Interactions of the Sulfonylurea Receptor 1 Subunit in the Molecular Assembly of \( \beta \)-Cell K\(_{\text{ATP}} \) Channels

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We have investigated protein interactions involved in pancreatic \( \beta \)-cell ATP-sensitive potassium channel assembly. These channels, which are of key importance for control of insulin release, are a hetero-oligomeric complex of pore-forming Kir6.2 subunits and sulfonylurea receptor (SUR1) subunits with two nucleotide-binding domains (NBD1 and NBD2). We divided SUR1 into two halves at Pro-1042. Expression of either the individual N- or C-terminal domain in a baculovirus expression system did not lead to glibenclamide binding activity, although studies with green fluorescent protein fusion proteins showed that both half-molecules were inserted into the plasma membrane. However, significant glibenclamide binding activity was observed when the half-molecules were co-expressed (even when NBD2 was deleted from the C-terminal half-molecule). Simultaneous expression of Kir6.2 resulted in enhanced glibenclamide binding activity. We conclude that the glibenclamide-binding site includes amino acid residues from both halves of the molecule, that there is strong interaction between different regions of SUR1, that NBD2 is not essential for glibenclamide binding, and that interactions between Kir6.2 and SUR1 participate in ATP-sensitive potassium channel assembly. Investigation of NBD1-green fluorescent protein fusion protein distribution inside insect cells expressing C-terminal halves of SUR1 demonstrated strong interaction between NBD1 and NBD2. We also expressed and purified NBD1 from Escherichia coli. Purified NBD1 was found to exist as a tetramer indicating strong homomeric attractions and a possible role for NBD1 in SUR1 assembly.

A central component of transmembrane signaling in the pancreatic \( \beta \)-cell is the ATP-sensitive potassium (K\(_{\text{ATP}}\)) channel that couples changes in plasma glucose concentration to insulin secretion and is also the target for the sulfonylurea drugs used to treat type I diabetes (1, 2) and for diazoxide, which inhibits insulin secretion and is used to treat insulinoma and familial hyperinsulinism. The \( \beta \)-cell K\(_{\text{ATP}} \) channel contains two subunits, Kir6.2, an inwardly rectifying potassium channel, and SUR1, which contains the high affinity sulfonylurea-binding site and whose presence in the complex is essential for regulated channel activity (4, 5). Channel closure is thought to be mediated by effects of ATP on Kir6.2 (6) whereas SUR1 endows the K\(_{\text{ATP}} \) channel with sensitivity to the inhibitory effects of sulfonylureas and the stimulatory effects of MgADP and potassium channel openers (7, 8). Studies on fusion constructs with fixed SUR1:Kir6.2 ratios suggest that the native channel has a (SUR1–Kir6.2)\(_4 \) stoichiometry (9, 10). A topographical model for SUR1 based on hydrophobicity plots proposes that SUR1 contains two tandem repeats of six transmembrane helices, each set followed by a large cytosolic loop, plus an additional N-terminal hydrophobic region containing five transmembrane helices (11). SUR1 is classified as a member of the ATP-binding cassette (ABC) superfamily, and the two cytosolic loops, each containing a Walker A and Walker B motif (12), are suggested to function as nucleotide-binding domains (NBDs). Disruption of either NBD results in the unregulated insulin secretion found in hypoglycemia and hyperinsulinism of infants (13, 14).

Only limited information is available on the regions of SUR1 and Kir6.2 important for channel assembly and ligand binding. Photoaffinity labeling of both SUR1 and Kir6.2 by radioactive sulfonylurea provided evidence for close association between the two channel subunits (10), and a direct physical association was demonstrated by immunoprecipitation (15). The proximal C terminus of Kir6.2 has been shown to be important for interaction with SUR1 (16). Such an association has been suggested to facilitate insertion of channel-containing vesicles into the plasma membrane or to stabilize channels once inserted into the plasma membrane (17). There is evidence that both N and C termini of Kir6.2 cooperate to form the ATP-binding site (18). Studies on SUR1-SUR2A chimeras have shown that the C-terminal set of transmembrane domains of SUR1 is implicated in binding of sulfonylureas (19) and that the first five transmembrane (TM) domains and the C terminus specify the spontaneous bursting pattern and sensitivity to inhibition by ATP, respectively (20).

