THE KCNQ1 (KV7.1) C-TERMINUS, A MULTI-TIERED SCAFFOLD FOR SUBUNIT ASSEMBLY AND PROTEIN INTERACTION

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The Kv7 subfamily voltage-dependent of distinct potassium channels, from subfamilies by dint of its large intracellular Cterminus, acts to regulate excitability in cardiac and neuronal tissues. KCNO1 (Kv7.1), the founding subfamily member, encodes a channel subunit directly implicated in genetic disorders such as the long QT (LQT) syndrome, a cardiac pathology responsible for arrhythmias. We have used a recombinant protein preparation of the Cterminus to probe the structure and function of this domain and its individual modules. The Cterminus proximal half associates with one calmodulin (CaM) constitutively bound to each subunit where CaM is critical for proper folding of the whole intracellular domain. The distal half directs tetramerization, employing tandem coiledcoils. The first coiled-coil complex is dimeric that undergoes concentration-dependent association to form a dimer of dimers. The outer coiled-coil is parallel tetrameric, whose details elucidated based on been 2.0 crystallographic data. Both coiled-coils act in a coordinate fashion to mediate the formation and stabilization of the tetrameric distal half. Functional studies including characterization of structure-based and LQT mutants prove the requirement for both modules and point to complex roles for these modules including folding, assembly, trafficking and regulation.

The KCNQ channels represent a subfamily of voltage-gated K⁺ channels (Kv), whose members (Kv7.1-5) are expressed in a wide variety of tissues. These channels play a major role in brain and

cardiac excitability through the modulation of the cardiac potential waveform, the regulation of action potential generation and propagation, the tuning of neuronal firing patterns and the modulation of neurotransmitter release (1,2). Mutations in human KCNQ genes lead to major cardiovascular and neurological disorders such as the cardiac long QT syndrome (LQTS) or neonatal epilepsy (3).

Like all Kv channels, the KCNQ α subunits share a common core structure of six transmembrane segments with a voltage-sensing domain (S1-S4) and a pore domain (S5-S6) (see figure 1A), likely to approximate the mammalian Kv1.2 channel α structure described by MacKinnon and coworkers (4). Often, KCNQ α is in complex with the integral membrane auxiliary subunit, known as KCNE1, IsK or MinK. This protein alters channel properties such as single channel conductance and activation kinetics while increasing channel density in the plasma membrane (5).

Several structural features of the Kv7 α family members distinguish them from the larger Kv superfamily. In particular, their primary sequence encodes a large (300 –500 residues), intracellular Cterminus (figure 1A). Sequence analysis predicts four helical regions (dubbed A - D) present in all family members (6). Helices C and D are thought to form coiled-coil assemblies. Yeast two-hybrid screens for proteins interacting with the C-terminus revealed CaM, the ubiquitous Ca²⁺ sensor protein, as binding partner (6-8). Moreover, constitutively associates with the channel (6-10). Helices A and B encode the binding site for CaM and CaM association is required for proper channel assembly and function (6,7,9). LQT mutations that disrupt or significantly weaken the CaM interaction result in little or no complex and little channel expression in live cells (8,11). Thus, CaM acts as additional auxiliary subunit of the KCNQ channel complex. Additionally, $\text{Ca}^{2^+}\text{-CaM}$ is a Ca^{2^+} sensor for KCNQ1 function, transducing Ca^{2^+} signals to stimulate I_{KS} channels and producing a Ca^{2^+} -dependent left shift in the voltage-dependence of channel activation. This $\text{Ca}^{2^+}\text{-sensitive }I_{KS}\text{-current}$ stimulation could increase the cardiac repolarization reserve, preventing the risk of ventricular arrhythmias.

Biochemical and functional studies have identified Kv7 C-terminal regions important for channel tetramerization and trafficking (12-16) based on deletion, truncation and mutagenesis. Little work has directly examined on a protein level the postulated structural modules and their functional correlates. What precisely is the C-terminus' tertiary and quaternary organization and what does it do that distinguishes it from the other Kv subfamilies? Using a recombinant bacterial co-expression system (8), we have dissected the KCNQ1 C-terminus protein complex. Our findings suggest that the KCNO1 C-terminus may be divided into two parts where the membrane proximal half is important for functional expression, folding, and gating of the channel but not oligomerization while the membrane distal half directs folding, oligomerization, partner specificity and trafficking to the plasma membrane. This C-terminus is a multifunctional platform, employing relatively simple structural modules to assemble a channel with high specificity in an apparent hierarchical manner.

