# Proximal C-terminal domain of sulphonylurea receptor 2A interacts with pore-forming Kir6 subunits in $K_{ATP}$ channels

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Functional $K_{ATP}$ (ATP-sensitive potassium) channels are hetero-octamers of four Kir6 (inwardly rectifying potassium) channel subunits and four SUR (sulphonylurea receptor) subunits. Possible interactions between the C-terminal domain of SUR2A and Kir6.2 were investigated by co-immunoprecipitation of rat SUR2A C-terminal fragments with full-length Kir6.2 and by analysis of cloned $K_{ATP}$ channel function and distribution in HEK-293 cells (human embryonic kidney 293 cells) in the presence of competing rSUR2A fragments. Three maltose-binding protein–SUR2A fusions, rSUR2A-CTA (rSUR2A residues 1254–1545), rSUR2A-CTB (residues 1254–1403) and rSUR2A-CTC (residues 1294–1403), were co-immunoprecipitated with full-length Kir6.2 using a polyclonal anti-Kir6.2 antiserum. A fourth C-terminal domain fragment, rSUR2A-CTD (residues 1358–1545) did not co-immunoprecipitate with Kir6.2 under the same conditions, indicating a direct interaction between Kir6.2 and a 65-amino-acid section of the cytoplasmic C-terminal region of rSUR2A between residues 1294 and 1358. ATP- and glibenclamide-sensitive $K^+$ currents were decreased in HEK-293 cells expressing full-length Kir6 and SUR2 subunits that were transiently transfected with fragments rSUR2A-CTA, rSUR2A-CTC and rSUR2A-CTE (residues 1294–1359) compared with fragment rSUR2A-CTD or mock-transfected cells, suggesting either channel inhibition or a reduction in the number of functional $K_{ATP}$ channels at the cell surface. Anti-$K_{ATP}$ channel subunit-associated fluorescence in the cell membrane was substantially lower and intracellular fluorescence increased in rSUR2A-CTE expressing cells; thus, SUR2A fragments containing residues 1294–1358 reduce current by decreasing the number of channel subunits in the cell membrane. These results identify a site in the C-terminal domain of rSUR2A, between residues 1294 and 1358, whose direct interaction with full-length Kir6.2 is crucial for the assembly of functional $K_{ATP}$ channels.

Key words: ATP-sensitive potassium channel, inwardly rectifying potassium channel Kir6.2, ATP-sensitive potassium channel ($K_{ATP}$ channel), subunit interaction, sulphonylurea receptor SUR2A.

## INTRODUCTION

ATP-sensitive potassium ($K_{ATP}$) channels are inhibited by cytoplasmic ATP and modulated by ADP levels, thus coupling the metabolic state of the cell to membrane excitability [1]. These channels comprise hetero-octamers of four pore-forming Kir6 (inwardly rectifying potassium) channel subunits and four SUR (sulphonylurea receptor) accessory subunits [2–4]. Kir6 subunits have intracellular N- and C-termini and two transmembrane segments M1 and M2, separated by a P-loop that forms the outer mouth of the pore [5]. $K_{ATP}$ channels of pancreatic $\beta$-cells contain Kir6.2 and SUR1 subunits, whereas those of cardiac muscle contain Kir6.2 and SUR2A. The SUR subunits are large proteins supposed to contain three transmembrane domains (TMD0, TMD1 and TMD2), each with several transmembrane $\alpha$-helical segments [6]. Nucleotide-binding folds are located on the intracellular loop between TMD1 and TMD2 and on the long intracellular C-terminus. Expression of functional $K_{ATP}$ channels requires both subunit types to be present, since ER (endoplasmic reticulum)-retention signals on each subunit are masked only when both subunits are expressed together [7,8]. Analysis of the interactions between chimaeric constructs of Kir6.2 and Kir2.1 subunits with SUR1 and SUR2A, using a combination of trafficking assays, has shown that the first transmembrane segment (M1) and the N-terminus of the Kir6.2 subunit are important for specifying assembly with SUR1 and SUR2A [8]. Similarly, analysis of the surface expression of chimaeras of SUR1 and MRP1 (multidrug-resistance-associated protein 1) with Kir6.2 revealed that replacement of any of the TMD0, TMD1 or TMD2 in SUR1 with those from MRP1 prevented the surface expression of Kir6.2. This suggested that each transmembrane domain was important in the assembly of the $K_{ATP}$ channel hetero-oligomer [8]. From these results, a model of subunit interaction, based on binding of the Kir6.2 M1 segment within a hydrophobic pocket created by the transmembrane domains of SUR1 within the membrane, was proposed [8].

In addition to the masking of ER retention signals, additional interactions between cytoplasmic domains of Kir6 and SUR polypeptides probably contribute to assembly and allosteric interactions within the channel. An interaction with SUR subunits involving the transmembrane M2 segment and the proximal C-terminal domain of Kir6.2 has been suggested from the prevention of anti-Kir6.2 antibody co-immunoprecipitation of SUR2A when this region in Kir6.2 was deleted [9] and the disruption of trafficking of Kir6.2/SUR1 hetero-oligomers to the cell surface when the equivalent C-terminal region of Kir2.1 was present in a Kir6.2/Kir2.1 chimaera [10]. When this interaction was investigated biochemically, the physical interaction site of SUR1 on Kir6.2 was mapped between residues 208 and 279 [11]. A further interaction between the cytoplasmic N-terminus

Abbreviations used: ABC transporter, ATP-binding-cassette transporter; EGFP, enhanced green fluorescent protein; EGFP-F, farnesylated EGFP; ER, endoplasmic reticulum; HEK-293 cells, human embryonic kidney 293 cells; $K_{ATP}$ channel, ATP-sensitive potassium channel; Kir6 channel, inwardly rectifying potassium channel; MBP, maltose-binding protein; MRP, multidrug-resistance-associated protein; NBD, nucleotide-binding domain; SUR, sulphonylurea receptor; TMD, transmembrane domain.

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of Kir6.2 and SUR1 was suggested by the loss of SUR1-mediated attenuation of channel open probability ($P_o$) [12,13] and loss of co-photolabelling of the Kir6.2 subunit by $^{125}$I-azidoglibenclamide [13] on deletion of the distal N-terminus of Kir6.2 in Kir6.2/SUR1 channels. Increased $P_o$ values and attenuation of sulphonylurea-mediated inhibition of $K_{ATP}$ channels on application of a peptide corresponding to the N-terminal amino acid sequence 2–33 of Kir6.2 to Kir6.2/SUR1 channels supported this putative interaction [13]. It was proposed that the Kir6.2 N-terminus and the cytoplasmic L0 linker in SUR1 might be in close proximity [13], since (i) the TMD0-L0 segment of SUR1 has been implicated in controlling $P_o$ [14–16], (ii) it is photolabelled by $^{125}$I-glibenclamide [17], (iii) TMD0 is co-immunoprecipitated with Kir6.2 [15,16], and (iv) in deletion mutants of SUR1, the L0 linker has been shown to be a determinant of high-affinity glibenclamide binding [18].

