Functional Clustering of Mutations in the Dimer Interface of the Nucleotide Binding Folds of the Sulfonylurea Receptor

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ATP-sensitive K⁺ (K\textsubscript{ATP}) channels modulate their activity as a function of inhibitory ATP and stimulatory Mg-nucleotides. They are constituted by two proteins: a pore-forming K⁺ channel subunit (Kir6.1, Kir6.2) and a regulatory sulfonylurea receptor (SUR) subunit, an ATP-binding cassette (ABC) transporter that confers MgADP stimulation to the channel. Channel regulation by MgADP is dependent on nucleotide interaction with the cytoplasmic nucleotide binding folds (NBF1 and NBF2) of the SUR subunit. Crystal structures of bacterial ABC proteins indicate that NBFs form as dimers, suggesting that NBF1-NBF2 heterodimers may form in SUR and other eukaryotic ABC proteins. We have modeled SUR1 NBF1 and NBF2 as a heterodimer, and tested the validity of the predicted dimer interface by systematic mutagenesis. Engineered cysteine mutations in this region have significant effects, both positive and negative, face by systematic mutagenesis. Engineered cysteine mutations erodimer, and tested the validity of the predicted dimer inter-

The abbreviations used are: KATP, ATP-sensitive potassium channel; Kir, inward rectifier potassium channel; SUR, sulfonylurea receptor; ABC, ATP-binding cassette; TMD, transmembrane domain; NBF, nucleotide binding fold; KCO, potassium channel opener; ABS, ATP-binding site; PKA, protein kinase A; DTT, dithiothreitol; WT, wild type.

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2 The abbreviations used are: K\textsubscript{ATP}, ATP-sensitive potassium channel; Kir, inward rectifier potassium channel; SUR, sulfonylurea receptor; ABC, ATP-binding cassette; TMD, transmembrane domain; NBF, nucleotide binding fold; KCO, potassium channel opener; ABS, ATP-binding site; PKA, protein kinase A; DTT, dithiothreitol; WT, wild type.

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In the absence of a high-resolution crystal structure, various approaches have been employed to assess NBF organization in SUR. Azido-ATP labeling experiments show that either MgATP or MgADP binding to NBF2 stabilizes ATP binding to NBF1 (29). Co-affinity purification of isolated NBF1 and NBF2 has been reported (30), and a soluble NBF1-GFP construct is recruited to the membrane by a TMD2-NBF2 construct when they are coexpressed in insect cells (31). Finally, a low-resolution crystal structure of the entire K<sub>ATP</sub> channel complex depicts two regions in the electron density map that may correspond to the SUR NBFs in close apposition; surprisingly, they are not located directly underneath the respective TMDs, but rather at a significant offset, suggesting a severe twisting of the TMD-NBF linkers or the thus far unexplored possibility of intersubunit NBF dimers (32).

In this study we have addressed the possibility of SUR NBF heterodimerization by homology modeling of SUR1 NBFs and cysteine mutagenesis of the predicted dimer interface. We have assessed the functional consequences of mutation by measuring MgADP stimulation of recombinant K<sub>ATP</sub> channels, as well as macroscopic fluxes in intact cells. Mutagenesis of predicted dimer interface residues has profound functional effects, especially at the center of the dimer, where hyperstimulating mutations are clustered in the D-loop region. These data support the hypothesis that SUR1 NBFs interact in a heterodimer configuration, and that this interaction is critical for SUR1-dependent nucleotide regulation of the K<sub>ATP</sub> channel.

**EXPERIMENTAL PROCEDURES**

**Homology Modeling of SUR NBFs—**NBF1 and NBF2 from hamster SUR1 (33) were modeled with the Linux-based version of Modeler6v2 (34) using MJ0796 (18) as a template. An initial sequence alignment between the template and the sequence of interest was obtained in Vector NTI and refined on the basis of our insight and on the crystal structures of Rad50 (21) and BtuD (20). The alignment was entered into Modeler6v2, and the resulting models were ranked in quality by a self-checking function in the program and independently by Prosa II (35) and Procheck (36). The three-dimensional structure of the models was analyzed with PyMol (37) and Swiss PDB Viewer (38).

