N-terminal transmembrane domain of the SUR controls trafficking and gating of Kir6 channel subunits

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The sulfonylurea receptor (SUR), an ATP-binding cassette (ABC) protein, assembles with a potassium channel subunit (Kir6) to form the ATP-sensitive potassium channel (K_{ATP}) complex. Although SUR is an important regulator of Kir6, the specific SUR domain that associates with Kir6 is still unknown. All functional ABC proteins contain two transmembrane domains but some, including SUR and MRP1 (multi-drug resistance protein 1), contain an extra N-terminal transmembrane domain called TMD0. The functions of any TMD0s are largely unclear. Using *Xenopus* oocytes to coexpress truncated SUR constructs with Kir6, we demonstrated by immunoprecipitation, single-oocyte chemiluminescence and electrophysiological measurements that the TMD0 of SUR1 strongly associated with Kir6.2 and modulated its trafficking and gating. Two TMD0 mutations, A116P and V187D, previously correlated with persistent hyperinsulinemic hypoglycemia of infancy, were found to disrupt the association between TMD0 and Kir6.2. These results underscore the importance of TMD0 in K_{ATP} channel function, explaining how specific mutations within this domain result in disease, and suggest how an ABC protein has evolved to regulate a potassium channel.

Keywords: ABC transporter/ATP-sensitive K channel/PHHI/sulfonylurea receptor/trafficking

Introduction

The sulfonylurea receptors (SUR1 and SUR2) belong to the ATP-binding cassette (ABC) protein superfamily (Ames *et al.*, 1992; Higgins, 1992; Dean and Allikmets, 2001). Mutations in *SUR1* lead to persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (Kane *et al.*, 1996). Normally, high levels of blood glucose cause insulin release by the pancreatic β cells. However, β cells in individuals with PHHI secrete insulin despite low blood glucose levels (Aguilar-Bryan and Bryan, 1999; Miki *et al.*, 1999).

All functional ABC proteins or protein complexes have a similar domain organization, consisting of two

transmembrane domains (TMD1 and TMD2) and two nucleotide-binding domains (NBD1 and NBD2) (Figure 1A). A model of how these protein machineries operate has emerged (Ames *et al.*, 1992; Chang and Roth, 2001; Locher *et al.*, 2002). The NBDs catalyze the hydrolysis of ATP, and the resulting free energy is used to drive the transport of substrates across a specific pathway formed by TMD1 and TMD2 (Ames *et al.*, 1992; Chang and Roth, 2001; Locher *et al.*, 2002) or to regulate the gating of the cystic fibrosis transmembrane regulator (CFTR), the only ABC protein that functions as an ion channel (Gadsby and Nairn, 1999).

SURs associate with the inwardly rectifying potassium (Kir) channel subunits (Kir6.1 or Kir 6.2) to form the functional K_{ATP} channels (Figure 1A) (Clement *et al.*, 1997; Lorenz *et al.*, 1998; Lorenz and Terzic, 1999) and are prevalent in neurons, muscle and pancreatic β cells (Inagaki *et al.*, 1995; Aguilar-Bryan *et al.*, 1998; Seino, 1999). K_{ATP} channels are inhibited by intracellular ATP but stimulated by MgADP (Noma, 1983; Dunne and Petersen, 1986; Ashcroft and Gribble, 1998). Therefore, they couple the metabolic state of a cell to its membrane excitability and serve important functions such as insulin secretion, control of vascular tone and protection against ischemia and seizures (Quayle *et al.*, 1997; Miki *et al.*, 1999, 2002; Cohen *et al.*, 2000; Yamada *et al.*, 2001). Kir channels show a characteristic topology of two transmembrane segments (M1 and M2) flanking a pore-forming loop (Doupnik *et al.*, 1995). The N-terminus and M1 of Kir6 are important in the interactions with SURs (Schwappach *et al.*, 2000). The corresponding regions in SURs that interact with Kir6 have yet to be determined.

An endoplasmic reticulum (ER) retention sequence, the RKR motif, is present in each of the Kir6 and SUR subunits of the K_{ATP} channel complex (Zerangue *et al.*, 1999). Each subunit expressed alone is retained in the ER. When both subunits are coexpressed, their respective RKR motifs are masked, allowing trafficking of the SUR/Kir6 complex to the cell surface. The RKR motif of Kir6.2 is localized within the last 26 amino acids, deletion of which results in a truncation mutant (6.2 Δ 26) that, unlike Kir6.2, shows functional expression (Tucker *et al.*, 1997; Zerangue *et al.*, 1999). The last 13 amino acids of SUR1 also contain an anterograde signal which enhances the K_{ATP} channel complex to exit the ER (Sharma *et al.*, 1999).