EXPERIMENTAL PROCEDURES

General subcloning and mutagenesis. For bacterial expression vectors, PCR was used to engineer BamHI and NotI restriction sites onto the specific human KCNQ1 gene fragments. PCR product was ligated into doubly digested (BamHI and NotI) modified pETDuet vector. This pETDuet vector contains a 8xHis-Tag sequence and TEV protease recognition sequence upstream to multiple cloning site I with CaM inserted into multiple cloning site II. Positive clones were identified by restriction analysis and subsequently sequenced in all DNA constructs described below. In addition, all site-directed mutagenesis was performed by the QuikChange (Stratagene) method. Altered sequences were confirmed by DNA sequencing.

Subcloning of Δ helix C. The KCNQ1 sequence in a pcDNA3 vector was used as a template for QuikChange where primers were designed to delete helix C (residues 548-565). This modified vector then served as the template for PCR of an insert for the pETDuet vector. of $\triangle loop$. Construction was in two steps. First, PCR was used to engineer BamHI and EcoRI restriction sites onto a KCNQ1 gene fragment (residues 352-386). PCR product was ligated into a doubly digested (BamHI and EcoRI), modified pETDuet vector. Then, PCR was used to engineer EcoRI and NotI restriction sites onto an additional gene fragment (residues 504-622). This PCR product was ligated into doubly digested (EcoRI and NotI) modified pETDuet vector already contained the fragment encoding residues 352-386. of C-terminus-GCN4-LI chimera. Subcloning was in two steps. First, PCR was used to engineer BamHI and EcoRI restriction sites into the KCNQ1 gene fragment (residues 352-594). PCR product was ligated into doubly digested (BamHI and EcoRI) modified pETDuet vector. Then, the GCN4-LI sequence (encoding IEDKLEEILSKLYHIENELARIKKLLG) was amplified by primers containing EcoRI and NotI sites in the 5'and the 3' flanking regions, respectively. GCN4-LI PCR product was ligated into doubly digested (EcoRI and NotI) modified pETDuet vector that already contained DNA encoding residues 352-594. Subsequently, the EcoRI restriction site was deleted by mutagenesis.

Expression, and purification of the various KCNQ1 C-terminus/ CaM complexes. Transformed E. coli BL-21 Tuner (Novagen), containing the "RIL" Codon PlusTM plasmid (Stratagene) cells were grown at 37° C in LB media, containing 100 ug/ml ampicillin, and 34 µg/ml chloramphenicol. Upon reaching an A_{600} of 0.3, the temperature was lowered to 16° C, and growth continued until the culture reached an A_{600} of 0.6. Protein expression was induced with 135 µM IPTG. Cells were harvested after 14 h by centrifugation, frozen, and suspended in 120 ml of lysis buffer, buffer L (300 mM NaCl, 50 mM sodium phosphate pH 8), 1 mM PMSF). After lysis by microfluidizer (Microfluidics), cell debris was removed by centrifugation at 20,000 x g. The soluble fraction was loaded onto a preequilibrated metal chelate Ni²⁺-CAM (Sigma) column (buffer A: 300 mM NaCl, 50 mM sodium phosphate, pH 8), at a flow rate of 1.0 ml/min. The

column was washed with buffer A, containing 10 mM imidazole, until a stable base line was achieved. After elution with buffer A, supplemented with 125 mM imidazole, the protein eluate was then subjected to TEV protease in a ratio of 1:150. The proteolysis continued for 12 h. Subsequently, the sample was diluted 4-fold with 10 % glycerol and loaded onto a pre-equilibrated SP Sepharose (Amersham Biosciences) column (buffer S: 50 mM NaCl, 20 mM Tris pH 7.5). The column was then washed with buffer S, and fractions were eluted with a shallow linear gradient of buffer S, containing 50-600 mM NaCl. Fractions were pooled and applied to a pre-equilibrated Superose 6 gelfiltration column (Amersham Pharmacia) with buffer F (150 mM NaCl, 20 mM Tris pH 7, 5 1 mM DTT). The elution peak was concentrated to 3 mg ml⁻¹ using spin concentrators (Vivascience), divided into aliquots and flash-frozen in liquid N₂. For purification of Δ helix D, Δ helices C-D, and Δ loop/CaM complexes, a Superdex-200 gel-filtration column was employed instead of the Superose-6 gel-filtration column.

