Transmembrane Topology of the Sulfonylurea Receptor SUR1*

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Lisa R. Conti‡, Carolyn M. Radeke‡, Show-Ling Shyang‡, and Carol A. Vandenberge‡,¶

From the ‡Department of Molecular, Cellular, and Developmental Biology and the Neuroscience Research Institute, University of California, Santa Barbara, California 93106 and the ¶Center for Research on Occupational and Environmental Toxicology, Oregon Health & Science University, Portland, Oregon 97201

Sulfonylurea receptors (SURx) are multi-spanning transmembrane proteins of the ATP-binding cassette (ABC) family, which associate with Kir6.x to form ATP-sensitive potassium channels. Two models, with 13–17 transmembrane segments, have been proposed for SURx topologies. Recently, we demonstrated that the aminoterminal region of SUR1 contains 5 transmembrane segments, supporting the 17-transmembrane model. To investigate the topology of the complete full-length SUR1, two strategies were employed. Topology was probed by accessibility of introduced cysteines to a membrane-impermeable biotinylating reagent, biotin maleimide. Amino acid positions 6/26, 99, 159, 337, 567, 1051, and 1274 were accessible, therefore extracellular, whereas many endogenous and some introduced cysteines were inaccessible, thus likely cytoplasmic or intramembrane. These sites correspond to extracellular loops 1–3, 5–6, and 8 and the NH2 terminus, and intracellular loops 3–8 and COOH terminus in the 17-transmembrane model. Immunofluorescence was used to determine accessibility of epitope-tagged SUR1 in intact and permeabilized cells. Epitopes at positions 337 and 1050 (putative internal loops 3 and 6) were labeled in intact cells, therefore external, whereas positions 485 and 1119 (putative internal loops 5 and 7) only were accessible after permeabilization and therefore internal. These results are compatible with the 17-transmembrane model with two pairs of transmembrane segments as possible reentrant loops.

Sulfonylurea receptors (SURx)¹ are found in many tissues and play a pivotal role in synchronizing electrical excitability with cellular metabolic state. SURx, members of the ATP binding cassette (ABC) family of proteins, associate with inward rectifier Kir6.x in a heteroetrameric 4:4 stoichiometry to form ATP-sensitive (KATP) channels (for review, see Refs. 1–3). SURx subunits physically associate with pore forming subunits (Kir6.x) and regulate the flow of potassium ions through the channel. Together, SURx and Kir6.x coordinate ATP and ADP binding with channel opening and closing. Different isoforms of SURx and Kir6.x contribute to distinct nucleotide binding affinities and orchestrate tissue specific electrical responses to metabolism. The role of the channel is perhaps best understood in pancreatic β cells, where SUR1 and Kir6.2 form the KATP channel that is the key mediator of insulin secretion. Increases in blood glucose cause closure of pancreatic KATP channels, triggered by an increase in the cellular ATP to ADP ratio. This results in membrane depolarization, Ca2+ influx, and insulin release (1–3). In vascular smooth muscle, the KATP channel (SUR2B/Kir6.2) is important in the regulation of blood pressure, and in cardiac muscle cells, the channel (SUR2A/Kir6.2) is involved in the response to ischemia. Sulfonylurea drugs and potassium channel openers bind to SURx directly and have been used extensively to regulate KATP channel activity. Accordingly, SURx subunits are drug targets for the treatment of type II diabetes, persistent hyperinsulinemic hypoglycemia of infancy, as well as hypertension (1–3). Elucidating the structure of the SURx family is vital to understanding their functions and is a valuable tool to further drug design.

SURx are multi-spanning integral membrane proteins with predicted molecular masses of ~170 kDa. SUR1 and SUR2A/B are highly homologous with similar hydrophobicity profiles and amino acid sequences (69% identical, 76% similar). Hydrophobicity analysis reveals three hydrophobic domains (TM0, TM1, and TM2), with hydrophilic nucleotide binding folds (NBF1 and NBF2) following TM1 and TM2 (4). The polypeptide within each hydrophobic domain has been hypothesized to cross the plasma membrane between four and six times. Although the transmembrane topology of the TM0 domain has been explored (5), the TM1 and TM2 domains remain undefined. The topology of the SURs has been debated, and considerably different models have been proposed, ranging from 13 transmembrane segments (in a 4+5+4 arrangement in TM0, TM1, and TM2) to 17 transmembrane segments (in a 5+6+6 arrangement) (1, 4–6).