Some ABC proteins, such as MRP1–3, MRP6–7 and SUR, have an additional N-terminal transmembrane domain (TMD0) that is connected to TMD1 by a cytoplasmic loop (L0) (Figure 1A) (Cole and Deeley, 1998; Dean and Allikmets, 2001). Distinct TMD0s from

Fig. 1. The TMD0 and TMD2–NBD2 segment of SUR1 can physically associate with Kir6.2. (A) Domain organization of the two K_{ATP} channel subunits (see Introduction for description). Each subunit has an ER retention sequence motif (RKR), which is represented by a solid circle in the cartoon. (B) Epitope tagging of Kir6 and truncated SUR1 segments. SUR1 is severed into three epitope-tagged segments: F195, F196–917 and 918M. Segment F195 contains TMD0, which comprises amino acids 2–195, tagged at its N-terminus with a FLAG epitope. The last transmembrane segment of TMD0 has been shown to lie before amino acid 195 (Raab-Graham *et al.*, 1999) (Supplementary figure 1). F196–917 contains L0, TMD1 and NBD1, which comprises amino acids 196–917, and is tagged at its N-terminus with FLAG. Amino acid 917 is homologous with the last amino acid of the NBD1 in CFTR defined using a severed approach (Chan *et al.*, 2000) (Supplementary figure 1). 918M contains TMD2 and NBD2, which comprises amino acids 918–1582, and is tagged at its C-terminus with C-MYC. Kir6.2 is tagged with an HA epitope at its C-terminus (6.2HA). (C) Western blots showing the independent expression of 6.2HA, F195, F196–917 and 918M. Membrane extracts from uninjected oocytes were used as the control in each case. (D) Assay of association between Kir6.2 and truncated SUR1 segments by immunoprecipitation. Immunoprecipitations were performed, using either anti-FLAG or anti-CMYC antibodies, on digitonin-solubilized membrane extracts of oocytes expressing F195+6.2HA, F196–917+6.2HA and 918M+6.2HA. Anti-HA antibody was used for subsequent western blotting in all cases. Membrane extract from oocytes injected with 6.2HA alone was used as control.

different ABC proteins show different amino acid sequences (Gao *et al.*, 1998) and their functions are largely unclear. The TMD0 in MRP1 seems to be dispensable since its deletion affects neither the transport function nor the cellular targeting of MRP1 (Bakos *et al.*, 1998). The TMD0 of MRP2 is required for correct cellular targeting but is not necessary for transport function (Fernandez *et al.*, 2002). The N-terminal region containing the TMD0 and L0 (TMD0–L0) regions of SUR has been

implicated in controlling the gating of the K_{ATP} channel (Babenko *et al.*, 1999b).

Here we show that the TMD0 of SUR1 (or simply called TMD0, unless otherwise specified) strongly associates with Kir6.2 and plays an important role in its trafficking and gating. Two TMD0 mutations that are correlated with PHHI were shown to abrogate its association with Kir6.2, underscoring the importance of TMD0 in K_{ATP} channel function.

Results

Strong physical association between TMD0 and Kir6.2

We used a deletion approach to identify the molecular determinants in SUR1 responsible for interactions with Kir6.2. Three truncated SUR1 constructs were made: F195, F196-917 and 918M (Figure 1B; see description of these constructs in the legend to Figure 1). We will refer to the extracellular N-terminus and the first five transmembrane segments of SUR1 as its TMD0. An HA tag was added to the C-terminus of Kir6.2 (referred to as 6.2HA). To assay for expression of these channel constructs, cRNA for each construct was injected into *Xenopus* oocytes and western blotting was performed using antibodies against their tags. As shown in Figure 1C, all four constructs could be detected by their respective antibodies. The predicted molecular weights for 6.2HA, F195, F196-917 and 918M are 44.9, 23.4, 81.9 and 75.6 kDa, respectively. These results indicate that each of these channel constructs can be expressed independently of the others. To determine which of the three SUR1 segments can associate with 6.2, each segment was coexpressed with 6.2HA and immunoprecipitations were performed using the antibodies that recognize the SUR1 segments. A strong band corresponding to 6.2HA could be detected in the precipitate when 6.2HA was coexpressed with F195 (Figure 1D). In contrast, 6.2HA could not be coprecipitated by the FLAG-tagged TMD0 from MRP1 (data not shown). A much weaker 6.2HA protein band was also detected when 6.2HA was coexpressed with 918M. However, 6.2HA could not be coprecipitated by F196-917. These data indicate that there is strong physical interaction between the TMD0 of SUR1 and 6.2. The coprecipitation of a small amount of 6.2HA with 918M suggests the presence of weak interaction between them.

Functional expression of TMD0/6.2HA channels in *Xenopus* oocytes

If F195 and 918M can associate with 6.2HA, can the resulting complexes form functional channels on the cell surface? To address this question, whole-cell currents from oocytes expressing the SUR1 segment complexes with 6.2HA were measured using two-electrode voltage-clamp (TEVC). Oocytes expressing SUR1+6.2 showed no basal currents (Figure 2A). Sodium azide, a metabolic inhibitor, activated the inwardly rectifying SUR1/6.2 currents reversibly. Washout of sodium azide led to a transient increase in the current, which is caused by the removal of a direct inhibitory effect of sodium azide on the SUR1/6.2 channels (Gribble *et al.*, 1997a; Trapp and Ashcroft, 2000). For uninjected oocytes, no current was activated by sodium azide (Figure 2C). Oocytes expressing F195+6.2HA, unlike the SUR1+6.2 controls, exhibited large basal currents which could be further activated by sodium azide in a reversible manner (Figure 2B). Complete activation and deactivation were about five times faster than those from oocytes expressing SUR1+6.2 (50 s versus 280 s). Both the basal and the azide-stimulated currents from the F195/6.2HA channels showed weak inward rectification (Figure 2B) and the basal current could be blocked by 3 mM BaCl₂, a typical Kir-channel blocker. The currents from the

