Secondary Structure of the Human Cardiac Na⁺ Channel C Terminus

EVIDENCE FOR A ROLE OF HELICAL STRUCTURES IN MODULATION OF CHANNEL INACTIVATION\(^*\)

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Little is known about the structure of the C terminus of the human cardiac voltage-gated sodium channel α subunit (SCN5A), but disease-linked mutations within this 244-amino acid intracellular region of the channel have marked effects on channel inactivation. Here we report a structural analysis of the C-terminal tail of the cardiac Na⁺ channel that sheds new light on mechanisms that control its inactivation gating. Homology modeling of the SCN5A C terminus predicts predominant α-helical structure (six helices) in the proximal half of this intracellular tail but little structure in the distal half. Circular dichroism of isolated and purified C terminal supports this prediction. Whole cell and single channel patch clamp recordings of wild type and mutant α subunits co-expressed with the hβ1 subunit in HEK 293 cells indicate that truncation of the distal, nonstructured, C terminus (L1921stop mutant) reduces current density but does not affect channel gating (n = 6). In contrast, truncation of the sixth helix containing a concentration of positively charged residues along with the distal C terminus (S1885stop mutant) also reduces current density but, in addition, has profound and selective effects on inactivation (no effect on activation). Channel availability is shifted (~11 ± 0.6 mV), and there is a 10-fold increase in the percentage of channels that burst (fail to inactivate) during prolonged depolarization (0.025% S1885stop (n = 7) versus 0.0028% wild type (n = 9), p < 0.005). These results suggest that the charged structured region of the SCN5A C terminus plays a major role in channel inactivation, stabilizing the inactivated state.

Voltage-gated Na⁺ channels are integral membrane proteins (1, 2) that not only underlie excitation in excitable cells but determine the vulnerability of the heart to dysfunctional rhythm by controlling the number of channels available to conduct inward Na⁺ movement (3). The Na⁺ channel α subunit, which forms the ion-conducting pore and contains channel gating components, consists of four homologous domains (I–IV) (4). Each domain contains six α-helical transmembrane repeats (S1–S6), for which mutagenesis studies have revealed key functional roles (5). Na⁺ channel inactivation 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 (6–9). NMR analysis of this inactivation linker (gate) in solution has revealed a rigid helical structure that is positioned such that it can block the pore, providing a structural explanation of the functional studies (10). Inherited mutations of the III/IV linker in the cardiac Na⁺ channel disrupt normal fast inactivation and cause cardiac rhythm disturbances in the Long QT syndrome (11–13).

Subsequent analysis of additional Na⁺ channel mutations linked both to Long QT syndrome and another inherited arrhythmia, the Brugada syndrome, has revealed a critical and unexpected role of the C-terminal tail of the channel in the control of inactivation (14–19). Point mutations in the C terminus shift the voltage dependence of inactivation, change the kinetics of both the onset of and recovery from inactivation, alter drug-channel interactions, and reduce entry of channels into an absorbing inactivated state (14, 17, 20, 21).

Here we report a structural analysis of the C-terminal tail of the cardiac Na⁺ channel that sheds new light on mechanisms that control inactivation gating of this channel. Homology modeling of the C terminus, assuming similarity to the N-terminal domain of calmodulin, predicted that the C terminus would adopt a predominantly α-helical structure, a prediction verified by CD of a purified C terminus fusion protein. Functional studies revealed that only the proximal region of the C terminus, which contains all of the helical structure, markedly modulates channel inactivation but not activation. The distal C-terminal tail, which is largely unstructured, does not affect channel gating but affects the density of functional Na⁺ channels in the surface membrane. Our results suggest interactions between the structured region of the C terminus and other components of the channel protein that act to stabilize the channel in a pore-blocked inactivated state during membrane depolarization.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise noted, chemicals and reagents were obtained from Fisher or Sigma.

Subcloning/Expression—Oligonucleotide primers used to make PCR products for the C terminus (of SCN5A) expression systems are as follows (restriction sites underlined): 5’-primer (sense), GCATACGTCATATGGGAACATCTCAGGTGCGC, sequence beginning with SCN5A Ghu\(^{1735}\) including an NdeI restriction site; 3’-primer (antisense), CGTCAAGCTGCATACAGGTGAAGCTCACTAGT, sequence ending with SCN5A C-terminal Val\(^{2016}\); and CGTCAGCTCGAGTCACACGATGGACTCACG, sequence ending with NdeI restriction site and upstream stop codons. The NdeI/XhoI nucleotide segments were subcloned into corresponding sites of pET-28a\(^{+}\) (Novagen, Madison, WI). These constructs synthesize the protein with 21 additional amino acids at the N termini, including a poly(His) tag and a thrombin cleavage site. The pET-28a\(^{+}\) vectors were transformed into competent BL21(DE3) (Novagen) cells containing the T7 RNA polymerase trans-
ion and plated with kanamycin to select for the pET-28a(+) insert. Large scale (1-liter) cultures were grown at 37 °C in 2 X YT (yeast extract (5 g/liter), tryptone (8 g/liter), and salt (2.5 g/liter NaCl)) medium with kanamycin sulfate (30 μg/ml; Invitrogen). Once culture optical density at 600 nm (A600) was greater than 0.6, cells were induced with 1 mM isopropanol-β-D-thiogalactoside and further grown at 30 °C for 4 h.

