Molecular Architecture of a Sodium Channel S6 Helix

RADIAL TUNING OF THE VOLTAGE-GATED SODIUM CHANNEL 1.7 ACTIVATION GATE*

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Background: In-frame deletion mutation (Del-L955) in Na$_v$1.7 sodium channel from a kindred with erythromelalgia hyperpolarizes activation.

Results: Del-L955 twists the S6 helix, displacing the Phe$^{960}$ activation gate. Replacement of Phe$^{960}$ at the correct helical position depolarizes activation.

Conclusion: Radial tuning of the activation gate is critical to the activation of Na$_v$1.7 channel.

Significance: Structural modeling guided electrophysiology reveals the functional importance of radial tuning of the S6 segment.

Voltage-gated sodium (Na$_v$) channels are membrane proteins that consist of 24 transmembrane segments organized into four homologous domains and are essential for action potential generation and propagation. Although the S6 helices of Na$_v$ channels line the ion-conducting pore and participate in channel activation, their functional architecture is incompletely understood. Our recent studies show that a naturally occurring in-frame deletion mutation (Del-L955) of Na$_v$1.7 channel, identified in individuals with a severe inherited pain syndrome (inherited erythromelalgia) causes a substantial hyperpolarizing shift of channel activation. Here we took advantage of this deletion mutation to understand the role of the S6 helix in the channel activation. Based on the recently published structure of a bacterial Na$_v$ channel (Na$_v$Ab), we modeled the WT and Del-L955 channel. Our structural model showed that Del-L955 twists the D11/S6 helix, shifting location and radial orientation of the activation gate residue (Phe$^{960}$). Hypothesizing that these structural changes produce the shift of channel activation of Del-L955 channels, we restored a phenylalanine in wild-type orientation by mutating Ser$^{961}$ (Del-L955/S961F), correcting activation by $\pm$10 mV. Correction of the displaced Phe$^{960}$ (F960S) together with introduction of the rescuing activation gate residue (S961F) produced an additional $\pm$6-mV restoration of activation of the mutant channel. A simple point mutation in the absence of a twist (L955A) did not produce a radial shift and did not hyperpolarize activation. Our results demonstrate the functional importance of radial tuning of the sodium channel S6 helix for the channel activation.

The Na$_v$1.7 channel,² a member of the voltage-gated sodium channel family, preferentially expressed in dorsal root ganglia and sympathetic ganglia neurons, plays a critical role in pain signaling (1, 2). The Na$_v$1.7 channel amplifies subthreshold membrane depolarizations, contributes to the generation of action potentials (3, 4), and may facilitate neurotransmitter release at the central terminals of dorsal root ganglia neurons within the spinal cord (5). Loss-of-function Na$_v$1.7 channel mutations cause congenital indifference to pain (6) whereas gain-of-function missense Na$_v$1.7 mutations cause several painful disorders including inherited erythromelalgia (7–9), paroxysmal extreme pain disorder (10, 11), and small fiber neuropathy (12–14). These gain-of-function missense mutations represent experiments of nature that may shed light on the structural basis of sodium channel function.

The large pore-forming $\alpha$-subunit of the mammalian Na$_v$1.7 channel, similar to all members of this channel family, consists of four homologous domains (I–IV) linked by three intracellular loops (L1–L3). Each domain of the $\alpha$-subunit has six transmembrane helices (S1–S6). The S1–S4 helices form the voltage sensor domain whereas the S5 and S6 helices, together with a membrane-reentrant pore loop (P-loop) between S5 and S6, form the ion-conducting pathway. The S6 helices are the backbone of the ion-conducting pathway, and multiple residues in the S6 helices have been implicated in channel activation and inactivation. However, there is still not a full understanding of the contribution of the S6 helices to channel activation. We recently identified a gain-of-function in-frame deletion (Del-L955) within S6 of domain II of Na$_v$1.7 channel from an inherited erythromelalgia family, which causes a robust hyperpolarizing shift of activation and slow inactivation (15). Here, we took advantage of the structural change within the S6 helix produced by Del-L955 to investigate the contribution of the S6 helix to the channel activation. Using structural modeling-guided mutagenesis and patch clamp electrophysiology, we