6.2HA channels were not significantly different from those of uninjected oocytes (Figure 2C). The difference in the currents between F195/6.2HA and 6.2HA was not due to an increase in the protein expression level of 6.2HA in the presence of F195 (Figure 2D). Basal and azide-stimulated currents from oocytes injected with F196-917+6.2HA, or 918M+6.2HA or even F196-917+918M+6.2HA were similar to those from uninjected oocytes (Figure 2C). Oocytes that were injected with F195+F196-917+918M+6.2HA gave both basal and azide-stimulated currents with activation and deactivation rates similar to those of F195/6.2HA (data not shown). The magnitude of the currents was slightly smaller than that of F195+6.2HA, which may be due to a saturation of the oocyte translational machinery caused by the excessive amount of RNA injected. Nevertheless, it is clear that even coexpressing F196-917 and 918M could not convert F195/6.2HA to an SUR1/6.2 channel. We conclude that F195/6.2HA, but not 918M/6.2HA, forms functional channels with characteristics different from SUR1/6.2 when expressed in oocytes.

Functional disruption of the RKR motif in Kir6 allows functional expression of TMD0/Kir6 complex

The successful functional expression of F195/6.2HA channels requires the masking of the RKR motif in 6.2. We tested for a possible effect of the C-terminal HA tag on the RKR motif by measuring currents from oocytes expressing F195+6.2 and comparing them with the F195/6.2HA currents (Figure 3A). Total current (basal plus azide-stimulated) from F195+6.2 was greatly reduced compared with that of F195+6.2HA. Removing the FLAG epitope from F195 had no effect on the current expression of F195+6.2HA. Functional disruption of the RKR motif in the F195/6.2 complexes, either by mutating the RKR to AAA (i.e. 6.2AAA) or by deletion (i.e. 6.2Δ26), also led to large current expression. These data suggest that the RKR motif in 6.2 reduces the surface expression of the F195/6.2 complex and the HA tag added to the C-terminus of 6.2 can disrupt the function of its RKR motif. Also, it is clear that TMD0 of SUR1 can enhance the functional expression of all of the 6.2 constructs listed in Figure 3A, particularly those where the function of the RKR motif is disrupted (i.e. 6.2Δ26, 6.2AAA, 6.2HA).

Since different tissue-specific K_{ATP} channels are formed by the combination of specific SUR and Kir6 subunits, we proceeded to test whether an equivalent TMD0–6.2 interaction exists between a different TMD0 and Kir6 subunits. We expressed wild-type Kir6.1 (6.1), RKR disrupted 6.1 (6.1Δ61 and 6.1AAA) and 6.2Δ26 with the TMD0 from SUR2 (S2-TMD0) (Figure 3B). Large total currents were obtained from oocytes expressing S2-TMD0+6.1Δ61, S2-TMD0+6.1AAA and S2-TMD0+6.2Δ26. Oocytes expressing 6.1Δ61 alone gave currents similar to those of uninjected oocytes. Therefore, TMD0 from SUR2 can functionally interact with 6.1Δ61, 6.1AAA and 6.2Δ26, and can enhance their current expressions. S2-TMD0+6.1 gave rise to total currents similar to those of uninjected oocytes, presumably due to retention of the S2-TMD0/6.1 complex in the ER.

Fig. 2. Functional expression of F195/6.2HA channels. (A and B) F195/6.2HA channels display currents that are distinct from those shown by SUR1/6.2 channels. The left panel shows a representative time course of current measured from an oocyte expressing (A) SUR1+6.2 or (B) F195+6.2HA. Current was measured at -80 mV by TEVC. The bath solution contains 96 mM K^+ , and 3 mM sodium azide or 3 mM $BaCl_2$ was included, as indicated, to cause metabolic inhibition or channel blockade, respectively. The right panels show the I-V relationships obtained at time points t_1 - t_3 as indicated on the time courses shown on the left panels. (C) Histogram of basal and azide-induced currents measured at -80 mV using TEVC. Only the basal currents from oocytes expressing F195+6.2HA or F195+F196-917+918M+6.2HA are shown. The basal currents from other groups of oocytes are similar to that of uninjected oocytes. Each bar is an average of at least five measurements ($n \geq 5$). (All current histograms in subsequent figures represent currents measured at -80 mV.) (D) Western blotting showing that the expression level of 6.2HA protein is not changed by the presence of F195.