The cytoplasmic NBD1 (nucleotide-binding domain 1) and NBD2 confer nucleotide diphosphate sensitivity to the $K_{ATP}$ channel hetero-oligomer. NBD1 from SUR1 forms homotetramers and a strong interaction occurs between NBD1 and NBD2 of SUR1, suggesting a role for the NBDs in channel assembly [19]. NBDs may also be involved in interactions between SUR and Kir6 subunits. Investigations have not revealed any evidence for an association between NBD1 or NBD2 and Kir6.2 [8,19]. Co-expression of NBD1 with Kir6.2 did not result in its surface membrane localization in Sf9 cells [19]. Replacement of the cytoplasmic loop of SUR1 containing NBD1 or the C-terminal domain containing NBD2 with corresponding domains from MRP1, which does not interact with Kir6.2, did not prevent Kir6.2/SUR1 assembly [8]. These observations suggest that NBD1 and NBD2 are not primary determinants of $K_{ATP}$ channel assembly, but do not rule out the possibility that interactions may occur between NBDs and Kir6.2.

In this study, possible interactions between the C-terminal domain of rat SUR2A (rSUR2A) containing NBD2 and Kir6 subunits were investigated. Interactions were investigated directly, by co-immunoprecipitation of MBP (maltose-binding protein)-tagged rSUR2A fragments with Kir6.2, and indirectly, by analysis of the function and distribution of $K_{ATP}$ channels in HEK-293 cells (human embryonic kidney 293 cells) stably expressing Kir6.2 and SUR2A subunits into which rSUR2A C-terminal domain fragments were transiently transfected. The results presented show that a small region of the proximal C-terminal domain of rSUR2A, amino acids 1294–1358, which contains the Walker A motif of NBD2, makes a direct interaction with Kir6.2 and that disruption of this interaction results in lowered cell-surface expression of the $K_{ATP}$ channel.

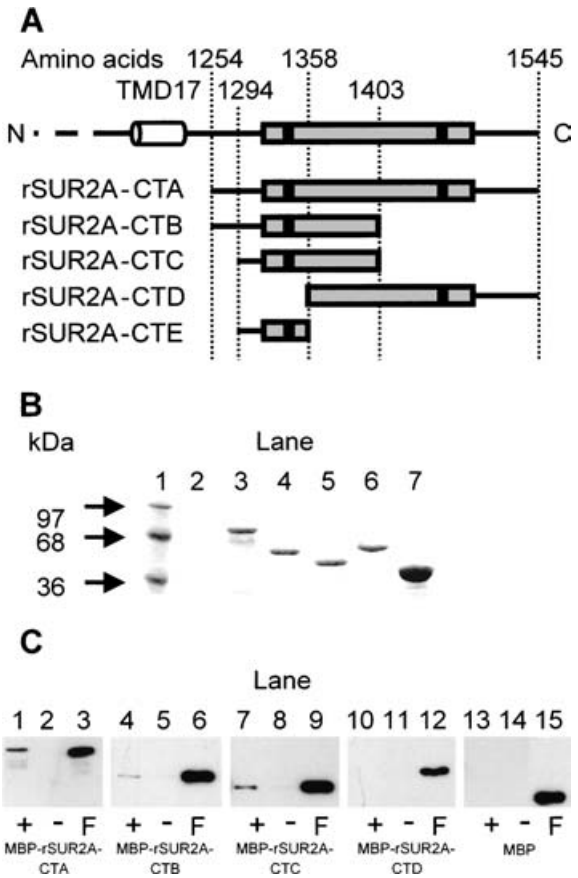


**Figure 1 Co-immunoprecipitation of MBP-rSUR2A-CT fragments with Kir6.2 subunits**

(**A**) The vertical lines show the amino acid boundaries of the rSUR2A-CT fragments within the C-terminal domain of rSUR2A: cylinder, TMD17; grey bar, NBD2; black boxes, Walker motifs A and B. (**B**) Purified MBP-rSUR2A-CT fragments analysed by SDS/PAGE (7.5 % gel). Lane 1, standard proteins; lane 2, 10 mM maltose in column buffer (20 mM Tris/HCl, pH 7.4/200 mM NaCl/1 mM EDTA/10 mM 2-mercaptoethanol); lane 3, MBP-rSUR2A-CTA; lane 4, MBP-rSUR2A-CTB; lane 5, MBP-rSUR2A-CTC; lane 6, MBP-rSUR2A-CTD; lane 7, MBP. (**C**) Co-immunoprecipitation using anti-Kir6.2 antiserum of MBP-rSUR2A-CT fusion proteins with Kir6.2. Lanes 1–3, rSUR2A-CTA; lanes 4–6, rSUR2A-CTB; lanes 7–9, rSUR2A-CTC; lanes 10–12, rSUR2A-CTD; lanes 13–15, MBP. Immunoprecipitation experiments were performed in the presence (+, lanes 1, 4, 7, 10 and 13) or absence (–, lanes 2, 5, 8, 11 and 14) of *in vitro*-translated Kir6.2. Lanes 3, 6, 9, 12 and 15, 1.2 $\mu$g of MBP-rSUR2A-CT fusion protein (F). Results are representative of 4–6 experiments.

## EXPERIMENTAL

### Polyclonal antibodies

Polyclonal site-directed antisera against C-terminal amino acid sequences in rat Kir6.2 (D86039), Kir2.1 (L48490) and SUR2A (D83598) $K_{ATP}$ channel subunits were raised and characterized as described previously [20,21].

### SUR protein fragments

Fragments of rSUR2A (Figure 1A) corresponding to the C-terminal domain (rSUR2A-CTA, amino acids 1254–1545), the proximal C-terminal domain (rSUR2A-CTB, amino acids 1254–1403, and rSUR2A-CTC, amino acids 1294–1403) and the distal C-terminal domain (rSUR2A-CTD, amino acids 1358–1545) and a small region spanning the Walker A motif of NBD2 of SUR2A (rSUR2A-CTE, amino acids 1294–1359) [22] and the equivalent region to rSUR2A-CTE from MRP1 (rMRP1-CTE, amino acids 1280–1344) were expressed as MBP fusion proteins using the pMAL protein fusion system (New England Biolabs, Hitchin, Herts., U.K.) in *Escherichia coli* BL21DE3 cells and purified by affinity chromatography on amylose–Sepharose (New England Biolabs) according to the manufacturer's instructions. Fusion protein preparations were analysed by SDS/PAGE (7.5 % gels) and stained with Coomassie Blue (Figure 1B).