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² The abbreviations used are: Na$_v$, voltage-gated sodium channel; PDB, Protein Data Bank.
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demonstrate that the radial orientation of the DII activation gate residue (Phe\textsuperscript{960}) within S6 is essential for channel activation. The displaced Phe\textsuperscript{960} in the Del-L955 mutant channel disrupts the activation gate, leading to a disease-causing hyperpolarizing shift of channel activation. Our results demonstrate the importance of radial tuning of the S6 helix for the Na\textsubscript{v}1.7 channel activation and suggest that structural modeling-guided mutagenesis can contribute to our understanding of the functional architecture of voltage-gated sodium channels.

**EXPERIMENTAL PROCEDURES**

*Structural Modeling*—Structural modeling was performed as described previously (16, 17). Briefly, a two-step method was used for construction of hNa\textsubscript{v}1.7 channel. First, four transmembrane domain structural models were generated by a membrane-bound protein prediction algorithm GPCR-ITASSER (18, 19). Then, each single domain model was aligned to the recently solved bacterial sodium channel tetramer (Protein Data Bank ID code 3RVY) (20) by TM-align (21). The four transmembrane domains models were assembled in a clock-wise order viewed from extracellular side as suggested previously (22, 23). The resulting four domain complex structural model was finally refined by Fragment-Guided Molecular Dynamics simulation (FG-MD) to remove interdomain clashes and improve model quality (19, 24, 25).

*Plasmid Preparation and HEK293 Cell Transfection*—Tetrodotoxin-resistant human Na\textsubscript{v}1.7 wild-type (WT) channel (hNa\textsubscript{v}1.7) was constructed based on the hNa\textsubscript{v}1.7 (mRNA, NM_002977.3; protein, NP_002968.1 of NCBI database) (26). Del-L955 and other mutant channels were constructed on the hNa\textsubscript{v}1.7r background. These channels were transfected into HEK293 cells together with human β-1 and β-2 subunits (27) using Lipofectamine (Invitrogen), as described previously (16). HEK293 cells were maintained in 1:1 Dulbecco’s modified Eagle’s medium (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS; Hyclone) in a humidified 5% CO\textsubscript{2} incubator at 37 °C. HEK293 cells were seeded onto poly-L-lysine-coated glass coverslips (BD Biosciences) in a 24-well plate 1 day before recording. The functions of all the mutant constructs of the Na\textsubscript{v}1.7 channel were screened using the PatchXpress automated parallel patch clamp system (Molecular Devices) (28).

*Voltage Clamp Recording*—Whole cell voltage clamp recordings were obtained after 1 day of transfection as described previously (16, 29). The extracellular solution contained the following: 140 mM NaCl, 3 mM KCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 20 mM dextrose, and 10 mM HEPES, pH 7.3, with NaOH (320 mosm adjusted with dextrose). The pipette solution contained the following: 140 mM cesium fluoride, 10 mM NaCl, 1.1 mM EGTA, 10 mM HEPES, 20 mM dextrose, pH 7.3, with CsOH (310 mosm adjusted with dextrose). Patch pipettes had a resistance of 1–2 megaohms when filled with pipette solution. After achieving whole cell recording configuration, the pipette and cell capacitance were manually minimized using the Axopatch 200B (Molecular Devices) compensation circuitry. Series resistance and prediction compensation (80–90%) were applied to reduce voltage errors. Recorded currents were digitized using pClamp software and a digidata 1440A interface (Molecular Devices) at a rate of 50 kHz after passing through a low pass Bessel filter setting of 10 kHz. The recording was initiated after a 5-min equilibration period after establishing whole cell configuration. To generate activation curves, cells were held at −140 mV and stepped to potentials of −80 to +40 mV in 5-mV increments for 100 ms. Peak inward currents were automatically extracted by Origin and fitted with Boltzmann function to determine the half-activation (V\textsubscript{1/2}) activation curve slope at half-activation (Z) and reversal potential (E\textsubscript{Na}) for each recording. Conductance was calculated as G = I/(V\textsubscript{m} − E\textsubscript{Na}) and were normalized by the maximum conductance value and fit with a Boltzmann equation.