TMD0 can traffic to the cell surface and enhances the surface expression of 6.2 Δ 26

How does TMD0 enhance the functional expression of RKR-disrupted Kir6 (e.g. 6.2 Δ 26)? F195 could cause this effect by increasing the number of 6.2 Δ 26 channels on the cell surface and/or by increasing the open probability of the 6.2 Δ 26 channels. To explore the first possibility, an HA epitope was engineered to the first extracellular loop of 6.2, 6.2 Δ 26 and 6.2HA (Figure 3C). This extracellular HA epitope (denoted by an asterisk in Figure 3C) allows quantitation of Kir6 channels expressed on the cell surface of a single oocyte using chemiluminescence (Zerangue *et al.*, 1999). When 6.2, 6.2 Δ 26 and 6.2HA were expressed alone, surface signals were low and were only slightly higher than that of the control (uninjected oocyte). Coexpression with F195 resulted in a slight increase in the surface signals for 6.2, but a striking increase in the surface signals for 6.2 Δ 26 and 6.2HA. The results from the surface detection assay correspond well to the current

measurements (Figure 3A), suggesting that TMD0 increases the functional expression of RKR-disrupted Kir6 by mainly increasing the number of channels expressed on the cell membrane. SUR1 could also increase the surface expression of 6.2 Δ 26, although the surface signal obtained for 6.2 Δ 26 was ~30% of that when 6.2 Δ 26 was coexpressed with F195.

Can TMD0 traffic to the cell surface in the absence of the other parts of the SUR molecule and 6.2 Δ 26? To investigate this, an HA tag (denoted by an asterisk in Figure 3D) was engineered to the N-terminus of TMD0 and its surface expression was measured. TMD0 could indeed traffic to the plasma membrane, as shown by the large surface signal obtained from oocytes expressing TMD0 (Figure 3D). Coexpressing 6.2, but not 6.2 Δ 26, caused a large reduction in the surface expression of TMD0, consistent with the trapping of TMD0/6.2 complexes in the ER caused by the RKR motif in 6.2. The surface expression of the TMD0 from another ABC

Fig. 3. TMD0 can traffic to the cell surface and enhance the surface expression of RKR-disrupted Kir6. (A) TMD0 of SUR1 can enhance functional expression of RKR-disrupted 6.2. Histogram of total currents (basal + azide-stimulated currents) measured from oocytes expressing different channels normalized to that from oocytes expressing F195+6.2HA ($n \geq 10$). (B) TMD0 of SUR2 (S2-TMD0) can enhance functional expression of 6.1Δ61, 6.1AAA and 6.2Δ26 ($n \geq 6$). (C) F195 can enhance the surface expression of RKR-disrupted 6.2. An HA tag was added to the first extracellular loop of 6.2, 6.2Δ26 and 6.2HA, and is indicated by an asterisk. Note that 6.2HA* also has a second HA tag at its C-terminus. Surface expression of different 6.2* constructs was assayed by single-oocyte chemiluminescence ($n \geq 10$). (D) High surface expression of both SUR1 and MRP1 TMD0. An extracellular HA tag (asterisk) was added to the N-terminus of the TMD0 from SUR1 or MRP1. Surface expression of TMD0* constructs was assayed by single-oocyte chemiluminescence ($n \geq 10$). The high surface expression of TMD0* is not affected by the presence of 6.2Δ26 but is decreased by the presence of 6.2.

protein, MRP1, was similarly high, indicating that MRP1-TMD0 can also traffic to the cell surface independently of the rest of the MRP1 molecule.

Single channels of SUR1-TMD0/6.2Δ26 and SUR1/6.2Δ26 are indistinguishable in the absence of nucleotides and drug modulators

In order to investigate whether TMD0 affects the gating of 6.2, single channels were recorded from oocyte membrane patches expressing 6.2Δ26, TMD0 + 6.2Δ26 and SUR1 + 6.2Δ26 (Figure 4). Single channels of TMD0/6.2Δ26 and SUR1/6.2Δ26 were very similar and were clearly different from those of 6.2Δ26 in that they displayed longer bursts of activity (Figure 4, left panels). Single-channel current amplitudes were similar in all three cases. The open and closed times, the burst duration and the open probability P_o were analysed and their values are listed in Table I. All these parameters were very similar for the TMD0/6.2Δ26 and SUR1/6.2Δ26 channels. Their τ_o were about twice those of the 6.2Δ26 channels, while the burst durations were ~25 times those of the 6.2Δ26 channels. The values of τ_{c1} and τ_{c2} were similar for all three channels. The values of P_o for the TMD0/6.2Δ26 and SUR1/6.2Δ26

channels were ~0.6, and were ~4 times those of 6.2Δ26, which can be explained by the lower frequency of openings and smaller τ_o for the 6.2Δ26 channels. These data indicate that TMD0 and full-length SUR1 exert equivalent effects on the single-channel behavior of 6.2Δ26 in the absence of nucleotides and drug modulators.