Sequence homology of the SURs to other ABC family members is limited to the two nucleotide binding folds (NBF1 and NBF2). However, hydrophobicity analysis suggests that ABC proteins may have topological similarities and form topologically distinct subfamilies. Comparing the structure of the SURs to other ABC family members is intriguing since the ABC family contains proteins that differ greatly in their function: channels, transporters, and channel regulators, with the SUR members being rare examples of channel regulators. The cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel involved in cystic fibrosis, and P-glycoprotein (P-gp), a transporter responsible for anticancer drug resistance have been proposed to typify the ABC proteins, with TM1 and

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† To whom correspondence should be addressed. Tel.: 805-893-8505; Fax: 805-893-2005; E-mail: vandenbe@lifesci.ucsb.edu.

‡ The abbreviations used are: SUR, sulfonylurea receptor; ABC, ATP-binding cassette; KATP, ATP-sensitive potassium channel; Kir, inwardly rectifying potassium channel; MRD, multidrug resistance-associated protein; NBF, nucleotide-binding fold; NEC, no external cysteines; rectifying potassium channel; MRP, multidrug resistance-associated protein; ATP-binding cassette (ABC) family of proteins, associate with inward rectifier potassium channel; CFTR, cystic fibrosis transmembrane conductance regulator; Fgp, P-glycoprotein; WT, wild-type; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PBSCM, phosphate-buffered saline plus CaCl2 and MgCl2; MTSET, [2-(tri-methylammonium)ethyl]methanethiosulfonate bromide; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis.
TM2 domains each followed by a nucleotide binding fold, NBF1 and NBF2 (4). Topological characterizations of CFTR and P-gp are compatible with a structural model that contains 12 transmembrane segments in a 6+6 arrangement of TM1 and TM2 (7–10). In contrast, the SURs, as well as multidrug resistance-associated protein (MRP), are members of a small subgroup of ABC proteins that are distinguished by the presence of three TM domains. Little is known of the structure of this subgroup, but partial characterization of MRP topology has been interpreted in the context of a 17-transmembrane segment model (11, 12). Attention to the similarities and differences in ABC protein structures may reveal information on the relatedness of proteins and the identification of functional domains.

There have been few structural studies of SURs. Glycosylation studies of SUR1 have indicated two N-linked glycosylation sites at Asn-10 (near the NH2 terminus in the TM0 domain) and Asn-1050 (in the TM2 domain), demonstrating these sites to be extracellular (5, 6). Additionally, we have shown previously, using an in vitro protease protection assay with SUR1-prolactin fusion proteins, that the TM0 domain consists of five membrane segments in a 6+6 arrangement of TM1 and TM2 (5). These data are consistent with the 17-transmembrane (5+6+6) model.

Experiments here describe a comprehensive dual strategy using a biotinylation assay in conjunction with immunofluorescence to elucidate the topology of full-length SUR1 expressed on the plasma membrane of cultured cells. An impermeant biotinylation reagent was used in a surface labeling assay to identify the external loops of SUR1. A SUR1 construct was created that lacks external cysteines, and then individual cysteine residues were introduced and assayed for accessibility to reagent. Additionally, epitope tags were inserted into postulated internal or external regions of SUR1 and were assayed for immunofluorescence of permeabilized and nonpermeabilized cells to evaluate the internal or external location of the epitope tags. Together, these data indicate that SUR1 spans the membrane 17 times with two of these pairs of transmembrane segments as reentrant loops.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—In all constructs, hamster SUR1 (a generous gift of Dr. J. Bryan) was tagged at the COOH terminus with the V5 epitope by subcloning into the vector pcDNA3.1/V5HisA (Invitrogen). To facilitate surface expression in the absence of Kir6.2, the SUR1 endoplasmic reticulum retention signal RKR (amino acids 648–650) was replaced with alanines (SUR1AAA) (13). A SUR1AAA construct lacking external cysteines was produced by replacing endogenous cysteines with serine or alanine. The resulting SUR1AAA construct containing C6S, C26S, C170A, C1051S, and C1057S was reintroduced to NEC (no external cysteines) and then individual cysteines was produced by replacing endogenous cysteines with serine (SUR1AAA-C6S, SUR1AAA-C26S, SUR1AAA-C170A, SUR1AAA-C1051S, and SUR1AAA-C1057S). NEC was subjected to PCR mutagenesis to individually create constructs that lacks external cysteines, and then individual cysteine residues were introduced and assayed for accessibility to reagent. Additionally, epitope tags were inserted into postulated internal or external regions of SUR1 and were assayed for immunofluorescence of permeabilized and nonpermeabilized cells to evaluate the internal or external location of the epitope tags. Together, these data indicate that SUR1 spans the membrane 17 times with two of these pairs of transmembrane segments as reentrant loops.

