A Carboxyl-terminal Hydrophobic Interface Is Critical to Sodium Channel Function

RELEVANCE TO INHERITED DISORDERS

Perturbation of sodium channel inactivation, a finely tuned process that critically regulates the flow of sodium ions into excitable cells, is a common functional consequence of inherited mutations associated with epilepsy, skeletal muscle disease, autism, and cardiac arrhythmias. Understanding the structural basis of inactivation is key to understanding these disorders. Here we identify a novel role for a structural motif in the COOH terminus of the heart NaV1.5 sodium channel in determining channel inactivation. Structural modeling predicts an interhelical hydrophobic interface between paired EF hands in the proximal region of the NaV1.5 COOH terminus. The predicted interface is conserved among almost all EF hand-containing proteins and is the locus of a number of disease-associated mutations. Using the structural model as a guide, we provide biochemical and biophysical evidence that the structural integrity of this interface is necessary for proper Na+ channel inactivation gating. We thus demonstrate a novel role of the sodium channel COOH terminus structure in the control of channel inactivation and in pathologies caused by inherited mutations that disrupt it.

Channelopathies, so named because they represent a set of diseases caused by mutations in genes coding for ion channels, are a new and growing class of human disorders that include but are not limited to diabetes, muscle disorders, neurological disease, and cardiac arrhythmias (1). A surprising number of channelopathies associated with a wide diversity of human disease are caused by similar mutation-induced changes in ion channel function. Inactivation of voltage-dependent Na+ channels is an example of a physiological process critically important in many tissues that, when altered by mutation, can result in muscle weakness, inherited epilepsies, autism, or cardiac arrhythmia (2–4). Clinical consequences of inherited mutations that disrupt Na+ channel inactivation provide the most direct link between ion channel biophysics and human physiology and pathophysiology. Conversely, investigation into the consequences of inherited mutations on ion channel function has, in many cases, provided insight into the physiological importance of novel regions and/or structures of ion channel proteins.

In the heart, Na+ channels (NaV1.5)4 primarily underlie action potential initiation and propagation but more recently have been shown to be critical determinants of action potential duration, particularly in the setting of certain inherited channelopathies. Inherited mutations in SCN5A, the gene coding for NaV1.5, are now known to underlie multiple inherited cardiac arrhythmias, including the congenital long QT syndrome variant 3, Brugada syndrome, and isolated conduction disease (5), and in most cases, these inherited mutations disrupt channel inactivation.

Fast inactivation of Na+ channels is due to rapid block of the inner mouth of the channel pore by the cytoplasmic linker between domains III and IV that occurs within milliseconds of membrane depolarization (6). Inherited mutations of the III/IV linker in the cardiac Na+ channel can disrupt fast inactivation, resulting in sustained current (I\textsubscript{CAS}), which can cause long QT syndrome variant 3 (5). However, the Na\textsubscript{V,1.5} COOH terminus also has been shown to play a role in inactivation both through chimeric studies (7), through the characterization of several disease-linked mutations found in the C terminus (8–11), and by direct biochemical evidence for COOH terminus interactions with the cytoplasmic peptide that links domains III and IV of the α subunit (III-IV linker) (12, 13).

Here we have tested the hypothesis that preservation of COOH terminus structure may also be critically important to Na\textsubscript{V,1.5} channel inactivation. Previously, we generated a structural model of the NaV1.5 C terminus based on homology to the amino-terminal lobe of calmodulin (14). The model predicts six α-helices (H1–H6), the first four forming two EF-hand pairs. EF hands are helix-loop-helix motifs that typically, although not always, bind Ca\textsuperscript{2+} in the loops between helices and generally occur in pairs. One helix from each EF-hand pair is predicted to form interhelical contacts with a helix from the opposite EF hand, H1 with H4 and H2 with H3. In the present experiments, we focus on a possible role of the putative interface between H1 and H4 in stabilizing the COOH terminus structure and, in turn, in the control of channel inactivation. The predicted