*Data Analysis*—Data were analyzed with Clampfit 9.2 (Molecular Devices) and OriginPro 8.5 (Microcal Software). Student’s t test was used, and statistical significance was accepted when p < 0.05. Data are presented as means ± S.E.

**RESULTS**

*The Del-L955 Deletion Mutation Hyperpolarizes Activation of Na\textsubscript{v}1.7 Channel*—Our previous work has demonstrated that an in-frame deletion (Na\textsubscript{v}1.7 Del-L955) within the DII/S6 of the Na\textsubscript{v}1.7 channel (Fig. 1A) produces inherited erythromelalgia (15). This deletion occurs in the highly conserved S6 region (Fig. 1A) and results in a dramatic (~25 mV) hyperpolarizing shift in the voltage dependence of activation (15). We evaluated channel activation from cells expressing WT (Fig. 1B) and Del-L955 mutant channels (Fig. 1C) using a recording protocol for voltage dependence of activation. We confirmed a robust shift...
in the activation $V_{1/2}$ (voltage at half-activation) for Na$_{v}1.7$ Del-L955 channel (Del-L955: $-50 \pm 1.6$ mV, $n = 11$) compared with Na$_{v}1.7$ WT channel ($-23.8 \pm 1.4$ mV, $n = 7$, $p < 0.001$, Fig. 1D). Inactivation kinetics was also analyzed. Within the range more positive than $-45$ mV, where WT channels begin to activate, inactivation kinetics was more rapid for Del-L955 mutant channel (e.g. fast inactivation time constant $\tau = 1.53 \pm 0.17$ at $-35$ mV, $n = 7$) compared with WT ($\tau = 2.79 \pm 0.32$ at $-35$ mV, $n = 6$, $p < 0.01$). Between $-35$ mV and 0 mV, where Del-L955 and WT channel are both activated, no significant differences were found. This result is consistent with our previous report (15).

Structural Modeling Reveals Radial Displacement of the Activation Gate (Phe$_{960}$) in the Na$_{v}1.7$ Del-L955 Mutant Channel—To understand the structural basis of this change of channel activation, we constructed an atomic-level structural model of the WT and Del-L955 Na$_{v}1.7$ channel based on the bacterial voltage-gated sodium channel Na$_{v}$Ab (PDB ID code 3RVY) (20) using our previously published methods (16). As can be seen from the structural model (Fig. 2A), the activation gate in WT Na$_{v}1.7$ channel consists of four aromatic residues, one from each S6 helix (DI, Tyr$_{405}$; DII, Phe$_{960}$; DIII, Phe$_{1449}$; and DIV, Phe$_{1752}$) (30). Key residues investigated in this study are colored and circled for highlighting; Phe$_{960}$, colored red, and Ser$_{961}$ is yellow. Leu$_{955}$ is located 5 residues upstream of the activation gate residue (Phe$_{960}$). When the Leu$_{955}$ residue is deleted, the orientation of Phe$_{960}$ (red) shifts radially toward the S6 of domain III (Fig. 2B). Del-L955 also rotates Ser$_{961}$ (yellow) to the previous location of Phe$_{960}$ so that the side chain of Ser$_{961}$ now points to the pore, facing the other three activation gate residues (Fig. 2B). Side (Fig. 2C) and top (Fig. 2D) views of the four S6 helices of Del-L955 channel show that the hydrophobic ring which forms the intact activation gate in WT channels is disrupted in Del-L955 channels because of the displaced Phe$_{960}$ (Fig. 2, C and D). According to our model, the DII/S6 helix extends a few residues below the activation gate residue and becomes a flexible loop as it transitions to L3 (the third intracellular loop). As we previously demonstrated that the intact activation gate is essential for wild-type channel gating (30), we hypothesized that the radial displacement of Phe$_{960}$ is a structural substrate that contributes strongly to the altered activation of the Del-L955 channel.

