Molecular Dissection of the Contribution of Negatively and Positively Charged Residues in S2, S3, and S4 to the Final Membrane Topology of the Voltage Sensor in the K⁺ Channel, KAT1*

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Voltage-dependent ion channels control changes in ion permeability in response to membrane potential changes. The voltage sensor in channel proteins consists of the highly positively charged segment, S4, and the negatively charged segments, S2 and S3. The process involved in the integration of the protein into the membrane remains to be elucidated. In this study, we used in vitro translation and translocation experiments to evaluate interactions between residues in the voltage sensor of a hyperpolarization-activated potassium channel, KAT1, and their effect on the final topology in the endoplasmic reticulum (ER) membrane. A D95V mutation in S2 showed less S3-S4 integration into the membrane, whereas a D105V mutation allowed S4 to be released into the ER lumen. These results indicate that Asp⁹⁵ assists in the membrane insertion of S3-S4 and that Asp¹⁰⁵ helps in preventing S4 from being released into the ER lumen. The charge reversal mutation, R171D, in S4 rescued the D105R mutation and prevented S4 release into the ER lumen. A series of constructs containing different C-terminal truncations of S4 showed that Arg¹⁷⁴ was required for correct integration of S3 and S4 into the membrane. Interactions between Asp⁹⁵ and Arg¹⁷¹ and between negative residues in S2 or S3 and Arg¹⁷⁴ may be formed transiently during membrane integration. These data clarify the role of charged residues in S2, S3, and S4 and identify posttranslational electrostatic interactions between charged residues that are required to achieve the correct voltage sensor topology in the ER membrane.

Voltage-dependent (gated) K⁺channels contain six transmembrane segments (S1–S6) and the pore (1). The fourth transmembrane segment, S4, which contains several positively charged residues and is only weakly hydrophobic, is part of the voltage sensor. To date, there have been several reports on the operation of the voltage sensor in Shaker-type channels (2–7). To express channel function, the voltage-dependent channel must be correctly integrated in the membrane, allowing the correct positioning of a set of amino acids involved in voltage sensing. Knowledge of the mechanism involved in the topogenesis of the voltage sensor in ion channels would provide great advances in our understanding of the voltage-sensing configuration. Only a limited number of reports have dealt with the membrane topogenesis of the different regions. Mutational analysis combined with electrophysiological measurements has identified the charged residues involved in the folding and function of the Drosophila Shaker K⁺ channel (8, 9). In addition, a model involving electrostatic interactions between charged residues in S2, S3, and S4 has been proposed on the basis of the time course of Shaker channel maturation in Xenopus laevis oocytes (10). To address the question of how the final membrane topology of the K⁺ channel is reached, more experimental data on transmembrane biogenesis are needed.

KAT1 is a well characterized plant hyperpolarization-activated K⁺ channel (11, 12). Plant K⁺ channels play an important role in signal transduction and regulation of ion homeostatics (13), and their membrane topology resembles that of animal Shaker channels (14). Studies of the topogenic function of each of the six transmembrane regions, S1–S6, and of the pore region of KAT1 have demonstrated that the four hydrophobic segments, S1, S2, S5, and S6, are integrated sequentially into the endoplasmic reticulum (ER) membrane (15, 16), whereas S3 and S4 are suggested to be synergistically and posttranslationally integrated into the membrane only after a specific interaction occurs between them (16). Since S4 does not possess a stop-transfer function and is not released into the ER lumen (16), we wished to identify the residues and intramolecular interactions involved in its retention in the membrane. The integration involving the pairing of transmembrane units has been recognized as a second type of biogenesis of polytopic membrane proteins (17). The biogenesis process seems to be essential for the integration of charged transmembrane segments, which have low hydrophilic property. Further detailed

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1 The abbreviations used are: ER, endoplasmic reticulum; DPP, dipeptidyl peptidase; H1, H1 segment of the E. coli leader peptide; PL, prolactin; RM, rough microsomal membrane; SA-I, type I signal-anchor sequence; SA-II, type II signal-anchor sequence.
study of the membrane insertion of the voltage sensor in the KAT1 channel will provide information on the dynamic membrane insertion process of closely spaced transmembrane segments throughout the polypeptide. To understand the molecular basis of the topogenic mechanism for the integration of S3 and S4 into the membrane, we have used in vitro translation and translocation experiments to evaluate the role of individual residues in KAT1 S2, S3, and S4 in the integration of the protein into the ER membrane and have identified specific interactions between charged residues that contribute to KAT1 membrane topology.

