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Molecular Basis for K_{ATP} Assembly: Transmembrane Interactions Mediate Association of a K^+ Channel with an ABC Transporter

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Summary

K_{ATP} channels are large heteromultimeric complexes containing four subunits from the inwardly rectifying K^+ channel family (Kir6.2) and four regulatory sulphonylurea receptor subunits from the ATP-binding cassette (ABC) transporter family (SUR1 and SUR2A/B). The molecular basis for interactions between these two unrelated protein families is poorly understood. Using novel trafficking-based interaction assays, coimmunoprecipitation, and current measurements, we show that the first transmembrane segment (M1) and the N terminus of Kir6.2 are involved in K_{ATP} assembly and gating. Additionally, the transmembrane domains, but not the nucleotide-binding domains, of SUR1 are required for interaction with Kir6.2. The identification of specific transmembrane interactions involved in K_{ATP} assembly may provide a clue as to how ABC proteins that transport hydrophobic substrates evolved to regulate other membrane proteins.

Introduction

ATP-binding cassette (ABC) proteins comprise a large superfamily of transport proteins present in prokaryotes and eukaryotes (Higgins, 1992; Holland and Blight, 1999). ABC transporters contain at least one nucleotide-binding domain (NBD) and a transport pathway residing in the membrane. During evolution, this ATP-coupled transport machinery has been adapted for the uptake and extrusion of diverse compounds, including sugars, lipids, peptides, and xenobiotics. Vertebrate ABC proteins, such as the multidrug resistance proteins (MDR1/3), peptide transporters (TAP1/2), and multidrug resistance-associated proteins (MRP1–6) are closely related to a subset of prokaryotic ABC proteins that function as efflux transporters of hydrophobic or amphipathic compounds (Allikmets et al., 1993; Decottignies and Goffeau, 1997; Saurin et al., 1999; see also the COGnitor database at http://www.ncbi.nlm.nih.gov/COG/ for COG1132 [ABC-type multidrug/protein/lipid transport system, ATPase component]; Tatusov et al., 1997). Sequence homology among ABC efflux proteins is concentrated in the NBDs and transmembrane domains. Conservation between ABC efflux proteins suggests that these transporters share a common set of conformational changes required for recognizing hydrophobic or amphipathic substrates and translocating them across the membrane.

Two vertebrate ABC proteins, the sulphonylurea receptor (SUR1/2) and the cystic fibrosis transmembrane regulator (CFTR), exhibit clear sequence homology to this superfamily of prokaryotic and eukaryotic efflux transporters yet are not known to transport substrates across the membrane. Rather, SUR1/2A/2B functions to regulate potassium ion channels (Kir6.1 and Kir6.2) belonging to the inwardly rectifying K^+ channel family (Aguilar-Bryan and Bryan, 1999). CFTR functions as a PKA-activated chloride channel and also regulates other ion channels (Schwiebert et al., 1999). The identification of ABC proteins functioning in a regulatory capacity raises the question of how a family of transporters evolved the ability to regulate other membrane proteins. To answer this question, it will be important to identify the domains that mediate interactions between ABC proteins and ion channels.

ATP-sensitive potassium channels (K_{ATP}) are large heteromultimeric complexes containing four SUR1/2A/2B subunits and four Kir6.1/2 subunits (for a recent review, see Aguilar-Bryan and Bryan, 1999). K_{ATP} channels couple the metabolic state of the cell to membrane excitability in many different cell types. They have been shown to control insulin secretion, regulate vascular tone, respond to leptin signaling, and precondition tissues against ischemic insult. Metabolic regulation and drug sensitivity of K_{ATP} channels is largely mediated by the interaction of SUR subunits with the Kir6.2 ion channel. It is therefore important to first determine which domains of Kir6.2 and SUR1/2A mediate K_{ATP} assembly. To do so, we have taken advantage of our understanding of K_{ATP} assembly and trafficking to develop two complementary trafficking-based assays for testing interactions between integral membrane proteins. In the trafficking enhancement assay, coassembly is necessary for the targeting of one subunit to the plasma membrane. In the trafficking trap assay, one of the subunits is normally present on the plasma membrane, but interaction with another subunit containing a strong dominant endoplasmic reticulum (ER) retention/retrieval signal causes the complex to be retained in the ER.

Using our trafficking-based approaches, we find that both the first transmembrane segment (M1) and the N terminus of the channel protein are important for specifying assembly with SUR1 and SUR2A. Changing as few as five amino acids in M1 of Kir2.1 (which does not assemble with SURs) to those found in Kir6.2 conferred the ability to assemble with SUR1/2A. Coassembly between the channel and SUR did not depend on the NBDs of SUR. Our finding that SUR1/2 interacts with a transmembrane segment of Kir6.2 may provide a clue as to how ABC proteins that transport hydrophobic substrates evolved to regulate other membrane proteins. These findings also raise the possibility that interactions in the plane of the membrane are important for transmitting gating information within the K_{ATP} channel complex.

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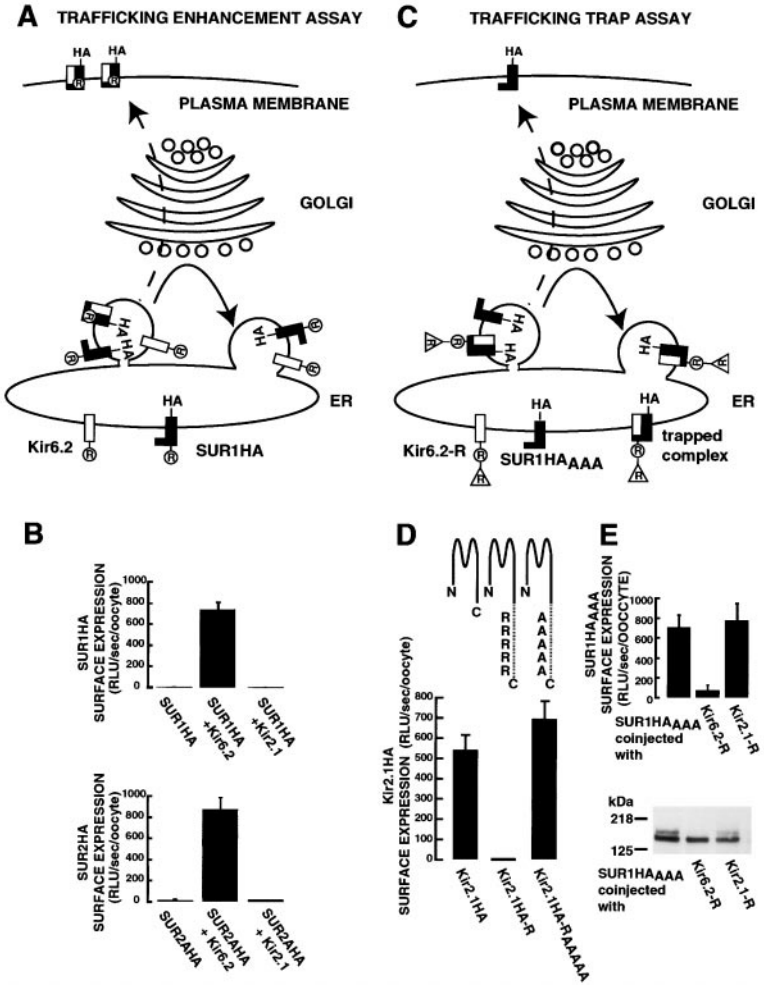

Figure 1. Trafficking Assays for Detecting Interaction between K_{ATP} Subunits

(A) Schematic depiction of the trafficking stimulation assay. Unassembled SUR1HA and Kir6.2 proteins do not leave the ER due to an exposed ER retention/retrieval signal ("R," in circle) (Zerangue et al., 1999). Once fully coassembled, the ER retention/retrieval signal in each of the SUR1HA and Kir6.2 subunits is hidden, and the assembled complex can traffick through the Golgi compartment and to the plasma membrane. Solid arrow indicates ER retention/retrieval, dashed arrow indicates forward transport through the secretory pathway.

(B) Chemiluminescent detection of SUR1 and SUR2A on the plasma membrane of *Xenopus* oocytes. An extracellular HA epitope was introduced into SUR1 and SUR2A, and surface protein on nonpermeabilized oocytes was labeled with an anti-HA epitope antibody and an HRP-conjugated secondary antibody. Bound antibody was quantitated using single oocyte chemiluminescence, as previously described (Zerangue et al., 1999). Error bars represent standard deviations for five to ten oocytes (pertains to all subsequent figures). Surface expression of SUR1HA (top) and SUR2AHA (bottom) was increased 200- to 500-fold by coexpressing Kir6.2 but not Kir2.1.

(C) Schematic depiction of the trafficking trap assay. Mutating the ER retention/retrieval sequence of SUR1HA to alanines ($SUR1HA_{AAA}$) allows surface expression in the absence of Kir6.2. Coexpression of Kir6.2 containing an additional ER retention/retrieval signal that is not masked by coassembly ("R," in triangle) traps $SUR1HA_{AAA}$ in the ER. Trapping is detected as a reduction in $SUR1HA_{AAA}$ surface expression.

(D) The last 14 amino acids of the α2C adrenergic receptor (-**KHILFRRRRRGFRQ**-COOH, indicated as "R") contains a strong ER retention/retrieval sequence. Fusing this sequence to the C terminus of Kir2.1HA prevents surface expression. Surface expression is restored when the five arginine cluster is mutated to alanines.

(E) Surface expression (top) and mature glycosylation (bottom) of $SUR1HA_{AAA}$ is reduced when coexpressed with Kir6.2-R (with the sequence from the α2C adrenergic receptor fused to the C terminus). Kir2.1-R does not reduce surface expression or affect glycosylation of $SUR1HA_{AAA}$. The upper band seen in the Western blot (detection with anti-HA antibody) has previously been shown to represent the mature glycosylated form of SUR1 (Raab-Graham et al., 1999).