Subcloning, expression, and purification of GST-helix C and GST-helices C-D. Both KCNO1 gene fragments (residues 535-572 or 535-622, respectively) were amplified by PCR with primers containing BamHI and NotI sites in the 5'and the 3' flanking regions, respectively. PCR product was ligated into doubly digested (BamHI and NotI) pGEX 4T-1 vector (Amersham Bioscience). Expression was as above. Cells were suspended in PBS (pH 7.4) with 1 mM PMSF. After lysis, cell debris was removed by centrifugation. The soluble fraction was loaded onto a pre-equilibrated glutathione sepharose column (buffer A: PBS pH 7.4), at a flow rate of 1.0 ml/min. The column was washed with buffer A, eluted with buffer B (100 mM Tris pH 8, 100 mM NaCl, 20 mM glutathione), and the protein applied to a pre-equilibrated Superdex 200 gel-filtration column with buffer F (150 mM NaCl, 20 mM Tris pH 7, 5 1 mM DTT). The elution peak was concentrated and stored as above.

Expression and purification of selenomethionine protein. The selenomethionine Δ loop/CaM complex was prepared by inhibition of the methionine pathway. Inoculum was grown from a single transformed colony in 10% LB medium. The cells were pelleted and media supernatant removed, prior to the addition to 2 L of New

Mininal Media, containing Kao and Michayluk vitamin solution (Sigma), 100µg/ml ampicillin, and 34μg/ml chloramphenicol. Upon reaching an A₆₀₀ of 0.3, the temperature was lowered to 16° C. Lysine, phenylalanine and threonine (100 mg/L), isoleucine, valine (50 mg/L) and DLleucine and selenomethionine (50 mg/L) were added 45 min before induction, when the culture reached an A₆₀₀ of 0.6. Protein expression was induced with 135µM IPTG over a 16 hr period. Purification of the SelenoMet protein was similar to that of the native protein, except that 5mM β-ME was added to all solutions to prevent oxidation of the Sel-Met derivative protein.

Peptide purification. The helix D synthetic peptide (residues 585-621, acetylated and amidated at the N and C-termini, respectively) was purified by reverse-phase HPLC chromatography using a C18 column (Vydac) with a shallow acetonitrile gradient 35–60% (both solvents were supplemented with 0.05% trifluoroacetic acid).

Analytical ultracentrifugation. Sedimentation velocity analysis of the different protein samples were carried out at 40000 or 50000 rpm and 5 °C (except Δ helices C-D and point mutants that were centrifuged at 10°C) in an XL-A analytical ultracentrifuge (Beckman-Coulter Inc.) with UV-VIS optics detection system, using an An60Ti rotor and 12 mm double-sector centerpieces. All the proteins were equilibrated in 20 mM Tris-HCl, 150 mM NaCl, 0.1 mM DTT, pH 7.5 buffer except CT-GCN4LI that was in PBS. The sedimentation velocity runs were done at different protein concentrations ranging from 0.1 to 1.5 mg/ml. Sedimentation profiles were registered every 5 minutes at the appropriated wavelength (ranging from 230 to 280 nm). The sedimentation coefficient distributions were calculated by least-squares boundary modeling of sedimentation velocity data using the c(s) method (17) as implemented in the SEDFIT program. The corresponding standard svalues (s_{20,w}) were obtained from the experimental svalues upon correction for density, viscosity and protein concentration (18) using the SEDNTERP program (19).

Sedimentation equilibrium experiments were carried out at multiple speeds (9000, 13000, 22000, 32000, 40000 and 43000 rpm) and wavelengths (230, 238, 250, 280 nm) with short-columns (80 μ l) using the same instrumentation and conditions as described above. In order to determine the stoichiometry of the

two KCNO/CaM complexes, the sedimentation equilibrium data of these macromolecular mixtures were analyzed assuming the linear approximation of the buoyant molecular weights (20,21): $bM_{w,ii} =$ $ibM_{w,A} + jbM_{w,B}$, where ij refers to the complex A_iB_i , and bMw,A and bMw,B are the buoyant molecular weights of pure A and pure B, respectively. In this case, the weight-average buoyant molecular masses (bM_w) were determined from the sedimentation equilibrium data by fitting a single species model to the experimental data using either a MATLAB program based on the conservation of signal (kindly provided by Dr. Allen algorithm (22) Minton, NIH) or the HeteroAnalysis program (20), both analytical methods gave essentially the same results. The molecular masses of the single solute components (helix D peptide and CaM) were determined from the corresponding experimental buoyant values using 0.734 and 0.732 cm/g as the partial specific volumes of peptide and CaM, respectively, calculated from the amino acid composition using the SEDNTERP program.