**Electrophysiology**—Cosm6 cells were transiently co-transfected with SUR1AAA constructs, Kir6.2, and green fluorescent protein (GFP) using Fugene6 (Roche Molecular Biochemicals), and were plated onto coverslips. Patch clamp recordings were made 48–72 h following transfection. The standard bath (intracellular) and pipette (extracellular) solutions contained 140 mM KCl, 20 mM Hepes, 1 mM K-EGTA, pH 7.3. Currents were recorded from excised inside-out membrane patches exposed to K-INT bath solution or K-INT solution containing 1 mM ATP (as the potassium salt) at –50 mV at room temperature as described previously (14). Micropipette resistance was typically 0.5–1 megohms.

**Biotinylation of Surface Proteins**—COS-1 cells were transiently transfected with SUR1AAA constructs using Fugene6 (Roche Molecular Biochemicals). Forty-eight hours after transfection, cells were washed three times with PBS/CM (phosphate-buffered saline (PBS), containing 0.1 mM CaCl2 and 1 mM MgCl2). Cells were then treated with 1 mM dithiothreitol for 10 min to reduce disulfide bonds. Following three washes with PBS/CM, cells were incubated in the presence or absence of 5 mM [2-trimethylammoniumethyl]methylenebisulfonate bromide (MTSET, Toronto Research Chemicals) for 30 min. Cells were then washed three times with PBS and incubated with 50 µM N-(maleimidypropionyl)biocytin (biotin maleimide, Molecular Probes). The reaction was quenched with 2% mercaptoethanol, and the cells were washed twice with PBS/CM. Cells were solubilized in lysis buffer (150 mM NaCl, 20 mM Hepes, pH 7.0, 5 mM EDTA, 1% Igepal CA-630 (Nonidet P-40) containing protease inhibitors (Complete™, Roche) by rotation at 4°C for 1 h, and then solubilized material was removed by centrifugation at 5 min × 20,800 × g. The relative concentration of SUR1 protein in cell lysates was determined by immunoblot, and equivalent amounts of complex-glycosylated SUR1 were used in each pull-down sample to compare surface accessibility of cysteines. Neuraminidase-agarose beads (30 µl; Pierce) were washed three times with lysis buffer and added to cell lysate to pull down biotinylated protein. The mixture was allowed to incubate overnight at 4°C with rotation. The beads were washed three times with lysis buffer, three times with high salt solution (500 mM NaCl, 10 mM Tris, pH 7.5, 0.1% Nonidet P-40), and then washed with 50 mM Tris, pH 7.5. Proteins were eluted in 50 µl of SDS sample buffer by incubation at 85°C for 5 min. Proteins were separated by SDS-PAGE (10%), transferred onto nitrocellulose, and blocked with 5% nonfat milk in TBS for 1 h. The blot was incubated with V5-rose peroxidase antibody (1:1000, Invitrogen) to detect SUR1 and visualized using enhanced chemiluminescence SuperSignal® West Femto (Pierce).

Biotinylation of Flag-tagged constructs was carried out essentially as described above. Anti-V5 antibody (1:4000, Invitrogen) was used to immuno precipitate Flag-SUR1AAA constructs after biotinylation. Whole cell lysates were precleared with Protein A-agarose beads (Zhongwei) for 1 h, incubated with anti-V5 antibody for 1–2 h, and then incubated with Protein A-agarose beads with rotation at 4°C overnight. Proteins were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, incubated with streptavidin- horseradish peroxidase (1:2000, Pierce), and visualized using enhanced chemiluminescence to detect surface Flag-tagged SUR1AAA constructs.