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4 The abbreviations used are: Na\textsubscript{V,1.5}, voltage-gated sodium channel isoform 1.5; GST, glutathione S-transferase; I\textsubscript{CAS}, sustained current; WT, wild type; TTX, tetrodotoxin; HEK, human embryonic kidney.
interface in Na\textsubscript{v,1.5} was initially of interest to us not only because several naturally occurring mutations predicted to be near it disrupt inactivation and cause multiple types of cardiac arrhythmias (8–10), but also because mutations in similar regions of a brain sodium channel isoform (Na\textsubscript{v,1.1}) have also been reported to be linked to inherited epilepsies (15–17). However, the importance of this interface may be more general than its role in sodium channel function, because in structures for EF hand proteins there are extensive side chain interactions between helices analogous to the first (H1) and fourth (H4) predicted helices in the Na\textsubscript{v,1.5} C terminus (see, on the World Wide Web, structbio.vanderbilt.edu/cabp_database/struct/cmaps/cmap_list.html). In addition, hydrophobic residues are conserved at this interface among EF-hand proteins (18, 19). Alignment of the first four helices of all voltage-gated sodium channels demonstrates significant homology in H1 and H4, whereas H2 and H3 are not well conserved among these channels (see supplemental data). We thus focused our experiments on possible interactions between helices H1 and H4. Our results indicate that mutation of hydrophobic residues integral to the H1/H4 interface disrupts protein stability and markedly alters channel inactivation, providing evidence that stabilization of the COOH terminus structure via the H1/H4 hydrophobic interface is necessary to preserve physiologically essential inactivation of the Na\textsubscript{v,1.5} channel.

**MATERIALS AND METHODS**

**Computational Analysis**—A homology model was generated as described previously (14). Alignments were performed using ClustalX (20) with default parameters.

**Molecular Biology and Tissue Culture**—Site-directed mutagenesis for electrophysiological studies was carried out on Na\textsubscript{v,1.5} in pcDNA3.1 (Invitrogen). Mutations were introduced using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocols. Mutations were confirmed by DNA sequencing. Wild type Na\textsubscript{v,1.5} and Na\textsubscript{v,1.5} mutants were transiently transfected with \(\beta\)1 subunits into HEK 293 cells using Lipofectamine (Invitrogen) as previously described (21).

The plasmid used to grow proteins for fluorescence experiments was generated using QuickChange mutagenesis on a previously generated plasmid of the Na\textsubscript{v,1.5} COOH terminus in the pGEX vector (14). The predicted unstructured proximal region (residues 1773–1785) was deleted using QuikChange reactions, and a stop codon was inserted following the residue at 1863. The resulting construct (Na\textsubscript{v,1.5} EF) contained those residues predicted to form the EF hands (residues 1786–1863) in the pGEX vector with a thrombin cleavage site following the coding region for GST and preceding residues 1786–1863.

**Electrophysiology**—Wild type and mutant human sodium channel \(\alpha\) subunits were co-expressed with human \(\beta\)1 subunits in HEK 293 cells, and currents were measured with whole cell patch clamp procedures as previously described (12, 21). In brief, whole cell Na\textsuperscript{+} current was recorded at room temperature (22 °C) using the following solutions. The internal solution contained aspartic acid (50 mmol/liter), CsCl (60 mmol/liter), Na\textsubscript{2}-ATP (5 mmol/liter), EGTA (11 mmol/liter), HEPES (10 mmol/liter), CaCl\textsubscript{2} (1 mmol/liter), and MgCl\textsubscript{2} (1 mmol/liter), with pH 7.4 adjusted with CsOH. The external solution contained NaCl (130 mmol/liter), CaCl\textsubscript{2} (2 mmol/liter), CsCl (5 mmol/liter), MgCl\textsubscript{2} (1.2 mmol/liter), HEPES (10 mmol/liter), and glucose (5 mmol/liter), with pH 7.4 adjusted with CsOH.

The voltage dependence of inactivation was determined after application of conditioning pulses (500 ms) applied once every 2 s to a series of voltages followed by a test pulse (20 ms) to voltages from −130 to −20 mV. In experiments designed to measure the voltage dependence of activation, external Na\textsuperscript{+} was reduced to 30 mm using \(n\)-methyl-glucamine as an Na\textsuperscript{+} substitute. Current was measured using test pulses (40 ms) from a holding potential of −100 mV to voltages ranging from −80 to +75 mV. Persistent Na\textsuperscript{+} channel current (\(I_{\text{sus}}\)) was measured as the tetrodotoxin (TTX; 30 \(\mu\)M)-sensitive current measured at 150 ms (Tyr\textsuperscript{1795} constructs) or 200 ms (all other constructs) during depolarization to −10 mV. Unless otherwise specified, the holding potential was −100 mV. \(I_{\text{sus}}\) was normalized to peak TTX-sensitive Na\textsuperscript{+} channel current measured at −10 mV and plotted as percentage of peak current in relevant figures. Membrane currents were measured using whole cell patch clamp procedures, with Axopatch 200B amplifiers (Axon Instruments, Foster City, CA). Capacity current and series resistance compensation were carried out using analog techniques according to the amplifier manufacturer (Axon Instruments, Foster City, CA). PClamp8 (Axon Instruments) was used for data acquisition and initial analysis. Data are represented as mean values ± S.E.