**Purification**—Bacterial pellets were resuspended and lysed in a denaturing buffer (8 mM urea, 100 mM NaH2PO4, 10 mM Tris, pH 8.0), 4 ml of buffer of wet pellet, rocking at room temperature for 2 h. The solution was spun at 12,000 × g for 25 min at 21 °C. The supernatant was retrieved and added to a Ni2+–nitrilotriacetic acid resin slurry (Novagen), 1 ml of resin slurry/4 ml of lysate, and rocked at room temperature for 1 h. Resin was washed twice with 1 lysis volume with 8 mM urea buffer (8 mM urea, 100 mM NaH2PO4, 10 mM Tris, pH 6.3) and spun at 1800 × g for 2 min at 21 °C, and supernatant was discarded. The wash step was repeated with each of 2, and 0 mM urea buffers (urea, 100 mM NaH2PO4, 10 mM Tris, 0.1% laryl sulfate, 10% ethanol, pH 8.0). Beads were resuspended in 1 l lysate volume of elution buffer (100 mM NaH2PO4, 10 mM Tris, 0.05% laryl sulfate, 5% ethanol, 250 mM imidazole, pH 5.0) and rocked for 1 h at 21 °C. Beads were spun, and supernatant was recovered. Purified protein was dialyzed into 0.1 M imidazole buffer for biophysical analysis.

**Circular Dichroism**—Purified proteins were prepared as above. CD spectra were measured from 200 to 250 nm using a Jasco J-810 spectropolarimeter (Easton, MD). All measurements were carried out at 22 °C using a 0.1-mm path length quartz cuvette. Data were acquired at a resolution of 0.5 nm, bandwidth 1 nm. Eight scans were averaged at 100 nm/min, and buffer spectra were subtracted from the raw data.

**Computational Analysis**—A sequence homology search was done with the PSI-BLAST program with default parameters (23). The checkpoint file was then used in a following one-iteration run of the PSI-BLAST on the Protein Data Bank. Two families of proteins in the Protein Data Bank were identified as possible templates for the modeling of the three-dimensional structure of the first half of the C terminus peptide (from residue 1785 to 1885): the N-terminal domains of calmodulin and troponin C. These two structural domains are sequence-related and have essentially identical folding topology. The N-terminal domain of calmodulin (Protein Data Bank code 1vrv, chain A, residues 4–95 (24)) is related to the C-terminal peptide with a p value of 10−5, and the N-terminal domain of the troponin C (Protein Data Bank code 1a2x, chain A, residues 11–105 (25)) is related to the C-terminal peptide with a p value of 10−6. Previous work has demonstrated that protein pairs with p values less than 10−5 are highly likely to adopt the same fold (26). We thus have compared the query sequence with more than 10,000 protein structures before selecting the appropriate template for modeling (27, 28). Hence, we use the N-terminal domain of the calmodulin 1vrv (chain A residues 4–95) as the template to build a model structure for the first half of the C terminus peptide (residues 1785–1885). Although the template structure has two EF hands for calcium binding, many of the key acidic residues that are used for specific calcium binding in calmodulin are not conserved in the C terminus peptide, suggesting that the C terminus peptide does not bind to calcium as in calmodulin. The structure of the second half of the C terminus peptide is not known. Nevertheless, the secondary structure predictions have suggested that the structure of the second half of the C terminus peptide adopts a long helical structure plus a long tail of nonstructural region.