**Immunofluorescence of Flag-SUR1AAA Constructs**—COS-1 cells grown on coverslips were transiently cotransfected with Flag-SUR1AAA constructs and pEGFP (CLONTECH), a plasmid encoding enhanced GFP, using Fugene6. Mock transfection consisted of co-transfection of SUR1 that contained no Flag epitope and pEGFP. Forty-eight hours after transfection, coverslips were washed twice with PBS and cells were fixed with 4% paraformaldehyde in PBS. To ascertain plasma membrane integrity of individual fixed cells, coverslips were incubated with DEAD® blue stain (Molecular Probes) for 30 min, washed with PBS followed by 1% bovine serum albumin. Coverslips then were incubated for 1 h at 4°C with Block (3% bovine serum albumin, 1% horse serum, in PBS with (permeabilized) or without (non-permeabilized) 1% Nonidet P-40). Cells then were incubated with anti-Flag M2 antibody (1:2000, Sigma) for 1 h at 4°C. Following four washes, cells were incubated with secondary donkey anti-mouse antibody conjugated to Cy3 (1:400, Jackson Laboratory). Coverslips were mounted onto slides with Pro-Long (Molecular Probes) mounting media. Cells were visualized with a 40× objective lens using an Olympus IX60 epi- fluorescent microscope, and images were recorded with an Optronics CCD camera (DEI-750). For data analysis, images were displayed and analyzed using Photoshop. Coverslips were washed with 0.05% Tween 20 three times with PBSCM and then incubated with 50 µM (3-maleimidopropionyl)biocytin (biotin maleimide, Molecular Probes). The reaction was quenched with 2% mercaptoethanol, and the cells were washed twice with PBS/CM. Cells were solubilized in lysis buffer (150 mM NaCl, 20 mM Hepes, pH 7.0, 5 mM EDTA, 1% Igepal CA-630 (Nonidet P-40) containing protease inhibitors (Complete™, Roche) by rotation at 4°C for 1 h, and then solubilized material was removed by centrifugation at 5 min × 20,800 × g. The relative concentration of SUR1 protein in cell lysates was determined by immunoblot, and equivalent amounts of complex-glycosylated SUR1 were used in each pull-down sample to compare surface accessibility of cysteines. Neuraminidase-agarose beads (30 µl; Pierce) were washed three times with lysis buffer and added to cell lysate to pull down biotinylated protein. The mixture was allowed to incubate overnight at 4°C with rotation. The beads were washed three times with lysis buffer, three times with high salt solution (500 mM NaCl, 10 mM Tris, pH 7.5, 0.1% Nonidet P-40), and then washed with 50 mM Tris, pH 7.5. Proteins were eluted in 50 µl of SDS sample buffer by incubation at 85°C for 5 min. Proteins were separated by SDS-PAGE (10%), transferred onto nitrocellulose, and blocked with 5% nonfat milk in TBS for 1 h. The blot was incubated with V5-rose peroxidase antibody (1:1000, Invitrogen) to detect SUR1 and visualized using enhanced chemiluminescence to detect surface Flag-tagged SUR1AAA constructs.

**RESULTS**

**Functional Characterization of SUR1AAA and SUR1AAA Mutants**—To assess the topology of SUR1, two approaches were employed. In one method, cysteine scanning was used with a

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biochemical surface labeling assay to determine the location of external loops. For these experiments, endogenous external cysteines were removed, and individual cysteine residues were introduced into the cysteineless background. In the second method, Flag epitope tags were introduced and evaluated for internal or external location using immunocytochemistry.

All mutations and Flag tags were introduced into a SUR1 background with the endoplasmic reticulum retention signal RKR mutated to AAA to promote surface expression (13). It has been demonstrated that SUR1AAA when co-expressed with Kir6.2 responds to ATP and ADP with a similar concentration dependence as does wild type SUR1 (WT SUR1) co-expressed with Kir6.2 (14), suggesting that mutation of the endoplasmic reticulum retention signal does not alter the structure of the receptor. To maximize surface expression and to avoid labeling of cysteine residues in Kir6.2, SUR1 constructs were expressed in the absence of Kir6.2.

Complex glycosylation was used as an indication of proper folding and surface expression. It has been shown that oligosaccharides play a significant role in the quality control of newly synthesized proteins (for review, see Refs. 15 and 16). All constructs that were evaluated for topological orientation displayed two bands on immunoblots when expressed in COS-1 cells (Fig. 1). The upper band (Fig. 1, filled arrow) migrated with an apparent molecular mass of ~250 kDa, and is the complex glycosylated form of SUR1 as shown previously (5, 17, 18). Upon treatment with the endoglycosidase peptide:N-glycosidase F, the upper band shifted to an apparent molecular mass of ~180 kDa (data not shown). The lower band of ~180 kDa (Fig. 1, open arrow) is the core glycosylated form of SUR1 and did not change significantly with peptide:N-glycosidase F treatment, in agreement with previous studies showing similar mobility of core glycosylated and unglycosylated SUR1 (5). Introduction of Flag tags in some sites of SUR1 did not produce a complex-glycosylated form when expressed in COS-1 cells (Flag-402, Flag-424, Flag-1189, and Flag-1232), and these were not further studied.

To test for the ability of SUR1 constructs to form functional channels when coexpressed with Kir6.2, patch clamp currents were recorded. As reported previously (14), SUR1AAA coexpressed with Kir6.2 produced functional ATP-sensitive potassium channels (Fig. 2). All Flag-tagged SUR1AAA constructs coexpressed with Kir6.2 also produced currents, and these currents were inhibited by ATP, consistent with WT SUR1 (Fig. 2). Conversely, NEC and introduced cysteine mutants did not yield detectable current with patch clamp analysis (data not shown). However, when a combination of cysteines 6, 26, and 170 were reintroduced into NEC, currents were equivalent to SUR1AAA. Introduction of Cys-170 alone did not rescue channel activity. This suggests that cysteines 6 and 26 may be important in channel activity. Another assay for normal folding is coassembly of SUR1 with Kir6.2. Although NEC and introduced cysteine mutants did not produce functional channels, all constructs that were tested (SUR1AAA, 6/26/170, and NEC)

FIG. 1. Glycosylation of SUR1AAA cysteine mutants and Flag-tagged constructs. SUR1AAA constructs were expressed in COS-1 cells and detected by immunoblotting with antibody to the V5 epitope. The solid arrow indicates the complex glycosylated form of SUR1; open arrow indicates core glycosylated form of SUR1. In the Flag-1050 SUR1 construct, the glycosylation site at position 1050 was removed, and thus the Flag-1050 construct shows lower apparent molecular mass of its complex glycosylated form.