Results

Development of Trafficking Enhancement and Trafficking Trap Assays

To analyze K_{ATP} assembly, we developed two trafficking-based interaction assays (Figures 1A and 1C). We have previously shown that coassembly between SUR1 and Kir6.2 is required in order for each subunit to express on the cell surface (Zerangue et al., 1999). This is due to the presence of an ER retention/retrieval signal in both subunit types that is masked upon assembly of the full octameric K_{ATP} channel complex. Like SUR1, SUR2A does not express well on the cell surface unless coexpressed with Kir6.2 (Figure 1B). In contrast to Kir6.2, the homologous protein Kir2.1 expresses at the cell surface by itself and does not stimulate surface trafficking of SUR1 or SUR2A (Figure 1B). Since Kir2.1 does not enhance trafficking of SUR proteins and does not interact with SUR proteins, as assayed by coimmunoprecipitation (Giblin et al., 1999; Figure 5B), we constructed chimeras between Kir2.1 and Kir6.2 to identify domains that specify assembly with SUR proteins. Five blocks were exchanged between the two homologous channel proteins: the distal N terminus; the membrane-proximal N terminus; transmembrane segment M1; the pore loop (H5), together with transmembrane segment M2; and the cytosolic C terminus (see Figure 2A for diagrams of the chimeras; the names of the chimeras consist of five digits that specify the origin of each of the five swapped regions). Chimeras were then tested for enhancement of SUR1HA or SUR2HA trafficking to the plasma membrane of *Xenopus* oocytes.

The trafficking enhancement assay (Figure 1A) requires that ER retention/retrieval signals present in each subunit are hidden in the assembled complex (Zerangue et al., 1999). Some channel chimeras may be capable of assembly, but the resulting heteromeric complex may not be well enough folded to reach the cell surface. To avoid this problem, we developed a complementary trafficking trap assay (Figure 1C). We reasoned that if

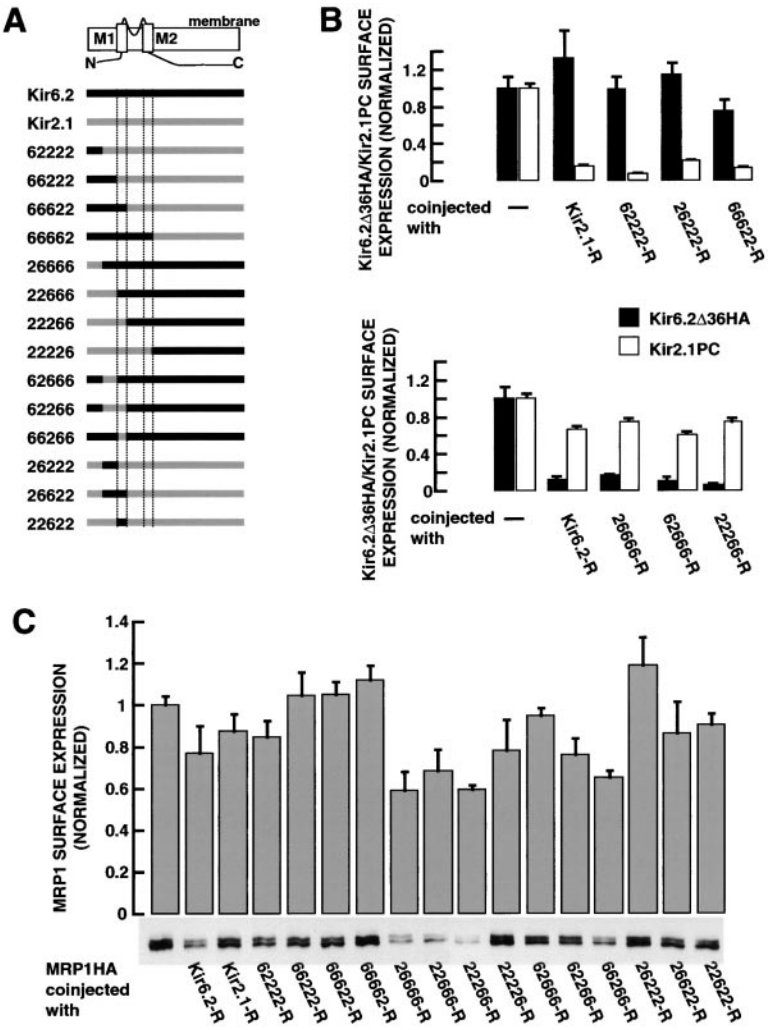

Figure 2. Characterization of Trap Assay

(A) Schematic representation of all channel chimeras tested in this study. Five blocks were exchanged between the two homologous channel proteins, Kir6.2 (black) and Kir2.1 (shaded). Swapping of the distal N terminus, membrane-proximal N terminus, transmembrane segment M1, pore loop (H5) plus transmembrane segment M2, and cytosolic C terminus resulted in the chimeras depicted.

(B) The trap assay confirms that the C termini of Kir6.2 and Kir2.1 determine subunit–subunit assembly compatibility. Coexpression of trap chimeras (5 ng/oocyte) with extracellular epitope-tagged Kir2.1PC or Kir6.2HA (2 ng/oocyte) is shown. Surface expression is normalized to Kir2.1PC or Kir6.2HA expressed alone. Chimeras containing the C terminus of Kir2.1 (top; 62222-R, 26222-R, and 66622-R) reduce surface expression of Kir2.1PC, but not Kir6.2Δ36HA, whereas chimeras containing the C terminus of Kir6.2 (bottom; 26666-R, 62666-R, and 22266-R) only reduce surface expression of Kir6.2Δ36HA.

(C) Trap chimeras (5 ng/oocyte) do not have strong effects on surface expression or protein levels of MRP1HA (5 ng/oocyte). Surface expression is normalized to surface signal of MRP1HA expressed alone. MRP1HA from total oocyte homogenates was detected by Western blotting with anti-HA antibody.

one subunit in a complex of membrane proteins contains a strong ER retention/retrieval sequence that is not hidden by coassembly, then the other proteins in the complex will also be trapped in the ER. Thus, if a membrane protein that can exit the ER by itself interacts with a strongly ER-retained protein, it is also trapped in the ER. To implement this strategy for analysis of K_{ATP} assembly, we used an extracellular HA-tagged SUR1 mutant ($SUR1HA_{AAA}$) that lacks an ER retention/retrieval signal and trafficks to the cell surface independent of coexpression with Kir6.2 (Zerangue et al., 1999). To create a Kir6.2 subunit that assembles with $SUR1HA_{AAA}$ and traps it in the ER, we added an additional ER retention/retrieval signal to the C terminus of Kir6.2 that is not masked by coassembly with SUR1 (Figure 1D). The ER retention/retrieval sequence we used was derived from the last 14 amino acids of the C terminus of the α2C adrenergic receptor. The tail of the α2C adrenergic receptor contains a potent five arginine ER retention/retrieval sequence (referred to as -R). When -R is added to the C terminus of Kir2.1, the protein is completely absent from the cell surface (Figure 1D), whereas total protein levels are unaffected (data not shown). When the five arginines are mutated to alanines, Kir2.1 trafficks to the cell surface (Figure 1D). We next fused the α2C tail to the C terminus of Kir6.2 (Kir6.2-R). Surface expression of $SUR1HA_{AAA}$ was reduced over 90% by coexpression of Kir6.2-R but was unaffected by Kir2.1-R (Figure 1E). In contrast to Kir6.2-R, which blocks surface expression of $SUR1HA_{AAA}$, wild-type Kir6.2 weakly stimulated surface expression of $SUR1HA_{AAA}$ (data not shown), indicating that reduced $SUR1HA_{AAA}$ surface expression is due to the presence of the trap tail on Kir6.2-R. Therefore, Kir6.2 can have two completely opposite effects on the surface expression of SUR1, depending on whether or not Kir6.2 contains the trap sequence. Western blotting revealed that Kir6.2-R caused a loss of mature complex-glycosylated $SUR1HA_{AAA}$ without reducing protein levels (Figure 1E). This result provides further evidence that Kir6.2-R traps $SUR1HA_{AAA}$ in the ER and prevents it from acquiring complex glycosylation in the Golgi apparatus (Raab-Graham et al., 1999).