**EXPERIMENTAL PROCEDURES**

**In Vitro Transcription, Translation, and Translocation**—The constructs used are shown in Figs. 2–6. Each PCR-amplified fragment was subcloned into the corresponding sites in the plasmid used for topogenic assays (15, 16). To produce S1–4–PLgly fusion proteins, DNA encoding Met1-Arg165, Met1-Arg171, Met1-Trp173, Met1-Arg184 from KAT1 was fused to DNA coding for PLgly using a two-step PCR approach (18). RNAs were translated in the reticulocyte lysate system (15, 22, 23) in either the presence or absence of dog pancreas rough microsomal membranes (RM) (15, 24); in some experiments, after translation, aliquots were treated with proteinase K (200 μg/ml) (16). The [35S]methionine-labeled proteins were then separated on SDS gels, which were subjected to autoradiography (15, 16).

Recording of Ion Currents in X. laevis Oocytes—Capped complementary RNA was injected into X. laevis oocytes prepared as described previously (25), and then the oocytes were kept for 1 day at 18 °C in standard culture solution (18). Two-electrode voltage clamp experiments were performed on oocytes suspended in 6 mM MgCl₂, 1.8 mM CaCl₂, 120 mM KCl, 10 mM HEPES-HCl, pH 7.3. Step voltage pulses (−30 to −170 mV with a 20-mV decrement) were applied from a holding potential of 0 mV, the duration of each pulse being 500 ms. Voltage pulse protocols and data acquisition were performed using a voltage clamp amplifier (AxoClamp 2B, Axon Instruments, Foster City, CA) and software (pCLAMP6, Axon Instruments).

**RESULTS**

S2, but Not S1, Is Required for Membrane Integration of S3–S4—The topology of KAT1 in the membrane is shown in Fig. 1. Transmembrane segments S1 to S4 (S1–S4), used in the present study, can be integrated correctly into the membrane in the absence of S5, pore, and S6 (16). The amino acid se-
quences of S2, S3, and S4 of Shaker-type K⁺ channels are highly conserved between animal and plant cells (Fig. 1B).

During topogenesis, integration of S1 and S2 occurs sequentially as S1 possesses a strong preference for a signal-anchor with an Ncyt/Cexo orientation (SA-II), and S2 has a stop-transfer function (16). In contrast, neither S3 nor S4 alone has significant topogenic function (16). Electrostatic neutralization of Asp141 in S3 by site-directed mutagenesis gives transmembrane function to S3 and allows it to be inserted into the membrane by itself. Interestingly, S4, which has low hydrophobic property, does not translocate into the lumen space when KAT1 protein is inserted into the membrane (16).

To dissect the molecular mechanism involved in the membrane integration of S3 and S4 in KAT1, we used the in vitro reticulocyte lysate protein synthesis system in the presence or absence of RM, which adds carbohydrate moieties to glycosylation sites exposed in the lumen. The constructs used consisted of the basic unit of S1–S4 coupled to a single prolactin (PL) molecule (200 amino acids) with a potential glycosylation site added to the linker between S3 and S4 (S1–4-PL), and in some experiments, a second potential glycosylation site was also generated in the PL molecule (S1–4-PLgly). PL gly-containing constructs were used for monitoring the release of the PL/PLgly protein into the ER lumen in comparison with PL-containing constructs, as used in the previous study (16). This basic unit was then modified by replacement of S1 or S1/H11001 S2 with other membrane-derived units or by mutation of residues in these constructs. If S3 and S4 are correctly integrated, the glyco-