CD spectroscopy. All measurements were done with an Aviv CD spectrometer model 202. Spectra were measured over the range of 260-180 nm at a scan rate of 1 nm/sec. A cell with 0.1 mm path length was used. Each spectrum is an average of five scans. The raw data were corrected by subtracting the contribution of the buffer to the signal. Then, data were smoothed and converted to molar ellipticity units. Protein concentration was determined using the predicted extinction coefficient at 280 nm. For melting experiments, CD was measured at 222 nm with a 1 mm path length cell. Temperature equilibrium time was two minutes, and integration time was 30 sec. The CD data were scaled from 0 to 1 with respect to the initial native form and the fully unfolded form using the equation:

$$CD_{scaled} = \frac{CD_{obs} - CD_{initial}}{CD_{final} - CD_{initial}}$$

Analytical gel filtration chromatography. Proteins were injected onto a Superose 6 10/300 GL or Superdex 200 10/300 GL or Superdex 75 10/300 GL (Amersham Biosciences) column. Proteins were eluted with buffer F (150 mM NaCl, 20 mM Tris pH 7. 5 , 1 mM DTT) at a flow rate of 0.5 ml/min, 0.7 ml/min, 0.8 ml/min for Superose 6, Superdex 200, Superdex 75 columns, respectively.

Crystallization. Crystals were grown in 12-16% PEG 8K, 0.1 M Tris, pH 7-8 at 19° C by sitting drop

vapor diffusion. Equal volumes (1 μ l) of frozen stock protein (15 mg/ml) were mixed with reservoir solution. Native crystals appeared after two weeks. Heavy atom soaks were prepared by adding 1 mM K_2ReCl_6 directly to drops containing crystals for 24 hours. Four selenomethionine protein crystals appeared after several months. Crystals were cryoprotected by sequential dilution with reservoir solution including 24% glycerol, then loop mounted and flash frozen in liquid N_2 .

Structure determination. Diffraction data was obtained under standard cryogenic conditions and processed with HKL (23). We executed a single wavelength anomalous diffraction experiment on the selenomet crystals, using the anomalous Se peak. Heavy atom site location and experimental phases calculation was performed with CNS (24) and SHARP (25,26), respectively. Multiple isomorphous replacement with anomalous scattering (MIRAS) from the selenium and rhenium derivative data sets against the native data set were used for experimental phase calculation. Subsequent density modification with SOLOMON (26) gave electron-density maps of excellent quality. An initial model was built with ARP/wARP (27) and subsequently refined with Refmac5 (28) with rounds of model-building.

Electrophysiology. Recordings were performed using the whole-cell configuration of the patchclamp technique. Signals were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments), sampled at 2 kHz and filtered at 800 Hz via a 4-pole Bessel low pass filter. Data were acquired using pClamp 8.2 software in conjunction with a DigiData 1322A interface. The patch pipettes were pulled from borosilicate glass (Warner Instrument Corp, USA) with a resistance of 4-7 M Ω . The intracellular pipette solution contained (in mM): 130 KCl, 1 MgCl₂, 5 K₂ATP, 5 EGTA, 10 HEPES, adjusted with KOH to pH 7.4 (290 mOsm). The external solution contained (in mM): 140 NaCl, 4 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 11 glucose, 5.5 HEPES, adjusted with NaOH to pH 7.4 (310 mOsm). Series resistances (8-15 M Ω) were compensated (75-90%) and periodically monitored.

Immunocytochemistry. COS 7 cells were grown on 13 mm diameter coated glass cover slips in 24-well plates. Cells were rinsed for 5 minutes in phosphate-buffered saline (PBS) and subsequently fixed for 20 minutes in 4% paraformaldehyde in PBS. Following extensive washes in PBS, the cells were permeabilized by incubation with 10% normal

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goat serum (NGS) in PBS containing 0.2% Triton X-100 for 20 minutes. Cells were then washed for 2X10 minutes in PBS containing 1% NGS. Cells were incubated at 4° C overnight with rabbit anti-KCNQ1 (Alomone labs) channel or mouse anti-FLAG (M2, Sigma) antibodies. After a 3x5 minutes wash in PBS, cells were incubated for an hour at room temperature with secondary antibodies, CY2-conjugated antirabbit IgG (1:200; Jackson Immunoresearch) and RRX-conjugated anti-mouse IgG (1:1000; Jackson Immunoresearch), respectively. Cells were viewed and digital images taken using a Zeiss 510 META confocal microscope using the 488 nm Argon or 543 nm HeNe excitation laser lines.