Several controls were performed to determine whether the trafficking trap assay detects specific interactions between membrane proteins. First, we tested whether the trafficking trap assay could demonstrate interactions that have been previously characterized by biochemical methods. Using a coimmunoprecipitation

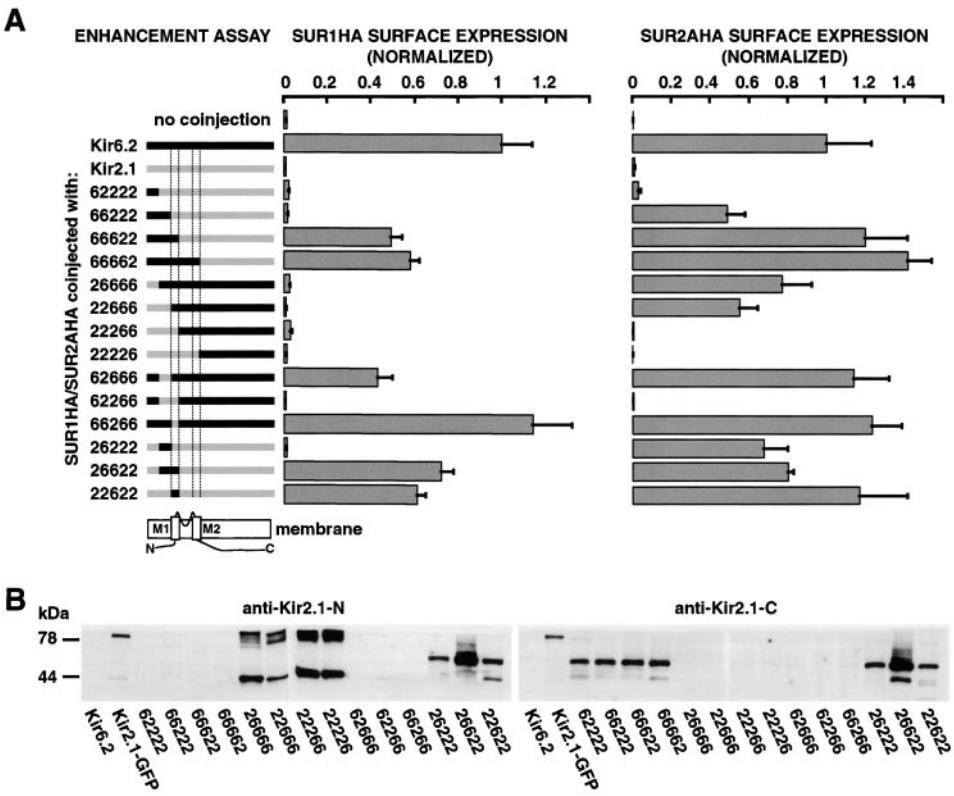

Figure 3. Trafficking Enhancement of SUR1HA and SUR2AHA by Kir2.1/Kir6.2 Chimeras

(A) Coexpression of SUR1HA (10 ng/oocyte) or SUR2AHA (10 ng/oocyte) with untagged chimeras (1 ng), as indicated. Surface signals were normalized to either SUR1HA or SUR2HA expressed alone.

(B) Western blot analysis of Kir2.1/Kir6.2 chimeras with antibodies against the N or C terminus of Kir2.1. For chimeras containing the C terminus of Kir6.2, aggregates with lower electrophoretic mobility were observed. For chimeras containing the C terminus of Kir2.1, proteolytic cleavage products with higher electrophoretic mobility were observed. Both phenomena are also observed with Kir6.2 and Kir2.1 wild-type proteins (B. S. et al., unpublished data).

assay, Tinker et al. (1996) characterized the major assembly domain in inward rectifier potassium channels that specify homotypic subunit association. The cytosolic C terminus was found to contain the primary compatibility determinant for subunit–subunit assembly. To determine whether our trap assay can detect the domains mediating the assembly of inward rectifiers, we expressed our trap chimeras (which do not contain an extracellular tag) with extracellular epitope-tagged Kir2.1PC (protein C epitope tag) or Kir6.2Δ36HA (a mutant form of Kir6.2 that is targeted to the plasma membrane when expressed alone [Tucker et al., 1997; Zerangue et al., 1999]). As predicted, coexpressing the trap versions of Kir2.1 and Kir6.2 (Kir2.1-R and Kir6.2-R) blocked surface expression of Kir2.1PC and Kir6.2Δ36HA, respectively (Figure 2B). Additionally, Kir2.1-R did not trap Kir6.2Δ36HA, and Kir6.2-R did not trap Kir2.1, thereby demonstrating that Kir2.1 and Kir6.2 do not coassemble. We next coexpressed other trap chimeras with Kir2.1PC and Kir6.2Δ36HA. As shown in Figure 2B, transmembrane segment M2 and the cytosolic C terminus of each trap chimera determined whether the camera trapped Kir2.1PC or Kir6.2Δ36HA. This result confirms that the C-terminal half of the protein contains the major determinant of homotypic subunit assembly for Kir2.1 and Kir6.2. We conclude that the trafficking trap assay provides information about interactions between membrane proteins that is consistent with biochemical studies.

As an additional control, we tested whether the trap constructs had nonspecific effects on expression or trafficking of an unrelated ABC protein, MRP1 (Cole et al., 1992). Figure 2C shows surface protein and total protein levels, as detected by single oocyte luminometry and Western blotting of total oocyte homogenates. In most cases, surface protein and total protein levels ranged between 70% and 115% of those of the control (MRP1HA expressed alone), indicating a lack of effect on MRP1HA surface expression. Several chimeras (26666-R, 22666-R, 22266-R, and 66266-R) reduced both overall protein levels and surface protein levels by ~50%, perhaps indicating a mildly toxic effect (Figure 2C). Even in cases in which less MRP1HA protein was detected, the ratio between the mature upper band and immature lower band was not grossly altered. The results from these control experiments are in contrast to what we observed for $SUR1HA_{AAA}$, where Kir6.2-R not only reduced surface expression but also eliminated mature glycosylation (Figure 1E) without affecting total protein levels.

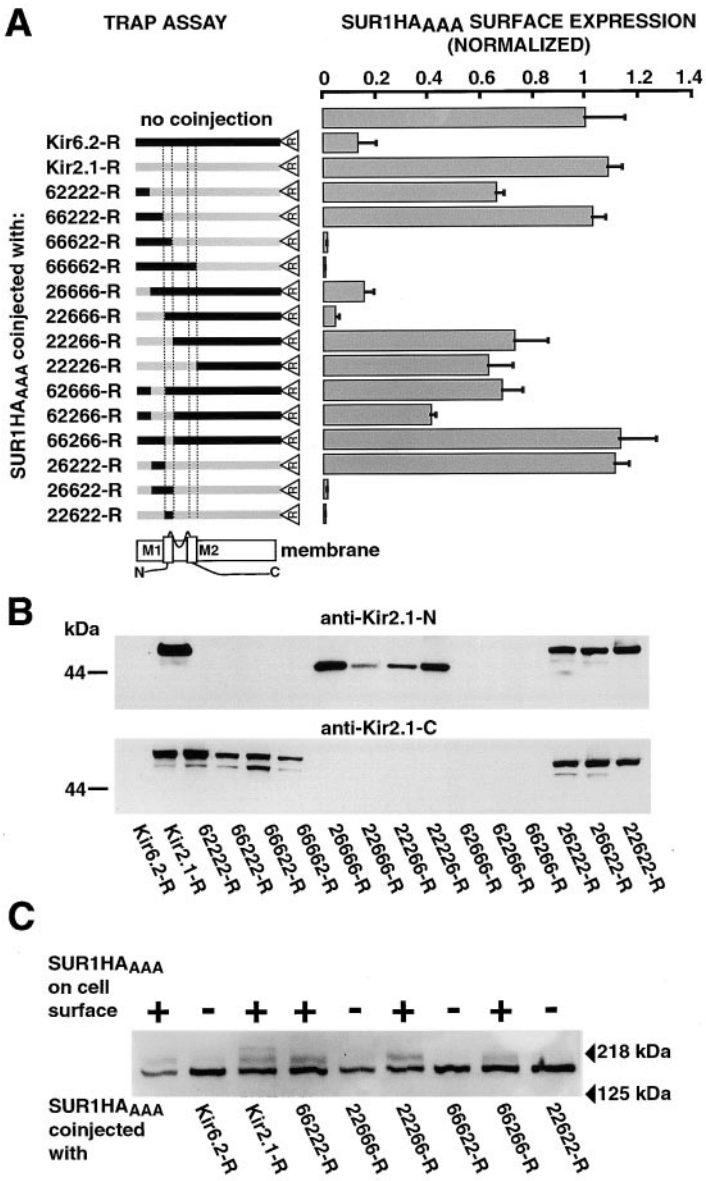

Figure 4. Trafficking Trap Analysis of SUR1HA$_{AAA}$

(A) Coexpression of SUR1HA$_{AAA}$ (10 ng/oocyte) with untagged trap chimeras (5 ng/oocyte), as indicated. Surface signals were normalized to SUR1HA$_{AAA}$ expressed alone.

(B) Western blot analysis of Kir2.1/Kir6.2 trap chimeras with antibodies against the N or C terminus of Kir2.1.

(C) Western blot analysis of SUR1HA$_{AAA}$ coexpressed with trap chimeras (detection with anti-HA antibody). Trap constructs that interfered with surface expression also reduced mature glycosylation of SUR1HA$_{AAA}$.

M1 and the N Terminus of Kir6.2 Contain Important Determinants for K_{ATP} Assembly

Having established our trafficking assay methodologies, we then tested the series of chimeras between Kir6.2 and Kir2.1 for interaction with SUR1HA or SUR2AHA (Figure 3A). Western blotting with antibodies against the N and C termini of Kir2.1 confirmed that all chimeras expressed protein (Figure 3B). Several chimeras containing the N terminus and transmembrane segment M1 of Kir6.2 (66662 and 66622) strongly stimulated the surface expression of SUR1HA (Figure 3A, left), but the enhancement was lost when M1 was changed to Kir2.1 (66222). This finding suggested that M1 may be important for assembling with SUR1. Indeed, a chimera consisting entirely of Kir2.1, except for M1 (22622), stimulated surface expression of SUR1HA, indicating that sequence determinants in M1 of Kir6.2 were sufficient to confer interaction with SUR1. However, M1 cannot be the only domain that interacts with SUR1, since the inverse chimera (66266) also stimulated the surface expression of SUR1HA. Neither the N nor the C terminus alone (66222 or 22266) conferred interaction with SUR1, yet both together (66266) stimulate surface expression. This suggests that interactions between the N and C termini of Kir6.2 are necessary for SUR1 to assemble with the cytoplasmic domains of Kir6.2.