TMD0/6.2Δ26 channels are less sensitive to ATP inhibition and are insensitive to MgADP, glibenclamide and diazoxide

In order to measure the ATP sensitivity of the 6.2Δ26, TMD0/6.2Δ26 and SUR1/6.2Δ26 channels, macropatches were excised from oocytes expressing each channel type and currents were measured at different concentrations of ATP in the bath (Figure 5A, B and C). Figure 5D compares the dose-response curves for the three channels. The ATP concentration required to cause 50% inhibition (K_i) for TMD0/6.2Δ26 channels was about twice that of 6.2Δ26 and ~20 times higher than that of SUR1/6.2Δ26. TMD0, unlike full-length SUR1, did not increase the ATP sensitivity of 6.2Δ26.

The sensitivity of F195/6.2HA channels to diazoxide and glibenclamide was tested (Figure 6A and B). Unlike

the SUR1/6.2 channels, F195/6.2HA channels were neither activated by diazoxide nor inhibited by glibenclamide. Currents recorded from inside-out macropatches also showed that F195/6.2HA channels, which have been inhibited by ATP, could not be activated by MgADP (Figure 6C). The lack of sensitivity of the F195/6.2HA channels to diazoxide, glibenclamide and MgADP is expected because of the deletion of critical regions of SUR1 (e.g. NBDs and TMD2), which have been shown to be important for the actions of these channel modulators (Nichols *et al.*, 1996; Gribble *et al.*, 1997b; Shyng *et al.*, 1997; Schwanstecher *et al.*, 1998; Babenko *et al.*, 2000; Moreau *et al.*, 2000; Zingman *et al.*, 2001, 2002).

Two PHHI mutations located in TMD0 abolish its association with 6.2

Two TMD0 mutations, A116P and V187D, have been reported to cause PHHI (Aguilar-Bryan and Bryan, 1999; Otonkoski *et al.*, 1999). The effects of these two mutations on the function of TMD0 were assessed. The current exhibited from the F195/6.2HA channels, the ability of F195 to enhance the surface expression of 6.2Δ26 and the ability of F195 to traffic to the cell surface were all abolished by both mutations (Figure 7A, B and C). Although these two mutations do not appear to have a gross effect on the protein expression of F195, immunoprecipitation showed that either mutation completely

abolished the association between TMD0 and 6.2HA (Figure 7D).

Discussion

The TMD0 of an ABC protein can couple to a potassium channel

Using a deletion approach, we set out to find the structural determinants in SUR1 that are important in its interactions with Kir6.2. Three truncated SUR1 proteins were used for this purpose. The results from F196-917 and 918M need to be interpreted with caution. It is possible that these two proteins are not inserted in the membrane correctly, which would explain why they show weak or no physical association with 6.2HA (Figure 1D). We found that the 918M segment is likely to insert into the membrane with the correct topology (Supplementary figure 2, available at *The EMBO Journal* Online). Another possibility is that the F196-917 and 918M proteins are misfolded. We have also deleted TMD0 from SUR1. The resulting construct did not show an effect on current expression and was not detected on the cell surface when coexpressed with the other channel segments, highlighting the deleterious effect of truncating TMD0 from SUR1 (Supplementary figure 3). Nevertheless, the inescapable conclusion from our results is that TMD0 interacts with Kir6.2Δ26 and affects its

Fig. 4. TMD0/6.2Δ26 and SUR1/6.2Δ26 channels have very similar single-channel properties under nucleotide-free conditions. Single-channel recordings of 6.2Δ26, TMD0/6.2Δ26 and SUR1/6.2Δ26 channels. Two different time scales are shown. Single channels were recorded at -80 mV from inside-out patches with 96 mM K⁺ on both sides.

Table I. Single-channel parameters for 6.2Δ26, TMD0+6.2Δ26 and SUR1+6.2Δ26

Channel	τ_o (ms)	τ_{c1} (ms)	τ_{c2} (ms)	Burst duration (ms)	P_o
6.2Δ26	0.76 ± 0.04	0.44 ± 0.031	6.7 ± 1.1	2.33 ± 1.2	0.15 ± 0.095
TMD0+6.2Δ26	1.81 ± 0.03	0.48 ± 0.026	9.8 ± 3.4	52 ± 15	0.63 ± 0.086
SUR1+6.2Δ26	1.83 ± 0.09	0.48 ± 0.021	6.3 ± 2.5	69 ± 7	0.65 ± 0.083

Fig. 5. ATP sensitivity of SUR1-TMD0/6.2Δ26 channels is similar to that of 6.2Δ26 channels. (A–C) Representative macropatch recordings from oocytes expressing 6.2Δ26, TMD0+6.2Δ26 and SUR1+6.2Δ26. Patch excisions are indicated by arrows. Different concentrations of ATP (in mM) were applied to the inside of the patches as indicated. Both the pipette and bath solutions contain 140 mM K⁺. The holding potential was –80 mV. (D) Dose–response curves for ATP inhibition. The K_i values (μM) for the 6.2Δ26, TMD0+6.2Δ26 and SUR1+6.2Δ26 channels are 138.4 ± 57.48 ($n = 3$), 308.92 ± 13.83 ($n = 9$) and 14.19 ± 1.07 ($n = 2$), respectively. Their Hill coefficients are 1.45 ± 0.10 , 1.27 ± 0.03 and 1.05 ± 0.08 , respectively.

gating and trafficking through strong direct physical association.

