

Membrane Topology of the Amino-terminal Region of the Sulfonylurea Receptor*

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The sulfonylurea receptor (SUR) is a member of the ATP-binding cassette family that is associated with Kir 6.x to form ATP-sensitive potassium channels. SUR is involved in nucleotide regulation of the channel and is the site of pharmacological interaction with sulfonylurea drugs and potassium channel openers. SUR contains three hydrophobic domains, TM₀, TM₁, and TM₂, with nucleotide binding folds following TM₁ and TM₂. Two topological models of SUR have been proposed containing either 13 transmembrane segments (in a 4+5+4 arrangement) or 17 transmembrane segments (in a 5+6+6 arrangement) (Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., Clement, J. P. t., Boyd, A. E., III, González, G., Herrera-Sosa, H., Nguy, K., Bryan, J., and Nelson, D. A. (1995) *Science* 268, 423–426; Tuszáný, G. E., Bakos, E., Váradi, A., and Sarkadi, B. (1997) *FEBS Lett.* 402, 1–3; Aguilar-Bryan, L., Clement, J. P., IV, González, G., Kunjilwar, K., Babenko, A., and Bryan, J. (1998) *Physiol. Rev.* 78, 227–245). We analyzed the topology of the amino-terminal TM₀ region of SUR1 using glycosylation and protease protection studies. Deglycosylation using peptide-*N*-glycosidase F and site-directed mutagenesis established that Asn¹⁰, near the amino terminus, and Asn¹⁰⁵⁰ are the only sites of *N*-linked glycosylation, thus placing these sites on the extracellular side of the membrane. To study in detail the topology of SUR1, we constructed and expressed *in vitro* fusion proteins containing 1–5 hydrophobic segments of the TM₀ region fused to the reporter prolactin. The fusion proteins were subjected to a protease protection assay that reported the accessibility of the prolactin epitope. Our results indicate that the TM₀ region is comprised of 5 transmembrane segments. These data support the 5+6+6 model of SUR1 topology.

ATP-sensitive potassium channels (K_{ATP} channels)¹ coordi-

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¹ The abbreviations used are: K_{ATP}, ATP-sensitive potassium channel; ABC, ATP-binding cassette; HR, hydrophobic region; Kir, inwardly rectifying potassium channel; MRP, multidrug resistance-associated protein; NBF, nucleotide-binding fold; PL, prolactin; SUR, sulfonylurea receptor; N_{exo}, amino terminus extracellular; N_{cyt}, amino terminus cytoplasmic; PAGE, polyacrylamide gel electrophoresis; TCEP, tri(2-carboxyethyl)phosphine hydrochloride; TM, transmembrane; PNGase F, peptide-*N*-glycosidase.

nate the metabolic state of the cell with the electrical activity of the membrane. K_{ATP} channels are weak inward rectifiers that are modulated by intracellular ATP/ADP ratios. The open state of the channel stabilizes the resting potential of the cell membrane near the potassium equilibrium potential. Inhibition of K_{ATP} channels by high levels of intracellular ATP or pharmacological reagents such as sulfonylureas leads to membrane depolarization, which in turn induces insulin secretion from pancreatic β -cells and contraction in vascular smooth muscle. These channels have been implicated in the etiology or treatment of several pathophysiological disorders including persistent hyperinsulinemic hypoglycemia of infancy, ischemia, and hypertension (for review, see Refs. 1–3).

K_{ATP} is a heteromultimeric channel consisting of pore-forming subunits Kir 6.x, which are members of the inwardly rectifying potassium channel family, and regulatory subunits, the sulfonylurea receptors (SUR), which are members of the ATP-binding cassette (ABC) family. The Kir 6.x subunit dictates permeation and rectification properties of the channel (2) and may contain the site for ATP inhibition (4, 5). SUR subunits are involved in channel regulation by Mg²⁺-nucleotides, inhibition of channel activity by sulfonylurea drugs used in the treatment of diabetes, activation of the channel by potassium channel openers used in the treatment of hypertension and persistent hyperinsulinemic hypoglycemia of infancy, and trafficking of the channel to the plasma membrane (6–12).

In light of the genetic and pharmacological importance of the SUR subunit, understanding the topological organization of the protein will assist in crucial structure-function and drug binding studies. Based on sequence analysis, SUR is a multispanning transmembrane protein. It has three hydrophobic domains, TM₀, TM₁, and TM₂, each of which is followed by a hydrophilic region (Fig. 1A) (13). Within the TM₀, TM₁, and TM₂ domains are multiple hydrophobic segments, each of which could potentially span the membrane. Initial biochemical purification and amino-terminal microsequencing of SUR1 indicated a glycosylation site near the amino terminus (14, 15). Moreover, recent studies of the cloned receptor suggest that the glycosylated form of the SUR1 protein is physically associated with Kir 6.2 (16), suggesting an extracellular localization for the amino terminus of the SUR1 subunit in the K_{ATP} channel. The two nucleotide binding folds (NBFs) are found within the two hydrophilic domains following TM₁ and TM₂ (Fig. 1A). Mutational analysis and binding studies have determined that both NBFs are sensitive to changes in intracellular concentrations of Mg²⁺-ADP and Mg²⁺-ATP (8, 17–19). These data therefore suggest that the two NBFs are on the cytoplasmic face of the membrane; however, the topology of the rest of the protein has yet to be determined directly.

To date, two topological models have been proposed in the literature based on hydrophobicity analysis and sequence