**Protein Expression and Purification**—Fusion proteins were transformed in BL21 (DE3) cells (Stratagene). Cells were grown to an \(A_{\text{600}}\) of ~0.6, and then expression was induced with the addition of isopropyl-D-1-thiogalactopyranoside and shaking for 72 h at 16 °C. After induction, the cells were harvested and resuspended in 20 mM Tris-Cl, 100 mM NaCl, pH 7.4, supplemented with EDTA-free protease inhibitor tablets (Roche Applied Science), DNase, MgCl\textsubscript{2}, and lysozyme. Following incubation at room temperature, the samples were sonicated, and the lysates were cleared by ultracentrifugation. The Na\textsubscript{v,1.5} EF-GST fusion proteins were further purified through affinity purification on GSTrap FF columns (Amersham Biosciences). GST eluate was thrombin-digested, and proteins were then further purified by gel filtration chromatography using a Superdex 75pg 16/60 column (Amersham Biosciences). Fractions that eluted at the appropriate time relative to previously analyzed protein standards were collected for fluorescence measurements. Mass spectrometry (matrix-assisted laser desorption ionization time-of-flight) was carried out to confirm sample purity and that the protein samples were the full-length polypeptide. Samples were collected at all stages, run on 4–20% SDS-polyacrylamide precast gels (Bio-Rad), and analyzed by Coomassie Blue staining. Protein concentration for the Na\textsubscript{v,1.5} EF was determined by absorbance at 280 nm using an extinction coefficient of 8370 M\textsuperscript{−1} cm\textsuperscript{−1}.

**Fluorescence Spectroscopy**—Fluorescence spectra were obtained on a PTI QuantaMaster spectrofluorometer in a 2-ml quartz cuvette (Hellma). Protein samples were at a concentration of 5 \(\mu\)M in buffer containing 20 mM Tris-Cl, 100 mM NaCl, pH 7.4, or the denatured protein in the same buffer plus urea at a concentration of 7.6 M. Intrinsic tryptophan fluorescence was excited at \(\lambda_{\text{ex}}\) = 295 nm and monitored for fluorescence emis-
Fluorescence quenching data were collected with the sequential addition of the 5 M acrylamide as the quencher. Stern-Vollmer plots were constructed according to the Stern-Vollmer equation,

\[ \frac{F_0}{F} = 1 + K_{SV} [Q] \]  

(Eq. 1)

where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of the quencher acrylamide at concentration \([Q]\), and \( K_{SV} \) is the Stern-Vollmer constant.

Analysis of Experimental Data—Analysis was carried out in Excel (Microsoft), Origin 7.0 (Microcal Software, Northampton, MA), and programs written in Matlab (The Mathworks, Natick, MA). Data are represented as mean ± S.E. Statistical significance was determined using an unpaired Student’s t test; \( p < 0.05 \) was considered statistically significant.

RESULTS

Computational Analysis of Na\(^{+}\) \( \alpha \)-Subunit COOH Terminus Predicts a Hydrophobic Interface—We previously generated a structural model of the Na\(^{+}\) \( \alpha \)-Subunit COOH terminus based on homology to the CaM amino-terminal lobe (14). The model predicts a structured proximal region containing six \( \alpha \)-helices (H1–H6), in which the first four helices are predicted to form two EF-hand pairs. One helix from each of these EF hands is predicted to form an interface with a helix from the opposite hand pair, H1 with H4 and H2 with H3 (Fig. 1A). Alignments using ClustalX (20) with default parameters predict significant structural homology between the first four predicted helices of the Na\(^{+}\) \( \alpha \)-Subunit COOH terminus and EF-hand proteins. The boxed regions indicate helical regions conserved between our model of the Na\(^{+}\) \( \alpha \)-Subunit COOH terminus and structures solved by NMR (1C07 (EGFR substrate), 1C7V (calcium vector protein), and 1TRF (troponin C:Apo)) or x-ray crystallography (1EXR (Ca\(^{2+}\)-bound calmodulin), 1BR1 chain B (smooth muscle myosin), and 1BJF chain A (neurocalcin)). Nonconserved helical regions extend beyond the boxed regions for individual proteins. Residues are shaded purple when identical among all proteins examined, red for conserved amino acids, pink for similar residues, and red text for weakly similar amino acids.