The identity of the M1-independent assembly domain in Kir6.2 became clearer when we tested the chimeras with SUR2AHA (Figure 3A, right). Like SUR1HA, the chimera containing only M1 of Kir6.2 (22622) and the inverse chimera (66266) both stimulated SUR2HA. In contrast to what was observed for SUR1HA, chimeras containing only the conserved membrane-proximal region of the N terminus of Kir6.2 (66222 and 26222) potently enhanced surface expression of SUR2AHA. Chimeras containing the C-terminal portions of Kir6.2 (22266 and 22226) did not stimulate surface expression of SUR2AHA.

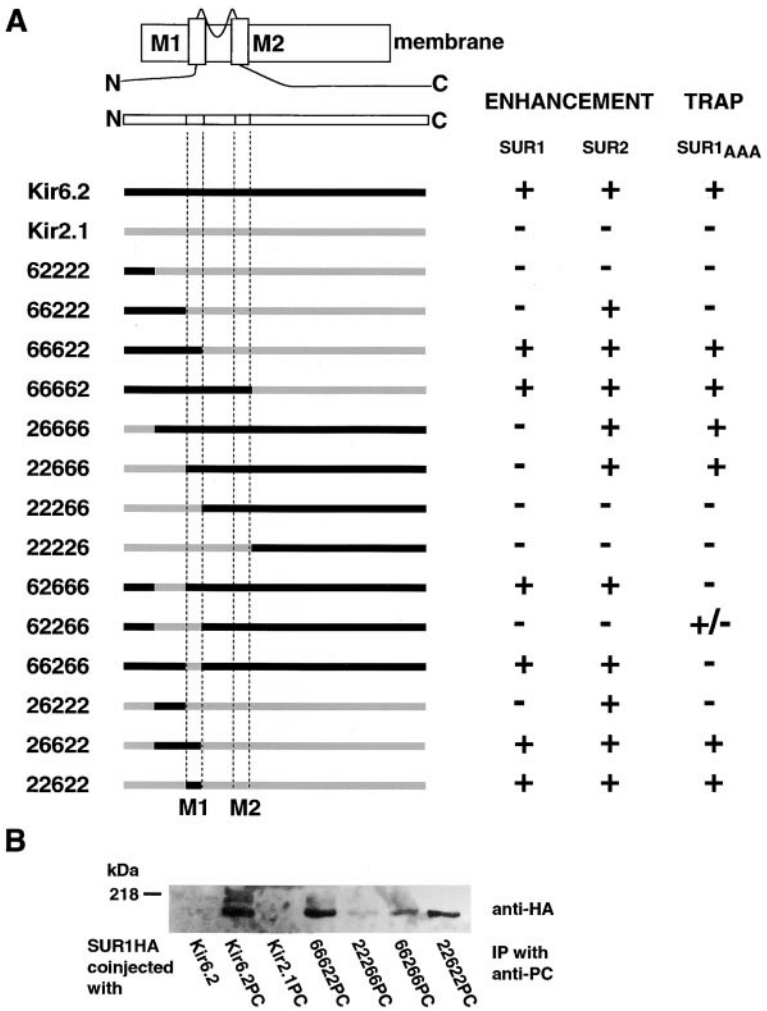

Figure 5. Summary of Data from Trafficking-Based Interaction Assays

(A) Schematic depiction of Kir6.2/2.1 topology and representation of chimeras. Results for enhancement of SUR1/2A surface expression and trapping of $SUR1_{AAA}$ are summarized to the right (compare Figures 3 and 4).

(B) Coimmunoprecipitation of SUR1HA with epitope-tagged versions of Kir6.2, Kir2.1, 66622, 22266, 66266, and 22622. The tag contains four copies of the protein C (PC) epitope. The first lane shows a control using an untagged form of Kir6.2. Channel proteins were precipitated from total oocyte homogenate using an antibody against the protein C epitope; the resulting immunopreciptate was resolved by SDS–PAGE and assayed for the presence of SUR1HA by Western blotting with an anti-HA antibody.

As an additional test for domains that mediate assembly of K_{ATP}, we examined whether the trap version of the chimeras reduced $SUR1_{AAA}$ surface expression (Figure 4A). Protein expression of trap chimeras was confirmed by Western blotting of total oocyte homogenates using antibodies directed against the N and C termini of Kir2.1 (Figure 4B). In contrast to what we observed for MRP1HA (Figure 2C), $SUR1HA_{AAA}$ was retained from the cell surface by several chimeras (Figure 4A). Consistent with what we observed for trafficking enhancement, chimera 22622-R reduced $SUR1HA_{AAA}$ surface expression to $<1\%$ of control levels. Therefore, M1 of Kir6.2 is sufficient to confer interaction with SUR1 in both the trafficking enhancement and trafficking trap assays. Several chimeras (26666 and 22666) that did not stimulate SUR1HA surface expression were observed to block surface expression of $SUR1HA_{AAA}$ when tested in the trap assay. Western blots of $SUR1HA_{AAA}$ provide additional evidence that reductions in surface expression reflect specific interactions and trapping in the ER (Figure 4C). Chimeras that reduced surface expression of $SUR1HA_{AAA}$ also prevented mature glycosylation without altering protein levels. Figure 5A summarizes the results with the different chimeras for the two types of trafficking-based interaction assay.

To verify our trafficking-based results using a biochemical method, we performed coimmunoprecipitation experiments with four of the chimeras and SUR1HA (Figure 5B). A multicopy protein C epitope tag was fused to the C terminus of the channel constructs to facilitate immunoprecipitation. As expected from previous studies (Giblin et al., 1999), Kir6.2 but not Kir2.1 coprecipitated SUR1HA. Consistent with our trafficking results, chimera 66622 coprecipitated SUR1HA as efficiently as Kir6.2, whereas chimera 22266 only weakly coprecipitated SUR1HA. Additionally, chimera 22622 coprecipitated SUR1HA more efficiently than chimera 66266 did, indicating that the transmembrane interactions between Kir6.2 and SUR1 are important for stable detergent-resistant interactions.

A consistent finding from both trafficking stimulation and trafficking trap assays was that M1 of Kir6.2 contains sequence determinants for interaction with SUR1 and SUR2A. To localize these determinants more precisely, we exchanged groups of just a few residues in M1 of 22622 and tested for enhancement of SUR1HA and SUR2AHA surface expression. The M1 transmembrane segment of Kir6.2 differs from Kir2.1 at 11 out of 23 positions. We divided these 11 amino acids into four groups, which were then changed in 22622 to the corresponding amino acid in Kir2.1 (Figure 6A). Changing

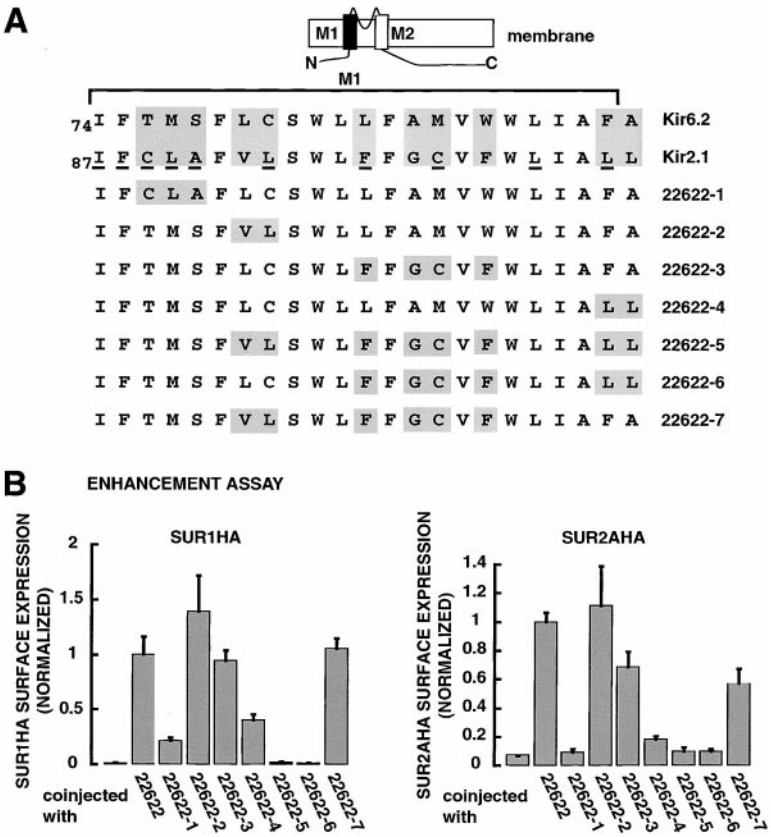

Figure 6. Residues in M1 of Kir6.2 Important for Assembly with SUR1 and SUR2A

(A) Alignment of the first transmembrane domain of Kir2.1 and Kir6.2. Sequence differences are highlighted (shaded boxes); Kir2.1 residues that have been found by Minor et al. (1999) to be highly tolerant toward substitution are underlined. In the chimera 22622, different amino acids in M1 were changed back to the corresponding amino acids in Kir2.1.

(B) Surface stimulation of SUR1HA (10 ng/oocyte) and SUR2AHA (10 ng/oocyte) by 22622 mutants (1 ng). Surface signal was normalized to expression of SUR1HA or SUR2HA alone.

the TMS (threonine-methione-serine) sequence at the beginning of M1 to CLA (22622-1) had the largest effect. Assembly with SUR2AHA was completely abolished, and assembly with SUR1 was reduced by 85% (Figure 6B). Assembly with SUR1HA and SUR2AHA were also reduced when the last two amino acids of M1 (FA) were changed to LL (22622-4). Other changes in M1 (22622-2 and 22622-3) had no effect. To define the minimal set of changes in M1 required for assembly, we first changed the CLA sequence in Kir2.1 to TMS (22622-5), but no stimulation was observed. When the TMS sequence and the FA sequence were introduced into M1 of Kir2.1 (22622-7), clear stimulation of both SUR1HA and SUR2AHA was observed. We conclude that changing five amino acids near the beginning and end of M1 of Kir2.1 to the corresponding amino acids in Kir6.2 is sufficient to allow assembly with SUR1HA and SUR2AHA.