**Transfection of COSm6 Cells with Cloned and Mutated cDNA—**DNA of interest was transfected into COSm6 cells using FuGENE 6 Transfection Reagent (Roche Applied Sciences). 1.1 μg of total DNA (0.3 μg of mouse Kir6.2 (39) plus 0.5 μg of hamster SUR1 (33) plus 0.3 μg of GFP as a marker for transfection) was mixed with 3 μl of FuGENE 6; cells were incubated in the presence of the transfection mixture for 12–24 h, and then plated on sterile glass coverslips for growth prior to patch-clamp experiments.

Point mutations in SUR1 were engineered by two-step PCR (a segment of the clone was synthesized by PCR with mutagenic primers, and then subcloned into the full-length SUR1 clone) or by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). All clones were confirmed by direct sequencing.

**Electrophysiological Methods—**Inside-out membranes were patch-clamped in a chamber mounted on the stage of an inverted microscope (Nikon, Garden City, NY). The chamber consists of four lanes (each containing different solutions) that run into the same end-pool (40). A float connected to a tension transducer controls the solution level by varying the outflow from the end-pool. COS cells (2–5 days post-transfection) that fluoresced green under UV illumination were selected for patch-clamping. Membranes were voltage-clamped with a CV–4 headstage and an Axopatch 1-D amplifier, and currents were digitized with a Digidata 1322A board (all from Axon Instruments, Union City, CA). Bath and pipette control solutions (K<sub>ATP</sub>) contained, in mM: 150 KCl, 10 HEPES, and 1 EGTA (pH 7.4). ATP and ADP were added to the bathing solution as dipotassium salts. Where indicated, MgCl<sub>2</sub> was added to the bathing solution to a calculated [Mg<sup>2+</sup>]<sub>free</sub> of 0.5 mM. Diazoxide (0.3 mM) was dissolved in K<sub>INT</sub> plus 0.1 mM MgATP from a stock solution in DMSO.

Data were analyzed off-line with the pClamp 8.2 software suite (Axon Instruments, Union City, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA). Results are presented as mean ± S.E. Statistical tests and p values are noted in the figure legends.

**Macroscopic 68Rb<sup>+</sup> Efflux Assays—**COS cells in 12-well plates were incubated for 24 h in culture medium containing 68RbCl (1 μCi/ml), 1–2 days after transfection. Before measurement of 68Rb<sup>+</sup> efflux, cells were washed twice with Ringer’s solution (in mM: 118 NaCl, 2.5 CaCl<sub>2</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 4.7 KCl, 25 NaHCO<sub>3</sub>, 1.2 MgSO<sub>4</sub>, 10 HEPES; pH 7.4) plus Metabolic Inhibition (1 mM 2-deoxy-D-glucose and 2.5 μg/ml oligomycin). At selected time points, the solution was aspirated from the cells and replaced with fresh solution; after completion of the assay, cells were lysed with 1% SDS, aspirated, and assayed in a scintillation counter. Raw data are shown as 68Rb<sup>+</sup> efflux relative to total counts (including all time points and lysate). The rate constant for K<sub>ATP</sub>-specific 68Rb<sup>+</sup> efflux (k<sub>e</sub>) was obtained by fitting the data with the single-exponential Equation 1,

\[
\text{Relative flux} = 1 - \exp\left[-(k_1 + k_2) \times t\right]
\]

where the apparent rate constant for nonspecific efflux (k<sub>e</sub>) was obtained from untransfected cells, for which k<sub>e</sub> was assumed to be 0.