**Electrophysiology**—Methods for mutagenesis of the human heart Na+ channel α subunit, transient transfection of HEK 293, and patch clamp analysis of expressed channels were as described in previous publications (29). Single channel currents were recorded using the cell-attached configuration of the patch clamp with the following bath...
The body was raised. The residues against which the anti-channel antibody, because it does not contain the proximal His tags and the other against the C-terminal 17 amino acids of SCN5A (schematic) are shown in Fig. 1. His-tagged proteins are shown in B, and those containing the C terminus of SCN5A are shown in C. The shorter construct is not recognized by the sodium channel antibody, because it does not contain the residues against which the antibody was raised.

solution: 140 mM KCl, 5 mM HEPES, 1 mM MgCl₂ (pH 7.4). Electrode resistance was typically 5–7 megaohms when filled with internal solution (110 mM NaCl, 10 mM HEPES, pH 7.4). After establishing the cell-attached configuration (seal resistance >10 gigaohms), the membrane was held at a holding potential of −120 mV. Test pulses (−30 mV, 100 ms) were applied every 0.5 s. The probability of bursting (Pb) is estimated by the equation, \( Pb = (1 - (nb/t)^{1/n}) \times 100 \), where \( nb \), \( t \), and \( n \) represent the number of nonbursting sweeps and total sweeps and channels, respectively. Sweeps with long lasting and repetitive opening activity are defined as bursting sweeps.

### RESULTS

**Theoretical Structure**—We used the sequence of the C terminus of SCN5A for homology modeling. Since this section of the sodium channel is naturally present intracellularly (5), we assumed that it would fold similarly to proteins that are entirely cytoplasmic. Fig. 1A shows the PSI-BLAST alignment of the query sequence to the template (see “Experimental Procedures”). Secondary structure predictions of the C terminus peptide in Fig. 1A were obtained from the servers of PSI-PRED (30) and PHD (31). The predicted structure of the first half of the C terminus domain consists of six helices (H1–H6; Fig. 2). The three-dimensional model of the first half of the C terminus peptide is shown in Fig. 1B. Only the first five helices’ three-dimensional locations can be predicted. Although the sixth, long helix is present, its relative location cannot be calculated with accuracy.

The tertiary structure of this region of the channel (Fig. 1B) has been modeled with PrISM (32). As expected for a cytoplasmic protein, hydrophilic residues are generally facing the outside of the structure, while hydrophobic amino acids are protected in the globular core. One particularly interesting property of this globular structure is that it has a density of acidic residues on one end (H1 and H2) and basic residues, primarily in helix H6, that are likely to be distributed on the opposite side of the structure to form a bipolar arrangement of the real charges in the structure. However, since the predicted structure does not reveal the exact positions of the basic residues in the structure, the hypothesis of the charge distribution remains to be tested. Sequence comparison between SCN5A and other human sodium channel isoforms (Fig. 2) reveals that those regions responsible for this suggested polarity are highly conserved, particularly the acidic rich domain, containing H1 and H2. On the other hand, the sequences of the distal half of the C-terminal domain after H6 are diverse with less conserved sequence features and extensive insertions and deletions. This is in agreement with the expectation that the distal half of the protein is not structured. If the functional properties of the C terminus are structurally dependent, then the distal half of the tail is not likely to be involved in channel gating (see below). Fig. 1C illustrates, in schematic format, the relationship be-

### Table 1

|                | α-Helix | β-Sheet | Nonstructure | Program |
|----------------|---------|---------|--------------|---------|
| 1774–1884      | 60.7    | 2.8     | 36.4         | CONTINLL |
| Full-length    | 69.3    | 13.7    | 16.4         | CDSSTR  |

FIG. 3. Purification of SCN5AC terminus. Two C terminus peptides were expressed in BL21(DE3) cells, with N-terminal His tags, and purified over a nickel affinity column. Coomassie stain of the purified proteins is seen in A. Lane 1, the full-length construct; lane 2, the proximal half of the C terminus. Protein identity was confirmed by Western blot using two separate antibodies: one against the His tag and the other against the C-terminal 17 amino acids of SCN5A (schematic). His-tagged proteins are shown in B, and those containing the C terminus of SCN5A are shown in C. The shorter construct is not recognized by the sodium channel antibody, because it does not contain the residues against which the antibody was raised.

FIG. 4. CD spectra of C terminus regions of SCN5A. Comparison of the C terminus constructs’ CD spectra. Each spectrum is represented as the mean residue molar ellipticity. The spectrum of the predicted structural region (residues 1774–1885, closed squares) shows more structural components, particularly α-helical content, relative to the full C terminus (residues 1774–2016, open squares), as seen by the increase in the ellipticity at 222 nm. The spectra were obtained by the subtraction of the buffer spectra from that of the sample.
tween the α subunit C-terminal tail and other previously identified functionally relevant sequences in the protein.