Functional Analysis of Chimeras Assembled with SUR

We next characterized the basic functional properties of Kir2.1/Kir6.2 chimeras. Most chimeras (66222, 66622, 66662, 22666, 22266, 22226, 62666, 62266, and 26622) did not exhibit functional channels when expressed alone or with SUR1 (data not shown). Several chimeras (62222, 62622, and 22622) exhibited large currents when expressed alone (Figure 7A; data not shown). Since chimera 22622 assembles with SUR1, we next attempted to investigate the properties of these channels. However, 22622 does not require SUR1 to express at the cell surface, making it impossible to record only channels formed by 22622 and SUR1. To circumvent this problem, we developed a method to restrict surface expression to channels that are fully assembled with four SUR1 subunits. We have previously shown that an RKR sequence near the C terminus of Kir6.2 prevents it from trafficking to the cell surface unless assembled with SUR (Zerangue et al., 1999). We reasoned that a channel chimera containing the RKR sequence at the C terminus would also require assembly with SUR for trafficking to the cell surface. When the nonconserved last 60 amino acids of Kir2.1 were replaced with the last 34 amino acids from Kir6.2, no currents were observed due to the presence of the RKR sequence in the Kir6.2 sequence (Figure 7A). Coexpressing SUR1 with Kir2.1-RKR did not restore current expression, consistent with a lack of interaction between SUR1 and Kir2.1. Like Kir2.1-RKR, no currents were observed when 22622-RKR was expressed alone. However, when SUR1 was coexpressed with 22622-RKR, strongly inwardly rectifying potassium currents were present (Figure 7A).

Having restored the trafficking checkpoint mechanism to 22622, we next tested whether channel complexes containing both SUR1 and 22622-RKR exhibited channel properties characteristic of K_{ATP}. Wild-type K_{ATP} channels containing SUR1 and Kir6.2 are potently activated by metabolically inhibiting the cell with 3 mM azide (Gribble et al., 1997a; Figure 7B). Furthermore, SUR1/Kir6.2 currents are completely blocked by sulphonylurea drugs, such as 10 μM glibenclamide. However, currents arising from 22622-RKR assembled with SUR1 did not respond to either azide treatment or glibenclamide. When the first 30 amino acids of Kir6.2 (which are not sufficient for assembly) are introduced into 22622-RKR

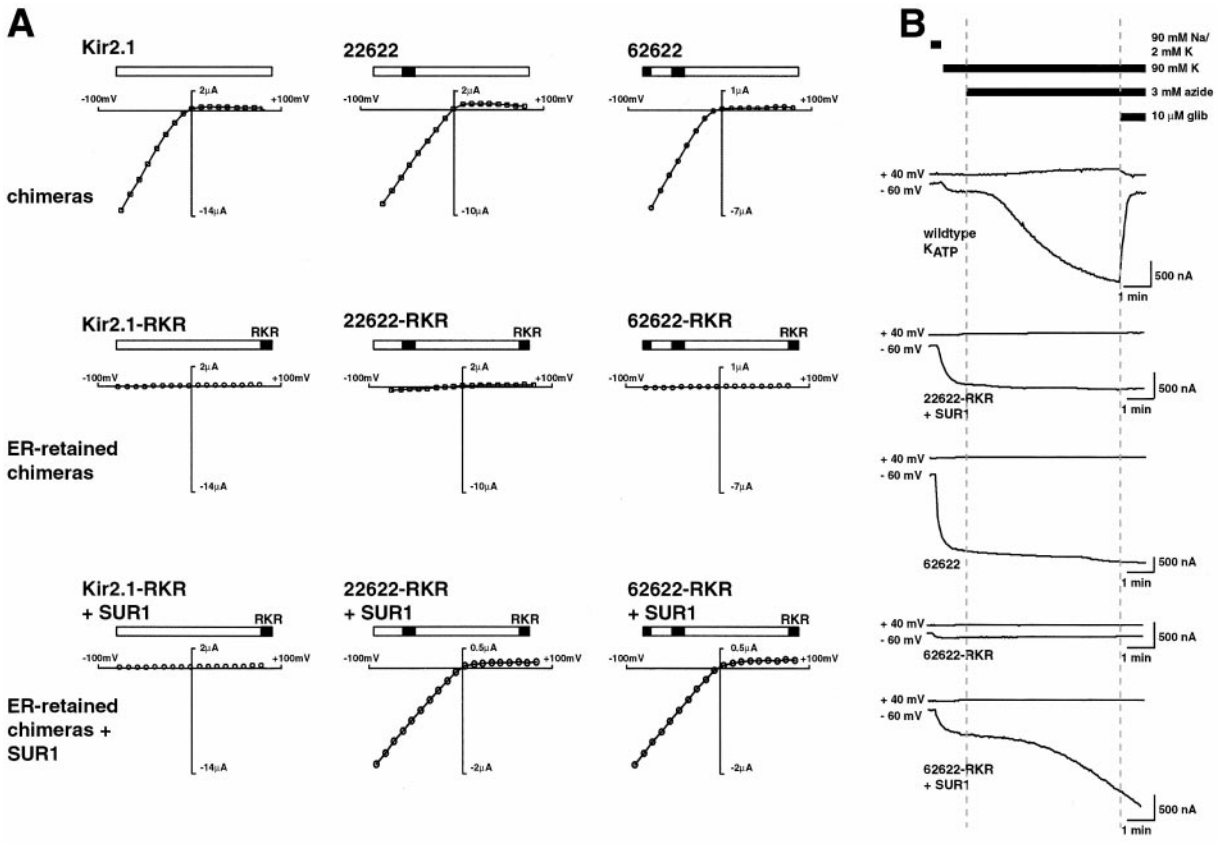

Figure 7. Analysis of Currents from Kir6.2/Kir2.1 Chimeras Assembled with SUR1

(A) Two-electrode voltage-clamp analysis of various constructs expressed in *Xenopus* oocytes. All constructs (1 ng/oocyte) exhibited large, strong, inwardly rectifying K^+ currents when expressed alone. Addition of the RKR sequence to the C termini of Kir2.1, 22622, and 62622 (1 ng/oocyte each) abolished current expression. Coexpression of SUR1 (10 ng) restored current expression for 22622-RKR and 62622-RKR but not Kir2.1-RKR.

(B) Effect of metabolic inhibition and K_{ATP} blockers on Kir6.2/Kir2.1 chimeras assembled with SUR1. Time course of currents at +40 mV and −60 mV recorded from oocytes expressing the combinations of constructs are indicated. At the times indicated, 3 mM azide or 10 μM glibenclamide was added to the bath solution (dashed lines).

to form 62622-RKR, channels resulting from coexpression with SUR1 were activated by azide treatment but not inhibited by glibenclamide application (Figure 7B). Sensitivity to azide treatment was dependent on assembly with SUR1, since channels formed by 62622 alone were not affected by azide treatment. Furthermore, we tested the effect of mutations in the NBDs of SUR1 that are expected to reduce ATPase activity (SUR1 K719A and K1385M; Gribble et al., 1997b). When the mutated SUR1 was coexpressed with Kir6.2HA, the level of surface expression was similar to that of wild-type SUR1, but azide-stimulated currents were reduced by 95% (data not shown). However, channels containing 62622-RKR were still activated to the same extent when coexpressed with SUR1 containing the NBD mutations (data not shown). Coexpressing Kir6.2 with SUR2AHA results in high levels of channel expression on the cell surface, but currents are not activated by azide (data not shown). When 62622-RKR is expressed with SUR2AHA, basal K^+ currents are present but are not further activated by azide (data not shown). Together, these results suggest that assembly with SUR1, but not SUR2A, confers on 62622-RKR the ability to respond to metabolic inhibition.

Assembly of SUR1-MRP1 Chimeras with Kir6.2/Kir2.1 Chimeras

To address which domains of SUR1 are important for assembly with transmembrane segment M1 and the cytoplasmic domains of Kir6.2, we created a series of chimeras between SUR1 and MRP1, a homologous ABC protein that does not assemble with Kir6.2. We chose junctions for the chimeras based on the previously characterized domain structure of MRP proteins (Tusnády et al., 1997). As shown in Figure 8A, these include transmembrane domain 0 (TMD0), which contains five transmembrane segments (Raab-Graham et al., 1999) and is unique to members of the MRP family; transmembrane domain 1 (TMD1); nucleotide-binding domain 1 (NBD1); transmembrane domain 2 (TMD2); and nucleotide-binding domain 2 (NBD2). These five domains from MRP1 were introduced independently into SUR1 (MSSSS, SMSSS, SSMSS, SSSMS, and SSSSM). SUR1-MRP1 chimeras were then tested for their ability to stimulate the surface expression of Kir6.2HA, 22622HA-RKR, and 66266HA (which contains the C terminus of Kir6.2 with the RKR motif; Zerangue et al., 1999). These Kir2.1-Kir6.2 chimeras were chosen to examine transmem-