### Co-immunoprecipitation of MBP-rSUR2A-CT protein fragments

[$^{35}$S]Methionine-labelled and unlabelled Kir6.2 and Kir2.1 subunits were produced in the TNT rabbit reticulocyte lysate Quick-coupled transcription/translation system (Promega, Chilworth, Southampton, U.K.), in the presence of canine pancreatic microsomal membranes (Promega) and either 0.74 MBq

of [$^{35}$S]methionine (Amersham Biosciences, Little Chalfont, Bucks., U.K.) or 1 mM methionine respectively. The samples were incubated at 30 °C for 90 min. *In vitro* translated [$^{35}$S]methionine-labelled (3 $\mu$l) and unlabelled (1 $\mu$l) Kir6.2 or Kir2.1 were mixed with 20 $\mu$l of 0.1 $\mu$g/ml rSUR2A or rMRP1 fragment MBP fusions (MBP-rSUR2A-CT and MBP-MRP1-CT respectively), sonicated for 15 s and incubated for 1 h at 37 °C, followed by 16 h at room temperature (22 °C). Two volumes of 1.5 % Triton X-100 and 20 mM Tris/HCl (pH 7.5) were added and the sample was incubated for 30 min at 4 °C. After centrifugation at 11 000 ***g*** for 15 min, the supernatant was made up to 100 $\mu$l to contain 20 mM Tris/HCl (pH 7.5), 5 % (w/v) BSA and 500 mM KCl. Anti-Kir6.2 antiserum (4 $\mu$l) and 50 $\mu$l of Protein A–Sepharose were added, and the reaction was incubated with mixing for 16 h at 4 °C. The resin was recovered by centrifugation at 1000 ***g*** for 1 min, and the pellet washed three times with 0.5 M KCl, 0.1 % Nonidet P40 and 20 mM Tris/HCl (pH 7.4). The final pellet was resuspended in SDS/PAGE loading buffer (62 mM Tris/HCl, pH 8.8/2 % (w/v) SDS/42.8 % (w/v) glycerol/6 mM dithiothreitol/0.01 % Bromophenol Blue) and run on an SDS/PAGE (7.5 %) mini-gel at 120 V for approx. 1.5 h. Proteins were transferred to PVDF transfer membrane (Hybond P; Amersham Biosciences) for 2 h at 150 mA using a wet-blot technique. The membrane was incubated in blocking solution (5 % dried milk in 10 mM Tris/HCl, pH 7.5/0.15 M NaCl) for 1 h and incubated overnight at 4 °C in blocking solution containing a 1:5000 dilution of anti-MBP antibody (New England Biolabs). The membrane was washed twice with TBST [50 mM Tris/HCl/0.15 M NaCl/0.5 % (v/v) Tween 20, pH 7.5] and once with TBS (50 mM Tris/HCl, pH 7.5/0.15 M NaCl), and then incubated for 2 h in blocking solution containing a 1:7000 dilution of anti-rabbit IgG–horseradish peroxidase conjugate (Sigma–Aldrich, Poole, Dorset, U.K.). The blot was then washed three times in TBST and twice in TBS, and peroxidase activity was detected by ECL® (Amersham Biosciences).

### Tissue culture

HEK-293 cells stably expressing Kir6.2/SUR2A [23] (a gift from Dr A. Tinker, University College London, London, U.K.) or transiently transfected with Kir6.2/SUR2B (GenBank® accession nos. D86039/AF087838), Kir6.2/SUR1 (D86039/L40624), Kir6.1/SUR2B (D42145/AF087838) or Kir2.1 (L48490)/SUR2A (D83598) were transiently transfected with rSUR2A-CT or rMRP1-CTE subunit fragments using LIPOFECTAMINE™ 2000 (Invitrogen, Paisley, Renfrewshire, Scotland, U.K.). rSUR2A-CT subunit fragments (rSUR2A-CTA, rSUR2A-CTC, rSUR2A-CTD or rSUR2A-CTE) and rMRP1-CTE were cloned in the expression vector pIRES2-EGFP (EGFP stands for enhanced green fluorescent protein; BD Biosciences Clontech, Oxford, U.K.). In this vector, the cloned cDNA and EGFP sequences were expressed as a common transcript (with EGFP second), so that EGFP intensity could be used as a reliable indicator of rSUR2A-CT fragment mRNA expression. For confocal microscopy experiments, rSUR2A-CT fragments were cloned into pIRES2-EGFP-F, a vector that encodes EGFP-F (farnesylated EGFP) located on the inner face of the plasma membrane. This vector, in which the C-terminus of EGFP is fused to the 20-amino-acid farnesylation signal from c-Ha-Ras [24], is a modified form of pIRES2-EGFP (BD Biociences Clontech) produced by overlap PCR. Transfection efficiencies of 70 % were achieved routinely for all fragments. Transiently transfected cells were maintained in minimal essential medium with Earle's salts, supplemented with 10 % (v/v) foetal calf serum and 5 % (w/v) L-glutamine. For the Kir6.2/SUR2A-expressing stable cell line, the medium was supplemented with 727 $\mu$g/ml Zeocin and 364 $\mu$g/ml G418 (Invitrogen).

### Electrophysiology

At 2 days after transfection, cells expressing the rSUR2A-CT or rMRP1-CTE fragments were identified by their green fluorescence at 488 nm under mercury lamp illumination. Inside-out patches were pulled using thick-walled borosilicate filamented glass, coated with Sylgard and fire-polished to a resistance of 8–12 MΩ. The bath and perfusing solution contained 140 mM K (110 mM KCl, 30 mM KOH), 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM EGTA and 5 mM Hepes (pH 7.2) and were maintained at $22 \pm 2$ °C. The pipette solution contained 140 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$ and 5 mM Hepes (pH 7.4). The inside-out patches were held at 0 mV (approximately equal to the potassium equilibrium potential $E_K$) and pulsed to $-80$ mV for 100 ms at 1 Hz. For each solution change, 20 recordings were made and a mean current was calculated from the last 50 ms of each voltage pulse. The mean data for all 20 pulses of each solution change were then exported to Microsoft Excel XP, where the mean current and S.E.M. were calculated. Inhibition by ATP was quantified by normalizing the current in the presence of ATP (10, 30 and 1000 $\mu$M) to the control current obtained after excision of the inside-out patches into bath solution containing no ATP. After washout, glibenclamide (10 and 100 nM) was also applied to the same patch to investigate whether pharmacological differences in $K_{ATP}$ channel current occurred between fragment-transfected and control-transfected HEK-293 cells. Statistical comparisons were made by ANOVA with a Bonferroni *post hoc* test for multiple comparisons.

### Immunocytochemistry

Immunocytochemistry in Triton X-100-permeabilized cells was performed as described previously [20] 2 days after transfection, using either anti-Kir6.2 or anti-SUR2A subunit antisera at a dilution of 1:250 and secondary detection with anti-rabbit IgG Alexafluor 568 conjugate, 1:1000 dilution (Molecular Probes, Leiden, The Netherlands). Anti-$K_{ATP}$ channel antibody-labelled cells were washed three times with 0.05 % Triton X-100, 150 mM NaCl and 15 mM sodium citrate for 10 min before microscopy.

### Confocal microscopy

Confocal microscopy was performed as described previously [20] with excitation wavelengths of 488 nm for the detection of EGFP-F and 568 nm for anti-$K_{ATP}$ channel subunit antibody binding, respectively, and an emission cut-off of $< 610$ nm. All images were analysed using a background subtraction method off-line. The background was defined as a region proximal to the cell and subtracted automatically from subsequent images.