FIGURE 1. Structural homology between the first four helices of the Na\(^{+}\) \( \alpha \)-Subunit COOH terminus and EF-hand proteins. A, structural model of the proximal Na\(^{+}\) \( \alpha \)-Subunit COOH terminus. Shown are the first four predicted helices (H1–H4), which are predicted to form two EF-hand pairs. One helix from each of these EF hands is predicted to form an interface with a helix from the opposite hand pair, H1 with H4 and H2 with H3. B, sequence alignment of the Na\(^{+}\) \( \alpha \)-Subunit COOH terminus (residues 1783–1859) demonstrates structural homology with EF-hand proteins. The boxed regions indicate helical regions conserved between our model of the Na\(^{+}\) \( \alpha \)-Subunit COOH terminus and structures solved by NMR (1C07 (EGFR substrate), 1C7V (calcium vector protein), and 1TRF (troponin C:Apo)) or x-ray crystallography (1EXR (Ca\(^{2+}\)-bound calmodulin), 1BR1 chain B (smooth muscle myosin), and 1BJF chain A (neurocalcin)).
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We thus first focused on this locus. Mutation of this residue to glutamate (Y1795E) alters channel inactivation, as reflected in an increase in sustained channel activity. Current traces are illustrated in Fig. 3A, and the mutation-induced increase in sustained current (I\textsubscript{sUS}) in response to prolonged depolarization (right traces) is evident when compared with traces from wild type (WT) channels (left traces). Space-filling models of the side chain substitution at this residue within the model (Fig. 3B) illustrate the relative difference in size of the side chains of these amino acids (197 Å\textsuperscript{3} for tyrosine and 134.8 Å\textsuperscript{3} for glutamate (22)). Replacement of Tyr\textsuperscript{1795} by Glu increases I\textsubscript{sUS} in a manner that is very similar to previously reported changes in I\textsubscript{sUS} caused by the naturally occurring mutation Y1795C (10) (Fig. 3C and Table 1) that results in a slightly larger change in the size of the residue side chain (108.5 Å\textsuperscript{3} for the cysteine side chain (22)). We thus asked whether mutation-altered inactivation correlated with possible structural alteration of the H1/H4 interface and constructed a series of mutations to test this hypothesis (Fig. 3C). Substitution of Y1795 by either nonpolar residues (Y1795A) or nonaromatic, charged residues (Y1795E, Y1795R), in addition to small nucleophilic residues (Y1795C, Y1795S), significantly increased I\textsubscript{sUS} (Fig. 3C). However, substitution with other aromatic residues (Y1795F or Y1795W) showed no significant alteration in persistent current. Substitution gating.

**FIGURE 2.** Conservation of predicted hydrophobic interface between the first (H1) and fourth (H4) helices of Na\textsubscript{v}1.5 CT and EF-hand proteins. A, sequence alignment of predicted structural regions examined in this study. Shown are the predicted first and fourth helices. Asterisks below the alignment indicate hydrophobic residues conserved among aligned EF-hand proteins. B, structural model of Na\textsubscript{v}1.5 CT showing the first and fourth predicted helices. Side chains for residues examined in this study (Tyr1795, Trp1798, Ile1853, Leu1854) are shown as stick models.