**Fig. 2.** S2 is essential for the membrane integration of S3-S4. As shown in A, PL or PLgly was fused C-terminal to S1-S4. DPP indicates that S1 (Met¹-Thr⁹⁵ of KAT1) was replaced with the mouse DPP IV segment, whereas H1 indicates that S1 and S2 (Met¹-Asp¹¹⁵ of KAT1) was replaced with the H1 segment of the E. coli leader peptidase. In all constructs, the endogenous N-glycosylated residue at position 158 was removed, and the N-glycosylation loop (shown as a circle) of human band 3 was inserted between S3 and S4. The other possible glycosylation sites in PLgly and in the N-terminal region of H1 are indicated by squares. B, effect of substitution of S1 or S1 + S2 on membrane topogenesis in the in vitro system. Upper section, autoradiographs of SDS gels of the different preparations synthesized in the absence or presence of RM. The monoglycosylated form is indicated by a single dot. Lower section, membrane topologies deduced from the results. Glycosylated and non-glycosylated sites are shown as filled and empty symbols, respectively. PK, proteinase K treatment.

**Fig. 3.** Role of negative residues of S2 in the membrane integration of S3-S4. A and B, effect of mutation of aspartate residues. Asp at positions 95, 105, or 141 in the S1–4-PL construct was replaced with Val. The mono-glycosylated and di-glycosylated forms are indicated by single and double dots, respectively.
sytlation site lies within the RM lumen and is glycosylated, whereas the PL moiety is external, and the glycosylation site on PLgly is not glycosylated. Correct integration of S3 and S4 in the presence of RM can therefore be monitored on SDS gels by glycosylation of the PL construct and mono-glycosylation of the PLgly construct. Since external regions of the molecule are sensitive to proteinase K digestion, in some experiments, proteinase K sensitivity of the constructs was also tested. Results using the starting construct, S1–4-PL, are shown in Fig. 2B (lanes 1–6) (16). In the absence of RM, the PL and PLgly fusion proteins were synthesized as single bands with the expected molecular mass (lanes 1 and 4), whereas in the presence of RM, single glycosylated bands were seen with both constructs (lanes 2 and 5). Proteinase K treatment resulted in the loss of these bands (lanes 3 and 6) since the regions of KAT1 proteins external to the RM and the fused PL were hydrolyzed by the added proteinase K. These control experiments showed that the PL or PLgly domain was external to the RM, that the S3-S4...
linker glycosylation site was internal since it was glycosylated in the PL construct, that the additional glycosylation site on PLgly was external since it was not glycosylated, and that much of the protein was external since it was degraded by proteinase K.

We first determined whether S1 was required for S3-S4 integration by replacing the N-terminal segment of KAT1 (Met1-Thr85), including S1, with mouse DPP IV, a region containing a transmembrane segment unrelated to S1 (19, 20), but which, like S1, has an SA-II function (Ncyt/Cexo orientation) (Fig. 2A (16)). As shown in Fig. 2B (lanes 7–12), the results for the DPP-S2–4-PL/PLgly constructs were identical to those for the S1–4-PL and S1–4-PLgly constructs, showing that S2–S4 was correctly integrated using DPP IV instead of S1.

We then determined whether S2 was essential for the integration and stabilization of S3-S4 in the membrane by constructing fusion proteins in which both S1 and S2 were replaced by the H1 segment of the E. coli leader peptidase (Fig. 2A). H1 has a strong preference for the N exo/Ccyt orientation (SA-I) (16, 21) and contains a possible glycosylation site, Asn, at position 3 in the N-terminal region (Fig. 2A, square). When translated in the presence of RM, both the H1-S3–4-PL and H1-S3–4-PLgly proteins appeared as a mono-glycosylated band, suggesting that only one of the three glycosylation sites was glycosylated (Fig. 2B, lanes 14 and 17), and proteinase K treatment resulted in the degradation of both fusion proteins (lanes 15 and 18). When the glycosylation site at Asn³ was removed by conversion to Gln, the glycosylated band disappeared (lanes 20 and 22), indicating that the constructs were only glycosylated at this site, i.e. the S3-S4 linker was external. These results show that S2 is essential for membrane integration of S3-S4 and cannot be replaced by other types of transmembrane sequence.