**RESULTS**

**Homology Modeling of SUR1 NBFs as a Heterodimer—**Using the crystal structure of the bacterial NBF MJ0796 as a template (18), we generated a homology model of NBF1 and NBF2 from SUR1 as an ATP-bound dimer (Fig. 1A). The Mg<sup>2+</sup> ion site is occupied by Na<sup>+</sup>, as the MJ0796 crystal structure was obtained bound to Na<sub>ATP</sub> rather than Mg<sub>ATP</sub>. A key feature of the model is that the nucleotide binding sites are located at the dimer interface, such that each nucleotide is interacting with residues from both NBFs. This feature is in agreement with similar NBF homology models of SUR1 (41, 42) and SUR2B (43). For clarity, we have defined each ATP-binding site (ABS) as follows: ABS1 is formed by Walker A, Walker B, Q-loop, and H-loop from NBF1, and the Signature Sequence from NBF2, while ABS2 is formed by Walker A, Walker B, Q-loop, and H-loop from NBF2, and the Signature Sequence from NBF1 (Fig. 1, B and C).

In addition to providing a detailed prediction of protein-nucleotide interactions, the model predicts residues outside the
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There is the possibility that the effects of the cysteine mutations are due to formation of a disulfide bond with native cysteines. However, DTT applied to excised patches had no effect on the MgADP stimulation of several representative mutants (data not shown), suggesting that either their effects are not due to disulfide bond formation, or, if a disulfide bond is indeed formed, it is not accessible to DTT. Additional attempts to detect disulfide bonds using oxidizing and cross-linking reagents in both excised patches and intact cells were unsuccessful, suggesting that either no disulfide bonds are formed by the introduced cysteines, or any disulfide bonds formed in the dimer interface are inaccessible to the employed reagents.

Effects of Predicted Dimer Interface Mutations on Diazoxide Stimulation—Stimulation of $K_{ATP}$ channels by both MgADP and potassium channel openers (KCOs) is hypothesized to occur through convergent pathways (i.e. by energetically favoring the same "activated" configuration of SUR1). For a few representative mutations, we tested the ability of a KCO (diazoxide) to stimulate $K_{ATP}$ channels (Fig. 2C). Consistent with expectations, two mutations that increased MgADP stimulation (A1510C and S1511C) also increased diazoxide stimulation; a mutation that decreased MgADP stimulation (D1513C) also decreased diazoxide stimulation; and a mutation that had no effect on MgADP stimulation (S1356C) had no effect on diazoxide stimulation (Fig. 2C).

Effects of Predicted Dimer Interface Mutations on Macroscopic $^{86}$Rb$^+$ Fluxes—To test the effects of dimer interface mutations on $K_{ATP}$ channel activity in the physiologically relevant intact cell rather than in excised patches, we measured macroscopic $^{86}$Rb$^+$ fluxes from transfected COS cells, under conditions of metabolic inhibition, for WT and 25 mutants (Fig. 3A). Flux data were fitted with Equation 1 (see “Experimental Procedures”) to determine the rate constant of $K_{ATP}$-specific efflux, $k_2$ (Fig. 3B). Two previously reported SUR1 NBF mutations were also included in the data set: F1388L, which results in increased MgADP stimulation (44), and D1506A, which abolishes MgADP stimulation (10). Because they represent two ends of the SUR1 functional spectrum, we have included them here as reference points. To compare these data with MgADP stimulation data, normalized $k_2$ values were plotted versus normalized MgADP stimulation (Fig. 3C). Overall, there is a good