**Protein Purification**—Two constructs of the C terminus of SCN5A were made and subclassed into an expression vector containing a T7 viral promoter. One construct contained the entire length of the C terminus, residues 1774–2016, and the other included simply the proximal half, the region predicted to be enriched in structure, residues 1774–1920. Expressed in bacterial cells containing a viral polymerase transposon, the T7 promoter is constitutively transcribed when induced by isopropyl-β-D-thiogalactoside. We included an N-terminal His tag to be used for affinity purification over a nickel column. The constructs were difficult to purify under native conditions, due to insolubility of the protein as well as its sensitivity to proteolytic degradation, particularly in the case of the full-length construct. Consequently, bacterial pellets were lysed under denaturing conditions and progressively washed into more native solutions while bound to the nickel resin. This gave high yields of purified protein in solution. Protein identity was confirmed by Western analysis with both antibodies raised against the poly(His) tag as well as the C-terminal 17 amino acids of SCN5A (Fig. 3). The purification of recombinant proteins from insoluble pellets under denaturing conditions has been used previously in studies in which structures have been solved through refolding, including the glutamate receptor (33), a membrane protein. However, in the case of the full-length construct for the SCN5A C terminus, it was also possible to purify a very small amount of protein from the soluble fraction.

**Circular Dichroism**—Purified proteins were dialyzed to 30 mM buffer without imidazole, since the compound is known to rotate light in the ultraviolet range. Each protein spectrum is the average of eight scans minus the spectrum of the buffer solution. The CD spectra for the two constructs are shown in Fig. 4. The full-length C terminus (Fig. 4, open...
two-thirds/H9251/H11002
prolonged depolarizing step (150 ms at WT (n = 14) and S1885stop (n = 24) (A) and L1921 stop (n = 5) (B) currents along with insets of nonnormalized currents at high gain. C, summary of the effects of C terminus truncation on average current density measured at the end of 150 ms for each construct. WT = -0.24 ± 0.05 pA/pF (WT n = 14), S1885stop = -1.78 ± 0.2 (n = 24, *), and L1921 stop = -0.27 ± 0.07 pA/pF (n = 5, p < 0.001), *, not significantly different from WT.

Fig. 6. Truncation of H6 enhances maintained Na\(^+\) channel current. A, averaged and normalized TTX-sensitive traces recorded upon a prolonged depolarizing step (150 ms at -10 mV) reveal changes in inactivation caused by H6 truncation. The panels show superimposed averaged channel currents (20 pA/50 ms (A) and 50 ms (B)) for WT and S1885stop (n = 24) (A) and L1921 stop (n = 5) (B). The insets show superimposed averaged traces recorded at high gain (20 pA/50 ms (A) and 50 ms (B)).

The abbreviations used are: WT, wild type; pA/pF, picoamps/picofarads.

squares) exhibits some definitive structure; however, it is not clearly dominated by any of the known secondary structures. As predicted by the computational model, the CD of the proximal half of the C terminus (Fig. 4, closed squares) is more clearly ordered and is very similar to spectra of all-helical proteins (known to have two distinct negative peaks at 208 and 222 nm (34)). In order to estimate the relative secondary structure content, we employed two computer algorithms, CONTINLL and CDSSTR (35, 36). The two programs showed the full-length construct to be composed of an even mixture of \(\alpha\)-helix and nonstructure, while the shorter construct is almost two-thirds \(\alpha\)-helical content (Table I). These data provide biophysical evidence that supports the predicted structure of the C terminus; the proximal half is predominantly helical, and the distal half is largely unstructured.

Functional Roles of C Terminus Structures: Electrophysiological Experiments—To further study the predictions of the model, we investigated the functional consequences of cleaving 1) a predicted nonstructured segment (construct L1921stop) and 2) a predicted helical portion of the C terminus along with the above unstructured region (S1885stop) using electrophysiological properties as an assay. The L1921stop mutation leaves the entire predicted structural region intact, while removing the nonstructured distal portion of the tail, and corresponds to the shorter construct studied above. Biophysical properties of channels encoded by this construct are similar to those encoded by full-length (WT)\(^1\) \(\alpha\) subunits (Fig. 5), not differing significantly from wild type in any observed property of the channel, with the exception of reduced peak current (-180.07 ± 19.7 pA/pF (n = 17) versus -403.18 ± 43.0 pA/pF (n = 21), respectively, p < 0.05). This rules out the role of the distal portion of the C terminus in channel gating. The S1885stop mutation, which cleaves positively charged residues between amino acids 1885 and 1921 and the predicted sixth helix, reduces peak currents (266.17 ± 17.3 pA/pF (n = 37) versus -403.18 ± 43.0 pA/pF (n = 21) for WT; p < 0.05) but, in addition, changes channel gating. The S1885stop mutation shifts steady state inactivation (-73.2 ± 0.6 mV (n = 12) for mutant versus -61.9 ± 0.2 mV (n = 8) for WT, p < 0.001) and slows the recovery from inactivation (time to half-recovery 3.01 ± 0.6 ms for WT (n = 8); 5.92 ± 0.06 ms for S1885stop (n = 5), p < 0.05; not shown). Perhaps most striking, however, is the pronounced effect of this mutation on Na\(^+\) channel currents measured during prolonged depolarization (Fig. 5, arrow, S1885stop traces). As can be seen more clearly in the inset of Fig. 6A and in the summary graph of Fig. 6C, this truncation induces a significant increase in sustained channel activity compared with both WT and L1921stop channels (1.78 ± 0.2 pF/pA (n = 24) for WT, 2.47 ± 0.05 pF/pA (n = 14) for mutant versus 0.24 ± 0.05 pF/pA (n = 14) for WT, p < 0.001; 0.27 ± 0.07 pA/pF (n = 5), L1921stop), suggesting in-