Cell surface biotinylation assays. Biotinylation of surface proteins was carried out by incubating cells with 1 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) in PBS containing 1 mM phenylmethylsulfonyl fluoride and 5 mM EDTA (solution A), for 30 min at room temperature. The reaction was terminated by incubating cells for 5 min in solution A containing 20 mM glycine followed by three washes in solution A. Cells were lysed in buffer P (50 mM Tris-HCl, pH 7.5; 20 mM HEPES, pH 7.5; 150 mM NaCl, 5 mM EDTA, 1.5 mM MgCl₂, 10% glycerol(w/v), 1% Triton X-100(v/v)), containing 1 mM phenylmethyl-sulfonyl fluoride and 10 µl/ml protease inhibitor cocktail (Sigma), for 1 hour at 4° C under rotation. Cell lysate was cleared by centrifugation and biotinylated proteins were precipitated by incubation with streptavidin agarose beads (Pierce) overnight at 4° C. The beads were washed six times with buffer P. Proteins were eluted by incubation with sample buffer at room temperature, and then resolved by SDS-PAGE and Western blotted.

ICP (inductively coupled plasma) spectrometry. Peptide samples were suspended at RT in a cocktail of metal chloride salts containing Tris 10 mM pH 7.5, 100 mM NaCl and 20 μM of the indicated metals for two hrs at 0.35 mg/ml. Subsequently, peptide was dialyzed for 16 hrs against the same buffer without metals. Samples were prepared for analysis by incubation in a hot water bath at 80° C for two hours with the following addition. One ml volumes of concentrated nitric acid were added to 1-2 ml sample batches in polypropylene 50 ml tubes. Liquid residues were taken up in deionized water and made up to 10 ml volume with the same solvent. Metal concentration in the acid extract was determined by ICP atomic emission spectrometry.

An ICP atomic emission spectrometer with cross flow nebulizer was used (Spectroflame Modula E from Spectro, Kleve, Germany.)

RESULTS

Oligomeric state of the KCNQ1 C-terminus/CaM complex

Work by Pitt and coworkers suggested previously that a soluble KCNQ1 C-terminus/CaM complex is tetrameric (11) using crosslinking, consistent with the universal tetrameric structure of Kv channels. Using a similar molecular comprising soluble C-terminus and complex. constitutively bound CaM (figure 1B and C), we probed its oligomerization state i.e. its molecular weight by sedimentation equilibrium analysis. The results indicate that the complex is best modeled as tetramer with four subunits of KCNQ1 C-terminus and four bound molecules of CaM (figure 1D). Data analysis, assuming a single sedimenting solute species, yields a buoyant molecular weight of 54,000 \pm 3000 (solid line). This value is very close to that calculated from the addition of the buoyant molecular weights of four molecules of C-terminus (4 x 8400) and four of CaM (4 x 4500). It is not compatible with a 4:2 complex (dotted line).

Sedimentation velocity studies that evaluate homogeneity/dispersity of the same C-terminus/CaM complex, provide convincing evidence that the primary species (89-95 % of the total loading concentration, varying on experiment) is this very same tetrameric complex (8.2 S) with a minor species that represents a higher molecular weight aggregate (11.4 S) (figure 1E). Importantly, there is no evidence of a free CaM species, indicating that CaM is stably bound since measurements of CaM alone exhibit a symmetrical sharp distribution with a standard s-value of 2.8 S, compatible with the expected value for monomeric CaM (figure 1E). No such peak is detected in the profile of the Cterminus/CaM complex. The hydrodynamic behaviour of the 4:4 C-terminus/CaM complex (frictional ration $f/f_0 = 1.8$; see (29)) is compatible with the solute being an elongated, non-globular protein. Moreover, calcium is not required for this assembly as size-exclusion chromatography of a Cterminus/CaM₁₂₃₄ complex, utilizing a CaM mutant that does not bind calcium, behaves like a Cterminus/CaM_{WT} complex (figure 1F).