![FIGURE 1. Model of SUR1 NBFs as a heterodimer, with the ATP-binding sites located at the dimer interface. A, homology model of SUR1 NBF1 (gray) and NBF2 (light blue) as a heterodimer; as seen from below (top), and from the side (bottom). Highly conserved sequences are colored as follows: Walker A, red; Walker B, dark blue; Signature Sequence, green; Q-loop, light green; H-loop, magenta; D-loop, orange. ABS1 and 2: ATP-binding sites 1 and 2 (each occupied by a Na$^+$ ion and an ATP molecule, in space-filled view). N and C termini for each NBF are indicated by N and C, respectively. B, schematic representation of NBF dimer model, with conserved sequences colored as in A. C, interactions between NBF residues (sticks) and NaATP (spheres) at ABS1 (top) and ABS2 (bottom) in the dimer model.](image)
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FIGURE 2. Cysteine mutations in the predicted SUR1 NBF dimer interface have significant effects on $K_{\text{ATP}}$ channel regulation. A, representative current traces recorded at −50 mV from inside-out excised membrane patches from COS cells transfected with Kir6.2 and SUR1 NBF cysteine mutants. Patches were exposed to varying concentrations of ATP and ADP, in the presence of 0.5 mM free Mg$^{2+}$, as indicated. Mutations were classified into three groups on the basis of their effects on ADP stimulation; for simplicity, only one representative from each group is shown. B, mean currents in 0.1 mM MgATP and in 0.1 mM MgATP plus 0.5 mM MgADP relative to control (no nucleotides) for all mutants. Bars indicate mean ± S.E.; n = 3–17 patches. Bar colors indicate effect on ADP stimulation: decreased (red), unchanged (yellow), or increased (green); p < 0.05 compared with WT SUR1 by Student’s t test. NE, did not express measurable $K_{\text{ATP}}$ channel current. Residues located in highly conserved sequences are indicated by horizontal bars underneath (WA, Walker A; Sig, Signature Sequence; ABS1 and 2: ATP-binding sites 1 and 2; see Fig. 1). C, diazoxide stimulation of SUR1 NBF single cysteine mutants. Mean currents (±S.E.) in 0.1 mM MgATP plus 0.3 mM diazoxide relative to control (no ligands). n = 3–13 patches. *, p < 0.05 compared with WT SUR1, by Student’s t test. Bar colors indicate effect when applied to excised patches (data not shown), suggesting that, either the effects are not due to disulfide bond formation or, if a disulfide bond is indeed formed, it is not accessible to DTT.

DISCUSSION

Perturbation of the Predicted Dimer Interface Alters $K_{\text{ATP}}$ Channel Function—Multiple mutations in the predicted dimer interface result in significant alterations of SUR1-dependent regulation of $K_{\text{ATP}}$ channel function. These effects are variable, both qualitatively (impairment or enhancement) and quantitatively (different degrees of impairment or enhancement). On average, NBF1 is more tolerant of mutation than NBF2 (56 versus 21% of mutations have no effect); additionally, out of the mutations that do have a significant effect, those in NBF2 tend to be more severe than the equivalent mutations in NBF1 (Fig. 2B). However, it is more revealing to consider the effects of these mutations in the context of the putative NBF heterodimer. When each mutation is mapped onto the homology model and color-coded according to its effect on MgADP stimulation, quite striking structure-function patterns emerge (Figs. 5 and 6).

The three hyperstimulating mutations (S858C, A1510C, and S1511C) are all in close proximity to one another at the center of the predicted dimer interface (Fig. 6, green), such that they are surrounded by protein and not exposed to solution. These residues are part of a highly conserved motif, the D-loop, consisting of a conserved aspartate and a less conserved group of three preceding nonpolar or uncharged polar residues (SALD in NBF1, ASID in NBF2). Despite the conservation of this D-loop in all ABC proteins, a consistent functional role has not been ascribed to it, in contrast to other conserved elements.