TMD0 is only found in certain members of the C subfamily of ABC proteins, including MRP1–3, MRP6–7 and SUR. There is no sequence homology among different TMD0s (Gao *et al.*, 1998). The function of TMD0 in any of these ABC proteins is not clear. Our finding demonstrates unambiguously a direct functional role for the TMD0 of an ABC protein, and raises the interesting possibility that, during evolution, some ABC proteins might have acquired TMD0 as an extra domain to serve a specialized purpose, such as regulating the function of a potassium channel. It is possible that TMD0s of other ABC proteins also associate with membrane proteins and modulate their function.

Two distinct functions of SUR in enhancing the trafficking of Kir6.2 to the cell membrane

The RKR motif present in each of the SUR and Kir6 subunits restricts their individual surface expression. Each RKR motif is masked when the two subunits associate in the correct stoichiometry. This leads to successful

Fig. 6. SUR1-TMD0/6.2HA channels are insensitive to MgADP, diazoxide and glibenclamide. (A) SUR1/6.2 channels are activated by diazoxide but inhibited by glibenclamide. Representative time course of currents measured from an oocyte expressing SUR1+6.2. Currents were measured at –80 mV by TEVC. Additions of 3 mM azide (Az), 340 μM diazoxide (DZX) and 10 μM glibenclamide were made to the bath solution as indicated. (B) SUR1-TMD0/6.2HA channels are insensitive to diazoxide and glibenclamide. Conditions as in (A) except that the oocyte was expressing F195+6.2HA. (C) SUR1-TMD0/6.2HA channels are insensitive to MgADP. Macropatch recording from oocyte expressing F195+6.2HA. The arrow indicates the time of patch excision (inside-out patch). Applications of 1 mM ATP, 340 μM diazoxide and 1 mM ADP were made to the inside of the patch as indicated.

Fig. 7. Two PHHI mutations located in TMD0, A116P and V187D disrupt the association between TMD0 and 6.2. (A) A116P and V187D mutations completely abolish the total current expressed from TMD0+6.2HA. Total currents were measured from *Xenopus* oocytes using TEVC and values from the mutated F195+6.2HA channels are normalized to that of the wild-type F195+6.2HA channels. (B) A116P and V187D mutations completely abolish the ability of TMD0 to enhance the surface expression of 6.2Δ26. Surface expression of 6.2Δ26* was measured and values obtained from the mutated F195+6.2Δ26* were normalized to that of the wild-type F195+6.2Δ26*. (C) A116P and V187D mutations completely abolish the ability of TMD0 to traffic to the cell surface. Surface expression of the mutated TMD0* is normalized to that of the wild type. (D) A116P and V187D mutations disrupt the association between TMD0 and 6.2. Total oocyte membranes (lysates) were assayed for expression of 6.2HA and wild-type or mutated-F195 proteins (upper two panels). Anti-HA antibody was used for immunoprecipitations and the precipitated proteins were assayed for the presence of F195 and 6.2HA (lower two panels).

trafficking of the K_{ATP} channel complex to the cell surface. However, disrupting the RKR motif in Kir6.2 increases its surface expression to a level much lower than that achieved when TMD0 or SUR is present (Figure 3C). Immunostaining of Kir6.2Δ36 in transfected COS7 cells also indicated that the majority of Kir6.2Δ36 proteins were localized in the ER (Ma *et al.*, 2001). Therefore, the RKR-mediated ER retention mechanism is not the only factor determining the trafficking of Kir6.2 to the plasma membrane.

Compared with previous reports (Tucker *et al.*, 1997; Zerangue *et al.*, 1999), we detected relatively smaller whole-cell currents from Kir6.2Δ26 (Figure 3A; Supplementary figure 4). However, conspicuous currents could clearly be detected from macropatches expressing Kir6.2Δ26 under ATP-free conditions (Figure 5A). One possible explanation is that whole-cell currents are dependent on the metabolic states of the oocytes, which can vary among different laboratories.

TMD0 plays an important role in the trafficking of Kir6.2 since it greatly enhances the surface expression of Kir6.2 following masking or disruption of the RKR motif in Kir6.2. The RKR motif of Kir6.2 must be masked by SUR1 domains other than TMD0, because TMD0 alone cannot override its function. Therefore, SUR1 has two distinct roles in ensuring the high surface expression of Kir6.2: (i) to mask the RKR motif of Kir6.2; (ii) to greatly increase the surface expression of Kir6.2 by a mechanism involving association with TMD0. Because TMD0 can be localized to the plasma membrane efficiently (Figure 3D), we hypothesize that it contains a forward signal that is responsible for its high surface expression and its ability to enhance the surface localization of an associated protein, such as Kir6.2Δ26.

Both the functional and surface expression data indicate that the HA epitope added to the C-terminus of Kir6.2 can disrupt the function of its RKR motif (Figure 3A and C). Fusing a FLAG epitope or green fluorescent protein (GFP) to the C-terminus has a similar effect, whereas a FLAG epitope engineered to the N-terminus does not affect the function of the RKR motif (data not shown). It has been shown that Kir6.2 could be expressed on the cell membrane independent of SUR (John *et al.*, 1998; Makhina and Nichols, 1998). In some of these studies, Kir6.2 was tagged at one of its termini. In light of our findings, epitopes introduced to Kir6.2 at its C-terminus might enhance its surface expression in the absence of SUR.