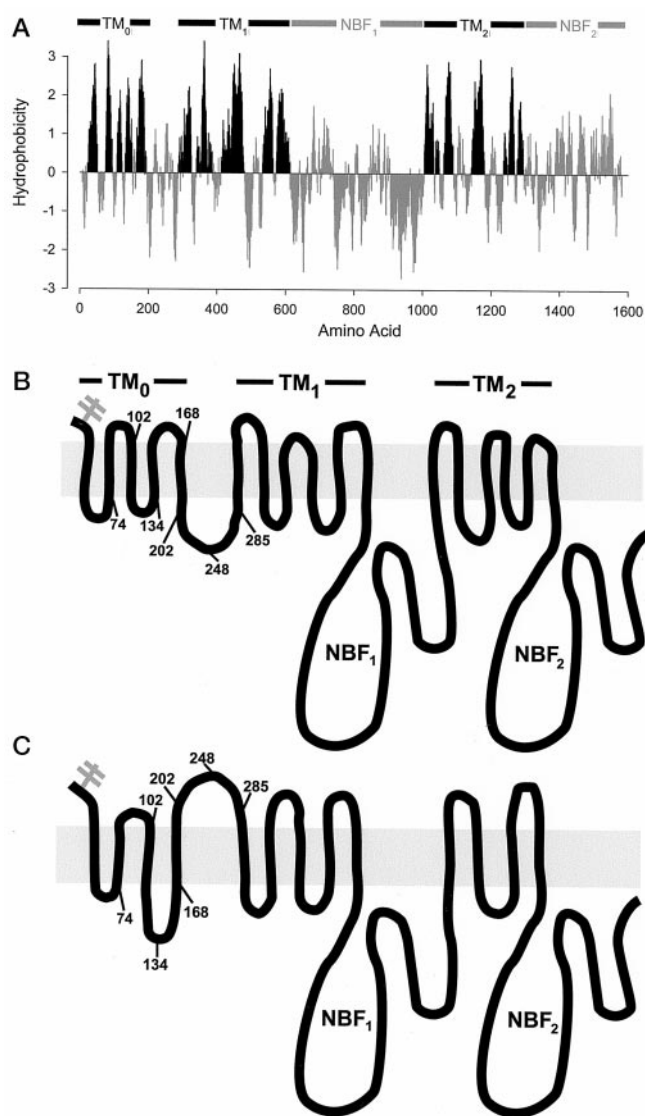


FIG. 1. **Hydrophobicity plot of SUR1 and proposed models.** Panel A, hydrophobicity plot of hamster SUR1 was constructed with a window size of 11 amino acids (24). Hydrophobic domains (TM₀, TM₁, and TM₂) and nucleotide binding folds (NBF₁ and NBF₂) are indicated above. Panel B, Tusnády *et al.* (13) model of SUR1 suggests a total of 17 transmembrane segments with a 5+6+6 arrangement. Panel C, Aguilar-Bryan *et al.* (14) model of SUR1 suggests a total of 13 transmembrane segments with a 4+5+4 arrangement.

alignment with other ABC proteins (Fig. 1, B and C). Tusnády *et al.* proposed a total of 17 transmembrane segments grouped within the three hydrophobic modules TM₀, TM₁, and TM₂ with a 5+6+6 arrangement, respectively (Fig. 1B) (2, 13). An alternative model suggested 13 transmembrane segments (4+5+4) (Fig. 1C) (14). Tusnády *et al.* (13) proposed that all ABC proteins possess the “core” TM₁ and TM₂ modules typified by the cystic fibrosis transmembrane regulator and P-glycoprotein. A subset of the ABC proteins, however, has an additional hydrophobic amino-terminal TM₀ domain. This subset includes SUR, multidrug resistance-associated protein (MRP), MRP-related liver canalicular multispecific organic anion transporter, rabbit epithelial basolateral chloride conductance regulator, and yeast cadmium factor 1. It has been suggested that the topological organization of the ABC proteins may reveal common functional domains within the superfamily. Recently, it has been speculated that the unique TM₀ region might influence drug binding affinities and/or transport properties because all of these proteins bind or transport drugs (13, 20).

We report here the membrane topology of the TM₀ domain of SUR1. We chose to investigate the TM₀ domain for several reasons. First, the membrane topology of the amino-terminal region often influences the folding of the rest of the protein (21–23). Second, this domain is unique compared with that of most ABC proteins; and finally, the models proposed in the literature describing the topology of SUR differ in the number of hydrophobic regions specified in this domain (Fig. 1, B and C) (2, 13, 14). Our findings suggest that the TM₀ region of SUR1 associated with Kir 6.2 consists of five transmembrane segments with the amino terminus localized to the extracellular side of the membrane and the hydrophilic region between TM₀ and TM₁ on the cytoplasmic side of the membrane.

EXPERIMENTAL PROCEDURES

Computer Analysis—The locations of membrane-spanning helices in hamster SUR1 were predicted using hydrophobicity analysis (24), hidden Markov methods HMMTOP (25) and TMHMM (26), the SOSUI algorithm (27), the neural network PredictProtein (28), and the TopPred2 method (29).

Plasmid Constructions—Hamster SUR1 cDNA (14) was subcloned into pcDNA3.1/HisC (Invitrogen) at the *EcoRI* site to create full-length SUR1 with an amino-terminal His tag. Full-length SUR1 with a carboxyl-terminal V5-fusion tag was created by replacement of the stop codon with an *XhoI* site and subcloning into the *EcoRI-XhoI* sites of pcDNA3.1/V5-HisA (Invitrogen).

Construction of SUR-Prolactin Fusion Proteins—cDNAs coding for the amino-terminal region of SUR1 were fused to the soluble portion of bovine prolactin, resulting in SUR74PL, SUR102PL, SUR134PL, SUR168PL, SUR202PL, SUR248PL, and SUR285PL. Each fusion protein contained the amino terminus of SUR1 through the amino acid number indicated followed by a 1-amino acid Gly linker, then the carboxyl-terminal 142 amino acids of bovine prolactin, omitting the prolactin signal sequence. First, a cDNA (SUR296-pcDNA) coding for the first five hydrophobic segments of SUR1 was made by truncating SUR1 after amino acid 296 via digestion of SUR1-pcDNA3.1/HisC with *BsmBI*, treating with Klenow then with *EcoRV*, and ligating. *SacII* sites were introduced individually directly following the nucleotides encoding Arg⁷⁴, Arg¹⁰², Arg¹³⁴, Lys¹⁶⁸, Arg²⁰², Arg²⁴⁸, and Arg²⁸⁵ in SUR296-pcDNA, and just before Cys⁵⁸ of bovine prolactin by site-directed mutagenesis (CLONTECH). The amino acid sequence at the fusion site was preserved with the exception of a Lys¹⁶⁸ to Arg¹⁶⁸ change in the SUR168PL construct. The cDNA encoding the carboxyl-terminal 142 amino acids of bovine prolactin was fused to SUR1 at the inserted *SacII* site and the vector *XbaI* site. All constructs were sequenced over the region of fusion (Sequenase 2.0, Amersham Pharmacia Biotech). Constructs lacking the amino-terminal His tag were then subcloned into the high expression vector pGEMHE at the *EcoRI* and *XbaI* sites. Thus, two sets of SUR-prolactin fusion proteins were generated: SURxxxPL and His-SURxxxPL, with the latter group containing an amino-terminal His tag.

Deletion of Glycosylation Sites—Candidate *N*-linked glycosylation sites at Asn¹⁰ and/or Asn¹⁰⁵⁰ were mutated to create proteins lacking one (SUR-N10Q and SUR-N1050Q) or both (SUR-N10Q,N1050Q) putative glycosylation sites.

Cloning of Kir 6.2—Rat Kir 6.2 was cloned by polymerase chain reaction from a rat heart cDNA library² using primers for the nucleotide sequences 52–70 and 1463–1479 of mouse Kir 6.2 cDNA (6). The polymerase chain reaction product ends were blunted, ligated into the *SmaI* site of Bluescript KS– (Stratagene), sequenced on both strands, then subcloned into the *BamHI-EcoRI* sites of pcDNA1/Amp (Invitrogen).