**TABLE 1**

| Construct | I\textsubscript{sUS} (pM) | Activation V\textsubscript{s50} (mV) | Inactivation V\textsubscript{s50} (mV) |
|-----------|----------------|----------------|----------------|
| WT        | 0.07 ± 0.01 (14) | −25.1 ± 0.8 (5) 100 mV | −71.6 ± 1.7 (7) |
| Y1795E    | 0.24 ± 0.04 (6)  | 21.3 ± 1.8 (6) 100 mV  | −81.9 ± 0.9 (6) |
| Y1795S    | 0.19 ± 0.04 (8)  | −20.9 ± 0.5 (6) 100 mV  | −62.1 ± 1.3 (6) |
| Y1795H    | 0.24 ± 0.04 (5)  | 20.1 ± 2.1 (6) 100 mV  | −83.3 ± 0.7 (6) |
| Y1795A    | 0.34 ± 0.03 (8)  | 19.2 ± 1.5 (6) 100 mV  | −78.6 ± 1.1 (6) |
| Y1795F    | 0.28 ± 0.02 (6)  | −19.8 ± 1.1 (6) 100 mV  | −78.8 ± 0.7 (6) |
| Y1795C    | 0.36 ± 0.04 (14) | −24.6 ± 1.6 (6) 100 mV  | −72.1 ± 1.7 (6) |
| Y1795N    | 0.09 ± 0.01 (6)  | −25.9 ± 0.7 (6) 100 mV  | −70.5 ± 1.3 (6) |
| W1798E    | 0.46 ± 0.11 (4)  | 19.6 ± 1.8 (7) 100 mV  | −77.5 ± 0.7 (4) |
| W1798A    | 0.27 ± 0.02 (6)  | −24.9 ± 1.7 (9) 100 mV  | −82.2 ± 1.5 (4) |
| W1798F    | 0.10 ± 0.02 (4)  | −20.0 ± 2.1 (4) 100 mV  | −79.8 ± 0.5 (5) |
| W1798L    | 0.07 ± 0.01 (5)  | −22.5 ± 0.6 (4) 100 mV  | −83.0 ± 0.4 (4) |
| I1853E    | 1.85 ± 0.09 (14) | −17.5 ± 2.4 (2) 100 mV  | −95.6 ± 3.3 (4) |
| I1853A    | 0.06 ± 0.01 (4)  | −27.7 ± 2.0 (6) 100 mV  | −71.0 ± 0.8 (6) |
| I1854E    | 0.18 ± 0.04 (4)  | −23.8 ± 1.2 (2) 100 mV  | −74.6 ± 1.7 (6) |
| I1854A    | 0.09 ± 0.01 (4)  | −28.5 ± 1.5 (6) 100 mV  | −66.1 ± 1.7 (6) |
| Y1795A/11853E | 1.65 ± 0.10 (5)   | −21.4 ± 1.0 (6) 100 mV  | −97.2 ± 2.3 (7) |

\* p < 0.05.
Brugada syndrome (10), results in an increase in $I_{\text{SUS}}$ despite its aromatic nature (Fig. 3C and Table 1). This can be attributed to the at least partial positive charge that histidine would be expected to have in the cytoplasm. Thus, our data suggest that there is a spatial and hydrophobic importance to residues predicted to be near the interface.

The effects of the Tyr1795 mutations on sustained current primarily reflect changes in inactivation that follow channel openings, but we also detected mutation-induced changes in the voltage dependence of steady-state inactivation with only modest changes in the voltage dependence of activation (Table 1). The importance of the presence of an aromatic ring or charged residue at position 1795 in the maintenance of inactivation gating raises the possibility that spacing between helices H1 and H4, determined in part by the presence of an aromatic ring at residue 1795, may be critical to channel gating and, in turn, that a putative H1/H4 interface may play a key role in the structural integrity of the COOH terminus domain.

**Mutation of Trp1798 Disrupts Channel Gating**—If integrity of an H1/H4 interface is key to control of inactivation, mutations of other hydrophobic residues predicted to form the interface would be expected to cause similar or more severe alteration in gating (inactivation), depending on the importance of the mutated residue to the integrity of the interface. We thus systematically studied the functional consequences of mutation of additional residues predicted to be within (Fig. 2B), and possibly critical to, the interhelical interface: Trp1798 (helix 1) and Ile1853 and Leu1854 (helix 4).

The functional consequences of the Trp1798 mutations are very similar to those that accompany mutation of Tyr1795, affecting inactivation with relatively minor effects on channel activation (Table 1). Introduction of nonaromatic residues (W1798A and W1798E) produced significant increases in $I_{\text{SUS}}$ (Fig. 4B) with accompanying negative shifts in steady-state inactivation (Fig. 4C). Similar to changes made at residue 1795, conservation of the aromatic ring at residue 1798 (W1798F) did not alter $I_{\text{SUS}}$ but, in contrast with the Y1795F mutation, did shift steady-state inactivation. Because Trp1798 is predicted to be more integral to the putative hydrophobic interface than Tyr1795, we also replaced the native tryptophan by a leucine, which is a large hydrophobic, but not aromatic, residue. We found that the W1798L mutation did not increase $I_{\text{SUS}}$ (Fig. 4B). However, this mutation, similar to the W1798F mutation, did produce a negative shift in the voltage dependence of steady state in activation (Table 1).