FIG. 2. Patch clamp current recordings of Flag-tagged SUR1AAA constructs. COSm6 cells were transfected with Kir6.2 and SUR1AAA, Flag-337, Flag-484, Flag-1050, or Flag-1119. Representative records are shown for excised inside-out patches that were exposed to K-INT solution with or without ATP as indicated by the bar above the records. Inward currents are shown as downward deflections and were inhibited by ATP.
were able to coassemble with Kir6.2 as assayed by coimmunoprecipitation (data not shown). Thus, the introduced cysteine SUR1 constructs displayed normal complex glycosylation, coassembly with Kir6.2, and surface expression (see below). They were used to further characterize SUR1 transmembrane topology relying on the stringent glycosylation standard and agreement with results from the Flag-tagged constructs as indications of correct folding.

**Biotinylation of Endogenous Cysteines**—The accessibility of endogenous cysteines of SUR1 to extracellular biotinylation was determined in order to evaluate the position of external loops and to establish boundaries for internal cysteines. Identification of endogenous external cysteines also allowed us to create a SUR1 construct lacking external cysteines for subsequent introduction of individual cysteines. SUR1 contains 30 endogenous cysteines, but few are in putative external regions.

Surface labeling of transiently transfected COS-1 cells with the cysteine modifying reagent biotin maleimide was used to determine the extracellular accessibility of endogenous cysteines. Following labeling with biotin maleimide, cells were solubilized and a fraction of the whole cell lysate was analyzed by in-gel biotinylation to measure relative amounts of glycosylated SUR1 protein in each sample. Surface-biotinylated proteins from whole cell lysates containing equivalent amounts of complex glycosylated SUR1 were pulled down with Neutravidin beads and visualized by immunoblot following SDS-PAGE.

When cells expressing SUR1AAA were biotinylated by this procedure, a band of 250 kDa was labeled, corresponding to the complex glycosylated form of SUR1 (Fig. 3A, arrow). Additional upper bands also were sometimes observed, most likely a result of aggregation that occurs due to the high temperature required to elute biotinylated SUR1 from the Neutravidin beads. To demonstrate that only external cysteines of SUR1 were labeled, and that biotin maleimide did not permeate the plasma membrane at the concentration used, control samples were prelabeled with the well-established impermeant sulphydryl reagent MTSET. SUR1AAA biotinylation was blocked in an MTSET-dependent manner, demonstrating that biotin maleimide does not permeate the plasma membrane (Fig. 3A). This demonstrates that WT SUR1AAA contains endogenous cysteines that are external and accessible to biotin maleimide.

To assess which SUR1 cysteines contribute to surface labeling, five putative external cysteines (cysteines 6, 26, 170, 1051, and 1057) were replaced with serine or alanine. This construct, named NEC, was not biotinylated with biotin maleimide (Fig. 3A). When Cys-6/Cys-26/Cys-170 were introduced back into NEC, biotinylation was restored (Fig. 3A). Conversely, when Cys-170 was reintroduced alone, no biotinylation was detected, indicating that position 170 is inaccessible, possibly residing in the transmembrane region. When Cys-1051 was introduced into NEC, again, biotinylation was restored (Fig. 3A). These data, in parallel with glycosylation and protease protection data (5), establish that the NH₂ terminus and position 1051 (putative external loop 6) are external. Furthermore, the absence of labeling in NEC indicates that all other endogenous cysteines are likely to be either internal or transmembrane.

The location of identified endogenous external cysteine residues (Cys-6, Cys-26, and Cys-1051) and glycosylation sites, and predicted location of Cys-1057 are depicted in the context of a 17-transmembrane model (Fig. 3B, green). The diagram also illustrates
regions that are proposed to be internal (Fig. 3B, orange) based on inaccessibility of endogenous cysteines in hydrophilic segments.