Expression of SUR1 and Kir 6.2 in COS-1 Cells—COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. SUR1, SUR-N10Q, SUR-N1050Q, and SUR-N10Q,N1050Q (20–30 μ g/100-mm plate) were cotransfected with Kir 6.2 (1–4 μ g/100-mm plate) using pF_x-2 (Invitrogen). 48 h after transfection, COS-1 cells were rinsed twice with phosphate-buffered saline and lifted with 5 mM EDTA, 5 mM EGTA in phosphate-buffered saline. Cells were pelleted, homogenized in buffer A (0.25 M sucrose, 20 mM Hepes, pH 7.4, 5 mM TCEP, 1 mM EDTA, 15 μ g/ml DNase, 10 μ g/ml RNase, 1 \times CompleteTM protease inhibitor mixture (Roche Molecular Biochemicals)), and the nuclear fraction was pelleted at 80 \times *g* for 10 min. The

² F. Périer and C. A. Vandenberg, unpublished data.

supernatant was saved, and the pellet was rehomogenized in buffer A and centrifuged as above. Supernatants were combined, and membranes were centrifuged at $100,000 \times g$ for 30 min at 4 °C and then resuspended in buffer A and stored at -80 °C.

Enzymatic Deglycosylation—Membrane protein (5–20 µg) from COS-1 cells transfected with Kir 6.2 and SUR1 or *in vitro* translated SUR-prolactin fusion proteins (10 µl, see methods below) were treated with the glycosidase PNGase F. In brief, protein was denatured by boiling for 10 min in 0.5% SDS, 1% β-mercaptoethanol. Sodium phosphate, pH 7.5, and Nonidet P-40 were added to a final concentration of 50 mM and 1%, respectively. Samples were incubated with 5 units (for membrane protein) or 2.5 units (for fusion protein) of PNGase F (New England Biolabs) at 37 °C for 1 h. Products were analyzed by SDS-PAGE followed by autoradiography or Western blot.

Immunoblot Analysis of SUR1—Samples containing 5–20 µg of total membrane protein isolated from COS-1 cells cotransfected with Kir 6.2 and SUR1 were solubilized in TCEP-SDS sample buffer (15 mM Tris, pH 9.0, 2.5% glycerol, 2% SDS, 0.1 mM EDTA, 2 mM TCEP) and boiled for 20 min. After separation by SDS-PAGE (2–12% gradient), proteins were transferred to nitrocellulose (Hybond, Amersham Pharmacia Biotech), analyzed by immunoblotting with V5 antibody conjugated to horseradish peroxidase (1:1,000; Invitrogen), and visualized by chemiluminescence (Super Signal™ West Dura; Pierce).

Protease Protection Assay of Fusion Proteins—SUR-prolactin fusion proteins were translated *in vitro* using a coupled transcription/translation system in the presence of [³⁵S]Met and canine pancreatic microsomal membranes (4 equivalents/25 µl, Promega). 10-µl samples of *in vitro* translated fusion protein were treated with 10 µg/ml proteinase K (Stratagene) in the presence and absence of 1.2% Triton X-100 for 1 h on ice. The reaction was quenched by the addition of 25 mM phenylmethylsulfonyl fluoride. Microsomes were pelleted at $16,000 \times g$ for 20 min at 4 °C. The supernatant was removed, and the membranes were resuspended in 1% SDS, 50 mM Tris, pH 8.0. Samples were incubated at 100 °C for 10 min, diluted to a final concentration of 0.1% SDS with buffer B (1% Nonidet P-40, 20 mM Hepes, pH 7.0, 150 mM NaCl, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride), and immunoprecipitated with anti-prolactin antibody. Positive control fusion protein samples were subjected to the same protocol omitting the proteinase K and Triton X-100 treatment. To assess the integrity of the microsomes, a parallel sample containing *in vitro* translated bovine prolactin was similarly subjected to proteinase K treatment as an additional control that was included in each experiment.

Immunoprecipitation of Products—The prolactin reporter, ± proteinase K treatment, was immunoprecipitated, separated by SDS-PAGE, and detected by autoradiography. In brief, polyclonal rabbit anti-sheep prolactin antibody (ICN) was preabsorbed to protein A or G agarose beads (Pierce) at room temperature for 2 h. The antibody-protein A/G complex was washed once in buffer B, mixed with solubilized protein for a final antibody dilution of 1:500, and incubated at room temperature for 1–2 h with constant agitation. The protein-antibody-protein A/G complex was washed twice with buffer B followed by a final wash with H₂O. Protein was eluted by boiling in SDS sample buffer for 5 min and then analyzed by SDS-PAGE on a reducing 13.5 or 15% gel followed by autoradiography.

RESULTS

Predicted Topologies of SUR1—Analysis of the amino acid sequence of SUR1 using algorithms that identify putative transmembrane helices predicted several topologies for SUR1. The various algorithms that were tested (25–29), together with the models in the literature (13, 14), produced a total of six models containing 13, 15, 16, 17, or 18 transmembrane helices, with none of the models identical. Of the three algorithms designed to predict helix orientation, all suggested that the amino terminus resided extracellularly. These predictions are consistent with the significant positive charge difference Δ(C-N) of residues surrounding the first hydrophobic segment of SUR1 which typify an extracellular amino terminus (23).

Many transmembrane helices were predicted consistently, whereas others were identified only by some of the algorithms. In the TM₀ region, between four and six transmembrane segments were predicted. The Tusnády *et al.* model suggested five transmembrane segments in this area (13). The third of these (HR3) was skipped in some models, and in one case an additional transmembrane segment was added following HR5. In

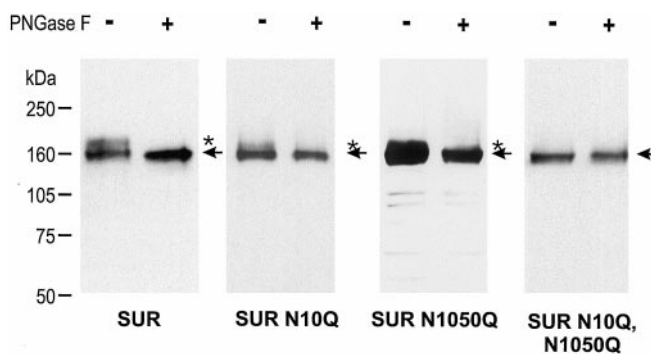


FIG. 2. Glycosylation of full-length SUR1 indicates that Asn¹⁰ and Asn¹⁰⁵⁰ are extracellular. Full-length SUR1, SUR-N10Q, SUR-N1050Q, and SUR-N10Q,N1050Q were coexpressed with Kir 6.2 in COS-1 cells and identified by immunoblotting with an antibody to a carboxyl-terminal V5 epitope tag. Asn¹⁰ and/or Asn¹⁰⁵⁰ of SUR1 was mutated to Gln to remove the N-glycosylation acceptor site. The samples were treated with and without PNGase F to remove the N-linked carbohydrate. The arrow indicates the mobility of the immature and unglycosylated forms of the protein; the asterisk indicates the mature glycosylated protein.

contrast to the Tusnády *et al.* model, which predicted an intracellular orientation for the large hydrophilic loop between TM₀ and TM₁, the models with four and six transmembrane segments in the TM₀ region predicted an extracellular location for this loop.