**Mutations of Hydrophobic Residues on the Partner Helix Alter Inactivation**—We next tested two H4 residues predicted to be critical to the interface within the framework of our computational model: an isoleucine at residue 1853 (Ile1853) and a leucine at residue 1854 (Leu1854). Residues at similar loci on E4-hand proteins are conserved hydrophobic residues (Fig. 2A). Based on the linear sequence of the protein, one might expect similar effects when either residue is mutated. However, the modeling of the protein structure places residue Ile1853 at a location that is more critical to the putative interhelical interface and thus predicts that mutation of Ile1853 may have a greater impact on the hydrophobic interface than mutation of residue Leu1854. Our functional experimental data support the predictions of the model. We find that mutation of each of these residues has marked consequences on channel gating; however, mutation of Ile1853 causes much greater disruption of inactivation, as reflected in increased $I_{\text{SUS}}$, as well as the voltage dependence of steady-state inactivation, than comparable mutation of residue Leu1854. Replacement of Leu1854 by a hydrophilic glutamate residue (L1854E), but not a nonpolar alanine residue (L1854A), results in a small, but significant, increase in $I_{\text{SUS}}$ with modest effects on steady-state inactivation (Fig. 5, A, C, and D). Similarly, mutation of residue I1853 to a nonpolar residue (I1853A) did not have significant effects of inactivation, but mutation to the polar residue glutamate (I1853E) results in a dramatic increase in $I_{\text{SUS}}$ as well as marked changes in the voltage dependence of steady-state inactivation (Fig. 5, A, C, and D). $I_{\text{SUS}}$ recorded for the I1853E mutation is almost 10-fold greater than that recorded for L1854E mutant channels. As with other mutations of hydrophobic residues in the putative H1/H4 interface, the functional consequences of the I1853E mutation are much more pronounced for inactivation than for activation (Table 1).

**Thermodynamic Cycle Analysis Is Consistent with Predicted Residue Interactions**—We next tested the proximity of residues on opposite helices using thermodynamic cycle analysis with methodology described by McPhee et al. (23). Briefly, changes in the free energy ($\Delta G$) of the inactivation process of individual mutations and a double mutation are computed from the percentage of $I_{\text{SUS}}$ reported in Table 1. The change in $\Delta G$ caused by these mutations relative to wild type is then calculated and compared. If the changes in $\Delta G$ of the inactivation process for
the individual mutants equal the change in free energy of the double mutant, then these processes are independent, and the residues mutated do not interact. If the changes in $\Delta G$ of inactivation for the mutants are significantly greater than or less than additive, this indicates that these changes in $\Delta G$ are dependent on one another, and these residues interact. As described by McPhee et al. (23), the equilibrium constant for the transition between the open and the inactivated state, $K_{eq}$, can be determined by the equation, $K_{eq} = (1/I_{SUS}) - 1$, and the free energy of inactivation is then $\Delta G = -RT \ln K_{eq}$. We used this approach, and calculated values for $K_{eq}$ and $\Delta G$ were from the values reported in Table 1, and the S.E. was propagated throughout the subsequent calculations.

We were limited in double mutants that expressed functional current but were able to test the double mutation containing Y1795A and I1853E. Mutations at both of these loci caused significant increases in sustained current, and the model predictions (Fig. 2B) placed them close to one another. The single mutations Y1795A (Fig. 6, A and B) and I1853E (Figs. 5, A and B, and 6B) by themselves both resulted in significant increases in sustained current (as shown previously) as well as shifting steady-state inactivation in the depolarizing direction (Table 1). Expression of the construct containing both mutations (Y1795A/I1853E) resulted in increases in $I_{SUS}$ and depolarizing shifts in steady-state inactivation (Fig. 6, A–C) similar to that of the I1853E mutation by itself. In the thermodynamic cycle, the individual mutations resulted in $\Delta(\Delta G)$ of 0.94 ± 0.10 kcal/mol for Y1795A and 1.93 ± 0.09 kcal/mol for I1853E (Fig. 6D). If these mutations acted independently on the free energy of inactivation, we would expect a $\Delta(\Delta G)$ of inactivation for the double mutation similar to the sum of the free energy changes in either direction around the thermodynamic cycle, 1.87 ± 0.09 kcal/mol. However, we calculated a $\Delta(\Delta G)$ of 2.87 ± 0.13 kcal/mol for the construct containing both mutations (Y1795A/I1853E), a value significantly larger than additive. The results of this analysis provide evidence that these residues, Tyr$^{1795}$ and Ile$^{1853}$, interact with one another in support of an H1/H4 interhelical interface.