Biotinylation of Introduced Cysteines—To identify additional external loops of SUR1, cysteines were introduced into NEC systematically and evaluated as described above for endogenous cysteines to assess surface accessibility of putative external sites. Amino acids Thr-99, Asp-159, Ala-161, Lys-337, Arg-388, Tyr-454, Lys-567, Thr-1161, Ser-1186, Pro-1162, Arg-1274, and Arg-1300 were individually replaced with cysteines and tested for accessibility to reagent. Several of the mutations (A161C, R388C, P1162C, and R1300C) produced SUR1 proteins that did not show a complex glycosylated form, and these were not evaluated further. However, all others produced both complex and core glycosylated forms (Fig. 1). Positions 99, 159, 337, 567, and 1274, corresponding to putative external loops 1, 2, 3, 5, and 8 in the 17-transmembrane model, were all accessible to biotin maleimide and blocked by pretreatment with MTSET (Fig. 4A). Position 1186, corresponding to an area postulated to reside in the cytoplasmic side of the membrane (putative internal loop 8) did not label with biotin maleimide. These results, summarized in Fig. 4B, are consistent with the proposed 17-transmembrane topology of SUR1 (4, 5). However, positions 454 and 1161, hypothesized to be in short segments, were all accessible to biotin maleimide, with high concentrations of biotin maleimide (up to 0.5 mM) used with the Cys-454 and Cys-1161 constructs. Labeling did not exceed that of NEC, indicating the cysteines to be well protected and potentially residing within the plasma membrane (data not shown). Additionally, attempts to “boost” the cysteines in both positions into a more aqueous accessible environment by inserting two alanines before and after Cys-454, one alanine before and after Cys-1161, or two glycines before Cys-1161 and one following, resulted in constructs that did not glycosylate (data not shown).

These results demonstrate putative external loops 1, 2, 3, 5, and 8 to be accessible to biotinylating reagent and external. Putative external loops 4 and 7 were inaccessible, possibly residing in or near the transmembrane region (Fig. 4B).

Immunofluorescence of Flag-tagged SUR1AAA Constructs—To confirm the location of external loops of SUR1 that were determined by surface biotinylation, as well as to investigate internal regions, Flag tags were inserted into various locations of SUR1 and probed for accessibility to antibody in the presence and absence of detergent. Labeling in the absence of detergent reflects positions external to the plasma membrane, whereas labeling that requires detergent indicates positions that are internal to the plasma membrane.

Transiently transfected COS-1 cells expressing Flag-tagged SUR1AAA constructs and GFP, to indicate transfection, were fixed with paraformaldehyde and incubated with DEAD blue. Cells were stained blue when the plasma membrane integrity was compromised during fixation, and were excluded from analysis. Cells expressing GFP that were not stained blue were scored for antibody labeling in the presence or absence of detergent. Flag tags inserted at positions 337 and 1050 showed labeling both in the presence and absence of detergent, indicating the external position of these sites (Fig. 5). In contrast, Flag tags inserted at position 485 and 1119 were only accessible to antibody labeling upon permeabilization, indicating cytoplasmic orientations of these sites (Fig. 5). For statistical comparison of labeling, Flag-485 and Flag-1119 were used as a positive control for external labeling, as site 1050 has been established to be external by antibody accessibility (Fig. 3) and glycosylation (5, 6). Quantification of labeling in Table I shows that the difference in labeling for Flag-485 and Flag-1119 in permeabilized and non-permeabilized cells is statistically significant.
when compared with Flag-1050 using a Student’s t test; thus, sites 485 and 1119 are internal. There was no significant difference, however, between labeling of the normalised percentage of cells labeled with permeabilization. The mean values ± S.E. are shown for n = 3 (constructs Flag-1050, Flag-337, and Flag-1119) and n = 2 (Flag-485). A Student’s t test was performed to compare external accessibility of each construct with Flag-1050, an established external site of SUR1 that has similar accessibility to labeling independent of permeabilization. The accessibilities of Flag-485 and Flag-1119 were significantly different from that of Flag-1050, establishing the 485 and 1119 sites as internal. In contrast, Flag-337 accessibility was not significantly different from Flag-1050, indicating site 337 to be external. *, statistically significant, indicating that these sites are not localized on the external side.

**Table 1**

| Flag tagged SUR1 construct | Average percentage of cells labeled | Normalized percentage of cells labeled (nonpermeabilized/permeabilized) | p value |
|----------------------------|------------------------------------|------------------------------------------------------------------------|---------|
|                            | Nonpermeabilized                   | Permeabilized                                                          |         |
| SUR1, no Flag              | 2.3 ± 1.2                          | 7.7 ± 4.1                                                              |         |
| Flag-1050                  | 46.0 ± 3.1                         | 43.0 ± 2.5                                                             | 1.0     |
| Flag-337                   | 34.8 ± 3.7                         | 41.7 ± 2.8                                                             | 0.33    |
| Flag-485                   | 5.5 ± 1.1                          | 22.0 ± 2.8                                                             | <0.001* |
| Flag-1119                  | 2.7 ± 1.1                          | 25.3 ± 4.3                                                             | <0.001* |