Effect of TMD0 on the channel properties of Kir6.2Δ26

Besides affecting the trafficking of Kir6.2Δ26, TMD0 also modulates its gating. SUR affects the gating of Kir6.2Δ26 by increasing its burst duration and P_o (Babenko *et al.*, 1999a). Our data demonstrated that TMD0/Kir6.2Δ26 and SUR1/Kir6.2Δ26 showed indistinguishable single-channel kinetics (Figure 4), indicating that TMD0 alone is sufficient to confer the characteristic long bursts of activity on Kir6.2Δ26. Using a chimeric approach (Babenko *et al.*, 1999b), the TMD0-L0 region of SUR was implicated as being responsible for this gating effect on Kir6.2. Our data unambiguously assign this gating

effect of SUR1 on Kir6.2 to a direct interaction between TMD0 and Kir6.2.

Since TMD0/Kir6.2Δ26 and SUR1/Kir6.2Δ26 show indistinguishable single-channel kinetics, this must also mean that, under nucleotide-free conditions, the other SUR1 domains do not functionally couple to Kir6.2Δ26. However, the other SUR1 domains are required for coupling the effects of MgADP, sulfonylureas and potassium channel openers to the Kir6.2Δ26 subunit because TMD0/6.2Δ26 channels are not sensitive to these pharmacological agents (Figure 6). Whether these SUR1 domains functionally couple to Kir6.2Δ26 directly or indirectly (e.g. through TMD0) remains to be determined.

Since SUR increases the ATP sensitivity of Kir6.2, does TMD0 confer this characteristic on Kir6.2Δ26? Our data indicate that TMD0 does not, because the ATP sensitivity of TMD0/Kir6.2Δ26 is slightly less than that of the Kir6.2Δ26 channels, which can be explained by their higher P_o compared with the Kir6.2Δ26 channels (Enkvetchakul *et al.*, 2000). Taken together, these data suggest that TMD0 increases the current expression of Kir6.2Δ26 by decreasing its ATP sensitivity and increasing its surface expression and P_o .

PHHI mutations affect K_{ATP} channels by disrupting the function of TMD0

Two mutations, A116P and V187D, have been reported to correlate with PHHI (Aguilar-Bryan and Bryan, 1999; Otonkoski *et al.*, 1999). Both are located in the transmembrane segments of TMD0. While detailed analysis of A116P has not been reported, V187D has been shown to abolish the function of the pancreatic K_{ATP} channels both in native β cells and when expressed in *Xenopus* oocytes (Otonkoski *et al.*, 1999). We found that both mutations can greatly impair the function of TMD0 by disrupting its association with Kir6.2. How do these two mutations affect the association between TMD0 and Kir6.2? Because these two mutations abolish the ability of TMD0 to traffic to the cell membrane, it is possible that they cause misfolding in TMD0, resulting in ER retention. Mutations causing cystic fibrosis have been found in the transmembrane segments of CFTR that lead to misfolding of the CFTR protein (Denning *et al.*, 1992; French *et al.*, 1996). These two PHHI-correlated mutations found in TMD0 further underscore the importance of TMD0 in the physiological function of the K_{ATP} channel.

Materials and methods

Molecular biology

Constructs were made by either standard PCR or quick-change site-directed mutagenesis (Stratagene). FLAG (MDYKDDDDK), C-MYC (EKLISEEDL) and HA (YPYDVPDYA) epitopes were introduced by incorporating their coding sequences into PCR primers. F195, F196-917, 918M and 6.2HA are described in Figure 1. S2-TMD0 contains the first 193 amino acids of SUR2A. MRP1-TMD0 contains the first 195 amino acids of MRP1. The extracellular HA tag was introduced between amino acids P¹⁰² and G¹⁰³ of Kir6.2, and the peptide DLYAYMEKGIT was also inserted between G⁹⁸ and D⁹⁹, as described (Zerangue *et al.*, 1999). All constructs were subcloned into pGEMHE (Liman *et al.*, 1992).

Oocyte preparation, western blotting and immunoprecipitation

Oocytes were surgically removed from female *Xenopus laevis* and were treated with collagenase as described (Chan *et al.*, 2000). All constructs in

pGEMHE were linearized by *NheI* and cRNAs were synthesized using the mMessage mMachine kit (Ambion). Four nanograms of SUR1, 1 ng of TMD0 [including F195, F195(A116P), F195(V187D), TMD0*, 1-195, S2-TMD0 and MRP1-TMD0*], 3 ng of F196-917, 3 ng of 918M and 2 ng of Kir6 (including various 6.2 and 6.1 constructs) RNAs were used in independent or coexpression experiments for TEVC, macropatch recording, western blotting and immunoprecipitation. For single-channel recording, the ratio of TMD0 or SUR1 RNA to 6.2Δ26 RNA was 10:1 and the amount of RNA injected was reduced to ~0.05–0.1 ng of 6.2Δ26 RNA.