### Data acquisition and image processing

Confocal image processing was performed using the Ultra-view software (v4.0) (PerkinElmer, Beaconsfield, U.K.). To represent distribution of fluorescent intensity in rSUR2A-CT-transfected cells, data were presented as a line created from individual cell images drawn horizontally through the confocal plane at the centre of the cell. Peak fluorescent intensities for EGFP-F and anti-$K_{ATP}$ channel antibody binding were normalized. Plasma-membrane-associated fluorescence was defined by the peaks of EGFP-F fluorescence at the edge of cells. The normalized fluorescence signal associated with anti-$K_{ATP}$ channel antibody binding at the plasma membrane was expressed as a ratio of the plasma-membrane EGFP-F fluorescent signal. Statistical analysis was by

ANOVA and Tukey's HSD (honestly significant difference) test, and $P < 0.05$ was considered significant.

## RESULTS

### Co-immunoprecipitation of MBP-rSUR2A-CT fragments with full-length Kir6.2 subunit

Co-immunoprecipitation experiments were conducted to investigate direct physical association between fragments of the C-terminal domain of the SUR2A subunit and full-length Kir6.2 subunits. In initial experiments, co-immunoprecipitation of full-length Kir6.2 and SUR2A subunits from mixtures of $^{35}$S-labelled *in vitro*-translated subunits using anti-Kir6.2 subunit antiserum was established (results not shown). This co-immunoprecipitation technique was used to screen for interaction between a panel of C-terminal fragments of SUR2A, expressed as MBP-rSUR2A-CT, and full-length Kir6.2 subunits (Figure 1C). Three MBP-rSUR2A-CT fusion proteins, MBP-rSUR2A-CTA, MBP-rSUR2A-CTB and MBP-rSUR2A-CTC were co-immunoprecipitated with full-length Kir6.2 using anti-Kir6.2 C-terminal antiserum. A fourth fusion protein, MBP-rSUR2A-CTD, and MBP alone, did not co-immunoprecipitate with Kir6.2 under the same conditions (Figure 1C). A further construct, MBP-rSUR2A-CTE, was constructed to define further the interaction site, but this construct expressed poorly, at levels well below the limiting concentration for co-immunoprecipitation of MBP-rSUR2A-CTC, thereby preventing direct immunoprecipitation experiments with this fragment.

To control non-specific interactions with Kir subunits, similar experiments were conducted to investigate the co-immunoprecipitation of MBP-rSUR2A-CT fragments with full-length Kir2.1, using an anti-Kir2.1 antiserum previously shown to immunoprecipitate this subunit specifically [21]. None of the MBP-rSUR2A-CT fragments was co-immunoprecipitated with [$^{35}$S]-methionine-labelled Kir2.1, even when concentrations of Kir2.1 subunits and rSUR2A-CT fragments were doubled in the experiment (results not shown). In further control experiments, no co-immunoprecipitation with full-length Kir6.2 or Kir2.1 of an MBP-rMRP1-CTE fragment from the non-interacting MRP1 protein [8], corresponding to the minimal interacting sequence rSUR2A-CTE, was observed (results not shown)

### Disruption of Kir6.2/SUR2A hetero-oligomer assembly by rSUR2A-CT fragments

To investigate whether the putative interaction domain identified in the biochemical assays described above makes important contacts in native channel oligomers, the ability of various rSUR2A-CT fragments to modify $K_{ATP}$ channel function was examined (Figure 2). cDNAs encoding rSUR2A-CT fragments were transiently transfected into HEK-293 cells stably expressing full-length Kir6.2 and SUR2A. The expression of functional $K_{ATP}$ channels in the cell membrane was examined by measuring the current amplitude in excised inside-out patches of cell membrane activated by excision into the bath solution containing no ATP. Inhibition of this current by ATP and glibenclamide was quantified to characterize any pharmacological differences between $K_{ATP}$ channel currents in the presence of different rSUR2A-CT fragments and $K_{ATP}$ current in HEK-293 cells expressing Kir6.2/SUR2A alone. Kir6.2/SUR2A-expressing cells that were mock-transfected with the pIRES2-EGFP vector containing no inserts had a mean $K_{ATP}$ channel current of $1283 \pm 182$ pA ($n = 6$), which was inhibited by 11, 69 and 100 % in the presence of 10, 30 and 1000 mM ATP respectively (Figure 2C), and 57 and 80 % in the presence of 10 and 100 nM glibenclamide (Figure 2D) respectively. When expressed as a fraction of currents obtained from the mock-transfected cells, the currents recorded after excision of patches from cells transiently expressing rSUR2A-CTA, rSUR2A-CTC or rSUR2A-CTE fragments were decreased significantly to 6 % ($64 \pm 6$ pA, $P < 0.001$), 13 % ($165 \pm 22$ pA, $P < 0.001$) and 18 % ($225 \pm 70$ pA, $P < 0.001$) respectively (Figures 2A and 2B). In each case, the residual $K_{ATP}$ channel currents were unmodified with respect to their sensitivity to ATP [Figure 2C, $K_i$ ($\mu$M) $= 19.7 \pm 0.1$, pIRES2-EGFP; $21.4 \pm 0.2$, rSUR2A-CTA; $18.9 \pm 0.3$, rSUR2A-CTC; $19.9 \pm 0.6$, rSUR2A-CTD; $20.6 \pm 0.1$, rSUR2A-CTE; $21.1 \pm 0.3$, rMRP1-CTE] and glibenclamide (Figure 2D). In contrast, no change in the fractional current was observed when the rSUR2A-CTD or rMRP1-CTE fragments were expressed (93 %, $P > 0.05$, and 96.7 %, $P > 0.05$ respectively, control current $= 1197 \pm 121$ pA, Figures 2A and 2B). In cells expressing Kir2.1 together with SUR2A, no change in potassium current was observed in the presence of 1 mM ATP when either rSUR2A-CTD ($343 \pm 46$ versus $312 \pm 48$ pA, $n = 6$) or rSUR2A-CTE ($366 \pm 38$ versus $364 \pm 25$ pA, $n = 6$) was co-expressed. In addition, no change in potassium current was observed in the presence of glibenclamide (100 nM) when either rSUR2A-CTD ($352 \pm 45$ versus $312 \pm 48$ pA, $n = 6$) or rSUR2A-CTE ($339 \pm 51$ versus $364 \pm 25$ pA, rSUR2A-CTE, $n = 6$) was co-expressed.

