial NotI and Sall sites were introduced into Kir6.2, changing positions S363 and S364 to A as well as positions F382 to S and S383 to T. The corresponding Kir6.2 protein behaved like wild type in all assays (Figure 3B [a]). Mutants Kir6.2 $\Delta 12$, Kir6.2 $\Delta 14$, Kir6.2 $\Delta 16$, Kir6.2 $\Delta 18$, R365A, GP366/367AA, LR368/369AA, KR370/371AA, L368A, R369A, R369K, K370A, K370R,

K370H, K370N, K370Q, K370W, K370I, K370E, R371A, R371K, RR369/371KK, and S372A were then constructed by ligating in complementary oligonucleotides with appropriate overhangs (Figure 3B [b–d and f]). For additional constructs, a NotI site was engineered at position 355 of the Kir6.2 cDNA, allowing for the deletion of residues 355 to 364 by using the 3' NotI site created at residues S363/364 (Figure 3B [c]); the NotI site creates an insertion of three alanines at position 355 in this construct and was further used to insert an alanine/glycine linker of ten residues (Figure 3B [c]) or residues 355 to 364 of Kir6.2 with LL355/356 mutated to AA (Figure 3B [f]). The HA epitope was inserted at position 117 of Kir2.1 and at the N terminus of the β 2 adrenergic receptor (the N-terminal amino acid sequence reads MGYDYDVPDYAQ⁺PGN). Artificial NotI sites were engineered immediately after the last codon of the mouse Kir2.1 and the hamster β 2 adrenergic receptor cDNAs. These NotI sites were used to fuse the last 36 codons of Kir6.2 (wild-type and LRKR368/369/370/371AAAA) to the respective cDNAs. An artificial NotI site was also introduced immediately after the last codon of the SUR1 cDNA. For the fusions between SUR1 and Kir6.2, a linker encoding six glycines was introduced between the two cDNAs. The 6.2 portion contained the 11HA epitope (see above). The last 36 residues of Kir6.2 were fused to the N terminus of Kir2.1 by insertion of a PCR fragment into the respective 5' cloning site BamHI. The N-terminally fused sequence reads M³⁵⁵L⁻⁻⁻390SSRSA. Tails encoding the last 36 codons of Kir6.2 or the sequence KKLETFKKTN were fused to the human CD4 cDNA via an artificial NotI site inserted after the last codon. PCR-derived sequences and inserted oligonucleotides were entirely sequenced. For oocyte expression, all constructs were in pGemHE (Liman et al., 1992), and cRNA was transcribed by T7 RNA polymerase. Yeast expression constructs were in p416MET25 (Mumberg et al., 1994). Mammalian expression constructs were all in pcDNA3 (Invitrogen).

Preparation and Surface Labeling of Oocytes

Xenopus oocytes were prepared and maintained as previously described (Collins et al., 1997). Unless otherwise noted, oocytes were injected with 1 ng cRNA for Kir6.1/2 constructs, β 2-AR constructs, and Kir2.1 constructs and 5 ng for SUR1 constructs. For surface labeling, oocytes were blocked for 30 min in ND96 with 1% bovine serum albumin (BSA) at 4°C, labeled with 1 μ g/ml rat monoclonal anti-HA antibody (3F10, Boehringer Mannheim, in 1% BSA for 30–60 min at 4°C), washed at 4°C, and incubated with HRP-coupled secondary antibody (goat anti-rat Fab fragments) (in 1% BSA for 30–60 min at 4°C). Cells were extensively washed (1% BSA, 4°C, 60 min) and transferred to ND96 without BSA. Individual oocytes were placed in 50 μ l Power Signal Elisa (Pierce) and incubated at room temperature for 1 min. Chemiluminescence was quantitated in a Monolight 2010 luminometer.

Electrophysiology

Currents were measured by standard two electrode voltage-clamp recording (Degan Amplifier, pCLAMP software). K_{ATP} currents were recorded in modified ND96 containing 40 mM K^+ /60 mM Na^+ and determined by subtracting control current (–80 mV) from currents recorded after 4 min application of 3 mM sodium azide. Glibenclamide (final concentration, 10 μ M) was added to the oocyte perfusion solution, and stable current block was measured 1–2 min after application. Kir2.1 currents (–60 mV) were measured in 90 mM K^+ /10 mM Na^+ .