Asp⁹⁵ and Asp¹⁰⁵ in S2 Have Different Functions in Stabilizing S3-S4 Topology—On the basis of the alignment of K/H11001 channels (Fig. 3B), KAT1 has conserved negatively charged residues at positions 95 and 105 in hydrophobic transmembrane segments of S2. We therefore tested the role of these residues in the integration of S3-S4 into the membrane. When the function of the complete K/H11001 channel protein containing a single mutation of these residues (D95V or D105V) was tested by two-electrode voltage clamp measurement using X. laevis oocytes, no detectable K/H11001 currents were seen (see Fig. 4C). Asp⁹⁵ and Asp¹⁰⁵ were therefore likely to be crucial residues for K/H11001 channel activity and/or in the generation of KAT1 membrane topology.

We then examined the membrane integration of KAT1-PL fusion proteins containing the D95V or D105V mutations (Fig. 3, A and B). In the presence of RM, the D95V mutation resulted in a significant decrease in intensity of the mono-glycosylated band on gels (lanes 10 and 12 compared with lanes 2 and 4), which was even more marked for the double mutant (lanes 18 and 20). Since glycosylation of PL only occurs when S3 and S4 are correctly inserted into the membrane, these results indicate that Asp⁹⁵ is required for the integration of these segments. In contrast, S1–4D105V-PLgly gave rise to both mono- and di-glycosylated bands (lane 16), indicating that the PL moiety of at least a proportion of the construct was internal.
and suggesting that Asp\textsuperscript{105} acts to prevent S4 from translocating into the RM lumen and to stabilize S4 in the membrane. When Asp\textsuperscript{105} was replaced by arginine (mutant D105R; used in experiments below), both glycosylated bands were still seen (lane 24). As reported previously (16), replacement of Asp\textsuperscript{141} with valine (D141V) did not affect membrane integration of S3 and S4, only the mono-glycosylated band being seen in the presence of RM with both S1–4\textsubscript{D141V}-PL and S1–4\textsubscript{D141V}-PL\textsubscript{gly} (Fig. 3B, lanes 6 and 8).

Arg\textsuperscript{177} in S4 Is Involved in Stabilization of S4 Topology—The interaction between negatively charged residues in S2 and S3 and positively charged residues in S4 is important in the voltage sensor operation (1, 3, 5, 7). Since mutation at Asp\textsuperscript{105} led to interaction between negatively charged residues in S2 and S3 Arg\textsuperscript{176}, o r Arg\textsuperscript{177} in S4 was replaced by valine was constructed contrast, the R165V and R171V mutants had no KC\textsubscript{adj} (Fig. 4 and R177V differed from that of the wild-type (Fig. 4). In though the voltage dependence of current amplitude of R176V that R174V, R176V, and R177V had the channel activity, al- opening and closing of the pore. activity, showing that these two residues play an important role in the gating charge movement, which is coupled to the release of S4 into the ER lumen (Fig. 3B). We predicted that a single neutral mutation at a

**Fig. 6. Contribution of Arg\textsuperscript{174} and negatively charged residues in S2 to S4 membrane spanning.** As shown in A, N-terminal Arg\textsuperscript{174} was fused to PL\textsubscript{gly} and Asp at position 95, 105, or 141 was replaced with Val. B, assay of the constructs using the \textit{in vitro} system. C, as in panel A, but using N-terminal Arg\textsuperscript{177} instead of N-terminal Arg\textsuperscript{174}. D, assay of the constructs using the \textit{in vitro} system.

As shown in Fig. 4D, the four D105R mutants containing R165D, R174D, R176D, or R177D gave a mixture of mono- and di-glycosylated bands, whereas that containing R171D gave only the mono-glycosylated band. Moreover, we confirmed that the R171D mutation alone (without D105R) led to some translocation of S4 into the ER lumen (Fig. 4E). This suggests that the Asp\textsuperscript{105}-Arg\textsuperscript{171} interaction is involved in supporting the membrane spanning of S4 during membrane integration.