The TM₁ region was predicted to have five to eight transmembrane segments (six in the Tusnády *et al.* model (13)), with HR8 most frequently skipped. The TM₂ region was predicted to have four to six transmembrane segments (six in the Tusnády *et al.* model (13)), with HR14 and HR17 most frequently skipped. Only three of the six models predicted an even number of transmembrane segments in the TM₂ region, which resulted in placement of the nucleotide binding folds on the same side of the membrane for only half of the models. Overall, the topology predictions showed that although several features of the SUR1 topology can be predicted, there was no agreement on the transmembrane assignments or orientations by the modeling algorithms.

Analysis of Endogenous Glycosylation Sites—To begin our study of the topology of SUR1 we investigated the endogenous sites of N-linked glycosylation. Purification and microsequencing of the sulfonylurea receptor had suggested previously that the amino terminus of SUR1 is glycosylated, indicating an extracellular location (14, 15). To explore this possibility further we examined the glycosylation state of the consensus glycosylation acceptor sites (Asn-Xaa-Ser/Thr) at Asn¹⁰ and Asn¹⁰⁵⁰ in SUR1. In both topological models presented in the literature these two sites are predicted to reside on the extracellular side of the membrane (Fig. 1, B and C). Immunoblot analysis of SUR1 using an epitope-specific V5 antibody detected the presence of two major bands of approximately equal intensities at apparent molecular masses of 187 and 164 kDa when SUR1 was coexpressed with Kir 6.2 (Fig. 2, first lane), consistent with previous reports of mature “complex” glycosylation and immature “core” glycosylation forms of SUR (15). Upon treatment of SUR1 with the endoglycosidase PNGase F, which is specific for N-linked glycosylation, SUR1 migrated as a single band (Fig. 2, second lane) at a relative mobility similar to the lower band in the untreated sample. The mobility of immature SUR1 was not easily differentiated from that of unglycosylated SUR1 in Fig. 2 (first two lanes) because of the minor difference of ~3 kDa in molecular mass between the two forms (15). When SUR1 was expressed in the absence of Kir 6.2, the resulting band comigrated with the immature form of the protein (data not shown), consistent with reports that the

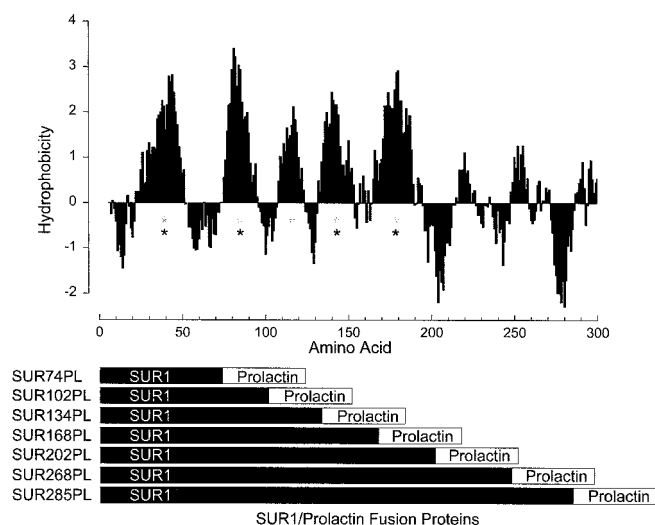


FIG. 3. Hydrophobicity plot of TM₀ region of SUR1 and schematic diagram of chimeric constructs. *Panel A*, a hydrophobicity plot of the TM₀ region of SUR1 was constructed with a window size of 11 amino acids (24). Asterisks indicate the location of transmembrane segments proposed in two models (gray, Aguilar-Bryan *et al.* (14); black, Tuszányi *et al.* (13)). Amino acids included in the five hydrophobic regions are as follows: HR1, 30–51; HR2, 75–94; HR3, 106–124; HR4, 135–155; and HR5, 169–190. *Panel B*, SUR-prolactin fusion proteins. The prolactin epitope was fused to SUR1 following each of the predicted hydrophobic regions. Three fusion proteins were engineered following the hydrophilic tail after HR5.

highly glycosylated form of SUR1 is found when Kir 6.2 is coexpressed (16).

To determine which residues were glycosylated, the predicted glycosylation acceptor sites Asn¹⁰ and Asn¹⁰⁵⁰ were mutated either individually (SUR-N10Q, and SUR-N1050Q) or together (SUR-N10Q,1050Q). Expression of SUR-N10Q (Fig. 2, *third and fourth lanes*) and SUR-N1050Q (Fig. 2, *fifth and sixth lanes*) demonstrated that elimination of either of the Asn¹⁰ or Asn¹⁰⁵⁰ glycosylation consensus sites resulted in a significant decrease of mature glycosylated receptor compared with wild type SUR1. Furthermore, simultaneous removal of both the Asn¹⁰ and Asn¹⁰⁵⁰ sites in SUR-N10Q,N1050Q resulted only in a band that comigrated with the unglycosylated form of the protein (Fig. 2, *seventh and eighth lanes*), indicating that both Asn¹⁰ and Asn¹⁰⁵⁰ were glycosylated in wild type SUR1. A reduction in glycosylation on SUR-N10Q relative to wild type SUR1 is in agreement with previous studies suggesting that Asn¹⁰ on the SUR1 amino terminus is glycosylated (14, 15). These results place both the amino terminus and the hydrophilic region containing amino acid 1050 on the extracellular side of the membrane. Asn¹⁰⁵⁰ is located in the TM₂ domain and is predicted to reside extracellularly between the 12th and 13th putative transmembrane segments in the 5+6+6 model of Tuszányi *et al.* (13).