\(^1\)The abbreviations used are: WT, wild type; pA/pF, picoamps/picofarads.
creased bursting activity in these channels. Single channel experiments reveal that this is the case (Fig. 7). Shown in Fig. 7 are consecutive current traces showing activity of a small number (10) of channels in cell-attached membrane patches. Recordings from WT channels reveal few reopenings during prolonged depolarization, consistent with channels entering an absorbing inactivated state. Infrequently, however, reopenings are observed for WT channels, reflecting a low probability of entrance into a bursting mode of channel activity, even with the full-length C-terminal tail. However, bursting is observed much more frequently in the case of recordings from S1885stop channels (Fig. 7), causing a significant increase in the probability that channels burst when compared in a large number of experiments (Fig. 7). This type of channel activity, shown to be arrhythmogenic in inherited cardiac disorders (11), indicates that a significant fraction of channels no longer enter an absorbing inactivated (nonconducting) state during prolonged depolarization and underlies sustained whole cell channel activity illustrated in Figs. 5 and 6. While only a fraction of the peak channel activity, this small fraction of noninactivating channels can conduct currents that are sufficient to prolong cellular action potentials and trigger fatal cardiac arrhythmias (37).

DISCUSSION

We have combined theoretical modeling, CD measurements, and functional expression studies to probe the structure and functional role of the C terminus of the human voltage-gated Na⁺ channel α subunit in the control of channel gating. CD measurements provide strong support for the computational predictions of the C terminus structure, and the functional studies provide new insights into mechanisms by which these structures may contribute to the control of channel gating.

The agreement between theoretical prediction and experimental measurement of the C terminus structure is strong. The CD data show that the first half of the protein is well structured and mostly helical, and the distal half of the protein is largely unstructured, supporting the predictions of the homology modeling. Because the distal half does not affect either activation or inactivation gating, our results suggest that without coordinated structure, this region of the C terminus does not interact with other channel components responsible for gating. On the other hand, the computational work suggests that the proximal half of the protein adopts a fold similar to calmodulin with the charged residues distributed in a bipolar pattern. Thus, the computational work predicts that the proximal helices may play important roles in controlling channel gating.

The predicted structure reveals several observations regarding the charge distribution within the C terminus. First, similarly charged residues in general occupy positions on a common side of helices. This is particularly true in H1 and H2 as
the last few years, several mutations linked to these diseases disturbances, Long QT syndrome and Brugada syndrome. Within inactivation gate forms a hydrophobic interaction with its re- inactivation with no reported effects on channel activation; and all mutations are in the acid-rich first five helices of the proximal region of the C terminus; all cause changes in channel inacti- region of the C terminus specifically modulates channel inacti-

Functional studies provide support for this speculation and suggest that C-terminal structures play an important and specific role in controlling channel inactivation. In our experiments, truncation (S1885stop mutation) of the model-predicted sixth helix, which contains positive charges in the end of the proximal half of the C terminus, caused significant changes in inactivation but not activation gating. Most notable was a significant increase in the fraction of channels that failed to enter an absorbing inactivated state during maintained depolarization. This fraction of channels is evident as the mutation-induced increase in maintained current in Fig. 6 and the increase in bursts of single channel activity seen in Fig. 7. These changes in inactivation occur despite the fact that the voltage dependence of activation of the channel is not affected. In contrast, however, the voltage dependence of inactivation is shifted toward more negative potentials by the S1885stop mutation. This means that less energy is expended (more modest depolarization) in reducing the availability (increasing the fraction of closed state inactivated channels). Additionally, this truncation slows the recovery of channel from the inactivated state by hyperpolarization. These latter effects of the truncation of H6 are consistent with stabilization of the closed inacti-

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