CaM organization

To explore the possibility that CaM may induce oligomerization by its association with the Cterminus proximal half, we coexpressed CaM with a protein segment spanning helix A and B but truncated afterwards (Δ helices C-D plus CaM) (figure 1B and C). Sedimentation velocity and sizeexclusion chromatography indicates that it behaves as a 1:1 complex of CaM to Δ helices C-D i.e. CaM does not crosslink C-terminus subunits inducing their oligomerization (figure 1E, supplementary fig. 1A), neither in the presence of calcium (1 mM) nor in its absence (5 mM EDTA). Since the intervening loop between helix A and B is long, binding by CaM of these two helices may be in either parallel or antiparallel helical arrangements. Nevertheless, the loop is not necessary for CaM binding since its removal does not prevent formation of the Δ loop/CaM complex (figure 1B-C), consistent with an antiparallel helix bundle as CaM's target.

Role of the distal C-terminus (helices C and D)

Earlier studies on KCNQ have assigned regions from the distal C-terminus, encompassing sequences predicted to form the two helices C and D with what has been called an assembly or tetramerization domain. Both of these predicted helices are amphipathic and have varying probabilities to form coiled-coils. We tested the basic prediction of helicity for helix C. Using CD spectroscopy, we measured the spectrum of helix C peptide (figure 2A and B). The results indicate clearly that helix C takes an α-helical conformation (figure 2C). Having established that this module is helical, we then asked if it self-associates. Sedimentation equilibrium studies demonstrate that helix C peptide is dimeric and appears to undergo concentration-dependent dimer-tetramer association with a K_D of 123±3 µM (figure 2D). Therefore, the isolated helix C dimerizes, presumably by a coiled-coil interaction, as supported by reversible thermal denaturation a property characteristic of coiled-coils (figure 2Cinset and supplementary figure 2 for the heptad repeat assignment), while further associating as a dimer of dimers.

We then asked what helix C will do in the context of the C-terminus/CaM but without the confounding action of helix D. Sedimentation studies, both equilibrium and velocity, of C-terminus Δ helix D/CaM (figure 2A and B) unambiguously demonstrate that the complex is best modeled as a

dimer i.e. two C-terminus subunits bound to two CaMs with a buoyant molecular weight half that of the full length C-terminus/CaM complex (solid line gradient in figures 2E). The data cannot be accommodated by a gradient expected for a 4:4 complex (figure 2E, dashed line). The hydrodynamic behaviour of Δ helix D/CaM complex (s-value of the main sedimenting species was 5 s, open circles in figure 2F) also deviates from that expected for a rigid globular solute (frictional ratio f/ f_0 = 1.7). We conclude that, like the isolated helix C module, helix C in its C-terminal context directs dimerization. It seems likely that during biosynthesis of the intact channel, this dimeric module forms a dimer of dimers due to high local concentration.

To probe the function of helix C in a cellular context, we recorded currents from CHO cells transfected with a helix C deletion channel construct. This deletion obviates functional expression i.e. production of a detectable K⁺ current (figure 2G). The expressed protein appears to be retained intracellularly, as shown by immunocytochemical labeling and cell surface biotinylation assays (figure 2H, I, respectively).

What function does helix D perform? To address this question, we measured CD spectra of helix D peptide (residues 585-621) confirming its helical conformation (data not shown). We examined the peptide's oligomeric state using both sedimentation equilibrium and velocity. The results show a tetrameric species (single species with molecular weight of 18400 ± 1500 ; see figure 3A and supplementary figure 1B) over a wide range of concentrations (10-100 µM) versus a monomeric one (molecular weight = 4200), proving that helix D forms a stable tetrameric coiled-coil with a globular shape (frictional ratio $f/f_0 = 1.3$), in contrast to helix C. Next, we purified a KCNQ1 C-terminus Δ helix C/CaM complex (figure 3B and C), allowing us to directly examine helix D's action without the potential confounding effects of helix C, shown above to also self-associate. Sedimentation velocity establishes that the Δ helix C/CaM complex tetramerizes much like the WT C-terminus/CaM complex (figure 3D). Therefore, helix D induces a tetramerization state upon the KCNQ1 C-terminus, independent of the helix C segment.