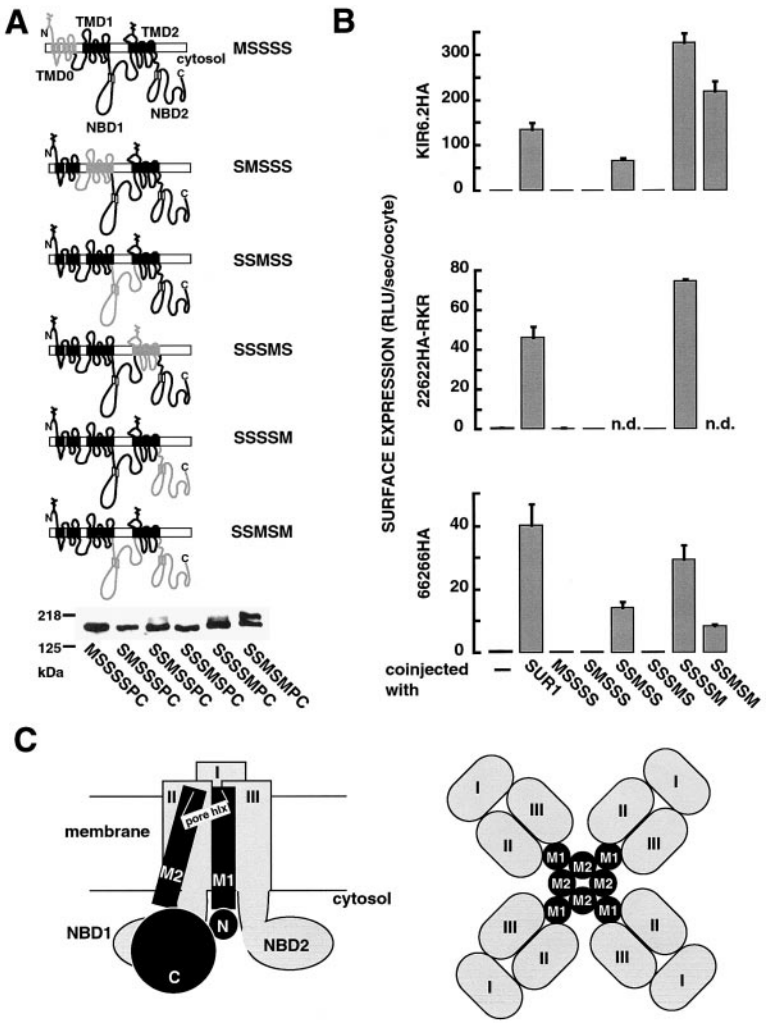

Figure 8. Analysis of SUR1-MRP1 Chimeras

(A) Diagrams illustrate chimeric constructs in which TMD0, TMD1, NBD1, TMD2, and NBD2 of SUR1 (black) were replaced with the corresponding domain from MRP1 (shaded). Protein levels for SUR1-MRP1 chimeras were determined by Western blotting using an antibody against a protein C epitope fused to the C terminus of each of the chimeras.

(B) Surface signal for Kir6.2HA, 22622HA-RKR, and 66266HA (1.5 ng/oocyte) coexpressed with SUR1-MRP1 chimeras (10 ng/oocyte).

(C) Schematic illustration of interactions in the K_{ATP} channel complex based on data in Figures 3–7. Left diagram, view of one SUR1 and one Kir6.2 protein from within the membrane; right diagram, top view of the assembled octameric complex. M1 of Kir6.2 is a major assembly determinant and must, therefore, interact with SUR transmembrane domains. The N terminus of Kir6.2 assembles with a cytosolic portion of SUR, probably not via the NBDs but rather with the cytosolic face of one of the transmembrane domains. In addition, the cytosolic domains of Kir6.2 interact with each other (Tucker and Ashcroft, 1999).

brane (22622HA-RKR) or cytoplasmic (66266HA) interactions between SUR1 and Kir6.2. Surface signals for each of these chimeras was increased at least 200-fold by coexpression of SUR1 (Figure 8B).

Since NBD1 and NBD2 are the largest cytoplasmic domains in SUR1 and are highly conserved between SUR1 and SUR2, we expected that the 66266HA chimera would be unable to assemble with chimeras in which NBD1 or NBD2 are from MRP1 (SSMSS and SSSSM). Conversely, 22622HA-RKR was predicted to require one or more of the transmembrane domains from SUR1. Interestingly, SSMSS and SSSSM both stimulated surface expression of Kir6.2HA, 22622HA-RKR (SSMSS not determined), and 66266HA (Figure 8B), suggesting that the NBDs of SUR1 may not be required for assembly with the cytoplasmic domains of Kir6.2. Even replacing both NBDs in SUR1 (SSMSM) did not prevent assembly with 66266HA. While the NBDs of SUR1 were not essential for assembly, changing the transmembrane blocks to MRP1 had a more drastic effect. Little or no surface stimulation for any channel construct was observed with these chimeras. Although these negative results could potentially be due to misfolding of the SUR1-MRP1 chimeras, total protein levels were similar for all of the chimeras (Figure 8A).

Discussion

Trafficking-Based Assays for Detecting Interactions between Membrane Proteins

Traditional approaches to studying oligomerization of membrane proteins primarily have relied on copurification and, in the case of ion channels, dominant-negative functional assays. While both approaches can provide valuable information, copurification requires detergent solubilization of membrane proteins, potentially destroying some hydrophobic interactions or causing aggregation artifacts. Dominant-negative functional assays require prior knowledge of mutations that ablate function of the heteromultimeric complex without disrupting assembly. An approach that avoids these problems is to take advantage of the fact that many membrane protein complexes are not competent to traffick to the cell surface unless properly oligomerized. Trafficking-based assays have the advantage of detecting protein–protein interactions in the undisturbed native environment of the ER membrane and may allow the detection of weaker interactions than does traditional biochemistry. For example, assembly of $GABA_A$ and nicotinic receptors has been successfully studied using antibody staining or toxin binding to detect protein maturation (Green and

Claudio, 1993; Connolly et al., 1996). We have used a variant of this approach to study K_{ATP} assembly. Since SUR1/2 does not traffick to the cell surface unless assembled with Kir6.2, we were able to create chimeras between Kir6.2 and Kir2.1 and test their ability to promote surface expression of extracellular epitope-tagged SUR1 and SUR2 (Figures 1 and 3). Chimeras between SUR1 and MRP1 were tested for stimulation of epitope-tagged Kir6.2/Kir2.1 chimeras (Figure 8). Furthermore, the general utility of this approach is greatly improved by the sensitivity and simplicity of the chemiluminescent surface protein assay we have developed (Zerangue et al., 1999).

Many membrane proteins can traffick independently to the plasma membrane alone and cannot be studied using the trafficking enhancement approach. For these proteins, the trafficking trap approach may be especially useful (Figures 1, 2, and 4). Strong ER retention/retrieval signals offer an unexplored resource for generating dominant-negative constructs for studying membrane proteins. We have found that the presence of an exposed ER retention/retrieval signal on one or more subunits in a membrane protein complex usually has a strong dominant-negative effect on the trafficking of the entire complex (Minor et al., 1999; Zerangue et al., 1999; Figures 1 and 2). Using ER retention/retrieval signals to create dominant-negative constructs offers several advantages. First, in most cases, fusing a short sequence containing the ER retention/retrieval signal to a cytoplasmic domain (usually the C terminus) is sufficient to create a dominant-negative construct and does not require any specific knowledge about the structure–function aspects of the protein of interest. Additionally, mutations previously used to create dominant-negative constructs are often in sensitive regions of the protein, such as the pore-forming domains in ion channels, and may impair assembly, thereby complicating interpretations.

Several lines of evidence suggest that our trafficking-based assays detect specific physical interactions between Kir2.1/Kir6.2 chimeras and SUR1/2. For the majority of chimeras, the trafficking stimulation and trafficking trap assays provided similar results, even though they measured opposite effects on the surface expression of SUR (Figures 3A, 4A, and 5A). Consistent with a previous study demonstrating that the C terminus of inwardly rectifying potassium channel subunits determines assembly compatibility (Tinker et al., 1996), wild-type Kir2.1 or Kir6.2 subunits were only retained by trap chimeras with the same C terminus (Figure 2B). This result demonstrates that the trafficking trap approach reproduces findings based on biochemical and dominant-negative functional assays. Additionally, trapping of $SUR1HA_{AAA}$ by some chimeras in the ER was independently confirmed by a biochemical parameter, disappearance of the mature glycosylated form of the receptor (Figures 1E and 4C). Results obtained by quantitation of surface protein were consistent with results from a current assay in which chimera 22622, containing an ER retention/retrieval motif at the C terminus, only expressed current in the presence of SUR1 (Figure 7). Finally, the results from the trafficking-based interaction assay could be confirmed by coimmunoprecipitation of SUR1HA with selected chimeras (Figure 5).

The Role of M1 and the N Terminus of Kir6.2 in K_{ATP} Assembly and Gating

Our finding that transmembrane segment M1 is an important assembly domain is consistent with structural studies showing that M1 does not directly contribute to the ion-conducting pathway and is exposed to the lipid membrane environment. A recent crystal structure of the bacterial potassium channel KcsA shows that the second transmembrane segment (M2) and the preceding pore domain (H5) from four subunits form the ion permeation pathway (Doyle et al., 1998). The first transmembrane segment (M1) from each subunit surrounds the inner core and is therefore accessible from within the lipid bilayer. A model of Kir2.1 transmembrane structure recently obtained by Minor et al. (1999) using a genetic approach is consistent with M1 being the most accessible part of the protein within the bilayer. Our mutagenesis within the minimal chimera 22622 revealed that the TMS and FA residues at the beginning and end of the M1 helix were most critical for the interaction with SUR (Figures 6A and 6B). The side chains of each of these five residues in Kir2.1 are likely to be exposed, since these positions tolerated substitution to hydrophobic amino acids of varying sizes (Minor et al., 1999; Figure 6A). Furthermore, these residues fall on the face of the helix that is predicted to form the protein–lipid interface of Kir2.1 and are therefore in the right place to interact with a transmembrane domain of SUR.