Hydrophobicity Analysis and Construction of the Fusion Proteins—We chose a classical protease protection approach to investigate which HRs span the membrane. Seven chimeric cDNAs were constructed which code for the amino terminus of SUR1 fused to the prolactin reporter. The prolactin reporter, consisting of the carboxyl-terminal soluble portion of prolactin, has been shown to have no effect on upstream signal and stop transfer sequences (30, 31). The constructs were based on the calculated hydrophobicity plot encompassing the TM₀ domain of hamster SUR1 (Fig. 3A) (24). This plot identified five HRs ranging from 18 to 22 amino acids which could potentially span the membrane. Prolactin was fused after each putative membrane-spanning region at the carboxyl-terminal end of the downstream hydrophilic loop (Fig. 3B). The location of each

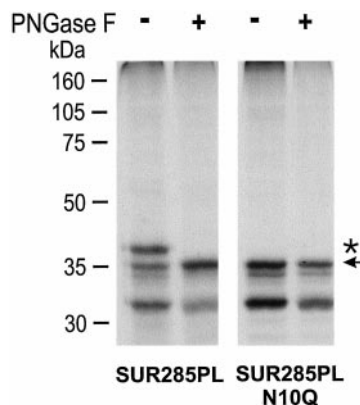


FIG. 4. Glycosylation of SUR285PL and SUR285PL-N10Q fusion proteins. Autoradiograms are shown of SUR285PL and SUR285PL-N10Q fusion proteins that were translated *in vitro* in the presence of [³⁵S]Met and canine pancreatic microsomes. Asn¹⁰ in SUR285PL-N10Q was mutated to remove the *N*-glycosylation acceptor site. The samples were treated with and without PNGase F to remove the *N*-linked carbohydrate and resolved on an 8.75% SDS-polyacrylamide gel. The arrow indicates the unglycosylated form of the protein; the asterisk indicates the glycosylated protein.

fusion site was chosen to preserve the charged residues surrounding the transmembrane segments because it has been suggested that charged regions flanking transmembrane segments may influence the orientation of the membrane-spanning region and thus, the native topology of the protein (32). Three of the constructs (SUR202PL, SUR248PL, and SUR285PL) contain increasing lengths of the hydrophilic linker following HR5 to ensure that this entire region resides on the same side of the membrane.

Glycosylation State of SUR-Prolactin Fusion Proteins—To ensure that the topological orientation of the amino terminus of the SUR-prolactin fusion proteins was consistent with that of full-length SUR1 expressed in cells, we analyzed the glycosylation state of Asn¹⁰ in the SUR285PL fusion protein. *In vitro* translation of SUR285PL in the presence of microsomes resulted in two major bands corresponding approximately to the molecular mass expected of the full-length fusion protein (Fig. 4, *first lane*): an upper band of apparent molecular mass of ~40 kDa and a lower band of ~37 kDa. An additional band below the full-length fusion protein is probably a truncated product of the protein, as commonly seen with *in vitro* translation (33).

Upon treatment with the glycosidase PNGase F, the 40-kDa band was reduced to the same relative mobility as the 37-kDa band (Fig. 4, *second lane*), demonstrating that the difference in molecular mass is caused by *N*-linked glycosylation. *In vitro* translation in the presence of microsomes results in proteins that are core glycosylated, but not mature glycosylated, indicating that the 40- and 37-kDa bands represent the immature core glycosylated and unglycosylated forms of the protein. These results are in agreement with previous reports that the immature form of SUR is approximately 3 kDa greater in mass than the unglycosylated receptor (15). The primary sequence of SUR285PL predicts only one consensus sequence for *N*-linked glycosylation at Asn¹⁰. Mutation of that site in SUR285PL-N10Q produced a single band at the same apparent molecular mass as the unglycosylated SUR285PL protein, and its mobility was not shifted by PNGase F treatment (Fig. 4, *third and fourth lanes*), showing that Asn¹⁰ is glycosylated in the SUR285PL fusion protein. The relative amounts of the glycosylated and the unglycosylated fusion protein varied, but typically they were approximately equal. Because the glycosylated protein must be oriented with the amino-terminal extracellularly, this suggests that at minimum, half of the protein is translated in this orientation. It is likely that much of the

unglycosylated protein also assumes the same orientation because glycosylation is known to be incomplete in this assay (33).

The TM_0 Domain of SUR1 Can Assume Two Topologies in Microsomal Membranes with Opposite Transmembrane Orientations of the Protein—To examine the transmembrane topology of the TM_0 domain, fusion proteins were translated in the presence of microsomes and [35 S]Met, treated with the proteolytic enzyme proteinase K, and immunoprecipitated with a prolactin-specific antibody. If the prolactin reporter were fused to an amino acid that places it within the microsome, a position destined to become extracellular, then prolactin would be protected from proteolytic digestion, and a radioactive band should be detected by autoradiography; however, if the prolactin reporter is on the outside of the microsome, in a cytoplasmic orientation, it should be digested by the protease, and consequently no radioactive band should be detected. Thus, constructs with an increasing number of hydrophobic regions might be expected to alternate between protection and degradation of the prolactin reporter each time the prolactin fusion site crosses the membrane (30, 31, 34).

In contrast to our expectation of alternating prolactin protection and accessibility, we observed two proteolysis patterns of SUR-prolactin constructs. Both patterns retained prolactin protection from protease digestion, but the sizes and proteolytic pattern of each group were suggestive of two populations of *in vitro* translated fusion proteins: one with the amino terminus destined to be extracellular (N_{exo} ; intramicrosomal) and one with the amino terminus intracellular (N_{cyt} ; cytoplasmic), each with five transmembrane segments. To address each separately, we will describe first the constructs with an even number of SUR hydrophobic regions (SUR102PL and SUR168PL), then those with an odd number of hydrophobic regions (SUR74PL, SUR134PL, SUR202PL, SUR248PL, and SUR285PL).

Protection of Prolactin Reporter in Fusion Proteins Consisting of an Even Number of Hydrophobic Regions—Fig. 5 illustrates the proteolytic pattern of the fusion proteins comprised of two and four hydrophobic regions. Schematic diagrams represent the two proposed topologies of the corresponding fusion proteins in the assay. The *top* diagram shows the protected prolactin reporter (intramicrosomal), consistent with the fusion protein in the N_{exo} orientation. The *bottom* diagram illustrates the digested prolactin reporter (extramicrosomal) which suggests the alternate, N_{cyt} orientation of the fusion protein.

To determine if an immunoprecipitated peptide was a protected fragment of the SUR-prolactin fusion protein, we heeded the following criteria: 1) the peptide must be specifically immunoprecipitated with the anti-prolactin antibody in a reproducible manner; 2) the peptide must be protected from proteolysis in the absence of detergent and must be degraded when proteolysis is performed after solubilization of the microsomes; and 3) the peptide must not be present in the prolactin control samples or the samples that were not treated with protease.