Deletion of helix D abrogates KCNQ1 current (12), underlining its requirement for proper function. If the helix is perturbed by substitution of proline at

position 609 (I to P), Roden and colleagues report that no functional current is detectable and the channel is found intracellularly (15). Presumably, this mutation disturbs the helix sufficiently to disrupt predicted coiled-coil. These investigators ascribed the elimination of functional current to helix D's essential role in trafficking the channel to the plasma membrane. We, therefore, examined the consequences of this mutation in our expression system. The mutated protein was insoluble (data not shown), similar to that seen when CaM is not coexpressed or its binding sites are perturbed (8,11). This result hints that viable channel folding and assembly requires properly folded helix D.

If helix D directs tetramerization then, perhaps we could replace it with an analogous coiled-coil that performs the same task. Such coiled-coils have been used in similar contexts (30). We utilized GCN4-LI, a modified GCN4 coiled-coil that forms a parallel, tetrameric coiled-coil (31). The GCN4-LI module was introduced into KCNQ1 C-terminus at residue 594, preserving completely the first helical turn of helix D (figure 3B). This chimeric protein was both soluble (figure 3C) and a fraction of it behaved like WT in sedimentation velocity experiments (figure 3D), corroborated by size-exclusion chromatography (supplementary figure 1C), but when transfected into produced no current. was intracellularly without evidence of cell surface expression (figure 3E-G).

Crystallographic studies of the helix D tetramer

What is the molecular mechanism by which helix D acts? We obtained the atomic details of this molecular complex using x-ray crystallography. Crystallization trials with C-terminus Δ loop/CaM complex (Figure 1B-C) yielded small crystals after several weeks. N-terminal sequencing of the crystallized protein identified the sample as KCNQ1 starting with residue 574, just upstream of helix D. We conclude that a contaminating protease cleaved the Δ loop protein in the predicted random coil region between helices C and D, allowing the helix D assembly to crystallize. SAD and MIRAS diffraction experiments using a selenomethionine protein crystal and a rhenium soaked crystal respectively, produced excellent electron density maps to 2.0 Å Bragg spacings (table 1). The current refined model includes KCNQ1 residues 585 to 621 with good stereochemistry, 52 solvent molecules and a R_{free} of 25.5 %. Residues 574 to 584 are not observed and must be disordered.

The crystallographic studies reveal a tetrameric parallel-oriented coiled-coil quaternary structure (figure 4A). The crystallographic asymmetric unit contains a dimeric coiled-coil, whereupon a two-fold crystallographic dyad generates the presumed biological tetrameric unit. Thus, formally the helix D assembly is a dimer of dimers, with pseudo four-fold symmetry that is close to perfect C4 symmetry. The coiled-coil cylinder is 50 Å long with a diameter of about 27 Å comprised of helices of nine turns or four and half heptads. about Coiled-coil parameterization analysis by the program TWISTER (32) shows the assembly to have the canonical heptad (7/2) repeat, left-handed supercoiling and other Crick parameters (table 2) with the signature of a classical tetrameric parallel coiled-coil. The buried residues of the a and d heptad positions maintain alternating perpendicular and parallel knobs-in-hole packing, respectively, as computed by Socket (33). The perpendicular and parallel terms refer to packing angles for the side-chains as defined by Harbury et al (31). This arrangement was observed for GCN4-LI. The tetramer is capped at its carboxy-terminal end with a histidine ring coordinating a metal ion (figure 4B).

The coiled-coil interactions constituting the hydrophobic core and the solvent exposed residues involved in interhelical interactions are summarized schematically in figure 4C-D. The a and d positions of the heptad are taken by branched, apolar residues generally conserved in both the KCNQ1 orthologs and the Kv7 subfamily. Notably, KCNQ1's helix D, as seen in the structure and sequence alignment (supplementary figure 2), has a longer coiled-coil, by one heptad repeat or two helical turns longer than the other KCNO members. The one exception to the branched hydrophobic residues in positions a and d is the capping histidine found in position a. Kim and coworkers, in studies using the dimeric GCN4 as a scaffold, proposed a set of rules for predicting the oligomeric state of a coiled-coil (31). They posited that unbranched CB (Leu) residues at the a position with branched Cβ residues (Ile, Val) at the d position will favor tetrameric assemblies. Contrary to this proposal, the tetrameric KCNO1 helix D d positions have almost as many unbranched residues as branched.