There is relatively high tolerance for mutation of residues predicted to be located toward the top of the dimer (i.e. near the membrane), whether in the vicinity of ABS1 or ABS2 (Fig. 6, yellow). By contrast, multiple residues located toward the bot-
The abolition of MgADP activation by mutation of the H-loop histidines (His-889 and His-1538) may be explained by their involvement in coordination of Mg$^{2+}$ and stabilization of the attacking water molecule during ATP hydrolysis. Consistent with this idea, H-loop mutations severely impair ATP hydrolysis in some prokaryotic NBFs (45). In SUR, biochemical studies indicate that ABS1 (unlike ABS2) does not significantly hydrolyze ATP (24, 25). In our study, mutation of the NBF1 H-loop histidine (in ABS1) abolished stimulation to an extent similar to mutation of the NBF2 H-loop histidine (in ABS2), suggesting that coordination of Mg$^{2+}$ in ABS1 may be required for tight MgATP binding in ABS2. However, it is interesting that mutation of nearby residues in both NBFs are also deleterious (K890C in NBF1, R1539C and V1540C in NBF2). While these residues may help position the conserved histidine appropriately, it is also possible that, given their close proximity to residues on the other NBF (especially the D-loop), the effects caused by H-loop perturbation are not due to disruption of nucleotide hydrolysis or binding, but rather of inter-NBF interactions (see below).

The dimer interface mutations have similar effects on MgADP stimulation and diazoxide stimulation (Fig. 2, B and C). Previous mutagenesis of SUR1 NBF residues has yielded paired effects on both functional properties (10, 14, 44). Although we did not pursue a systematic characterization of mutational effects on diazoxide stimulation, we did obtain data for a few selected residues to confirm that these mutations have similar effects on both MgADP and diazoxide stimulation, as predicted if both exert their stimulatory effects by stabilizing the same “activated” configuration of SUR1 (9). Our results are therefore in agreement with this trend.

Sensitivity to Stimulatory MgADP Correlates with Channel Activity in the Intact Cell—By characterizing a large number of mutations, we have effectively generated a graded spectrum of alterations of MgADP stimulation, from completely absent to significantly increased. This permits a detailed correlation between MgADP stimulation and $K_{\text{ATP}}$ channel activity in the intact cell, by using macroscopic $^{86}$Rb$^+$ fluxes in metabolically inhibited COS cells. Previous studies show that mutations causing loss of MgADP stimulation also result in decreased fluxes, indicating that sensitivity to MgADP stimulation is a key determinant of $K_{\text{ATP}}$ channel activity in the intact cell (1, 14, 46). Consistent with this, we observe a general trend where mutations
that decrease MgADP stimulation lead to decreased $^{86}$Rb$^+$ fluxes in an almost “dose-dependent” manner (Fig. 3C). There are, however, two exceptions to this trend (V862C and T1381C), indicating that, although channel activity is critically dependent on sensitivity to Mg-nucleotides, mutations in the SUR NBFs may conceivably also alter the response to other intracellular regulators of channel activity (which range from membrane phospholipids to exocytotic proteins) (3).

Interestingly, none of the three hyperstimulating mutations (S858C, A1510C, and S1511C) exhibits fluxes greater than WT (Fig. 3B), and as a result the relationship between MgADP stimulation and activity in the intact cell reaches a plateau (Fig. 3C). Thus, under the conditions of metabolic inhibition employed here (1 mM 2-deoxyglucose and 2.5 $\mu$g/ml oligomycin), WT channels appear to be maximally activated, and increased sensitivity to MgADP is inconsequential. However, when the activity of the hyperstimulated mutation F1388L was tested with less severe metabolic inhibition (0.5 or 1 mM 2-deoxyglucose, with no oligomycin), F1388L activity was indeed greater than WT (44). Thus, under physiological conditions, one would expect to see differences between WT SUR1 and the three hyperstimulating mutations reported here.

Putative Role of the D-loop in NBF Dimerization—The non-additive effects of double cysteine mutation at the center of the NBF dimer (Fig. 4A). Representative current traces recorded at $-50$ mV from inside-out excised membrane patches from COS cells transfected with Kir6.2 and WT SUR1 or SUR1 NBF cysteine mutants. Patches were exposed to varying concentrations of ATP and ADP, in the presence of 0.5 mM free Mg$^{2+}$, as indicated. B, mean relative currents (± S.E.) in 0.1 mM MgATP plus 0.5 mM MgADP. $n = 5$–19 patches. C, location of mutated residues in NBF dimer model (see Fig. 1). Side chains of introduced cysteines are shown as sticks. Orange: D-loop; magenta: H-loop; light blue: other NBF1 residues; light gray: other NBF2 residues; dark gray spheres: ATP molecules.