Yeast Complementation Assay

The yeast strain SGY1528 lacks both potassium uptake systems, Trk1p and Trk2p (*MATa; ade2-1; can1-100; his3-11,15; leu2-3,112; trp1-1; ura3-1; trk1::HIS3; trk2::TRP1*), and does not grow on low- K^+ media but can be rescued by heterologous expression of Kir2.1 (Tang et al., 1995). Yeast were transformed and plated on standard dropout media without uracil containing 100 mM KCl (pH adjusted to 6.5 with Tris). Transformants were then grown and plated on minimal media with limiting K^+ concentrations (1, 0.75, and 0.5 mM) to test for complementation (10 mM L-arginine base, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, trace minerals, vitamins, all amino acids except uracil and methionine [to allow for maximal expression from the *MET25*

promoter], 1% dextrose, and 1.5% Sea-KEM agarose, pH adjusted to 6 with phosphoric acid) (Uozumi et al., 1995).

Western Blot Analysis

Oocytes were homogenized essentially as described (Tucker et al., 1996), separated by SDS-PAGE electrophoresis (8%–10% gels), and transferred to nitrocellulose. Blots were blocked in Tris-buffered saline (TBS) containing 5% milk powder and 0.2% NP-40. Primary (rat anti-HA monoclonal 3F10, 200 ng/ml) and secondary (HRP-conjugated goat anti-rat IgG, 1:1000) antibodies were diluted in TBS-blocking solution. Washes were in TBS, 0.1% NP-40. Detection was performed with the enhanced chemiluminescence system (Amersham).

Immunofluorescence and Flow Cytometry

COS-7 cells were transfected with lipofectamine (GIBCO BRL) or Fugene (Boehringer Mannheim). For immunofluorescence, cells were grown on glass chamber slides. Cells were fixed in 4% formaldehyde in PBS and blocked with 5% goat serum in PBS with 0.3% Triton-X-100. Cells were labeled with a monoclonal anti-CD4 antibody (mAb 1779, Chemicon, 1:1000) and secondary antibody (Cy3-conjugated goat anti-mouse IgG, 1:1000) in 1% goat serum in PBS, 0.3% Triton-X-100. Immunofluorescence microscopy was performed with a BioRad confocal microscope.

For flow cytometry, cells were detached by treatment with 0.01% trypsin in PBS-based cell dissociation buffer (GIBCO) and labeled with the monoclonal anti-HA antibody 3F10 (2 μ g/ml, Boehringer Mannheim) in PBS containing 5% goat serum and 5% cell dissociation buffer for 1 hr at 4°C. After three washes in the same medium, cells were exposed to a FITC-conjugated anti-rat secondary antibody (1:500), washed briefly, and analyzed with a Facscan flow cytometer (Becton-Dickinson). Viability was assessed with propidium iodide (1 μ g/ml).

Acknowledgments

We thank Lydia Aguilar-Bryan for the SUR1 cDNA, Marc G. Caron for the HA-tagged β 2 adrenergic receptor cDNA, Anthony Collins for the HA-tagged Kir2.1 cDNA, Nigel Killeen for the CD4 cDNA, Steven Kurtz for the yeast strain SGY1528, Susumo Seino for the Kir6.1 and Kir6.2 cDNAs, and Holly Ingraham for advice and help with luminometry. Further, we wish to thank Sharon Fried for excellent technical assistance and the Howard Hughes Medical Institute sequencing and flow cytometry facilities at the University of California, San Francisco for support. Anthony Collins was the first to insert an HA epitope between transmembrane domains M1 and M2 of Kir2.1 in our lab. We thank many Jan lab members for stimulating discussions and comments on the manuscript. This work was supported by a National Institute of Mental Health Silvio Conte Center grant at the University of California, San Francisco. B. S. is supported by a Human Frontier Science Program postdoctoral fellowship. N. Z. is the recipient of a Howard Hughes predoctoral fellowship. L. Y. J. and Y. N. J. are Howard Hughes predoctoral fellows.

Received January 12, 1999; revised February 26, 1999.

