Voltage-dependent K⁺ channels consist of a voltage-sensing region and a pore-forming region. Here we have identified the negative residues of the second transmembrane segment in the plant voltage-dependent K⁺ channel, KAT1, which involves the function of voltage sensing. Point mutations at D95 and D105 but not D89 and D116 failed to complement the K⁺ uptake deficient properties of the mutant yeast. In vitro translation and translocation experiments showed that the membrane integration of the third and fourth segments involving voltage sensor were impaired by the replacement of D95 or D105 by serine. These data show that both the residues play a crucial role in the membrane topogenesis of the voltage sensor in KAT1.

Key words: channel; topology; potassium; topogenesis; transmembrane

Voltage-dependent (gated) K⁺ channels are integral membrane proteins with intrinsic voltage sensors, which are composed primarily of charged amino acid residues in the channel proteins. Molecular cloning has revealed that many voltage-dependent K⁺ channels have six transmembrane segments (S1-S6) and pore (P). The second and third transmembrane, S2 and S3, contain negatively charged amino acids and the fourth transmembrane segment, S4, contains several positively charged residues with low hydrophobic properties. Mutational analysis has identified some of the charged residues involved in folding functions of the Drosophila Shaker K⁺ channel. An Arabidopsis voltage-dependent K⁺ channel, KAT1, also contains four aspartate residues around S2 and these four residues are well conserved among plant voltage-dependent K⁺ channels (Fig. 1). There are no reports on which residues are a component of the voltage sensor. In this study, we have identified the negative residues in S2 required for K⁺ transport in yeast and have evaluated the role of negatively charged residues located in S2 of KAT1 on the proper integration into the endoplasmic reticulum membrane using in vitro translation and translocation experiments.

Single mutations, D89C, D95C, D105C, D116C, double mutation of D89S-D116S, and triple mutation of D89S-D95S-D116S were introduced into the wild-type KAT1 gene present in a pYES2-based vector constructed previously. Cysteine was not introduced into the double or triple mutated constructs to exclude the possibility of formation of disulfide bonds. We chose serine instead of cysteine because the volume of the side chain of serine is similar to that of cysteine. The KAT1 mutants and the wild-type were expressed in Saccharomyces cerevisiae CY162, which has mutations in the genes encoding the yeast K⁺ uptake systems, Trk1 and Trk2. The strain can grow in the synthetic minimal media containing 100 mM KCl but not in the media without KCl supplementation (selection medium).

To clarify the role of negative residues in S2 which are involved in the function of K⁺ permeation of KAT1, aspartate at 89, 95, 105 and 116 positions was replaced by cysteine. The resultant mutant channels were expressed in the K⁺ uptake deficient yeast strain (Fig. 2). The D89C and D116C grew in the medium charged with 100 mM KCl.
Fig. 2. Complementation Test for the Negative Residues around S2 in KAT1.

The wild type KAT1 and the mutant containing D89C, D95C, D105C, D116C, D89S-D116S, and D89S-D95S-D116S were expressed in a K⁺ uptake deficient yeast strain.

while D105C did not. The growth of the D95C-expressing yeast was lower. The previous reports suggested that the voltage sensor consists of some negative residues in S2 and S3 and positive residues in S4, which interact electrostatically with each other.¹⁵,¹⁶ Numerous interactions between negative and positive residues are likely to form the voltage-sensing region. Therefore some of the single substitutions could be compensated for by the neighboring negative residues in S2. To eliminate these possibilities, the double mutant, D89S-D116S, and the triple mutant, D89S-D95S-D116S were constructed. The D89S-D116S mutant complemented the yeast mutation. The D89S-D95S-D116S mutant rescued the mutation but their growth was very low. These results suggested that D95 and D105 play a crucial role in the K⁺ channel function. Based on these results, further experiments focused on the D95 and D105.

Fig. 3. Role of Negatively Charged Residues in S2 on the Membrane Integration of S3-S4.

(A) PLgly was fused at R177 in KAT1. The endogenous N-glycosylated residue at position 158 was removed. The N-glycosylation loop (circle) of human band 3 was placed between S3 and S4. Another possible glycosylation site in PL is shown by a square.