The fusion protein SUR102PL, which contains two hydrophobic regions, shows a doublet corresponding to the glycosylated and unglycosylated forms of the protein (Fig. 5A, *first lane*, *gray asterisk* and *arrow*). In separate experiments the *upper band* was shown to be glycosylated by its sensitivity to PNGase F treatment (data not shown), as was seen with all of the SUR-prolactin fusion proteins tested (e.g. Fig. 4). The unglycosylated protein had an apparent molecular mass of 27 kDa with a predicted molecular mass of 28 kDa.

The SUR102PL fusion protein treated with proteinase K produced a protease-protected peptide of an apparent molecular mass of 15 kDa ($n = 7$; Fig. 5A, *second lane*, *black arrow*).

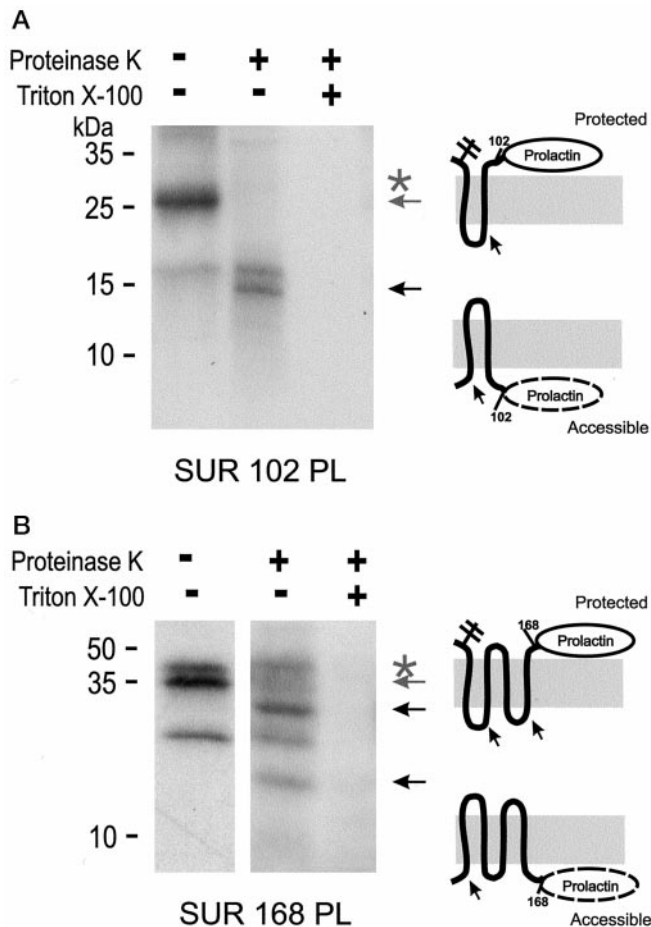


FIG. 5. Immunoproteolysis of the protected prolactin reporter in fusion proteins with an even number of hydrophobic regions. SUR102PL (panel A) and SUR168PL (panel B) chimeric cDNAs coding for two and four hydrophobic regions were translated *in vitro* in the presence of [35 S]Met and canine pancreatic microsomes. Fusion protein was left untreated (-) or treated (+) with proteinase K in the absence (-) or the presence (+) of Triton X-100, followed by immunoprecipitation with anti-prolactin antibody, SDS-PAGE, and autoradiography. The *gray asterisk* and *arrow* to the right of each autoradiogram indicate the glycosylated and the unglycosylated forms of the protein, respectively. The *black arrow* indicates the protease-protected peptide. The two topological orientations of the fusion protein are diagrammed to the right of the autoradiogram with intramicrosomal above and extramicrosomal below the diagrammed membrane. *Arrows* in the diagrams indicate the proposed protease-sensitive sites. Glycosylation is denoted by the tree-like structure on the amino terminus of the protein. The *broken line* outlining the prolactin reporter indicates digestion of the epitope by the protease. **Panel A**, proteinase K treatment of SUR102PL fusion protein resulted in a protected peptide with an apparent molecular mass of ~15 kDa (*black arrow*), suggesting a protease-sensitive site between HR1 and HR2 (*black arrows* in the *top* diagram, *panel A*). One-quarter of the SUR102PL fusion protein (-) protease (*first lane*) was loaded on the gel relative to the fusion protein (+) protease (*second and third lanes*) to visualize the glycosylated and unglycosylated form of the protein. **Panel B**, proteinase K treatment of SUR168PL fusion protein resulted in the protection of two peptides with apparent molecular masses of 26 and 17 kDa (*black arrows*). These peptides are consistent with the presence of two protease-sensitive sites between HR1-2 and HR3-4 (*black arrows* in the *top* diagram, *panel B*). The SUR168PL fusion protein (+) protease autoradiogram was exposed eight times longer than the sample (-) protease.

Upon treatment of the fusion protein with the protease in the presence of detergent, the prolactin epitope was completely degraded, eliminating the possibility that the protected peptide represented a protease-insensitive fragment (Fig. 5A, *third lane*). A protected peptide corresponding to a predicted molecular mass of ~20 kDa is consistent with a protease-sensitive site between HR1 and HR2 as shown in the N_{exo} orientation

(top diagram). The resulting protease-protected peptide of ~15 kDa is within a reasonable range to correspond to HR2 fused to the protected prolactin epitope. An alternative explanation, that the peptide is a proteolytic fragment of the minor band observed below the unglycosylated fusion protein (Fig. 5A, *first lane*), was unlikely because in other experiments the protected peptide was still present when the minor band was absent.

When SUR168PL fusion protein, containing four hydrophobic regions, was treated with proteinase K, two bands of apparent molecular masses of 26 and 16 kDa were immunoprecipitated with anti-prolactin, in addition to a band with the same mobility as the glycosylated full-length construct ($n = 5$; Fig. 5B). The peptide of 26 kDa is in close agreement with the predicted molecular mass of 27 kDa if the fusion protein were clipped at amino acid 74 between HR1 and HR2 (Fig. 5B, *top diagram*). These results are consistent with the protease-sensitive site observed for the SUR102PL construct (Fig. 5A). Similarly, the protected peptide with an apparent molecular mass of 16 kDa is consistent with a protease-sensitive site between HR3 and HR4 (predicted molecular mass of 20 kDa; Fig. 5B, *top diagram*). These data show that each of the first four HRs of SUR1 forms a transmembrane segment, and they further indicate that the protected prolactin reporter in fusion proteins comprised of an even number of hydrophobic regions represents a population of proteins that are in the N_{exo} orientation.

Protection of Prolactin Reporter in Fusion Proteins Consisting of an Odd Number of Hydrophobic Regions—Fig. 6 illustrates the protease protection pattern of the fusion proteins consisting of an odd number of hydrophobic regions. The schematic diagrams indicate that the prolactin reporter in fusion proteins with an odd number of hydrophobic regions will be digested in proteins having the N_{exo} orientation (*top diagram*) but will be protected in a fraction of the population if those proteins are translated in an N_{cyt} orientation (*bottom diagram*). Thus, protease-protected fragments will be present if some of the fusion protein is translated in an N_{cyt} orientation.