**Discussion**

Hydrophobicity analysis has led to many possible topology profiles for SUR1 (4–6). SUR1 topology has been predicted with the use of sophisticated algorithms that consider amino acid hydrophobicity, charge, polarity, and distributions. However, each prediction program yielded a different model of SUR1 topology, and the location and number of transmembrane segments varied considerably from 13 to 18 (4–6). Although hydropathy profiles are important and can offer insights, in the case of SUR1, experimental data are necessary to define the topology. Our previous study of SUR1 topology provided evidence to favor a 17-transmembrane-spanning model. That investigation targeted the amino-terminal region of the protein and showed that the amino-terminal TM0 domain con-
tained five transmembrane segments (5). The data presented here provide an extensive investigation of SUR1 topology. Using a surface labeling biotinylation assay in conjunction with epitope insertion, we mapped the topology of SUR1 in the context of the intact protein. We provide direct evidence to support the 17-membrane-spanning model and suggest that two pairs of these transmembrane segments may form reentrant loops (Fig. 7).

Seventeen-transmembrane Segment Model of SUR1 Topology—Surface biotinylation has been used successfully to probe the topology of many transmembrane proteins (9, 19–21). Although the number of endogenous cysteines made the assay impractical for directly examining internal regions, cysteine accessibility provided stringent boundaries for defining the position of external loops. Endogenous or introduced cysteines inserted into NEC (lacking cysteines 6, 26, 170, 1051, and 1057) were labeled with the cysteine modifying biotinylating reagent, biotin maleimide, and evaluated for external labeling. Our previous topology study of SUR1, limited to the TM0 domain, provided a good starting point for investigation (5).

Identification of endogenous external cysteines revealed positions Cys-6, Cys-26, and Cys-1051 to be external (Fig. 3). These data are in agreement with glycosylation and protease protection studies (5, 6). Examination of cysteines introduced into the NEC background further showed that positions Thr-99 and Asp-159 are external, in concurrence with results from protease protection assays (5). In addition, cysteine scanning revealed that sites Lys-337, Lys-567, and Arg-1274 also are external (Fig. 4). These positions, corresponding to the NH₂ terminus and external loops 1, 2, 3, 5, 6, and 8, support a 17-transmembrane topology (Fig. 7).

Direct analysis of the location of internal regions of SUR1 using the biotinylation assay is limited due to the large number of internal cysteine residues. Interpretation, however, can be made from the absence of cysteine labeling. Twenty-six of 30 endogenous cysteines did not label, suggesting their location to be intramembrane or cytoplasmic (Fig. 3). Additionally, introduction of Cys-1186 in putative internal loop 8, did not label, indicating this region to be internal (Fig. 4). The absence of biotinylation of cysteine residues in all of the hydrophilic internal loops 3–9 provides strong, although indirect, evidence of their cytoplasmic localization.

Insertion of epitope tags into integral membrane proteins has been used frequently to determine topology (8, 10–12). Flag tags were inserted into SUR1 AAA at several positions. Flags inserted at external loops 3 (position 337) and 6 (position 1050) were confirmed to be external with antibody labeling of non-permeabilized cells (Fig. 5). These data complement the biotinylation data by supporting the topology derived from the SUR1 cysteine constructs. Additionally, epitope labeling is useful to directly ascertain the position of putative internal loops. Flags inserted at position 485 and 1119, corresponding to internal loops 5 and 7, were shown only to label with permeabilization (Fig. 5). These loops flank NBF1, an ATP binding domain known to reside within the cytoplasm (22–24). There has been much interest in the binding and coordination of ATP. It has been shown that drug binding influences ATP binding (25, 26). Indeed, internal and transmembrane regions have been shown to be the primary sites involved with drug binding (27–29). Understanding the architecture of SUR1 may give insights to this mechanism.
Reentrant Loops in Membrane-Spanning Segments 8/9 and 14/15—The hydrophobic profile of SUR1 suggests that there may be two additional external loops: putative loop 4 bracketed by transmembrane segments 8 and 9, and putative loop 7 bracketed by transmembrane segments 14 and 15. Close inspection of the hydrophobic regions that make up the proposed membrane-spanning segments 8/9 and 14/15 reveals only a limited number of amino acids available to form external loops, of which few are hydrophilic. Based on position and hydrophilicity, Tyr-454 and Thr-1161 were the likeliest candidates for externally residing residues. When replaced with cysteines, these positions were inaccessible to external biotinylating reagent. Attempts to probe regions at the transmembrane interface using increased concentrations of biotin maleimide also indicated that these sites were inaccessible. Topology studies of P-gp used a comparable strategy to examine the two small analogous putative loops. As with SUR1, attempts to label these P-gp regions with increased reagent concentration or incubation time did not result in detectable biotinylation (9). Conversely, glycosylation studies with CFTR revealed the two analogous loops to be external (7). In those studies, however, amino acids were added to introduce glycosylation sites, which may have contributed to the accessibility of the region in CFTR. Epitope insertion studies of P-gp were unable to map the region equivalent to putative external loop 4 of SUR1, but showed that the region analogous to external loop 7 is external (10). In that study, the addition of three tandem hemagglutinin tags (27 amino acids) was required to obtain accessibility to labeling for the P-gp loop analogous to SUR1 external loop 7, and the site was inaccessible with the addition of only one to two tandem hemagglutinin tags (10). In SUR1, attempts at “boosting” positions 454 and 1161 to a more accessible position with the introduction of alamines or glycines on either side of the cysteine resulted in SUR1 constructs that lacked complex glycosylation and were not used in analysis.