References

- Ashcroft, S.J., and Ashcroft, F.M. (1990). Properties and functions of ATP-sensitive K-channels. *Cell. Signal* 2, 197–214.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1997). *Current Protocols in Molecular Biology* (New York: J. Wiley & Sons).
- Babenko, A.P., Aguilar-Bryan, L., and Bryan, J. (1998). A view of sur/KIR6.X, K_{ATP} channels. *Annu. Rev. Physiol.* 60, 667–687.
- Blount, P., Smith, M.M., and Merlie, J.P. (1990). Assembly intermediates of the mouse muscle nicotinic acetylcholine receptor in stably transfected fibroblasts. *J. Cell Biol.* 111, 2601–2611.
- Bonifacino, J.S., Cosson, P., and Klausner, R.D. (1990). Colocalized transmembrane determinants for ER degradation and subunit assembly explain the intracellular fate of TCR chains. *Cell* 63, 503–513.
- Bryan, J., and Aguilar-Bryan, L. (1997). The ABCs of ATP-sensitive

- potassium channels: more pieces of the puzzle. *Curr. Opin. Cell Biol.* **9**, 553–559.
- Ciechanover, A. (1998). The ubiquitin–proteasome pathway: protein death and cell life. *EMBO J.* **17**, 7151–7160.
- Clement, J.P.I., Kunjilwar, K., Gonzalez, G., Schwanstecher, M., Panten, U., Aguilar-Bryan, L., and Bryan, J. (1997). Association and stoichiometry of $K(ATP)$ channel subunits. *Neuron* **18**, 827–838.
- Collins, A., Chuang, H., Jan, Y.N., and Jan, L.Y. (1997). Scanning mutagenesis of the putative transmembrane segments of Kir2.1, an inward rectifier potassium channel. *Proc. Natl. Acad. Sci. USA* **94**, 5456–5460.
- Dunne, M.J., and Petersen, O.H. (1986). Intracellular ADP activates K^+ channels that are inhibited by ATP in an insulin-secreting cell line. *FEBS Lett.* **208**, 59–62.
- Fink, M., Duprat, F., Lesage, F., Heurteaux, C., Romey, G., Barhanin, J., and Lazdunski, M. (1996). A new K^+ channel beta subunit to specifically enhance Kv2.2 (CDRK) expression. *J. Biol. Chem.* **271**, 26341–26348.
- Geering, K. (1990). Subunit assembly and functional maturation of Na,K-ATPase. *J. Membr. Biol.* **115**, 109–121.
- Gribble, F.M., Ashfield, R., Ammalá, C., and Ashcroft, F.M. (1997). Properties of cloned ATP-sensitive K^+ currents expressed in *Xenopus* oocytes. *J. Physiol. (Lond)* **498**, 87–98.
- Harvey, J., McKenna, F., Herson, P.S., Spanswick, D., and Ashford, M.L. (1997). Leptin activates ATP-sensitive potassium channels in the rat insulin-secreting cell line, CRI-G1. *J. Physiol. (Lond)* **504**, 527–535.
- Hille, B. (1992). *Ionic Channels of Excitable Membranes*, Second Edition (Sunderland, MA: Sinauer Associates).
- Hui, N., Nakamura, N., Sonnichsen, B., Shima, D.T., Nilsson, T., and Warren, G. (1997). An isoform of the Golgi t-SNARE, syntaxin 5, with an endoplasmic reticulum retrieval signal. *Mol. Biol. Cell* **8**, 1777–1787.
- Inagaki, N., Gono, T., Clement, J.P.T., Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S., and Bryan, J. (1995). Reconstitution of I_{KATP} : an inward rectifier subunit plus the sulfonylurea receptor. *Science* **270**, 1166–1170.
- Inagaki, N., Gono, T., and Seino, S. (1997). Subunit stoichiometry of the pancreatic beta-cell ATP-sensitive K^+ channel. *FEBS Lett.* **409**, 232–236.
- Isom, L.L., De Jongh, K.S., and Catterall, W.A. (1994). Auxiliary subunits of voltage-gated ion channels. *Neuron* **12**, 1183–1194.
- John, S.A., Monck, J.R., Weiss, J.N., and Ribalet, B. (1998). The sulphonylurea receptor SUR1 regulates ATP-sensitive mouse Kir6.2 K^+ channels linked to the green fluorescent protein in human embryonic kidney cells (HEK 293). *J. Physiol. (Lond)* **510**, 333–345.
- Kast, C., and Gros, P. (1998). Epitope insertion favors a six transmembrane domain model for the carboxy-terminal portion of the multidrug resistance-associated protein. *Biochemistry* **37**, 2305–2313.
- Klausner, R.D., Lippincott-Schwartz, J., and Bonifacio, J.S. (1990). The T cell antigen receptor: insights into organelle biology. *Annu. Rev. Cell Biol.* **6**, 403–431.
- Kopito, R.R. (1997). ER quality control: the cytoplasmic connection. *Cell* **88**, 427–430.
- Kowalski, J.M., Parekh, R.N., Mao, J., and Wittrup, K.D. (1998). Protein folding stability can determine the efficiency of escape from endoplasmic reticulum quality control. *J. Biol. Chem.* **273**, 19453–19458.
- Letourneur, F., and Klausner, R.D. (1992). A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell* **69**, 1143–1157.