**The N-terminal Region of S4 up to Arg\textsuperscript{174} Is Required for S4 Membrane Spanning—**Although the Asp\textsuperscript{105}-Arg\textsuperscript{171} interaction is important in S4 membrane spanning, other forces and components must also be involved. To determine the minimal segment of S4 required for S3-S4 integration, a series of S1–4-PL\textsubscript{gly} constructs containing various segments of S4 C-terminally truncated at residues 165, 171, 173, 174, 176, 177, and 184 was constructed (Fig. 5A). In the presence of RM, S1-Arg\textsuperscript{165}-PL\textsubscript{gly} gave no glycosylated bands (Fig. 5B, lane 2), showing that it did not allow S3 integration into the membrane. S1-Arg\textsuperscript{171}-PL\textsubscript{gly} and S1-Trp\textsuperscript{173}-PL\textsubscript{gly} each gave two glycosylated bands (Fig. 5B, lanes 4 and 6), whereas S1-Arg\textsuperscript{174}-PL\textsubscript{gly} gave only the mono-glycosylated band (lane 8), suggesting that Arg\textsuperscript{174} is involved in retaining S4 in the membrane. The amount of the mono-glycosylated band increased when going from Arg\textsuperscript{174} to Arg\textsuperscript{184} (lanes 8–14), suggesting that the arginine residues in this region help stabilize S4 in the membrane.

Since D141V forced S3 to be transmembrane (14, 16), the same series of S4 truncations was generated in the D141V mutant (Fig. 5C). When translated in the presence of RM, S1-Arg\textsuperscript{165}_D141V-PL\textsubscript{gly} gave a dominant di-glycosylated band (lane 2). The presence of Arg\textsuperscript{171} resulted in both mono- and di-glycosylated forms, and the intensity of the mono-glycosylated form increased with the Trp\textsuperscript{173}-containing construct, an effect not seen using the Asp\textsuperscript{141} form (Fig. 5B). All constructs containing Arg\textsuperscript{174} gave only the mono-glycosylated band. These results showing the contribution of Arg\textsuperscript{174} (Fig. 5C) correlate with those obtained with the Asp\textsuperscript{141} constructs above (Fig. 5B).
The above results showed that, for the integration of S4, at least its N-terminal region up to residue Arg174 is required. To confirm this, we introduced D95V and/or D105V into the constructs truncated at Arg174 (Fig. 6, A and B) or at Arg177 (Fig. 6, C and D). Compared with the original S1-Arg174-PLgly and S1-Arg177-PLgly, those proteins containing D95V or D95V and D105V showed a weaker glycosylated band (Fig. 6, B and D, lanes 2, 4, and 6), whereas those containing only D105V showed two glycosylated bands (lane 8), confirming the association between Asp105 and Arg171 seen in Fig. 4D.

**DISCUSSION**

In this study, we used molecular dissection to assess the contribution of negatively and positively charged residues in S2–S4 to directing the final topology of the KAT1 voltage sensor. Single mutation of the negatively charged residues, Asp95, Asp105, and Asp141, had a clear effect on KAT1 topology, whereas single mutation of the positively charged residues, Arg165, Arg171, Arg174, Arg176, and Arg177, did not. However, the use of double mutations (Fig. 4D) or truncated proteins (Fig. 5) allowed us to analyze the role of S4 arginine residues in membrane topogenesis. For membrane integration of the voltage-sensing segments, negative amino acids in S2 are essential (Fig. 3B), but the positive residues in S4 can be compensated by neighboring positive residues (Fig. 4, A and B). Amino acid sequence alignment also showed that the negatively charged residues in S2 and in S3 are well conserved between animal and plant channels (Fig. 1B), whereas positive residues in S4 vary in number and spacing (Fig. 1B).