Do putative external loops 4 and 7 extend into the extracellular space? Evidence suggests that these short loops may not be exposed to the surface of the cell, and instead they may form reentrant segments that do not entirely cross the membrane. The hydrophobic putative membrane-spanning region 14/15 consists of only 33 amino acids (residues 1146–1178). This length of hydrophobic stretch is too small to traverse the membrane twice as an α-helix in which 18–20 amino acids per membrane span are required. In addition, the absence of a hydrophilic segment in the middle, and the lack of accessibility of this segment in SUR1 to modifying reagents, suggest that this region does not completely cross the membrane. Accessibility of the analogous site in P-gp and CFTR were only measured after insertion of additional amino acids (7, 10). Similarly, the hydrophobic stretch for putative membrane-spanning region 8/9 (residues 428–478) lacks a hydrophilic segment that may correspond to an external loop, and is inaccessible in SUR1 and P-gp (but accessible in CFTR after addition of amino acids). In contrast to the 14/15 segment, the 8/9 region is sufficiently long (50 amino acids) to span the membrane twice in an α-helical structure. However, its strongly hydrophobic makeup and lack of external accessibility suggest that it also forms a reentrant membrane loop. Interestingly, the putative 8/9 and 14/15 transmembrane regions each contain a pair of proline residues that is conserved among SUR, MRP, P-gp, and CFTR subfamilies, and we speculate that these prolines may participate in the unique structural arrangement of these segments.

Functional Implications of SUR1 Mutations—Experiments suggest that the two external cysteines 6 and 26 are required for expression of current. These cysteines are highly conserved, and are present in mammalian SUR1 and SUR2, Drosophila SUR, the MRP proteins, as well as Btp1p from yeast (6, 30–32). Since preincubation with a reducing agent is necessary for subsequent modification by sulfhydryl reagents in WT SUR1, it is possible that disulfide bonds are present and necessary for channel function. Although SUR1 constructs lacking Cys-6 and Cys-26 did not show detectable currents with patch clamp analysis, topological data from the cysteine mutants are consistent with epitope mapping of Flag-tagged constructs that do form functional channels (Fig. 2), suggesting that cysteine mutants retain normal topological structure.

Although the exact role of glycosylation of SUR1 is not understood, oligosaccharides have been shown to play an important role in protein folding (reviewed in Refs. 15 and 16) and have been correlated with surface expression of SUR1 (13). Consequently, glycosylation of SUR1 constructs was imposed as a requirement for analysis. In addition to the sites discussed, many sites subjected to mutation or Flag insertion resulted in constructs that did not glycosylate. Regions that showed sensitivity to disruption of glycosylation were found in internal loops 4, 5, 8, 9, and 9 external loop 2. These regions may have some importance in the proper folding of SUR1. Previous studies of insertion of epitope tags into MRP and P-gp similarly showed that function was more often impaired by insertion of tags into intracellular loops (10, 12).

Concluding Remarks—The data presented here support a 17-transmembrane model for the topological structure of SUR1 with two pairs of transmembrane segments as possible reentrant loops (Fig. 7). The transmembrane segments are arranged in a 5+6+6 topology in the TM0, TM1, and TM2 domains of SUR1. It is likely that SUR2A and SUR2B have identical topologies to SUR1, given their high sequence homology, similar hydrophobicity profiles, and similar functions. Comparison of the topological structure of SUR1 with other ABC proteins suggests structural similarities among the proteins despite significant functional differences. MRP, a transporter involved in drug resistance of cancer cells, is the protein most closely related to SUR1. Partial mapping of MRP topology is consistent with the 5+6+6 topology model proposed for SUR1 (11, 12). Determination of SUR1 topology also indicates the close relationship of SUR1 to the more common ABC proteins with the 6+6 conformation such as CFTR and P-gp, and may have broad implications for the structural relationships among ABC proteins.

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