We have previously shown that functional K\(_{\text{ATP}} \) channels can be expressed in insect cells using baculovirus (21). In the present study we have used the baculovirus system to express, both independently and in combination, the N- and C-proximal halves of SUR1, containing NBD1 and NBD2, respectively. We showed that neither half-molecule alone gave rise to detectable increase in intracellular [ATP]/[ADP] ratio; ATP blocks the channels whereas MgADP activates them. Closure of K\(_{\text{ATP}} \) channels in response to sulfonylureas and opening of the channels by diazoxide involves direct binding of the drugs to the channel.

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glibenclamide binding activity although using green fluorescent protein (GFP)-tagged constructs we were able to show that both half-molecules were targeted to the plasma membrane. Co-expression of both half-molecules, however, resulted in the simultaneous expression of Kir6.2. Deletion of the second NBD from the C-proximal half did not prevent assembly of the two halves to form a glibenclamide-binding site. These data indicate that there are strong interactions between two different regions of SUR1 that make possible self-association. Using GFP-tagged NBD1 we were able to provide evidence that such interactions occur between NBD1 and NBD2. We also demonstrated that NBD1 forms a tetramer, suggesting that homomeric interactions of this domain may be important in channel assembly.

**EXPERIMENTAL PROCEDURES**

**Cells and Viruses**—Sf9 (Spodoptera frugiperda) cells were propagated at 28 °C in TC100 medium containing 10% fetal calf serum. Cells were infected by each recombinant baculovirus at a multiplicity of infection of 0.1, and the supernatant was collected 5 days later. Penta-His antibody (QIAGEN) was used in Western blots (Western blot analysis). The GFP gene was also incorporated in this vector to give pACas2G so that GFP fusion proteins were obtained. A polymerase chain reaction product providing a C-terminal His6 tag was further increased by the simultaneous expression of pET24d (Novagen) to obtain expression of His-tagged NBD1 in Escherichia coli.

**Construction of Plasmid DNAs and Recombinant Baculoviruses**—We constructed transfer vectors containing DNA fragments encoding rat SUR1 (4) and mouse Kir6.2 (22) under control of the polyhedron promoter in the pAcYM1 vector. We designed a transfer vector for expression of the N-terminal half of SUR1 (pAcCas1) containing a SUR1 sequence (amino acids 1–55), an adapter containing BamHI and NotI sites, and sequences encoding a C-terminal His6 tag. The GFP gene (23) was inserted at the NotI site (by NotI–EcoRI) to create pAcCas1G to obtain a GFP fusion protein. pAcNSUR1 and pAcNSUR1G transfer vectors were obtained by cloning a polymerase chain reaction copy of the SUR1 amino acid 55–1042 sequence in pAcCas1 and pAcCas1G, respectively. For cloning of the C-terminal part of SUR1 we constructed a transfer vector (pAcCas2) containing a SUR1 leader sequence (amino acids 1–27), NotI restriction sites for cloning, and a C-terminal His6 tag. The GFP gene was also incorporated in this vector to give pACas2G so that GFP fusion proteins were obtained. A polymerase chain reaction product coding for amino acids 1042–1580 of SUR1 was cloned in both pAcCas2 and pAcCas2G. The GFP gene was inserted in that site to obtain pAcNBD1G and pAcNBD2G transfer vectors. Transfer vectors were used for co-transfection of Sf9 cells together with Autographa californica nuclear polyhedrosis virus (AcNPV PAK6) (24).