We found that several forces are involved in achieving the correct membrane integration of S3 and S4 as summarized in Fig. 7. Replacement of S1 and S2 with H1 did not allow S3 and S4 to form the transmembrane structure (Fig. 2, A and B). Studies of voltage sensor operation in animal channels have predicted that negative residues in KAT1 are essential for channel function (2, 3, 6, 7). In this study, we found that Asp95 and Asp105 are also involved in generating the correct membrane topology of the voltage sensor, each playing different roles. In the absence of a negative charge at Asp95 (D95V), the amount of S3 and S4 in the membrane was reduced (Figs. 3 and 6), suggesting that Asp95 assists in pulling the S3-S4 complex into the membrane. Replacement of Asp105 with valine or arginine caused release of S4 into the ER lumen (Figs. 3 and 6), and in the case of the D105R mutant, this effect was overcome by additional mutation of Arg171 in S4 to aspartate (Fig. 4D), showing that pairing of Asp105 and Arg171 occurs during the step of membrane integration of S3 and S4. Previous studies have indicated that electrostatic neutralization by Asp141 is a critical event for the initiation of membrane integration of S3 and S4 (14, 16). Fig. 7 illustrates the interactions possibly involved in the membrane integration of S3 and S4.

Interaction probably begins at N-terminal residues of S4 during their membrane insertion (17). Membrane integration of voltage sensor segments is thought to involve a series of interactions between positively and negatively charged residues. Arg174, Arg176, Arg177, and/or Arg184 helped stabilize S4 in the membrane at the end of the S4 insertion event (Fig. 5). Since these residues reside within the membrane and Asp105 is located on the cytosolic side of the membrane according to a map of charged residues in the animal Shaker channel (2–6), Asp105 and Arg171 may not be in close proximity to one another in the final configuration of KAT1 in the membrane. Moreover, in the animal Shaker channel, electrostatic interaction between charged residues in S2, S3, and S4 is involved in operating voltage sensing (10). In the *Drosophila* Shaker K+ channel, residues Glu285, Arg368, and Arg371 (corresponding to Asp95, Arg171, and Arg174 in Kat1; Fig. 1B) form one charge network, whereas residues Glu283, Asp316, and Lys374 (corresponding to Asp105, Asp141, and Arg177 in KAT1) form the other (10). The charged residues that participate in the network are important for channel integration and function (3, 9, 10, 26, 27). If the same applies to KAT1, Asp105 and Arg171 would belong to different networks (pairing groups) in the final topology, and the Asp105-Arg171 interaction, which was shown to affect the retention of S4 in the membrane (Fig. 4D), may represent a transient initial step in the insertion process. Further information on the final structure of voltage sensor segments will help us to uncover the membrane integration process completely.

The use of proteins containing truncated S4 (Fig. 5) provided evidence for another electrostatic interaction between voltage-sensing segments and showed that Arg174 was a determining factor in S4 retention in the membrane (Fig. 5).
determine which negative residue in S2 or in S3 interacted with Arg$^{171}$ using the mutant containing R174D, but this did not give the di-glycosylated band (data not shown). Taking into consideration the loss of function of D95V-, D105V-, and D141V-containing KAT1 and the retention of activity of R174V-containing KAT1 (Fig. 4C), Arg$^{174}$ is unlikely to contribute a gating charge movement in the final topology by its interaction with Asp$^{105}$, Asp$^{105}$, or Asp$^{11}$. The interaction of Arg$^{174}$ with negative residues may be a transient event during the acquisition of the final topology. We estimate that, during membrane integration, the interaction between Arg$^{171}$ and Asp$^{105}$ is stronger than that between Arg$^{174}$ and as yet undefined negative residues since the former interaction could be detected by the double mutation approach (Fig. 4D).

Unlike the classical insertion of hydrophobic transmembrane segments into the membrane, the insertion process of S3-S4 in KAT1 into the membrane involves the posttranslational peptide binding of transmembrane segments, a newly recognized second type of membrane protein integration process (17). The charged residues in S2, S3, and S4 have been reported to be crucial to the voltage-sensing operation (2). In this study, we have identified interactions between charged residues in voltage sensor segments and demonstrated their contribution to the membrane integration of S3 and S4, which shows that the charged residues also play a crucial role in the biogenesis of the voltage dependent KAT1 channel.

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Molecular Dissection of the Contribution of Negatively and Positively Charged Residues in S2, S3, and S4 to the Final Membrane Topology of the Voltage Sensor in the K⁺ Channel, KAT1

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