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

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Summary

Proper ion channel function often requires specific combinations of pore-forming \(\alpha\) and regulatory \(\beta\) subunits, but little is known about the mechanisms that regulate the surface expression of different channel combinations. Our studies of ATP-sensitive K\textsuperscript{+} channel (K\textsubscript{ATP}) trafficking reveal an essential quality control function for a trafficking motif present in each of the \(\alpha\) (Kir6.1/2) and \(\beta\) (SUR1) subunits of the K\textsubscript{ATP} complex. We show that this novel motif for endoplasmic reticulum (ER) retention/retrieval is required at multiple stages of K\textsubscript{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 \(\alpha\) subunits and, in many cases, additional regulatory \(\beta\) subunits. Coassembly among different \(\alpha\) subunits and further interaction with \(\beta\) subunits generate a large number of heteromultimeric 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 (Ison et al., 1994; Sheng et al., 1994; Wang et al., 1994; Rhodes et al., 1997). Additionally, there is a growing list of \(\alpha\) subunits that require a specific \(\beta\) 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. Endoplasmic 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\textsuperscript{+} channels (K\textsubscript{ATP}). K\textsubscript{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\textsubscript{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\textsubscript{ATP} channels have an unusual octameric stoichiometry consisting of four pore-lining inward rectifier \(\alpha\) subunits (Kir6.1/2), like other K\textsuperscript{+} channels, but also contain four regulatory sulphonylurea-binding \(\beta\) 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\textsubscript{ATP} channels are intricately linked processes. Since only octameric K\textsubscript{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\textsubscript{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
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\(_{\text{ATP}}\) 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\(_{\text{ATP}}\) 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 \(\alpha\) Subunits**

Our studies revealed that K\(_{\text{ATP}}\) 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\(_{\Delta36}\) was 900-fold higher than full-length Kir6.2HA or uninjected oocytes (Figure 1C). As previously reported (Tucker et al., 1997),
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 [f]).

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 Kir6.2-HAΔ36. 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 3B). Mutating the RKR completely reversed the effect. After 5–6 days, oocytes expressing Kir2.1HAΔ36 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 Kir2.1CΔ36, 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 2. Sequence Determinants that Prevent Surface Expression of Kir6.2

(A) Sequence alignment of the C termini of Kir6.1 and Kir6.2. Conserved residues are shown in bold. The last 36 amino acids of Kir6.2 are underlined.

(B) Mutational analysis of the last 36 residues of Kir6.2. All measurements were normalized to values for Kir6.2HAΔ36 expressed in the same batch of oocytes.

(a) Mutations introduced to facilitate further cassette mutagenesis had no effect.
(b) Sequential C-terminal deletions (3, 10, 12, 14, 16, 18, 20, 26, and 36 residues) implicated residues 355 to 372 as crucial for preventing surface expression.
(c) Deletion and alanine substitutions indicated that only the RKR residues are critical.
(d) Additional RKR mutations.
(e) Potential ubiquitination sites were changed to arginines without effect.
(f) Mutating a dileucine motif (residues 355 to 356) to alanines did not result in surface expression of Kir6.2, but when combined with an LRKR alanine mutant, surface protein levels equaled those observed for Kir6.2Δ36.

staining of CD4CΔ36 indicated that the protein accumulated in a perinuclear compartment. Changing RKR to alanine resulted in weak or undetectable perinuclear staining but strong plasma membrane staining (Figure 5). The same dramatic change in pattern of staining was also observed when the well-established KKXX motif for ER retention/retrieval was fused to the C terminus of CD4 (Figure 5) and is similar to previous studies of the effect of KKXX on CD4 staining patterns (Nilsson et al., 1989). These results show that the RKR motif causes ER accumulation of membrane proteins containing the trafficking signal.
The Function of RKR during $\mathrm{K_{ATP}}$ 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 Kir6.2HAΔ36 RNA with various concentrations of Kir6.2 RNA. Coinjecting an equal concentration of Kir6.2 with Kir6.2HAΔ36 reduced surface expression of Kir6.2HAΔ36 by over 90%, and coinjecting a 2-fold higher concentration of Kir6.2 blocked 78% of Kir6.2HAΔ36 surface expression (Figure 6A). The strong dominant-negative effect of Kir6.2 on the surface expression of Kir6.2HAΔ36 implies that Kir6.2HAΔ36 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 Kir6.2HAΔ36 (data not shown). In contrast to the dramatic effects of coinjecting Kir6.2 with Kir6.2HAΔ36, coinjecting a 4-fold higher concentration of Kir6.2Δ36 or GIRK1 had only a small, presumably nonspecific, effect on surface expression of Kir6.2HAΔ36 (Figure 6B). Interestingly, full-length Kir6.1 also trapped Kir6.2HAΔ36, suggesting that the two subunits can form heteromultimers.