To investigate directly whether the lowered expression of functional channels in the presence of rSUR2A-CT fragments containing residues 1294–1358 was a result of disruption of channels on the cell surface or lowered surface expression, HEK-293 cells stably expressing Kir6.2/SUR2A and transiently transfected with the rSUR2A-CTE fragment were subjected to immunocytochemistry using anti-Kir6.2 antiserum to look for possible changes in the subcellular localization of this subunit. GFP co-expressed with the rSUR2A-CT fragments, while useful for identifying cells for electrophysiological analysis, was lost on cell permeabilization for immunocytochemistry. In these experiments, rSUR2A-CT fragments were expressed from pIRES2-EGFP-F vectors containing the cDNA for a membrane-localized form of EGFP. Membrane-localized expression of EGFP was used to identify transfected cells after permeabilization and immunocytochemistry. Optical sections of cells were taken systematically in the horizontal axis across the centre of all cells to determine the localization of the plasma-membrane-associated EGFP fluorescence (green) and anti-$K_{ATP}$ channel subunit fluorescence (red). Optical sections are presented as pixel profiles (Figure 3). In cells transfected with empty pIRES2-EGFP-F vector or rSUR2A-CTD, most of the Kir6.2 polypeptide-associated immunofluorescence co-localized with plasma-membrane-associated EGFP-F fluorescence (Figures 3A–3C and 3G). In comparison, plasma-membrane-located anti-Kir6.2-associated fluorescence was significantly lower in most of the cells transiently expressing fragment rSUR2A-CTE (Figures 3D–3F), with most of anti-Kir6.2-associated fluorescence shifted to an intracellular compartment close to the plasma membrane (Figures 3D–3F). To analyse further the relative levels of Kir6.2-associated immunofluorescence at the plasma membrane in these experiments, the fluorescent signals for EGFP-F and Kir6.2 were normalized to the highest peak for each signal. The ratio of Kir6.2:EGFP-F associated fluorescence was then determined at the plasma membrane, defined by the peak of EGFP-F fluorescence (Figures 3C and 3F). The fluorescence ratio thus determined was decreased significantly in cells transfected with rSUR2A-CTE compared with cells transfected with either empty vector or rSUR2A-CTD (Figure 3H). Very similar results were obtained when $K_{ATP}$ channel distribution was investigated using

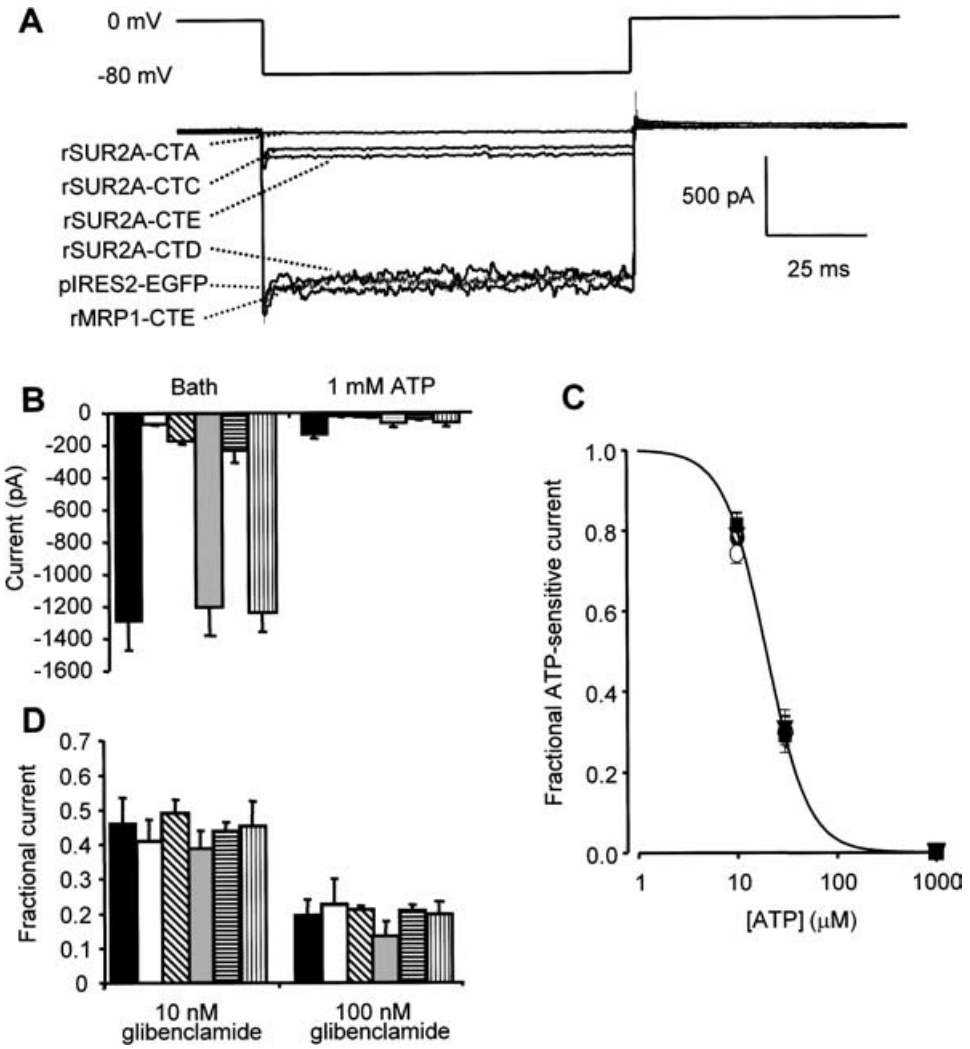


**Figure 2 Reduction of $K_{ATP}$ channel current in HEK-293 cells expressing Kir6.2/SUR2A channels in the presence of rSUR2A-CT fragments and rMRP1-CTE**

(**A**) Raw current traces (overlaid) from inside-out patches from HEK-293-transfected cells elicited by a square-wave pulse (top trace) from 0 mV (approx. $E_K$) to $-80$ mV for 100 ms at 1 Hz. (**B**) Mean current $\pm$ S.E.M. from inside-out patches of HEK-293-transfected cells ($n = 6$). Control current was 1283 $\pm$ 182 pA ($n = 6$). Cells transfected with empty pIRES2-EGFP vector (black bars), rSUR2A-CTA (open bars), rSUR2A-CTC (diagonal hatched bars), rSUR2A-CTD (light grey bars) rSUR2A-CTE (horizontal hatched bars) and rMRP1-CTE (vertical hatched bars). (**C**) Dose-dependent inhibition of $K_{ATP}$ channel current by ATP. Fractional current was defined as the difference between current in ATP-free control solution and the current measured in the presence of 1 mM ATP. Cells transfected with empty pIRES2-EGFP vector (●), rSUR2A-CTA (○), rSUR2A-CTC (▲), rSUR2A-CTD (△), rSUR2A-CTE (■) and rMRP1-CTE (stars). (**D**) Inhibition of $K_{ATP}$ channel current by glibenclamide. Mean fractional current $\pm$ S.E.M. from inside-out patches of HEK-293-transfected cells ($n = 6$) expressed as a fraction of mean current in control solution. Bars defined as in (**B**).

anti-SUR2A subunit associated fluorescence, except that some additional nuclear staining was observed in every cell (results not shown).