**Generation of Recombinant Baculoviruses**—Fig. 1 illustrates the predicted topology of SUR1 and the regions of SUR1 expressed by vectors used in this study. The N-proximal half-molecule, designated NSUR1, contained the first two sets of putative TM domains, NBD1 and the first TM helix of the second 6-TM set. A variant of this construct, designated

![FIG. 1. Predicted topology of SUR1 and design of the recombinant proteins containing regions of SUR1 used in this study. Membrane topologies are based on Ref. 11. Black boxes indicate the SUR1 leader sequence. Stippled bars show SUR1 sequences used in the recombinant proteins whose names are indicated on the right. Arrows indicate sites on SUR1 used for generation of SUR1 deletion mutants. White boxes (A and B) show the location of Walker A and B motifs that can form nucleotide-binding sites in each putative cytosolic nucleotide-binding domain (NBD1 and NBD2). The figure also shows the location of GFP in the GFP-tagged recombinant proteins used.](http://www.jbc.org/Downloaded_from)
NSUR1G, had a C-terminal GFP tag. The C-proximal half-molecule, designated CSUR1, contained the last five putative TM helices and NBD2 and had an artificial leader sequence to facilitate membrane insertion. The leader sequence was identical to that of SUR1 itself. A truncated variant, CSUR1tr, lacked NBD2. An N-terminal GFP tag and leader sequence were added to CSUR1 to produce CSUR1G. We also prepared the two nucleotide-binding domains, NBD1 and NBD2, with C-terminal GFP tags (NBD1G and NBD2G). All recombinant proteins were designed to contain a His6 tag. Fig. 2 shows Western blots (using Penta-His antibody) of Sf9 insect cells expressing the recombinant proteins. The positions of the main bands on the Western blot correspond to those predicted from amino acid sequences.

**Expression at the Plasma Membrane of SUR1 Half-molecules**—GFP was used as marker for investigation of recombinant protein localization. Fluorescence microscopy of Sf9 cells expressing recombinant proteins is shown in Fig. 3. GFP itself (Fig. 3A) was randomly distributed inside the insect cell and did not bind to the plasma membrane. Fig. 3B shows that NBD1G was also randomly distributed inside the insect cell. In contrast to NBD1G, NBD2G forms inclusion bodies (Fig. 3C). Fig. 3, D and E show that the N- and C-terminal halves of SUR1 tagged with GFP were both expressed at the plasma membrane of Sf9 insect cells infected with the corresponding recombinant baculovirus. These data show that individual regions of SUR1 possess plasma membrane insertion abilities.

**Interaction between NBD1 and NBD2**—NBD1 expressed in Sf9 cells as a GFP fusion protein was distributed throughout the cell (Fig. 3B). After co-expression of NBD1G and the C-terminal half-molecule CSUR1, NBD1G was found mostly near the plasma membrane (Fig. 3F). This effect disappeared after deletion of NBD2 from the C-terminal part of SUR1, i.e. when NBD1G was co-expressed with CSUR1tr (Fig. 3G). These data indicate strong interaction between NBD1 and NBD2. Co-expression of NBD1G with Kir6.2 did not localize NBD1G to the plasma membrane (Fig. 3H) suggesting a lack of strong interactions between Kir6.2 and NBD1.

**DISCUSSION**

Many ABC transporters contain four structural domains that include two sets of TM regions and two cytosolic NBDs (26). In prokaryotic ABC transporters the TM and NBD regions are often separate polypeptides. Moreover some ABC proteins contain only a single set of six TM helices and one NBD. It has been suggested, therefore, that many members of the ABC superfamily have evolved by duplication or fusion of previously autonomous half-molecules (27). P-glycoprotein consists of two homologous halves, each composed of 6 putative TM helices and an NBD. Either half-molecule, when expressed in Sf9 cells, exhibited ATPase activity that was not stimulated by drugs (28). However, drug stimulation of ATPase activity was present following co-expression of both halves of P-glycoprotein, indicating the ability of the two fragments to associate and couple ATPase activity to drug binding.