- Letourneur, F., Hennecke, S., Démolière, C., and Cosson, P. (1995). Steric masking of a dilysine endoplasmic reticulum retention motif during assembly of the human high affinity receptor for immunoglobulin E. *J. Cell Biol.* **129**, 971–978.
- Liman, E.R., Tytgat, J., and Hess, P. (1992). Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs. *Neuron* **9**, 861–871.
- Lorenz, E., Alekseev, A.E., Krapivinsky, G.B., Carrasco, A.J., Clapham, D.E., and Terzic, A. (1998). Evidence for direct physical association between a K^+ channel (Kir6.2) and an ATP-binding cassette protein (SUR1) which affects cellular distribution and kinetic behavior of an ATP-sensitive K^+ channel. *Mol. Cell. Biol.* **18**, 1652–1659.
- Makhina, E.N., and Nichols, C.G. (1998). Independent trafficking of K_{ATP} channel subunits to the plasma membrane. *J. Biol. Chem.* **273**, 3369–3374.
- Matter, K., Yamamoto, E.M., and Mellman, I. (1994). Structural requirements and sequence motifs for polarized sorting and endocytosis of LDL and Fc receptors in MDCK cells. *J. Cell Biol.* **126**, 991–1004.
- McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M.G., and Foord, S.M. (1998). RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* **393**, 333–339.
- Mumberg, D., Müller, R., and Funk, M. (1994). Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res.* **22**, 5767–5768.
- Nichols, C.G., and Lederer, W.J. (1991). Adenosine triphosphate-sensitive potassium channels in the cardiovascular system. *Am. J. Physiol.* **261**, H1675–H1686.
- Nilsson, T., Jackson, M., and Peterson, P.A. (1989). Short cytoplasmic sequences serve as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell* **58**, 707–718.
- Rhodes, K.J., Strassle, B.W., Monaghan, M.M., Bekele-Arcuri, Z., Matos, M.F., and Trimmer, J.S. (1997). Association and colocalization of the Kvbeta1 and Kvbeta2 beta-subunits with Kv1 alpha-subunits in mammalian brain K^+ channel complexes. *J. Neurosci.* **17**, 8246–8258.
- Rorsman, P., and Trube, G. (1985). Glucose dependent K^+ -channels in pancreatic beta-cells are regulated by intracellular ATP. *Pflügers Arch.* **405**, 305–309.
- Sakura, H., Ammalá, C., Smith, P.A., Gribble, F.M., and Ashcroft, F.M. (1995). Cloning and functional expression of the cDNA encoding a novel ATP-sensitive potassium channel subunit expressed in pancreatic beta-cells, brain, heart and skeletal muscle. *FEBS Lett.* **377**, 338–344.
- Sandoval, I.V., and Bakke, O. (1994). Targeting of membrane proteins to endosomes and lysosomes. *Trends Cell Biol.* **4**, 292–297.
- Schutze, M.P., Peterson, P.A., and Jackson, M.R. (1994). An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. *EMBO J.* **13**, 1696–1705.
- Scott, V.E., Muniz, Z.M., Sewing, S., Lichtinghagen, R., Parcej, D.N., Pongs, O., and Dolly, J.O. (1994). Antibodies specific for distinct Kv subunits unveil a heterooligomeric basis for subtypes of alpha-dendrotoxin-sensitive K^+ channels in bovine brain. *Biochemistry* **33**, 1617–1623.
- Sheng, M., Tsaur, M.L., Jan, Y.N., and Jan, L.Y. (1994). Contrasting subcellular localization of the Kv1.2 K^+ channel subunit in different neurons of rat brain. *J. Neurosci.* **14**, 2408–2417.
- Shi, G., Nakahira, K., Hammond, S., Rhodes, K.J., Schechter, L.E., and Trimmer, J.S. (1996). $Bet\alpha$ subunits promote K^+ channel surface expression through effects early in biosynthesis. *Neuron* **16**, 843–852.
- Shyng, S., and Nichols, C.G. (1997). Octameric stoichiometry of the K_{ATP} channel complex. *J. Gen. Physiol.* **110**, 655–664.
- Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997). Regulation of stability and function of the epithelial Na^+ channel (ENaC) by ubiquitination. *EMBO J.* **16**, 6325–6336.
- Tang, W., Ruknudin, A., Yang, W.P., Shaw, S.Y., Knickerbocker, A., and Kurtz, S. (1995). Functional expression of a vertebrate inwardly rectifying K^+ channel in yeast. *Mol. Biol. Cell* **6**, 1231–1240.
- Teasdale, R.D., and Jackson, M.R. (1996). Signal-mediated sorting of membrane proteins between the endoplasmic reticulum and the Golgi apparatus. *Annu. Rev. Cell Dev. Biol.* **12**, 27–54.
- Terzic, A., Jahangir, A., and Kurachi, Y. (1995). Cardiac ATP-sensitive K^+ channels: regulation by intracellular nucleotides and K^+ channel-opening drugs. *Am. J. Physiol.* **269**, C525–C545.