We conclude that Kir6.2 channels form tetramers in the absence of SUR1 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 $\mathrm{K_{ATP}}$ complexes containing both Kir6.2 and SUR1. To create partial $\mathrm{K_{ATP}}$ complexes with a defined subunit stoichiometry, we used a previously developed strategy (Clement et al., 1997; Inagaki et al., 1997;
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Inagaki et al., 1997), we observed no KATP 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 KATP 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 KATP 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 KATP function (Shyng and Nichols, 1997). We took advantage of this method of generating partial KATP 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 Tetrâmér is Controlled by the RKR Motif, and a 1:1 Stoi-
chiometry of SUR1 and Kir6.2 Is Required for Surface Expression

(A) Kir6.2(D36) containing an extracellular HA tag (Kir6.2(D36)) was coexpressed with different concentrations of untagged full-length Kir6.2. Surface levels of Kir6.2(D36) 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 Kir6.2(D36) 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).
systematically varied the number and position of RKR motifs in the partial complexes.

Coexpression of either Kir6.2 or Kir6.2.D36 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 Kir6.2.D36, 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 Kir6.2.D36 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 Kir6.2.D36 completely blocks surface expression of the R-A fusion, while A-A coexpressed with Kir6.2.D36 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 Kir6.2.D36, 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 Kir6.2.D36 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
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of channel stoichiometries that are active under normal metabolic conditions and do not respond to KATP channel blockers. These probably include tetramers of Kir6.2AAA. Indeed, expression of Kir6.2AAA 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 SUR1AAA and Kir6.2AAA are on the surface and are partially responsible for the atypical currents.

Discussion

Cell Surface Expression of K\(_{\text{ATP}}\) Subunits Is Regulated by Intracellular Trafficking Signals

Previous studies provide convincing evidence that the coexpression of both \(\alpha\) and \(\beta\) subunits of the KATP 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 \(\alpha\) and \(\beta\) 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 (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\(_{\text{ATP}}\) stoichiometry is necessary for K\(_{\text{ATP}}\) 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

previous studies (Inagaki et al., 1995; Gribble et al., 1997), coexpressing wild-type K\(_{\text{ATP}}\) subunits resulted in native-like K\(_{\text{ATP}}\) 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\(_{\text{ATP}}\) complexes. In contrast, coexpressing Kir6.2AAA and SUR1HAAAA 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 Kir6.2AAA and SUR1HAAAA are coexpressed. Rather, the RKR motifs function to prevent the surface expression...
are present on the cell surface, it remains an open question whether an octameric stoichiometry is necessary for \( K_{\text{ATP}} \) 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 (li) 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 \( K_{\text{ATP}} \) Subunits**

From a physiological perspective, there are important consequences of having retention/retrieval signals on the \( \alpha \) and \( \beta \) subunits of \( K_{\text{ATP}} \). Unlike wild-type \( K_{\text{ATP}} \) 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 Kir6.2ΔΔ 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 \( \beta \) 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 \( \beta \) 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 \( K_{\text{ATP}} \) channels to or from the cell surface. Perhaps slow trafficking rates create an intracellular reservoir of \( K_{\text{ATP}} \) channels. Such an intracellular 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 \( K_{\text{ATP}} \) is preferentially located on the basolateral surface of some epithelial tissues (Tsukihara et al., 1992), the dileucine signal could also be involved in the polarized trafficking of \( K_{\text{ATP}} \). Further experiments will be needed to determine whether these trafficking signals have additional roles in the regulation of \( K_{\text{ATP}} \) function.

One of the most surprising findings in our study is that individual \( K_{\text{ATP}} \) 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 \( \alpha \) and \( \beta \) subunits and allowed surface expression of partial complexes containing both SUR1 and Kir6.2. Therefore, at multiple stages of \( K_{\text{ATP}} \) 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 \( K_{\text{ATP}} \) 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 "GDLAYMEKGIT". For insertion of the HA epitope into the loop between the putative sixteenth and seventeenth transmembrane domains of SUR1 (Tsunada et al., 1997; Kast and Gros, 1998), complementary oligonucleotides with the appropriate overhangs were ligated into the minimal ER retention sequence we have identified. 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.
K370H, K370N, K370Q, K370W, K370E, R371A, R371K, RR369/371KK, and S372A were then constructed by ligating in complementary oligonucleotides with appropriate overlaps (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 β2 adrenergic receptor (the N-terminal amino acid sequence reads MGPYDPVDQYPAGN). Artificial NotI sites were then created immediately after the last codon of the mouse Kir2.1 and the hamster β2 adrenergic receptor cDNAs. These NotI sites were used to fuse the last 36 codons of Kir6.2 (wild-type and LRKKR369/370/371AAAA) 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 NHA 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 63 codons of Kir2.0 or the sequence KKKL$^\text{TFKTX}
$ 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, β2-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 μg/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-rabbit Fab fragments) in 1% BSA for 30-60 min at 4°C. Cells were then extensively washed (2% BSA, 4°C, 60 min) and transferred to ND96 without BSA. Individual oocytes were placed in 50 μl 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 μM) 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, Tkt1p and Tkt2p (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 KC1 (pH adjusted to 6.5 with Tris). Transformants were then grown and plated on minimal media with limiting K$^+$ concentrations (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-C4 antigen (mAb Ab 1779, Chemicon, 1:1000) and secondary antibody (Cy3-conjugated goat anti-mouse IgG, 1:10000) 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 μg/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 μg/ml).

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Bonifacino, J.S., Cosson, P., and Klausner, R.D. (1990). Colocalized minimal media with limiting K$^+$ concentrations (1, 0.75, and 0.5 mM) was added to the oocyte perfusion 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 μg/ml).

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