Biochemical Evidence for an H1/H4 Hydrophobic Interface—The predicted EF-hand pair in the Na$_v$1.5 COOH terminus contains a single tryptophan, Trp$^{1798}$, which is important not only because our model predicts it to be integral to the hydrophobic interface but also because its intrinsic fluorescence can be used to probe and report the nature of the environment in which it is located (13, 24, 25). We thus prepared a GST fusion protein consisting of residues predicted to form the EF-hand pair (residues 1786–1863) and carried out experiments using Trp$^{1798}$ as a reporter of its environment. Expression of this protein, cleavage of the GST tag, and subsequent size exclusion chromatography provided us with high yields of soluble protein (referred to as Na$_v$-1.5 EF) well suited for fluorescence studies (see Fig. 7).
First, we tested whether the I1853E and L1854E mutations affect the amount of soluble protein we were able to obtain for the Na\textsubscript{v1.5} EF hand construct (see “Materials and Methods”), relative to the wild type protein. Introduction of either mutation causes almost all of the fusion protein to be expressed in the insoluble pellet (see Fig. 7). Subsequent purification of the soluble fraction resulted in insignificant yield of purified protein containing either mutation (Fig. 7), possibly due to negative structural effects in addition to the adverse functional effects of these mutations.

Next we investigated fluorescence of Trp\textsuperscript{1798}. We could selectively excite (\(\lambda = 295\) nm) Trp\textsuperscript{1798} of the Na\textsubscript{v1.5} EF protein and monitor emission spectra. The Na\textsubscript{v1.5} EF protein, excited at 295 nm, has an emission maximum at 333 nm (Fig. 8B, solid line), consistent with Trp\textsuperscript{1798} located within a relatively hydrophobic environment (26). This is consistent with the model-predicted location of Trp\textsuperscript{1798}, which is near the C terminus of the first helix (H1) (Fig. 8A). The same protein in a urea concentration sufficient for complete denaturation (7.6 M) resulted in an emission maximum of 351 nm (Fig. 8B, dashed line), consistent with an unfolded protein where Trp\textsuperscript{1798} is fully exposed to an aqueous medium (26). This provides evidence that our preparation of Na\textsubscript{v1.5} EF in the native state is probably a folded protein in which Trp\textsuperscript{1798} is partially buried.

The fluorescence curves in Fig. 8B are normalized for the maximum fluorescence of either the native or the denatured protein. However examination of the fluorescence data before normalization provides additional information about the environment of residue Trp\textsuperscript{1798}. These data reveal that the denatured protein has significantly higher fluorescence intensity relative to the native preparation (Fig. 8C), indicating that, in the case of the folded protein, there is considerable endogenous “quenching” of the Trp fluorescence. Such an effect has been demonstrated previously in the hydrophobic core of homeodomains (27, 28), where a Trp has been shown to play a key role in structural integrity. In these proteins, the quenching of the Trp fluorescence was found to be the result of interactions with neighboring aromatic residues. In the case of Na\textsubscript{v1.5} EF, the structural model predicts several aromatic residues within the H1/H4 interface, with the tyrosine at 1795 and the phenylalanine at 1801 being closest to Trp\textsuperscript{1798} (Fig. 8A). Thus, the endogenous fluorescence quenching of the protein in the native state provides additional evidence that our protein is folded in a conformation in which there are neighboring aromatic residues to Trp\textsuperscript{1798}.

We further probed the environment of Trp\textsuperscript{1798} in the Na\textsubscript{v1.5} EF protein with experiments in which additional fluorescence quenching was assayed using a chemical probe. Here the ability of a compound to quench the intrinsic tryptophan fluorescence provides information about the accessibility of tryptophan to the quenching molecule. We used acrylamide as our quenching molecule, because it is a large neutral molecule that has limited ability to penetrate the protein matrix, limiting its access for quenching primarily through aqueous pathways. The Stern-Volmer plot generated from the acrylamide quenching data is illustrated in Fig. 8D. The Stern-Volmer constant, \(K_{SV}\), derived from the linear fit is 8.3 ± 0.5 M\textsuperscript{-1} for the native protein and 17.0 ± 3.3 M\textsuperscript{-1} for the denatured protein.
the tryptophan is not accessible and 17.5 for free tryptophan (24). The $K_{SV}$ we found for the native protein thus describes a tryptophan that is partially accessible, consistent with fluorescence emission maximum and the structural model.

Thus, our fluorescence emission spectra and acrylamide quenching experiments provide biochemical evidence in support of the model-predicted hydrophobic interface as well as the key location of residue Trp1798 in it, and a key role of this residue, and in turn the hydrophobic interface, in preserving channel gating is provided by our electrophysiological experiments.

**DISCUSSION**

In this study, using a structural model as a guide, we carried out biochemical and electrophysiological experiments, the results of which support the presence of a predicted EF-hand motif in the proximal COOH terminus of the NaV1.5 sodium channel and demonstrate the importance of its stabilization by an interhelical hydrophobic interface. Furthermore, we show that perturbation of this hydrophobic interface results in the destabilization of sodium channel inactivation, elucidating a novel mechanism through which sodium channel mutations may manifest their deleterious effects.