### Disruption of an assembly of hetero-oligomers of different combinations of Kir6 and SUR subunits by the rSUR2A-CTE fragment

The amino acid sequence of SUR2A between residues 1294 and 1358 is identical in SUR2B and relatively conserved in SUR1. It was hypothesized that interaction via the NBD2 of each of these isoforms with Kir6.2 would also be disrupted by the minimal rSUR2A-CTE fragment. Analysis of patches pulled from HEK-293 cells transiently transfected with rSUR2A-CTE along with Kir6.2/SUR2B or Kir6.2/SUR1 revealed markedly decreased ATP- and glibenclamide-sensitive currents compared with cells expressing the hetero-oligomer combinations alone (Table 1), indicating that a similar interaction is present between Kir6.2 and SUR2B and SUR1. As different combinations of SUR and Kir6 subunits can co-assemble [1], it was further hypothesized that an rSUR2A-CTE fragment-binding domain was also present in Kir6.1 subunits. Again, co-expression of rSUR2A-CTE fragment with Kir6.1/SUR2B resulted in substantially decreased ATP and glibenclamide-sensitive currents compared with cells expressing the Kir6.1/SUR2B hetero-oligomers alone (Table 1), indicating that a binding site for the rSUR2A-CTE domain is also present in Kir6.1. This also suggested that there are common cytoplasmic interactions between Kir6 and SUR subunits in all subunit combinations.

## DISCUSSION

The sarcolemmal $K_{ATP}$ channel in cardiac muscle comprises a hetero-octamer of four Kir6.2 channel subunits and four SUR2A subunits [2–4]. Kir6 subunits comprise two transmembrane

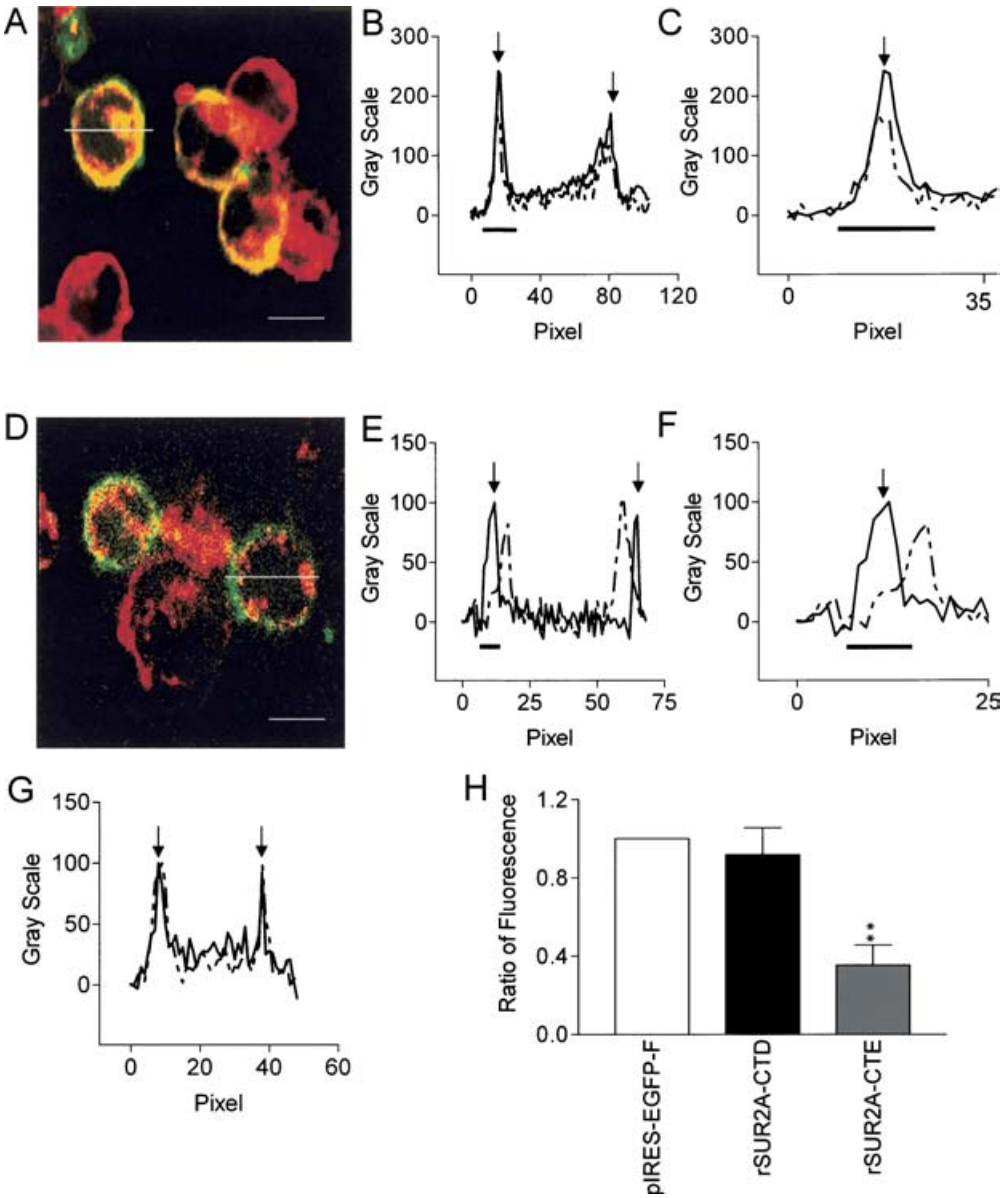


**Figure 3 Kir6.2 subunit distribution in HEK-293 cells stably co-expressing Kir6.2/SUR2A channels and transiently transfected with rSUR2A-CT fragments**

Representative images of EGFP-F (green) and anti-Kir6.2 subunit antibody-associated immunofluorescence (red) in Kir6.2/SUR2A expressing cells transiently transfected with rSUR2A-CTD (**A**) and rSUR2A-CTE (**D**). Co-localized signal appears yellow. Scale bar, 10 $\mu$m. The horizontal lines through the cells in (**A**, **D**) describe the optical sections shown in (**B**, **E**) respectively. (**B**, **E**, **G**) Optical sections (pixel profiles) of fluorescent intensity of EGFP-F (solid line) and anti-Kir6.2 subunit-associated fluorescence (broken line). Cells were transiently transfected with rSUR2A-CTD (**B**), rSUR2A-CTE (**E**) and empty pIRES2-EGFP-F vector (**G**) (representative of three separate experiments). The plasma membranes were defined by the localization of EGFP-F fluorescence (horizontal black line in **B**, **E**). (**C**, **F**) Expanded fluorescence intensity profiles close to the plasma-membrane section on the left-hand side of cells in (**A**, **D**) respectively (horizontal black line, plasma-membrane-associated fluorescence, as above). (**H**) Anti-Kir6.2:EGFP-F fluorescence ratio of plasma-membrane-associated normalized fluorescence signals ($n = 30$ cells), $^{**}P < 0.01$ compared with pIRES2-EGFP-F-transfected cells.