Total oocyte membranes were prepared from 20–40 oocytes. All steps were carried out on ice or at 4°C. Oocytes were homogenized, using a 261/2 gauge needle connected to a 1 ml syringe, in a lysis buffer containing 5 mM Tris, 1 mM EDTA, 1 mM EGTA and 1× protease inhibitor (Sigma). Homogenate was spun at 3000 g for 10 min and the resulting supernatant was further spun at 200 000 g for 30 min. The pellet containing the membrane fraction was resuspended in the lysis buffer (1 μl/oocyte).

Twenty microliters of membrane proteins were resuspended in 750 μl of an Ip buffer containing 1% digitonin (Calbiochem), 50 mM Tris pH 7.4, 150 mM NaCl and 1× protease inhibitor. This membrane protein suspension was rocked at 4°C for 1 h. Insoluble matter was removed by spinning at 200 000 g for 30 min. The supernatant was used for immunoprecipitation according to the standard protocol (Sambrook *et al.*, 2001). Either the M2-agarose (Sigma) or the anti-HA antibody (Roche) and protein G-Sepharose (Amersham) was used. The precipitants were washed once with Ip buffer and three times with the Ip buffer without digitonin, and eluted with SDS-PAGE sample buffer at 90°C. Membrane proteins or immunoprecipitants were resolved by 12% SDS-PAGE and transferred to nitrocellulose membrane. Standard western blotting, using Tris-buffered saline (TBS) and 1% non-fat milk powder, was performed (Sambrook *et al.*, 2001). Anti-M5 (Sigma; 1:500 dilution), anti-HA (Roche; 1:500 dilution) or anti-CMYC (Roche; 1:400 dilution) antibody was used. All washing steps were performed using 0.2% Tween containing TBS. Enhanced ECL reagents (Amersham) were used for detection.

Electrophysiology

TEVC recording was used to measure whole-cell currents from oocytes expressing various channel constructs. ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, 1.8 mM CaCl₂ pH 7.5) was used for the bath. Stock solutions of 3 M sodium azide (1000×) and 1 M BaCl₂ (333×) were dissolved in water, and 340 mM diazoxide (Sigma; 1000×) and 20 mM glibenclamide (Sigma; 2000×) were dissolved in dimethyl sulfoxide (DMSO). Oocyte membrane was held at –80 mV and a ramp from –100 to +100 mV was applied in 1 s. Currents at –80 mV from the ramp were used to plot the time course of the current and for comparing current expression among different channel constructs.

Inside-out macropatch recording was used to measure the response of the current to ATP inhibition. The currents were filtered at 0.2 kHz, sampled at 0.5 kHz and recorded using an EPC9 amplifier (HEKA). The pipette solution was 120 mM KCl, 1.2 mM MgCl₂, 2.5 mM CaCl₂ and 10 mM HEPES. The bath solution was 120 mM KCl, 2 mM MgCl₂, 5 mM EGTA and 10 mM HEPES. Pipette resistance was 0.3–0.7 MΩ. ATP stock solution was dissolved in water and pH was adjusted to 7.2. Because there was little rundown during the experiments, the steady current at a particular ATP concentration was measured and the averages of these were used to plot the ATP dose–response curve. The data were used to fit the Hill equation: $I/I_c = 100 \times \{K_i^n / (K_i^n + [ATP]^n)\}$, where I and I_c are the currents measured at the test ATP concentration and no ATP-containing solution, respectively, $[ATP]$ is the ATP concentration used, K_i is the inhibition constant and is equal to the ATP concentration when current inhibition is 50% and n is the Hill coefficient.

Single-channel recording was similar to the inside-out macropatch recording except that the pipette used was much smaller (resistance 5–10 MΩ) and the amount and ratio of RNAs injected were adjusted (see description of oocyte preparation). The bath solution was the same as for the macropatch. Single-channel currents were filtered at 5 kHz and sampled at 10 kHz. The digitized data were analyzed using a combination of TAC (HEKA) and pCLAMP 8 (Axon), helped with in-house software. The open probability of the channel was calculated by dividing the overall open probability in the patch by the number of channels seen opening in the patch. The channel open time can be well fitted with a single-exponential function. Burst duration was analyzed with a burst delimiter determined using pStat and Origin. At least three closed states can be seen in the single-channel data analysis. The shortest one represents the closed state within a burst and the medium one represents the closed state

between burst openings; a very long closed state can also be seen between burst openings, which varies significantly from patch to patch. Therefore, we did not include this state in the analysis.

Single-oocyte surface expression assay

Surface expression assay was performed using the single-oocyte chemiluminescence protocol as described (Schwappach *et al.*, 2000). We used 0.35 µg/ml rat monoclonal anti-HA antibody and a 1000× dilution of the HRP-conjugated secondary antibody (Roche). Individual oocytes were put into 50 µl Power Signal Elisa (Pierce) and incubated for 1 min. Chemiluminescence was quantitated using a Turner 20e luminometer.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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