Restoring Phe in the Activation Gate Location Depolarizes Activation—Our structural model reveals that in the Del-L955 channel, the side chain of Ser$_{961}$ at DII/S6 becomes aligned with the other activation gate residues from DI, DIII, and DIV (Fig. 2B). The side chain of Ser$_{961}$, however, is not likely to contribute to maintaining the hydrophobic ring needed for an effective activation gate (Fig. 2B) (30). We hypothesized that the disrupted activation gate contributes to the hyperpolarizing shift of activation in Del-L955 channel. We tested this hypothesis by asking whether restoration of the hydrophobic ring, by mutating Ser$_{961}$ back to Phe, would depolarize activation of the Del-L955 channel. Structural modeling (Fig. 3A) shows that this newly introduced Phe (yellow) would be situated in a location similar to the original Phe, so that it would be expected to interact with the other three hydrophobic residues to restore the activation gate (Fig. 3A). Note that the displaced Phe$_{960}$ is in red. For this experiment, we created Del-L955/S961F double mutation and compared it with the Del-L955 channel. Na$_{v}1.7$ WT channel activation is shown for comparison ($-23.8 \pm 1.4$ mV, $n = 7$). As shown in Fig. 3B, the Del-L955/S961F mutant channel displayed a robust $\sim +10$ mV depolarizing shift in activation compared with that of Del-L955 (Del-L955: $-50 \pm 1.6$ mV, $n = 11$, versus Del-L955/S961F: $-40.2 \pm 1.5$ mV, $n = 11$, $p < 0.001$), suggesting that introduction of a Phe to restore the activation gate depolarize channel activation.

Correcting the Displaced Phe Further Depolarizes Activation—Structural modeling also suggested that the displaced Phe$_{960}$ (red) may influence the conformation of S6 helix of DIII that contributes to altered activation of the Del-L955 channels (Fig. 3A). To test this hypothesis, we mutated this displaced Phe$_{960}$ into Ser (red, Fig. 3C), which was the original residue at this location. Voltage clamp analysis showed that this additional mutation further shifted the activation $V_{1/2}$ by $\sim +5$ mV (Del-L955/S961F: $-40.2 \pm 1.5$ mV, $n = 11$; Del-L955/S961F/F960S: $-35.1 \pm 1.5$ mV, $n = 9$, $p < 0.05$) (Fig. 3D). To further test the effect of residue size at this site, we mutated the Phe$_{960}$ to Ala, a small residue predicted to have a minimal structural effect, and created a Del-L955/S961F/F960A mutation. The activation $V_{1/2}$ of this mutation was comparable with that of Del-L955/S961F/F960S mutation, shifting the $V_{1/2}$ by $\sim 6$ mV ($-34.4 \pm 1.1$ mV, $n = 9$, $p < 0.01$ compared with Del-L955/S961F) (Fig.
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FIGURE 3. Restoring the activation gate and removing the displaced Phe depolarize activation. A, structural modeling suggests that mutating Ser\textsubscript{961} to Phe may restore the activation gate composition. Phe\textsuperscript{960} is shown in red, and S961F is shown in yellow; both of them are circled for highlighting. Note that Phe\textsuperscript{960} (red) is in a displaced position. B, Del-L955/S961F mutant channel was created and studied in parallel with Del-L955 channel in HEK293 cells. Del-L955/S961F displayed a robust +10 mV depolarizing shift in activation $V_{1/2}$ compared with that of Del-L955 (Del-L955: −50 ± 1.6 mV, n = 11, versus Del-L955/S961F: −40.2 ± 1.5 mV, n = 11, p < 0.001). Both Del-L955 and WT curves (here and in the following figures) are a replot of Fig. 1. C, structural model shows the correction of a displaced Phe\textsuperscript{960} into Ser (circled and red) together with an introduced Phe (circled and yellow) in the activation gate helical position. D, the displaced Phe\textsuperscript{960} was mutated to Ser, which was the original residue at this location. This additional mutation further shifted activation $V_{1/2}$ by −5 mV (Del-L955/S961F: −40.2 ± 1.5 mV, n = 11; Del-L955/S961F/F960S: −35.1 ± 1.5 mV, n = 10, p < 0.005). Phe\textsuperscript{960} was also mutated to Ala (Del-L955/S961F/F960A), and the activation $V_{1/2}$ of this mutation (−34.4 ± 1.1 mV, n = 9) was comparable with that of Del-L955/S961F/F960S mutation but significantly different from Del-L955/S961F mutation (p < 0.01).