segments M1 and M2, separated by a P-loop that forms the outer mouth of the pore [5]. Both N- and C-termini of the Kir6 subunits are located on the cytoplasmic side of the sarcolemma, as is the C-terminal domain of the SUR2A subunits [6]. In this study, evidence is presented that identifies a novel cytoplasmic region in the proximal C-terminal domain of SUR2A subunit, located between residues 1294 and 1358, which forms a direct interaction with the full-length Kir6.2 subunit. This proposal was supported by direct demonstration of co-immunoprecipitation with full-length Kir6.2 subunit of three MBP-rSUR2A-CT fragments containing residues 1294–1358 (MBP-rSUR2A-CTA, MBP-rSUR2A-CTB and MBP-rSUR2A-CTC), but not a fragment corresponding to the distal C-terminal domain (MBP-rSUR2A-CTD). The specificity of this interaction was investigated in parallel experiments performed in the presence of Kir2.1, a Kir subunit that does not associate with SUR subunits to form functional channels [25]. None of the rSUR2A-CT fragments was co-immunoprecipitated with the Kir2.1 subunit, suggesting that the interactions of MBP-rSUR2A-CT fragments demonstrated with the Kir6.2 subunit were specific. Specificity of the MBP-rSUR2A-CT fragment interactions with Kir6.2 was further suggested by the absence of co-immunoprecipitation with Kir6.2 of a fragment

**Table 1 Reduction of ATP-sensitive current in HEK-293 cells expressing different $K_{ATP}$ channel subunit combinations in the absence or presence of the SUR2A-CTE fragment**

Mean ATP-sensitive current ± S.E.M. ($n = 6$) was determined by the subtraction of residual current in 1 mM ATP from total current recorded in control solution. ATP-insensitive current was never more than 20 % of the total current.

| | ATP-sensitive current (pA) | |
|---|---|---|
| | Absence of rSUR2A-CTE | Presence of rSUR2A-CTE |
| Kir6.1/SUR2B | 124 ± 6 | 7 ± 3* |
| Kir6.2/SUR2B | 306 ± 24 | 12 ± 3* |
| Kir6.2/SUR1 | 271 ± 5 | 11 ± 4* |

* $P < 0.05$ compared with the absence of rSUR2A-CTE.

containing the equivalent region from the non-interacting MRP1 protein (rMRP1-CTE). The identified 65-amino-acid sequence between residues 1294 and 1358 in SUR2A is located over the proximal part of NBD2 and contains the Walker A motif [22]. Identification of an interaction site in this region of rSUR2A herein is consistent with the recent observation in immunoprecipitation experiments of a weak interaction between the TMD2-NBD2 segment derived from SUR1 and Kir6.2 subunits [16].

The interaction between the SUR2A region encompassed by residues 1294–1358 and Kir6.2 was shown, indirectly, to be important for the assembly of functional channels in experiments to assess the effects of competing rSUR2A-CT subunit fragments on channel function and cellular localization of Kir6.2/SUR2A subunit containing channels. When rSUR2A-CT fragments were transiently transfected into HEK-293 cells stably expressing Kir6.2/SUR2A hetero-oligomers, a substantial reduction in ATP- and glibenclamide-sensitive $K_{ATP}$ channel current was observed only in cells expressing rSUR2A-CT fragments containing the sequence between residues 1294 and 1358 (rSUR2A-CTA, rSUR2A-CTC and rSUR2A-CTE). The currents remaining in these cases were unmodified with respect to their sensitivity to ATP and glibenclamide, confirming that these currents, although small, were still flowing through $K_{ATP}$ channels. The specificity of interaction of the rSUR2A-CT fragments containing residues 1294–1358 to reduce currents conducted by surface Kir6.2/SUR2A channels was further suggested by the absence of a change in Kir6.2/SUR2A current density on co-expression of the rMRP1-CTE fragment. This fragment corresponded to the minimal interacting sequence of rSUR2A-CTE found in the ABC transporter (ATP-binding-cassette transporter) protein MRP1, which is known not to interact with Kir6.2 [8]. The specificity of the interaction of rSUR2A residues 1294–1358 with Kir6.2-containing channels was further confirmed by the absence of effect when rSUR2A-CT fragments were co-expressed with Kir2.1 and SUR2A. Functional Kir2.1 channels form in the absence of SUR2A [25]. The absence of functional association of Kir2.1 and SUR2A was confirmed in this study by the insensitivity of Kir2.1-containing channels to ATP and glibenclamide inhibition when Kir2.1 and rSUR2A were co-expressed. rSUR2A-CT fragments containing residues 1294–1358 were without effect on currents conducted by Kir2.1-containing channels. This was interpreted to indicate the absence of a direct interaction of these fragments with Kir2.1 channels and, by inference, specificity of their interaction with Kir6.2.

Whether the lowered expression of functional channels in the presence of rSUR2A-CT fragments containing residues 1294–1358 was a result of disruption of channels on the cell surface or lowered surface expression was investigated by immunocytochemistry. In HEK-293 cells stably expressing Kir6.2 and SUR2A subunits, transient transfection with the rSUR2A-CTE fragment containing the amino acid residues 1294–1359 resulted in significantly decreased Kir6.2 and SUR2A subunit-associated immunofluorescence at the plasma membrane and increased intracellular fluorescence compared with cells transiently transfected with empty pIRES2-EGFP-F vector or the non-interacting fragment rSUR2A-CTD. Reduction of cell-surface labelling in the presence of interacting fragment indicated that the decreased current density produced by fragments containing residues 1294–1358 was a consequence of a decreased number of channel oligomers rather than disruption of channel hetero-oligomers within the plasma membrane. Whether this was due to increased removal of $K_{ATP}$ channels from the cell membrane or decreased replenishment of $K_{ATP}$ channel hetero-oligomers, due to disruption of assembly in the ER during normal turnover, was not determined. For expression of either subunit at the cell surface, assembly of $Kir6.2_4/SUR2A_4$ hetero-oligomers in the ER is required to mask ER-retention signals in both subunits [8]. Our working hypothesis is that the fragments containing the 1294–1358-residue sequence disrupted normal assembly of the stably expressed channel hetero-oligomers, such that ER-retention signals remained unmasked, resulting in decreased incorporation at the plasma membrane.

In addition to disrupting the normal assembly of Kir6.2/SUR2A channels, ATP- and glibenclamide-sensitive currents, carried by both Kir6.2/SUR2B and Kir6.2/SUR1 channels, were markedly decreased on co-expression of the rSUR2A-CTE fragment. The interacting amino acid sequence 1294–1358 identified in SUR2A is identical in SUR2B and relatively conserved in SUR1. Disruption by the rSUR2A-CTE fragment of Kir6.2 channels containing SUR2A, SUR2B or SUR1 indicates that a similar interaction is present between Kir6.2 and the C-terminal domains of all three of these accessory subunits. In addition, ATP- and glibenclamide-sensitive currents carried by Kir6.1/SUR2B were substantially decreased on co-expression of the rSUR2A-CTE fragment. This result indicated that Kir6.1 also contains a binding site for rSUR2A-CTE domain and, thus, that there are common cytoplasmic interactions between Kir6 and SUR subunits in all subunit combinations.