(B) Effects of mutation of D at position 95 and/or 105 on membrane topogenesis in the in vitro system. D at position 95 and/or 105 in the S1-R177-PLgly construct was replaced with S. Upper section: Autoradiographs of SDS gels of the different preparations synthesized in the absence or presence of RM. The mono-glycosylated 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.

(C) Effects of mutation of the aspartate residues with D141V. In addition to the mutations in Fig. 3B, D at position 141 in the S1-R177-PLgly construct was replaced with V. The mono-glycosylated and di-glycosylated forms are indicated by single and double dots, respectively. Glycosylated and non-glycosylated sites are shown as filled and empty symbols, respectively.
There are two possible explanations for the loss of the function of K⁺ channel by mutations of D95 and D105; i) The mutation may impair the integration of S3 and S4 into the membrane because the interaction between negative residues and positive residues are needed for the membrane formation of S3 and S4, ii) By the mutations, the K⁺ channel gating function is disordered because the integration of the negative residues with some positive residues in S4 are needed for the voltage-sensing function. To evaluate the contribution of the residues 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 rough microsomal membranes (RM) from dog pancreas. The constructs used consisted of the basic unit of S1-S4 up to R177 coupled to a single prolactin (PL) molecule (200 amino acids) with a potential glycosylation site added to the linker between S3 and S4. A second potential glycosylation site was also generated in the PL molecule (S1-R177-PLgly).²⁻⁷ The RNAs were translated in a reticulocyte lysate³⁻⁴ in either the absence or presence of RM.²⁻⁷ This basic unit was then modified by replacement of D95 and/or D105 with serine. S. If S3 and S4 are correctly integrated, the glycosylation site lies within the RM lumen and is glycosylated, whereas PLgly is not glycosylated. Integration of S3 and S4 in the presence of RM can therefore be detected by mono-glycosylation of the PLgly construct. Results using the starting construct, S1-R177-PLgly, are shown in Fig. 3A and B.²⁻⁰ In the absence of RM, the PLgly fusion protein was synthesised as a single band with the expected molecular mass (lane 1), while, in the presence of RM, a single glycosylated band was seen with the construct (lane 2). The previous results using similar constructs that contain KAT1 regions up to the S4-S5 linker showed that the glycosylated band contained the glycosylation at the S3-S4 linker but not on PLgly.²¹ We considered therefore in these control experiments, the S3-S4 linker glycosylation site was in the ER lumen and the additional glycosylation site on PLgly was external (Fig. 3B).

Next, the membrane integration of KAT1-PLgly fusion proteins containing the D95S and/or D105S mutations (Fig. 3B) was examined. In the presence of RM, the D95S and D105S constructs resulted in a decrease in intensity of the mono-glycosylated band on gels (lanes 4 and 6 compared to lanes 2). The intensity was even more marked for the double mutants (lane 8). These results indicate that D95 and D105 are required for the integration of these segments.

The previous study reported that D141V forced S3 to be a transmembrane and S4 becomes a transmembrane segment.⁵⁻¹⁰ In this case, the glycosylation occurs at the possible glycosylation site at the S3-S4 linker. Accordingly, a mono-glycosylation band was seen in lane 2 in Fig. 3C. Interestingly, S1-D105S-D141V-PLgly gave a di-glycosylated band (lane 6) whereas S1-D95S-D141V-PLgly did not show the band (lane 4), indicating that D105 acts to prevent S4 from translocating into the RM lumen and to stabilize S4 in the membrane. This effect is observed in the substitution of D105V (unpublished data).

Figure 4 shows the possible interaction required for the membrane integration of S3 and S4. The charges of D95 and D105 contribute not only to achieve the proper topology of a voltage sensor but also to operate voltage sensing. In Drosophila Shaker K⁺ channel, the negative residues corresponding to D95 and D105 in KAT1 form the electrostatic interaction between positive residues in S4 and consists of a charge network which operates the opening and closing of the pore in K⁺ channels.¹¹ Based on the results and our results in this study, we predicted that D95 and D105 contribute not only to achieve the proper toplogy of a voltage sensor but also to operate voltage sensing.

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