A homology model of the sodium channel COOH terminus previously generated by us suggested a structured region in the proximal COOH terminus with homology to EF-hand pairs (14). The presence of EF hands in the sodium channel COOH terminus was first identified by Babitch (29). Subsequently, circular dichroism data (14) and NMR studies (30, 31) have confirmed both the secondary and tertiary structure consistent with the presence of EF-hand pairs. Experimental data have validated not only the predictions of the model (14) but also predictions of spatial separation between residues on two of the helices (H1 and H4) central to the present study (21). In addition, models generated via another methodology (supplemental Fig. 1) (see, on the World Wide Web, www.sbg.bio.ic.ac.uk/~phyre/) were very similar to our model, particularly in the predicted helices H1 and H4. Although the model was generated for the COOH terminus of the cardiac sodium channel (NaV1.5), this region is highly conserved among several voltage-gated sodium channels, particularly within the predicted first and fourth helices (H1 and H4) (supplemental Fig. 2). Importantly, several disease-associated mutations that cause inherited epilepsies have been reported in this region of the brain NaV1.1 sodium channels (15–17), and, like the mutations in NaV1.5, these mutations also disrupt inactivation, raising the possibility that a putative functional role of this region with respect to channel gating may be conserved among voltage-gated sodium channels.

The EF-hand pair domain is a commonly occurring tertiary structure where amphipathic helices pack against one another to form a hydrophobic core surrounded by polar residues on the outside surface of the structure. These polar residues aid in solubility but also may participate in electrostatic interactions that affect protein function (see below). Our homology model predicts a structure consistent with EF-hand structure. Furthermore, hydrophobic residues conserved among EF-hand proteins (Fig. 1A) and relatively conserved in our model of the NaV1.5 C terminus have been shown to play functional roles in other proteins. Several studies have examined the hydrophobic core residues of EF-hand proteins and have demonstrated functional roles, including effects on Ca$^{2+}$ binding exchange as well as roles in protein stabilization (32–35).

Our results consistently show that mutation of the H1/H4 interface preferentially affects inactivation gating (Table 1), and mutation-altered sustained Na$^{+}$ channel current ($I_{\text{sw}}$) tracks most closely with mutations that affect the interface. How might disruption of the packing of the proximal EF-hand pairs in the COOH terminus of the sodium channel have such a dramatic impact on inactivation gating? Stabilization of inactivation is dependent on intramolecular interactions between the NaV1.5 cytoplasmic III-IV linker and the COOH terminus domain, and this interaction is mediated through the predicted sixth a helix (H6) of the COOH terminus (12). Recent studies demonstrate that H6 interacts with the EF-hand domain (H1–H4) (30). It may be possible that while the III-IV linker binding region in the COOH terminus is distal to the EF-hand domain, allosteric coupling could affect III-IV linker/COOH terminus binding kinetics and hence inactivation gating.

Another possibility is that disruption of the EF hand packing in the proximal COOH terminus domain alters a surface of negative charge, predicted by the model of the COOH terminus that is presented to intracellular components of the sodium channel. Consequential altered electrostatic interaction might then cause the perturbation in gating that we detect. A third possibility is that stabilization of H1–H4 packing by the hydrophobic interface is necessary to coordinate intermolecular interactions between the sodium channel and auxiliary proteins. The COOH terminus of the sodium channel has been shown to interact with a number of proteins that can modulate various properties, typically gating or trafficking (36). In addition, a region containing H4 has been shown to interact with the COOH-terminal region of the $\beta$1 subunit, and a mutation in NaV1.1 that disrupts this interaction has been shown to cause epilepsy (17). Finally, paired EF-hand motifs have been implicated in several intra- and intermolecular protein interactions (37–41), including modulation of voltage-gated sodium channels. Taken together, this raises the possibility that at least some of the gating changes induced by mutation of the H1/H4 hydrophobic interface might be the result of adverse effects on protein-protein interactions, in addition to or superceding more direct effects on gating caused by disruption of the proposed hydrophobic core of the EF-hand motif. Furthermore, the preferential sensitivity of $I_{\text{sw}}$ versus steady-state inactivation to mutations that disrupt this hydrophobic core suggests differential roles of COOH terminus structure in the modulation of inactivation that results from transitions between open and/or closed states.

In summary, this study demonstrates that hydrophobic residues located within a predicted EF-hand motif play a critical role in sodium channel inactivation gating and provides a structural basis for the altered function of disease-linked mutations in the NaV1.5 COOH terminus as well as other sodium channel isoforms.

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