High-resolution structures for eukaryotic ABC transporter proteins remain to be determined, but crystal structures of a number of bacterial ABC transporter proteins or NBDs derived from these proteins reveal essentially the same basic two-lobed fold for NBD monomers (reviewed in [26]). Residues 1294–1358 of rSUR2A span a region of $\beta$-sheet followed by the Walker A motif which are located on opposite sides of lobe 1 of this structure. In all cases, NBDs were crystallized as dimers, although four distinct structural arrangements have been observed [26]. Functional co-operativity between NBDs in eukaryotic ABC transporter proteins suggests that an organization of domains similar to that in bacterial NBD dimers may occur. For an interaction to occur with Kir6.2, at least part of the 1294–1358 rSUR2A-CT interaction domain should be present at the surface of such an NBD dimer structure. This criterion could be satisfied in all four proposed NBD dimer structures. In the HisP arrangement, the Walker A motifs map to the surface, whereas the $\beta$-sheet region would be buried. Alternatively, in Rad50, the favoured common structure for ABC transporter NBD dimers [27], MalK and ArsA arrangements, the Walker motifs would be buried and the $\beta$-sheet region would be at the surface. This is consistent with a recently proposed model of NBD dimerization in SUR1 [28].

Similarities seen in several crystal structures [27] and comparison of the interface-to-non-interface sequence entropies [26]

favours a Rad50-like, or possibly MalK, arrangement of NBD dimers, in which the nucleotide-binding sites are buried. These models are consistent with fluorescence resonance energy transfer measurements of the distance between Walker A cysteine residues of 30–38 Å (1 Å = 0.1 nm) in P-glycoprotein [29]. Although not totally in agreement, two studies examining cross-linking of cysteine residues between NBDs in P-glycoprotein indicate a much closer proximity of Walker A motifs ($\leqslant$ 17 Å) as suggested by the ArsA model [30,31]. All three models predict, however, that rSUR2A-CT interaction with Kir6.2 would be mediated through the $\beta$-sheet structure. It is noteworthy that none of these models take account of additional constraints on NBD interactions that may be imposed by the transmembrane domains of eukaryotic ABC transporter proteins. High-resolution structures of eukaryotic ABC transporter proteins and more detailed analysis of the 1294–1358 rSUR2A-CT interaction domain are required to define the precise structure of the interaction domain.

The site(s) of interaction in the Kir6 subunit for the Kir6-binding domain in SUR2A NBD2 identified in this study remains to be determined. Analysis of the interaction between Kir6.2 and SUR2A subunits, assayed by co-immunoprecipitation with anti-Kir6.2 antiserum, demonstrated that deletion of the transmembrane domain TM2 and the proximal C-terminal domain of Kir6.2 prevented the association of SUR2A [9]. Similarly, targeting of hetero-multimers of Kir6.2/SUR1 to the plasma membrane was prevented when a chimaeric Kir6.2/Kir2.1 construct containing the M2/proximal C-terminal domain of Kir2.1 in a background of Kir6.2 was expressed [10]. Co-immunoprecipitation of various chimaeras of Kir6.2 and Kir2.1 with full-length SUR1 further localized a region of interaction in the proximal C-terminal domain of Kir6.2 between residues 208 and 279 [11]. In addition, mutation of residues Lys-176 and Lys-177 upstream in the proximal C-terminal domain prevented MgADP-dependent channel stimulation and sulphonylurea-induced inhibition of Kir6.2/SUR1 channels [32], further implicating this region in SUR contacts. The proximal C-terminal region, together with the M2 transmembrane segment, is important also in determining homotypic and heterotypic interactions between other Kir subunits [33–36] and represents an important region of biochemical contacts that could associate with the interacting SUR2A region identified herein. An alternative or complementary interaction of the Kir6-binding domain in SUR2A NBD2 with the cytoplasmic N-terminal domain of Kir6.2 cannot be ruled out, as evidence for a cytoplasmic interaction between the N-terminus of Kir6.2 and other parts of SUR1, namely the sulphonylurea-binding L0 loop, has been reported [12,13].

ATP-dependent inhibition of $K_{ATP}$ channel activity occurs through binding of ATP at a site formed by the intra-molecular association of the N- and C-terminal domains of Kir6 subunits [37,38]. ATP sensitivity is further modified in response to nucleotide binding to the SUR subunits (see [1] and references therein). Binding of MgADP to the nucleotide-binding folds, NBD1 and NBD2, which are located in the cytoplasmic loop between TMD1 and TMD2 and in the cytoplasmic C-terminal domain respectively [39] is sufficient to desensitize the pore-forming Kir6 subunits to the inhibitory effects of ATP [40,41]. NBD1 binds ATP with little hydrolytic activity [41–43], and NBD2 binds MgADP and/or binds and hydrolyses MgATP [42,44–46]. The activities of the two NBDs are interdependent and both appear to be required for optimal regulatory activity [40,41,46]. MgADP binding to NBD2 promotes binding of ATP to NBD1 [42,45,46], and the presence of NBD1 appears to be mandatory for the regulation of ATPase-dependent gating of $K_{ATP}$ channels mediated through NBD2 [47]. Chimaeras between SUR2A and SUR2B have shown that the nature of a seven-amino-acid segment within the C-terminal 42 amino acids of the SUR2 subtype further defines the differential activation of $K_{ATP}$ channels containing SUR2 subunits in response to ADP [48,49]. Homology-structure models based on HisP structure suggested a possible electrostatic interaction with Arg-1344 in NBD2, thereby implicating the C-terminus of these subunits in making a contribution to the sensitivity of channel activation by MgADP [49]. Consistent with this, mutations in NBD2 and in the C-terminal domain of SUR1, found in familial hyperinsulinaemia, reduce the sensitivity of Kir6.2/SUR1 channels to ADP (see e.g. [50] and references therein). Thus co-operative nucleotide binding to the two NBDs decodes the metabolic status of the cell by integrating responses to levels of nucleotides present.

Transfer of the regulatory information from the accessory SUR to pore-forming Kir6 subunits most probably entails a series of physical contacts between extramembranous domains of the two subunit types, in addition to the membrane domain contacts so far characterized [8]. Experiments in Sf9 cells showed that co-expression of a GFP-tagged NBD1 polypeptide of SUR1 with full-length Kir6.2 did not result in localization of NBD1 to the plasma membrane in association with Kir6.2 [19]. In the same series of experiments, the tagged NBD1 fragment was directed to the plasma membrane on co-expression with the C-terminal half of the SUR1 protein, but not when NBD2 was deleted from the truncated subunit. Taken together, these results indicated that, although an interaction was observed between NBD1 and NBD2, there was no evidence for a strong association between NBD1 and Kir6.2. In contrast, the results of the present study provide evidence for a direct contact between the proximal C-terminal NBD2-containing domain of SUR2A and Kir6.2. Whereas the disruption of plasma-membrane targeting of Kir6.2/SUR2A channels by co-expression of competing SUR2A-CT fragments demonstrated the structural importance of this interaction, more subtle mutagenesis studies focused in this area are required to determine whether allosteric information can be transduced through this contact.

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