Mutational analysis of K_{ATP} gating to date has focused on the cytoplasmic domains and transmembrane segment M2 of Kir6.2 (Drain et al., 1998; Trapp et al., 1998; Tucker et al., 1998; Koster et al., 1999; Proks et al., 1999; Reimann et al., 1999). Our results clearly demonstrate that SUR1/2 specifically recognizes sequence determinants in M1, and the observation that azide can stimulate 62622-mediated currents in an SUR1-dependent manner suggests that M1 may also participate in gating (Figures 5 and 7B). While it is clear from structural studies that M1 is unlikely to directly form part of the ion-conducting pathway, M1 may physically interact with the pore domain (H5). Therefore, conformational changes in M1 could affect gating by altering pore conformations. If transmembrane gating occurs during K_{ATP} activation, it raises the unexamined possibility that inward rectifier gating in general involves conformational changes in transmembrane domains. Recent studies suggest that rearrangements in the outer pore may be responsible for some forms of gating in voltage-dependent K^+ channels (Liu et al., 1996; Loots and Isacoff, 1998; Zheng and Sigworth, 1998).

We find that SUR not only recognizes M1, but also interacts specifically with the cytosolic N terminus (Figure 3A). A number of studies have addressed the role of the N terminus in ATP inhibition of the K_{ATP} channel (Tucker et al., 1998; Babenko et al., 1999; Koster et al., 1999; Proks et al., 1999; Reimann et al., 1999). Mutational analysis suggests that the N terminus, perhaps cooperatively with the C terminus, forms part of the ATP-binding site. Several groups have reported that various deletions in the N-terminal domain of Kir6.2 abolish the sensitization effect of SUR1 on Kir6.2 ATP inhibition (Babenko et al., 1999; Koster et al., 1999; Reimann et al., 1999), suggesting that SUR1 may interact with this domain. Consistent with these findings, the distal N terminus

of Kir6.2 was important for mediating SUR1-dependent responses to azide. However, when coexpressed with an SUR1 mutant expected to have impaired ATPase activity (Gribble et al., 1997b), metabolic inhibition still activated 62622-RKR fully, whereas channels formed by Kir6.2 and the SUR1 mutant exhibited weak activation (data not shown). It is possible that ATP hydrolysis is required to relieve the ATP inhibition of wild-type channels involving specific interactions between the cytoplasmic domains, which are likely to be lacking in the 62622-RKR chimera.

Our data clearly show that either M1 or the N terminus of Kir6.2 is sufficient for conferring assembly with SUR1/2. A previous analysis of chimeras between Kir6.2 and Kir2.1 found that the C terminus of Kir6.2 was required for coimmunoprecipitation of chimeras with SUR1 when expressed in stable transfected cell lines (Giblin et al., 1999). However, we found that chimeras containing only the C terminus of Kir6.2 (22226 and 22266) did not interact with SUR1 or SUR2 in either the trafficking stimulation or the trafficking trap assay (Figure 5A), and chimera 22266 coprecipitated much less SUR1HA than did any other chimera tested (Figure 5B). It is unlikely that our failure to detect such interactions was due to lack of 22226 and 22266 protein, since both of these chimeras were detectable by Western blotting, and 22266-R is capable of assembling with Kir6.2Δ36 (Figures 2–4). Although the N terminus of Kir6.2 was sufficient for interaction with SUR2A, our data are also consistent with SUR1 recognizing a cytoplasmic surface of Kir6.2 composed of both the N and C termini. Such an interaction between the N and C termini of Kir6.2 has recently been demonstrated (Tucker and Ashcroft, 1999). Since our experiments were performed at 16°C in *Xenopus* oocytes, it is possible that interactions between SUR1 and the C terminus alone could be temperature dependent or cell-type specific, or require coexpression over longer time periods.

Transmembrane Interactions between ABC Transporters and Ion Channels

Comparing the sequence of SUR1/2 to that of other ABC proteins reveals that SUR1/2 is most closely related to the multidrug resistance-associated protein family MRP1–6 (Tusnády et al., 1997; Cole and Deeley, 1998; Kool et al., 1999). Members of the MRP family specialize in transporting amphipathic substrates, such as glutathione-conjugated endogenous lipophilic molecules and xenobiotics (referred to as GS-X pump). MRP proteins are able to recognize their substrates via binding sites in the membrane. A substrate specificity domain of MRP1 has been mapped to the last transmembrane block of MRP (Stride et al., 1999). Interestingly, both sulphonylurea binding and opener binding specificity maps to the last transmembrane block of SUR1/2A/2B (Schwanstecher et al., 1998; Uhde et al., 1999). Opener binding, unlike binding of sulphonylurea drugs, also requires hydrolyzable Mg ATP, typical of substrate interactions with many ABC proteins (Schwanstecher et al., 1998). It has been proposed that K_{ATP} openers function to uncouple Kir6.2 from SUR1 inhibition (Shyng et al., 1997). These observations raise the possibility that K_{ATP} openers disrupt the interaction between SUR1 and M1 of Kir6.2, possibly competing for the same binding site in SUR.

The observation that SUR1/2 interacts specifically with a transmembrane segment of Kir6.2 may help explain how an ABC protein could assemble with and regulate another membrane protein, such as an ion channel. Substrates for MRP proteins are typically amphipathic, containing a polar moiety (e.g., glutathione) and a hydrophobic portion that partitions into the inner leaflet of the lipid membrane. We found that the most critical M1 determinant was the TMS sequence located at the beginning of the transmembrane domain, predicted to be close to the cytoplasmic lipid interface. Further experiments will be required to determine whether SUR1/2 recognizes M1 of Kir6.2 using a transmembrane-binding pocket similar to the substrate-binding pocket of MRP proteins. Such an interaction would provide a simple explanation for how an ABC transporter of hydrophobic compounds evolved the capacity to interact with other membrane proteins. Furthermore, such an interaction may also provide insight into how the transport cycle of ABC proteins has been utilized by SUR1/2 to regulate the gating of a potassium channel.

It will be interesting to compare the assembly domains we identified with other examples of ABC proteins interacting with ion channels. For example, CFTR has been shown to interact with a number of ion channels, such as epithelial sodium channels, outwardly rectifying chloride channels, and an inwardly rectifying potassium channel (Kir1.1) (Schwiebert et al., 1999, and references therein). It has been proposed that CFTR assembles with Kir1.1 to form a channel with properties similar to renal K_{ATP} channels (Ruknudin et al., 1998). These channels may utilize similar structural mechanisms for assembly, as we have observed for Kir6.2 and SUR1/2.

Experimental Procedures

Molecular Biology

General protocols were from Ausubel et al. (1997). All constructs were in pGEMHE (Liman et al., 1992). Tagging of Kir6.2 and SUR1 with extracellular HA epitopes has been described (Zerangue et al., 1999). HA-tagged MRP1 was a kind gift of Christina Kast and Philippe Gros (Kast and Gros, 1998, construct 5b). For SUR2AHA, the epitope was introduced in the same location as for SUR1HA (the protein sequence at the site of epitope insertion reads ^{1242}GLVHREG VYPYDVPDYAHRELSAGLV^{1244}). All chimeras were created by sequential overlapping polymerase chain reactions (PCRs). The protein sequences at the breakpoints read ^{28}TRER-RSRF^{47} (62XXX), ^{40}RQQC-RARF^{35} (26XXX), ^{70}HTLL-IFCL^{90} (X62XX), ^{83}WMLV-IFTM^{77} (X26XX), ^{98}GDLA-DTSK^{117} (XX62X), ^{111}GDLD-PGEG^{105} (XX26X), ^{164}LGCI-MAKM^{193} (XXX62), and ^{186}IGAV-FMKT^{171} (XXX26). The C-terminal 14 amino acids of the human α2C adrenergic receptor (-KHILFRRRRRGFRQ) were fused to the C termini of Kir6.2 and Kir2.1 by using an artificially introduced NotI site (described in Zerangue et al., 1999). The same NotI site was used to fuse four copies of the protein C epitope (EDQVDPRLIDGK) to the C termini of selected chimeras. To create the SUR1-MRP1 chimeras, artificial restriction sites were engineered into both cDNAs. Introduction of the sites introduced the following mutations into the corresponding proteins: SUR1 (insertion of two residues, ^{206}P**VD**P^{207}; insertion of two residues, ^{692}G**LIN**P^{694}; exchange of two residues, ^{963}R**EA**L^{966}—note that these changes are found in SUR2), MRP1 (insertion of one, exchange of one residue, ^{205}P**VD**P^{207}; insertion of three residues, ^{658}P**LIN**P^{659}; and insertion of three residues, ^{921}S**EAL**G^{922}; each individual chimera contains only the sites flanking the respective swapped piece). Four copies of the protein C epitope were fused to the C terminus of

each of the chimeras via an artificially introduced NotI site that replaces the stop codon. PCR-derived sequences were entirely sequenced. cRNA was transcribed using T7 RNA polymerase.

Oocyte Surface Protein Assays

Xenopus oocytes were prepared and maintained as previously described (Collins et al., 1997). Oocytes were injected with the amount of cRNA indicated in the figure legends. Surface assays were performed 3–5 days after injection. 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 [Roche], in 1% BSA for 30–60 min at 4°C), washed at 4°C, and incubated with horseradish peroxidase– (HRP-) coupled secondary antibody (HRP-conjugated goat anti-rat F[Ab′]2 fragments [Jackson], in 1% BSA for 30–60 min at 4°C). Cells were extensively washed (1% BSA, 60 min, 4°C) and transferred to frog Ringer solution 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 TD-20/20 luminometer (Turner Designs).