- Trimmer, J.S. (1998). Regulation of ion channel expression by cytoplasmic subunits. *Curr. Opin. Neurobiol.* *8*, 370–374.
- Trowbridge, I.S., Collawn, J.F., and Hopkins, C.R. (1993). Signal-dependent membrane protein trafficking in the endocytic pathway. *Annu. Rev. Cell Biol.* *9*, 129–161.
- Tsuchiya, K., Wang, W., Giebisch, G., and Welling, P.A. (1992). ATP is a coupling modulator of parallel Na,K-ATPase-K-channel activity in the renal proximal tubule. *Proc. Natl. Acad. Sci. USA* *89*, 6418–6422.
- Tucker, S.J., Bond, C.T., Herson, P., Pessia, M., and Adelman, J.P. (1996). Inhibitory interactions between two inward rectifier K⁺ channel subunits mediated by the transmembrane domains. *J. Biol. Chem.* *271*, 5866–5870.
- Tucker, S.J., Gribble, F.M., Zhao, C., Trapp, S., and Ashcroft, F.M. (1997). Truncation of Kir6.2 produces ATP-sensitive K⁺ channels in the absence of the sulphonylurea receptor. *Nature* *387*, 179–183.
- Tusnády, G.E., Bakos, E., Váradi, A., and Sarkadi, B. (1997). Membrane topology distinguishes a subfamily of the ATP-binding cassette (ABC) transporters. *FEBS Lett.* *402*, 1–3.
- Uozumi, N., Gassmann, W., Cao, Y., and Schroeder, J.I. (1995). Identification of strong modifications in cation selectivity in an *Arabidopsis* inward rectifying potassium channel by mutant selection in yeast. *J. Biol. Chem.* *270*, 24276–24281.
- Wang, H., Kunkel, D.D., Schwartzkroin, P.A., and Tempel, B.L. (1994). Localization of Kv1.1 and Kv1.2, two K channel proteins, to synaptic terminals, somata, and dendrites in the mouse brain. *J. Neurosci.* *14*, 4588–4599.
- White, J.H., Wise, A., Main, M.J., Green, A., Fraser, N.J., Disney, G.H., Barnes, A.A., Emson, P., Foord, S.M., and Marshall, F.H. (1998). Heterodimerization is required for the formation of a functional GABA_B receptor. *Nature* *396*, 679–682.
- Wilson, G.F., Wang, Z., Chouinard, S.W., Griffith, L.C., and Ganetzky, B. (1998). Interaction of the K channel beta subunit, Hyperkinetic, with eag family members. *J. Biol. Chem.* *273*, 6389–6394.