The predicted topology of SUR1 and other ABC transporters such as the multidrug resistance protein is not consistent with such a symmetrical arrangement because of the large additional N-terminal extension containing several additional TM segments. Nevertheless when the multidrug resistance protein was divided into two, each half-molecule expressed in Sf9 cells was able to integrate into the plasma membrane and associate to form a functional transporter (29). We have now applied such a complementation approach to SUR1.

We divided SUR1 at position Pro-1042 into two halves. These N- and C-terminal half-molecules were expressed separately and together in Sf9 cells using a baculovirus system that we
have previously applied to the expression of active \( \text{K}_\text{ATP} \) channels (21). Both half-molecules, when expressed in Sf9 cells as GFP-tagged proteins, were concentrated at the plasma membrane. However, glibenclamide binding activity could not be detected in cells expressing either half-molecule. When the two half-molecules were co-expressed, however, substantial glibenclamide binding activity was found. These data indicate that the two halves of SUR1 interact and that the glibenclamide-binding site requires residues from both halves of the molecule. This latter conclusion is consistent with the findings from a study of chimeric SURs, which concluded that glibenclamide interacts with two high affinity sites on SUR1 (19). We can exclude NBD2 from a significant contribution to the glibenclamide-binding site(s), because co-expression of NSUR1 with the truncated C-terminal half-molecule lacking NBD2 also resulted in expression of glibenclamide binding activity. This suggests interaction between the TM domains of the C-terminal half of SUR1 with either cytosolic or TM domains in the N-terminal half-molecule. The increased glibenclamide binding observed when Kir6.2 was co-expressed with NSUR1 and CSUR1 or CSUR1tr is indicative of interaction between SUR1 half-molecules and Kir6.2 to facilitate SUR1 assembly. There is evidence that the C-terminal region of Kir6.2 may be important in this interaction (16).

The density of glibenclamide-binding sites in cells co-expressing the two half-molecules was approximately 25% of that in cells expressing full-length SUR1. This may indicate that the binding activity of the reconstituted protein is less than that of the intact molecule. However, it is more likely to be attributable to heterogeneity of the infected cell population. We cannot determine the stoichiometry with which the half-molecules are expressed in individual cells; therefore the lower glibenclamide...
binding may reflect the proportion of half-molecules able to heterodimerize.

NBD1 is the major cytosolic domain in NSUR1. It therefore seemed possible that NBD1 interacted with CSUR1 to facilitate formation of functional SUR1. The GFP fusion protein NBD1G was used to test this possibility. NBD1G was distributed throughout the cell when expressed alone. However, when co-expressed with CSUR1 there was significant localization of NBD1G to the plasma membrane. According to this hypothesis, transmembrane membrane proteins attain their folded structure within the frame of a two-stage model for the process whereby polytopic integral assembly of SUR1. In cells co-expressing Kir6.2 and NBD1G there was no detectable membrane localization of NBD1G, suggesting that NBD1 does not strongly interact with Kir6.2.

When purified NBD1 was studied in vitro this protein domain was found to exist as a tetramer. Because four SUR1 molecules are believed to be present in the active K_ATP channel this observation suggests that homomeric NBD1 interactions may play a key role in channel assembly.

The complementation approach described here has been applied to several membrane proteins. For example, when C- and N-terminal halves of the GLUT1 glucose transporter were co-expressed in SF9 cells, a ligand-binding site was formed (30). Fragments of the human red cell anion exchanger (band 3; α1) that individually were not competent for transport, were able to assemble to form a functional anion transporter when co-expressed in Xenopus oocytes (31, 32). Functionally active β3-adrenergic (33) and muscarinic cholinergic (34) receptors have also been reconstituted from co-expressed N- and C-terminal domains. These studies have been interpreted in terms of a two-stage model for the process whereby polytopic integral membrane proteins attain their folded structure within the membrane (35). According to this hypothesis, transmembrane α-helices fold independently on insertion into the membrane where they are stabilized by main chain hydrogen bonds and hydrophobic interactions with the lipid bilayer. Subsequently these independent α-helical domains associate to form the final structure without significant secondary structure rearrangement. Our results with SUR1 are consistent with such a model.

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