SDS–PAGE and Immunoblotting

Oocytes were homogenized essentially as described (Tucker et al., 1996), separated by SDS–PAGE electrophoresis (4%–15% gradient gels [BioRad] and 8% gels), and transferred to nitrocellulose. Blots were blocked in TBS containing 5% milk powder and 0.05% NP-40. Primary (rabbit anti-Kir2.1 antibodies, 1:2000; rat anti-HA monoclonal 3F10, Roche, 200 ng/ml; mouse anti–protein C monoclonal HPC4, Roche, 500 ng/ml) and secondary (HRP-conjugated anti-rabbit polyclonal antibody, Amersham, 1:1000; HRP-conjugated goat anti-rat F[Ab′]2 fragments, Jackson, 1:1000) antibodies were diluted in TBS-blocking solution. The rabbit anti-Kir2.1 antibodies recognize the following peptide epitopes: anti-N2, ^{25}AVANGFGNGKSKVHTRQQC^{43}, and anti-C2, ^{351}NTPLCSARDLAEK KYILSNANSF^{374}. Washes were in TBS, 0.05% NP-40. Detection was performed using the ECL system (Amersham).

Coimmunoprecipitation

Oocytes were homogenized essentially as described (Tucker et al., 1996), and the resulting homogenate was solubilized in five volumes of lysis buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1 mM EDTA, and 1% NP-40, complemented with Complete protease inhibitors [Roche] and 1 mM PMSF) and cleared by centrifugation. Immunoprecipitates were obtained by incubation with 5 μg mouse monoclonal anti–protein C antibody (Roche) and protein G–Sepharose (Pharmacia) overnight, washed three times in lysis buffer with 0.1% NP-40 and once with phosphate-buffered saline (PBS), and eluted with SDS–PAGE sample buffer at 65°C.

Electrophysiology

Standard two-electrode voltage-clamp recording (Geneclamp 500, pCLAMP software) was performed on oocytes 2–5 days after injection. Unless otherwise stated, currents were measured in solution containing 90 mM KCl, 10 mM Na HEPES, 1.8 mM $CaCl_2$, and 1.0 mM $MgCl_2$ (pH 7.4). For time course measurements, steady-state currents were measured after a 50 ms voltage jump to +40 mV or −90 mV every 4 s.

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References

Aguilar-Bryan, L., and Bryan, J. (1999). Molecular biology of adenosine triphosphate-sensitive potassium channels. Endocr. Rev. *20*, 101–135.

Allikmets, R., Gerrard, B., Court, D., and Dean, M. (1993). Cloning and organization of the abc and mdl genes of Escherichia coli: relationship to eukaryotic multidrug resistance. Gene *136*, 231–236.

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 and Sons).

Babenko, A.P., Gonzalez, G., and Bryan, J. (1999). The N-terminus of KIR6.2 limits spontaneous bursting and modulates the ATP-inhibition of KATP channels. Biochem. Biophys. Res. Commun. *255*, 231–238.

Cole, S.P., and Deeley, R.G. (1998). Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP. Bioessays *20*, 931–940.

Cole, S.P., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart, A.J., Kurz, E.U., Duncan, A.M., and Deeley, R.G. (1992). Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science *258*, 1650–1654.

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.

Connolly, C.N., Krishek, B.J., McDonald, B.J., Smart, T.G., and Moss, S.J. (1996). Assembly and cell surface expression of heteromeric and homomeric gamma-aminobutyric acid type A receptors. J. Biol. Chem. *271*, 89–96.

Decottignies, A., and Goffeau, A. (1997). Complete inventory of the yeast ABC proteins. Nat. Genet. *15*, 137–145.

Doyle, D.A., Cabral, J.M., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T., and MacKinnon, R. (1998). The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. Science *280*, 69–77.

Drain, P., Li, L., and Wang, J. (1998). KATP channel inhibition by ATP requires distinct functional domains of the cytoplasmic C terminus of the pore-forming subunit. Proc. Natl. Acad. Sci. USA *95*, 13953–13958.

Giblin, J.P., Leaney, J.L., and Tinker, A. (1999). The molecular assembly of ATP-sensitive potassium channels. Determinants on the pore forming subunit. J. Biol. Chem. *274*, 22652–22659.

Green, W.N., and Claudio, T. (1993). Acetylcholine receptor assembly: subunit folding and oligomerization occur sequentially. Cell *74*, 57–69.

Gribble, F.M., Ashfield, R., Ammälä, C., and Ashcroft, F.M. (1997a). Properties of cloned ATP-sensitive K^+ currents expressed in Xenopus oocytes. J. Physiol. *498*, 87–98.

Gribble, F.M., Tucker, S.J., and Ashcroft, F.M. (1997b). The essential role of the Walker A motifs of SUR1 in K-ATP channel activation by Mg-ADP and diazoxide. EMBO J. *16*, 1145–1152.

Higgins, C.F. (1992). ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. *8*, 67–113.

Holland, I.B., and Blight, M.A. (1999). ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. J. Mol. Biol. *293*, 381–399.

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.

Kool, M., van der Linden, M., de Haas, M., Scheffer, G.L., de Vree, J.M., Smith, A.J., Jansen, G., Peters, G.J., Ponne, N., Scheper, R.J.,

et al. (1999). MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc. Natl. Acad. Sci. USA *96*, 6914–6919.

Koster, J.C., Sha, Q., Shyng, S., and Nichols, C.G. (1999). ATP inhibition of KATP channels: control of nucleotide sensitivity by the N-terminal domain of the Kir6.2 subunit. J. Physiol. *515*, 19–30.

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.

Liu, Y., Jurman, M.E., and Yellen, G. (1996). Dynamic rearrangement of the outer mouth of a K^+ channel during gating. Neuron *16*, 859–867.

Loots, E., and Isacoff, E.Y. (1998). Protein rearrangements underlying slow inactivation of the Shaker K^+ channel. J. Gen. Physiol. *112*, 377–389.

Minor, D.L., Jr., Masseling, S.J., Jan, Y.N., and Jan, L.Y. (1999). Transmembrane structure of an inwardly rectifying potassium channel. Cell *96*, 879–891.

Proks, P., Gribble, F.M., Adhikari, R., Tucker, S.J., and Ashcroft, F.M. (1999). Involvement of the N-terminus of Kir6.2 in the inhibition of the KATP channel by ATP. J. Physiol. *514*, 19–25.

Raab-Graham, K.F., Cirilo, L.J., Boettcher, A.A., Radeke, C.M., and Vandenberg, C.A. (1999). Membrane topology of the amino-terminal region of the sulfonylurea receptor. J. Biol. Chem. *274*, 29122–29129.

Reimann, F., Tucker, S.J., Proks, P., and Ashcroft, F.M. (1999). Involvement of the n-terminus of Kir6.2 in coupling to the sulphonylurea receptor. J. Physiol. *518*, 325–336.

Ruknudin, A., Schulze, D.H., Sullivan, S.K., Lederer, W.J., and Welling, P.A. (1998). Novel subunit composition of a renal epithelial KATP channel. J. Biol. Chem. *273*, 14165–14171.

Saurin, W., Hofnung, M., and Dassa, E. (1999). Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. J. Mol. Evol. *48*, 22–41.

Schwanstecher, M., Sieverding, C., Dörschner, H., Gross, I., Aguilar-Bryan, L., Schwanstecher, C., and Bryan, J. (1998). Potassium channel openers require ATP to bind to and act through sulfonylurea receptors. EMBO J. *17*, 5529–5535.

Schwiebert, E.M., Benos, D.J., Egan, M.E., Stutts, M.J., and Guggino, W.B. (1999). CFTR is a conductance regulator as well as a chloride channel. Physiol. Rev. *79*, S145–S166.

Shyng, S., Ferrigni, T., and Nichols, C.G. (1997). Regulation of KATP channel activity by diazoxide and MgADP. Distinct functions of the two nucleotide binding folds of the sulfonylurea receptor. J. Gen. Physiol. *110*, 643–654.

Stride, B.D., Cole, S.P., and Deeley, R.G. (1999). Localization of a substrate specificity domain in the multidrug resistance protein. J. Biol. Chem. *274*, 22877–22883.

Tatusov, R.L., Koonin, E.V., and Lipman, D.J. (1997). A genomic perspective on protein families. Science *278*, 631–637.

Tinker, A., Jan, Y.N., and Jan, L.Y. (1996). Regions responsible for the assembly of inwardly rectifying potassium channels. Cell *87*, 857–868.

Trapp, S., Proks, P., Tucker, S.J., and Ashcroft, F.M. (1998). Molecular analysis of ATP-sensitive K channel gating and implications for channel inhibition by ATP. J. Gen. Physiol. *112*, 333–349.

Tucker, S.J., and Ashcroft, F.M. (1999). Mapping of the physical interaction between the intracellular domains of an inwardly rectifying potassium channel, Kir6.2. J. Biol. Chem. *274*, 33393–33397.

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.

Tucker, S.J., Gribble, F.M., Proks, P., Trapp, S., Ryder, T.J., Haug, T., Reimann, F., and Ashcroft, F.M. (1998). Molecular determinants of KATP channel inhibition by ATP. EMBO J. *17*, 3290–3296.

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.

Uhde, I., Toman, A., Gross, I., Schwanstecher, C., and Schwanstecher, M. (1999). Identification of the potassium channel opener site on sulfonylurea receptors. J. Biol. Chem. *274*, 28079–28082.

Zerangue, N., Schwappach, B., Jan, Y.N., and Jan, L.Y. (1999). A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. Neuron *22*, 537–548.

Zheng, J., and Sigworth, F.J. (1998). Intermediate conductances during deactivation of heteromultimeric *Shaker* Potassium Channels. J. Gen. Physiol. *112*, 457–474.