3D). These results suggest that a displaced bulky Phe may influence the adjacent DIII/S6 helix lining the pore, thus affecting channel activation. Taken together, introducing a new Phe into the appropriate location within the activation gate and replacement of the displaced Phe with a smaller residue corrected the activation $V_{1/2}$ of Del-L955 channel by >60%.

A Point Mutation of Residue 955, in the Absence of a Twist, Does Not Cause a Hyperpolarizing Shift of Activation—As we hypothesized that the strong hyperpolarizing shift of the Del-L955 channel is due to an in-frame deletion causing the twist of S6 helix, especially the displacement of the activation gate residue Phe\textsuperscript{960} at the more distal portion of the S6 helix, we further asked whether a point mutation of Leu\textsuperscript{955}, in the absence of a twist, could hyperpolarize channel activation. If a point mutation of Leu\textsuperscript{955} were not able to hyperpolarize channel activation, it would further suggest that the twist of the S6 helix by Leu\textsuperscript{955} deletion, rather than a residue change of Leu\textsuperscript{955}, is likely to be the underlying mechanism of the activation shift. Alanine was chosen due to its small effect on the protein structure and because it has been found to be frequently located in transmembrane helices (31). Structural modeling showed that Leu\textsuperscript{955} in WT channel pointed toward a space formed by S4–5 linker and S6 helix (Fig. 4A). When Leu\textsuperscript{955} was mutated to Ala, the side chain was smaller and the structural effect was subtle (Fig. 4, A and B). Interestingly, the activation of L955A channel (L955A: −19.1 ± 2.2 mV, n = 7) was close to that of WT channel (WT: −23.8 ± 1.4 mV, n = 7, p > 0.05, Fig. 4C), which is markedly different from Del-L955/S961F/F960A channel or all the other mutant channels introduced in this study (p < 0.01, Fig. 4C). Therefore, our data strongly suggest that the twist of the S6 helix, rather than the local change of the Leu\textsuperscript{955} residue, underlies the strong shift of activation seen in Del-L955 mutant channels.

DISCUSSION

In this study, we took advantage of a naturally occurring deletion (Del-L955) mutation of the Na\textsubscript{v}1.7 sodium channel that produces a large hyperpolarizing shift in channel activation and employed a combined approach of structural modeling, mutagenesis, and voltage clamp electrophysiology to understand the contribution of the functional architecture of the S6 transmembrane helix to channel activation. Using structural modeling, we found that this in-frame deletion results in a twist of the DII S6 helix beginning several residues above the activation gate residue (Phe\textsuperscript{960}), causing a radial shift of this residue toward the S6 helix of DIII instead of the ion-conducting pore. Virtual mutagenesis predicted that the substitution Ser\textsuperscript{961} to Phe (Del-L955/S961F) might restore the WT composition of the activation gate and thus partially rescue activation. This manipulation indeed depolarized activation $V_{1/2}$ by −10 mV, supporting our hypothesis. As modeling further suggested that the originally displaced Phe\textsuperscript{960} might have a functional effect on the S6 of DIII, we additionally mutated the displaced Phe\textsuperscript{960} into a Ser (Del-L955/S961F/F960S) to mimic the original residue composition of this part of the DII/S6. This substitution resulted in an additional +5 mV shift of activation $V_{1/2}$ toward the WT value. Consistent with a steric influence, we observed a depolarization of the activation $V_{1/2}$ with Del-L955/S961F/F960A. To understand whether the local change of the Leu\textsuperscript{955} residue contributes to this hyperpolarizing shift of activation, we created and assessed a point mutation (L955A) that did not introduce a twist. This manipulation placed the activation $V_{1/2}$ within the range of that of the WT Na\textsubscript{v}1.7 channel, with a slight depolarizing tendency that did not reach statistical significance. Taken together, these results indicate that the structural integrity of the helix rather than a single amino acid change in this region is responsible for the large hyperpolarizing shift of activation. Our results support a model in which radial orientation of critical residues within S6 plays a key role in shaping channel activation.