Proteolysis of fusion proteins SUR74PL (HR1), SUR134PL (HR1–3), and SUR202PL (HR1–5) resulted in protected peptides (Fig. 6) but with a proteolytic pattern different from that observed previously for the even numbered hydrophobic regions (Fig. 5). For each of the SUR-prolactin constructs with an odd number of hydrophobic regions, a band migrating with a slightly faster mobility than the unglycosylated form of the protein was present after protease treatment (compare *first two lanes* in Fig. 6, A–C). The molecular mass of the protected peptide was ~2–5 kDa less than the apparent molecular mass of the unglycosylated protein (SUR74PL, $n = 4$; SUR134PL, $n = 5$; SUR202PL, $n = 6$). This difference in size is consistent with degradation of the hydrophilic amino terminus of ~3 kDa (amino acids 1–29).

To determine whether the amino terminus was removed by proteinase K treatment, the length of the amino terminus was increased by the addition of a His tag (Fig. 6, A–C, *fourth through sixth lanes*). *In vitro* translated proteins with the His tag were not glycosylated; and because of the addition of the tag, they migrated at an apparent molecular mass ~4 kDa more than the unglycosylated form of the protein without the His tag (compare *first and fourth lanes* of Fig. 6, A–C). Upon proteinase K treatment, each of the His-tagged proteins produced a band migrating at a size ~5–10 kDa smaller than the unproteolyzed fusion protein, showing that a larger amino terminus had been removed. Furthermore, the size of the fragment after proteolysis was the same whether it was derived from proteolysis of fusion proteins with or without an amino-terminal His tag. These data indicate that the amino terminus

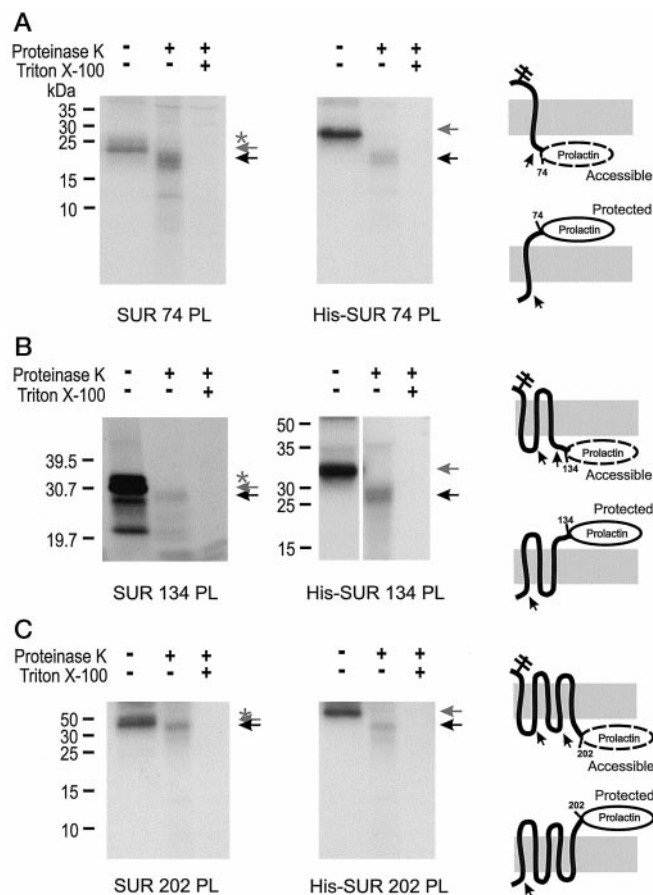


FIG. 6. Immunoprecipitation of the protected prolactin reporter in fusion proteins with an odd number of hydrophobic regions. Autoradiograms are shown for SUR74PL, SUR134PL, and SUR202PL fusion proteins containing one (*panel A*), three (*panel B*), and five (*panel C*) hydrophobic regions. Fusion proteins with or without an amino-terminal His tag were left untreated (–) or digested (+) with proteinase K in the absence (–) or the presence (+) of Triton X-100 and then immunoprecipitated with anti-prolactin; the peptides were separated on a 13.5 or 15% SDS-polyacrylamide gel. The *gray asterisk* and *arrow* to the right of the autoradiogram indicate the glycosylated and the unglycosylated form of the proteins, respectively. The *black arrow* indicates the protease-protected peptide. The diagrams to the right of the autoradiogram depict the two proposed topological models of the corresponding fusion protein, with the N_{exo} orientation (*top*) and N_{cyt} orientation (*bottom*). The *arrows* in the diagrams indicate protease-sensitive sites in the corresponding fusion protein. One-quarter of the samples (–) proteinase K were loaded on the gel relative to the samples (+) protease, except for *panel B* (left), where equal amounts of SUR134PL without the His tag were loaded.

was degraded and therefore, cytoplasmic (Fig. 6, A–C, *bottom diagrams*). Similar results were observed with fusion proteins SUR248PL and SUR285PL (containing HR1–5) with and without the amino-terminal His tag (SUR248, $n = 6$; SUR285, $n = 3$; data not shown). For the fusion proteins with an odd number of hydrophobic regions, data are consistent with each of these regions being transmembrane, in agreement with the results from constructs with an even number of hydrophobic regions.

Based on the protease protection results, the TM_0 region of SUR1 contains five transmembrane segments. Glycosylation studies and SUR-prolactin constructs with an even number of HRs suggest that the amino terminus is extracellular; however, the SUR-prolactin fusion proteins with an odd number of HRs indicate that some of the fusion protein can assume a topology with the amino terminus cytoplasmic. We considered an alternative interpretation of the data: that the protein was synthesized in one orientation but that some percentage of the microsomes ruptured and recircularized in an “inside-out”

orientation during handling. To test this alternative, we co-translated full-length prolactin with the SUR-prolactin fusion protein SUR248PL. Full-length prolactin was used as a control because it is processed to a smaller soluble form that is trapped within the microsome, thus permitting assessment of microsomal integrity. These controls showed that processed prolactin was not accessible to digestion by proteinase K, thus indicating that the microsomes remained intact (data not shown). These data argue against microsomal reorientation and suggest that the dual topology of TM_0 in the *in vitro* translated protein is a product of protein biogenesis.

DISCUSSION

The TM_0 domain of SUR1, and the analogous region in other MRP-related proteins, has been predicted to contain between four and six transmembrane segments (2, 13, 14). To date, the topology of this region of SUR has only been investigated by interpretation of the hydrophobicity profile and amino acid sequence, with the added constraint of an extracellular amino terminus (14). In this study we have used a combination of fusion protein constructs in a protease protection assay and analysis of endogenous *N*-linked glycosylation sites to determine systematically the number of transmembrane segments that encompass the TM_0 region of SUR1. Herein we report the first experimental determination that the TM_0 domain, common among MRP-related ABC proteins, consists of five transmembrane segments.