FIGURE 4. Non-additive effects of double cysteine mutation at the center of the NBF dimer. A, representative current traces recorded at $-50$ mV from inside-out excised membrane patches from COS cells transfected with Kir6.2 and WT SUR1 or SUR1 NBF cysteine mutants. Patches were exposed to varying concentrations of ATP and ADP, in the presence of 0.5 mM free Mg$^{2+}$, as indicated. B, mean relative currents (± S.E.) in 0.1 mM MgATP plus 0.5 mM MgADP. $n = 5$–19 patches. C, location of mutated residues in NBF dimer model (see Fig. 1). Side chains of introduced cysteines are shown as sticks. Orange: D-loop; magenta: H-loop; light blue: other NBF1 residues; light gray: other NBF2 residues; dark gray spheres: ATP molecules.

FIGURE 5. Location of interfacial residues in the SUR1 NBF dimer model. SUR1 NBF heterodimer model depicting the location of residues included in the perturbation scan. Native side chains are shown as sticks, and they are colored based on the functional effect of mutation to cysteine (from Fig. 2B): red (decreased ADP stimulation), yellow (no change in ADP stimulation), green (increased ADP stimulation), or blue (no measurable $K_{ATP}$ channel current). The model is shown from above (i.e. from the membrane). ATP-binding sites (ABS1 and ABS2) are indicated, and ATP molecules are shown as sticks.

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NBFs of the chloride channel Cystic Fibrosis Transmembrane conductance Regulator (CFTR) can be cross-linked by inducing disulfide bond formation at introduced cysteines in the D-loops; cross-linking is phosphorylation-dependent, and thus correlates with an active, conducting state of CFTR (48).

In a model of the ADP-bound Hemolysin B dimer, steric hindrance between the opposing D-loops suggests that the structure is unstable and that the D-loop is therefore also involved in the breaking apart of the dimer after hydrolysis (49). This suggests that the effects of the three hyperstimulating mutations described here may be due to improved inter-NBF interaction between these two domains in the MgADP-bound dimer, while the effects of the D-loop deleterious mutations may be due to increased steric hindrance, and thus weakened inter-NBF interactions.

Functional Relevance of the NBF Dimer Interface in the Context of the \( K_{ATP} \) Channel—The general picture that emerges from these results, beyond specific structural patterns or the role of specific residues, is that the interface predicted by the dimer model is a functionally critical region where single point mutations can have varying and dramatic effects on \( K_{ATP} \) channel function. Marked effects of mutations located at the dimer interface, but away from the nucleotide binding sites, suggest that protein-protein contacts are also important contributors to SUR1 NBF dimerization. It is interesting, for example, that MgADP stimulation can be completely abolished by mutations well outside the predicted nucleotide binding sites (e.g. D1513C), resulting in channel behavior that is remarkably like that resulting from catalytic site mutations expected to abolish ATP hydrolysis (10, 13). It is unclear whether these mutations prevent nucleotide binding or hydrolysis, or affect downstream events in the signal transduction pathway, but their location in the dimer model suggests they must exert any such effects indirectly and through other regions of the protein.

Interestingly, several SUR1 mutations that render \( K_{ATP} \) channels more sensitive to stimulation by Mg-nucleotides have recently been shown to cause neonatal diabetes in humans (15–17, 50, 51). All of these mutations share a common end-pathway (increased Mg-nucleotide stimulation), despite the fact that their mechanisms of action are markedly different and may operate at the level of ATP hydrolysis at the NBFs (16, 17), transduction of MgADP stimulation via TMD0 (51), or single-channel gating kinetics (52). Thus, the three hyperstimulating mutations described in the present study may well be identified as disease-causing mutations in the future, and may in turn represent an additional mechanism of neonatal diabetes caused by SUR1 mutations.

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