The S6 helix directly lines the ion-conducting pathway and contributes to several aspects of channel gating. Point mutations of the Na\textsubscript{v}1.7 channel in patients with inherited erythromelalgia reported so far all hyperpolarize activation (1, 2). These point mutations, although informative for understanding channel gating, do not provide insight into the functional architecture of the S6 helix. An in-frame deletion such as Del-L955, on the other hand, causes a more significant change to the overall architecture. We therefore used this deletion as a tool to identify key features of the S6 helix. We demonstrated that a radial displacement of Phe\textsuperscript{960} in the Del-L955 mutant channel disrupted the activation gate and that the displaced bulky Phe
appears to influence a neighboring helix. Interestingly, in our previous study, we found that direct mutation of activation gate Phe\textsuperscript{960} (F960V) leads to a $\sim$6-mV hyperpolarizing shift of activation (30), emphasizing the importance of intact activation gates. The current study reinforces this finding and additionally shows that for the DII activation gate, radial orientation is essential. Interestingly, our earlier study suggests that replacing Phe with a larger Trp would not produce additional effects regarding the channel activation (30). Although the mutations we introduced in this study depolarize activation significantly toward WT channel, the effect is incomplete. This may be because the in-frame deletion may cause more profound changes to channel structure in additional to the twist of the DII/S6 helix downstream of Phe\textsuperscript{960} was shifted as well). Addition of another residue after the Phe may not resolve this issue, because neither crystal structure nor molecular biophysical analysis has definitively identified the end of the S6 helix of the hNa\textsubscript{v}1.7 channel yet.

Following the initial report of the structure of the bacterial voltage-gated sodium channel (Na\textsubscript{r}Ab, PDB ID code 3RVY) (20), the crystal structures of other bacterial Na\textsubscript{r} channels (Na\textsubscript{r}Rh, PDB 4DXW) (32) and (Na\textsubscript{r}M, 4F4L) (33), as well as Na\textsubscript{r}Ab in an inactivated state (PDB 4EKW) (34) have been reported. These new structures suggest an asymmetric organization of the four S6 helices, despite the fact that these are homotetrameric channels. With the availability of new structures, we asked how they may affect our modeling of the activation gate region of the Na\textsubscript{v}1.7 channel. In Fig. 5, we align both the WT and Del-L955 Na\textsubscript{v}1.7 structural models with Na\textsubscript{r}Rh. Na\textsubscript{v}1.7 is shown in wheat and Na\textsubscript{r}Rh is shown in gray, cyan, or gray. Tyr\textsuperscript{405} of DI, Phe\textsuperscript{960} of DII, Phe\textsuperscript{1449} of DIII, and Phe\textsuperscript{1722} of DIV are shown in stick configuration. Activation gate of Na\textsubscript{r}Rh (Leu\textsuperscript{219}) is also shown in stick configuration. B, extracellular view of the alignment of Na\textsubscript{v}1.7 structural model with Na\textsubscript{r}Rh. C, cytosolic view of the alignment of Del-L955 mutant channel structural model with Na\textsubscript{v}1.7 and Na\textsubscript{r}Rh. Del-L955 is shown in cyan, and Na\textsubscript{v}1.7 is shown in gray, and Na\textsubscript{r}Rh is shown in gray, D, extracellular view of the alignment of Del-L955 mutant channel structural model with Na\textsubscript{r}Rh.

Traditional, systematic mutagenesis using alanine (36), cysteine (37), or tryptophan scanning (38, 39) has yielded important information regarding the structure-function relationship of sodium channels. However, this approach is relatively time- and resource-consuming. In the present study, structural modeling provided insights regarding which residues to change via site-directed mutagenesis to test our hypothesis. With the increasing availability of sodium channel crystal structures (20, 32, 34), structural modeling guided mutagenesis may provide an approach that is direct and time- and resource-efficient.

In summary, our studies indicate that a naturally occurring gain-of-function deletion mutation (Del-L955) causes a radial shift of the S6 helix of Na\textsubscript{v}1.7 channel, resulting in a disruption of the activation gate and displacement of a bulky residue which affects the neighboring DIII S6 helix. Our results demonstrate an important contribution of radial tuning of S6 to activation.
and suggest that structural modeling-guided mutagenesis may provide a useful tool for understanding the functional architecture of voltage-gated sodium channels.

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