Our analysis of wild type SUR1 and glycosylation mutants coexpressed with Kir 6.2 in COS-1 cells demonstrated that the receptor is glycosylated at two sites: Asn¹⁰ on the amino terminus and Asn¹⁰⁵⁰ located on the extracellular loop following the first NBF. Both glycosylation sites are conserved among the MRP-related ABC proteins (35, 36). In addition, other ABC proteins, such as the cystic fibrosis transmembrane regulator, are glycosylated at a position equivalent to Asn¹⁰⁵⁰ (37). These results therefore favor the hypothesis that members of the ABC protein family may share common transmembrane topologies.

Transmembrane Topology Mapping—The method of carboxyl-terminal truncation/fusion mapping has been used successfully for mapping the topology of many multispanning transmembrane proteins (31, 34, 38, 39). The two models proposed in the literature postulate that the TM_0 domain of SUR1 will consist of either four or five transmembrane regions (Fig. 1). The hydrophobicity profile (Fig. 3) shows five hydrophobic peaks, ranging from 18 to 22 amino acids, which are candidate regions for spanning the bilayer as an α helix, with the HR3 segment being the least likely based on its shorter, less hydrophobic sequence. We tested these hypotheses in a protease protection assay. If the TM_0 domain consists of four transmembrane regions with the amino terminus extracellular (14, 15), then the prolactin reporter would be protected when it was fused to sites following HR2, HR3, or HR5 (SUR102PL, SUR134PL, SUR202PL, SUR248PL, and SUR285PL). On the other hand, if the TM_0 domain has five transmembrane regions with the amino terminus extracellular (13), one would expect that the prolactin reporter fused to sites following HR2 or HR4 (SUR134PL and SUR168PL) would be protected. Our results agree with the latter prediction for a five-transmembrane segment model of TM_0 , with the addition that in the *in vitro* translation system, a fraction of the chimeric proteins was oriented in the N_{cyt} direction. Protease digestion of the even numbered HRs (SUR102PL and SUR168PL) resulted in protected peptides consistent with the Tusnády *et al.* model of five transmembrane segments (13). Additionally, protease digestion of the odd numbered HRs (SUR74PL, SUR134PL, SUR202PL, SUR248PL, and SUR285PL) resulted in protected fragments indicating five transmembrane segments, yet with a

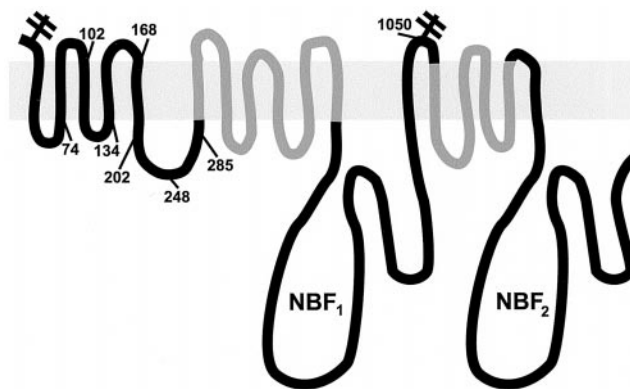


FIG. 7. Proposed topology of SUR1 associated with Kir 6.2. Protease protection and glycosylation (determined in this report), together with nucleotide binding and physiological studies have established the topological orientations of the SUR1 polypeptide regions outlined in *black*. The tree-like structures indicate the *N*-linked glycosylation sites at Asn¹⁰ and Asn¹⁰⁵⁰. Amino acid numbers designate the sites at which the prolactin reporter was fused. In the N_{exo} orientation, SUR102PL and SUR168PL were protected from the protease, whereas SUR74PL, SUR134PL, SUR202PL, SUR248PL, and SUR285PL were digested. NBF₁ and NBF₂ denote the two nucleotide binding folds. The topology of the remainder of the protein outlined in *gray* has not been determined.

proteolyzed amino terminus suggesting a N_{cyt} orientation for some of the protein. Taken together, our results suggest that all five hydrophobic regions serve as transmembrane segments.

These results are consistent with the topology proposed for the TM_0 region of MRP, which is based on glycosylation near the MRP amino terminus, and localization of epitope tags inserted on each side of HR5 (36, 40, 41). Those studies showed that in MRP, the fifth hydrophobic segment of TM_0 is a transmembrane segment and that the hydrophilic loop between TM_0 and TM_1 is on the cytoplasmic side of the membrane (40).

Topological Model of SUR1 Associated with Kir 6.2—Fig. 7 demonstrates our current view of the topology of SUR1 associated with Kir 6.2 (13). The regions outlined in *black* indicate the transmembrane orientation of regions supported by experimental evidence, whereas the regions outlined in *gray* have yet to be established. We favor this model because it is the only topology that agrees with all of the following experimental constraints: 1) the amino terminus, Asn¹⁰, is glycosylated and therefore extracellular (reported above (14, 15)), as is Asn¹⁰⁵⁰; 2) Kir 6.2 is associated with the glycosylated form of SUR1 (16), thus supporting the N_{exo} orientation over the N_{cyt} orientation; 3) the TM_0 domain has five transmembrane segments as indicated by the protease protection assay; 4) the NBFs reside on the cytoplasmic side of the membrane (8, 17–19); and 5) an epitope tag has indicated an extracellular orientation between putative transmembrane segments 16 and 17 (12). At this time, there is no evidence for more than one topology of SUR1 in cells, but we cannot rule out the possibility that SUR1 expressed in the absence of Kir 6.2 might assume an alternate topology, as alternate topologies have been reported for several other membrane proteins (39, 42–45).

Concluding Remarks—These data suggest that the TM_0 domain of SUR1 consists of five transmembrane segments. We have demonstrated that Asn¹⁰ is utilized as a glycosylation acceptor site both in cells and in our *in vitro* assay, suggesting that the amino terminus of SUR1 is on the extracellular side of the membrane. Because Kir 6.2 has been shown to associate with the glycosylated form of SUR1 to form the pancreatic K_{ATP} channel, our data favor the model that SUR1 assumes a 5+6+6 topology (13). This model is in agreement with the topology suggested for MRP from glycosylation and epitope insertion

studies (35, 40, 41, 46). These results suggest that SUR1 and members of the MRP-related subfamily of ABC proteins share a common membrane topology. Recent work has demonstrated that the TM₀ region of MRP is necessary for the transport of the organic anion substrate leukotriene C₄ (20). Future work will determine the functional significance of the TM₀ domain in SUR1.

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