The helix D assembly buries a total of 6540 Å², an area typical of coiled-coil architectures and

reflects a character more akin to globular protein core than standard protein-protein interfaces. We measured the stability of this coiled-coil using thermal denaturation, assayed by CD spectroscopy (figure 4E). The measurements indicate a Tm of \sim 35° C with reversibility of the process. We note this seemingly low value for tetrameric coiled-coils, despite efficient interior packing (34,35).

Having established the coiled-coil architecture of both helix C and D modules separately and determined their thermal stability, we examined the consequences of having them in the tandem arrangement found in the intact protein. Specifically, would the relatively low Tms and reversible folding/unfolding seen for the individual modules be the same in this type of tandem organization? To this end, we constructed a GST-fusion protein, beginning at helix C extending to just beyond the end of helix D, which was expressed, cleaved from the GST and purified (figure 4F-G). This protein characterized analytical bv size-exclusion chromatography that suggests it is most likely a tetrameric complex, as expected (supplementary figure 1D). We further analyzed the complex with CD spectroscopy where it shows the anticipated helical structure (figure 4H). Thermal denaturation experiments demonstrate a more complex denaturation curve than that seen for both of the individual modules with a marked increase in the Tm (about 45°) and two different phases in the unfolding, one in the 30-40° range with a later phase occurring between 55-65° (figure 4H-inset). Moreover, the folding was not reversible. We conclude that the tandem coiled-coil architecture confers appreciable stability, including a possible kinetic deterrent to unfolding/dissociation of the distal C-terminus.

From examination of the helix D complex architecture that is both elegantly simple and symmetric, we discern three pertinent molecular features. The first (i) is the interface by which each polypeptide chain folds with the others, forming the hydrophobic core of the complex, primarily but not exclusively mediated by the interior a and d heptad positions. The second feature (ii) is the intersubunit interface and surface that contains stereochemically specific interactions e.g. h-bond and van der Waal interactions with a special emphasis on salt bridge feature networks. This furnishes biological specificity in helix D complex formation, possibly specifying homotypic or heterotypic combinations in the Kv7 subfamily. The third feature (iii) is the solvent accessible molecular surface available for protein-protein interactions with other proteins, that is likely to play an important role in trafficking and regulation of the channel (15,36). We probed these three features with a functional survey of 17 different structure-based point mutations in the helix D region, designed to perturb relevant structural determinants (table 3). Certain residues are likely to contribute to more than one molecular feature.

When we sought to disrupt the coiled-coil hydrophobic core (molecular feature i), only specific changes have impact. We focused not just on residues of the a and d positions but also on buried residues from other heptad positions. For example, G589M, L602I or I609L had no significant effect, as assessed by current recorded from transfected cells (table 3). These results suggest a measure of plasticity in the helix D complex's folding determinants. In contrast, mutations previously performed by Roden and coworkers (L606P+L613P, L606D+L613D. I609P, L602D+I609D) essentially abrogate cell surface expression (15), are easily rationalized in light of the structure. All of these mutations change a or d heptad position hydrophobic residues to either helix-kinking proline or a negative charge, almost certainly disrupting the coiled-coil assembly. On the other hand, when these same residues were replaced by alanine in that same study, surface expression approached that of WT, underscoring the complex's plasticity. The alanine replacements are neutral; they do not negatively or positively select against heptad position partners, a principle clearly critical in coiled-coils (33,37).

Hydrogen-bonded salt bridges play a prominent role in the intersubunit interface (molecular feature ii). For example, R594 (c position in α subunit) hbonds to E596 (e position in β subunit), which concomitantly engages K598 (g position in α subunit) (figure 4C, D). This network is typical of coiled-coils and may facilitate specificity of assembly in two ways; one, determination of the partner subunit, and two, number of subunits in the assembly. Charged residues confer specificity by requiring an oppositely charged residue to be in proximity and by selecting against a partner whose proximal residue is like charged. Nonetheless, obvious empirical rules are not easily derived since many expected perturbations of this molecular feature like T600L, O601A or D603G appear to have little effect (table 3).

To probe the importance of the complex's solvent accessible residues vis á vis their potential role to generate a platform for interacting proteins (molecular feature *iii*), we mutated almost all of the helix D residues with accessibility greater than forty percent (table 3). The preponderance had little or no effect. However, three mutations, G589D, A590W, and N593G, located in the N-terminal third of helix D reduced the expressed K⁺ currents in the transfected cells.

KCNQ1 has been implicated genetically in LOTS. Lesions responsible for this pathophysiological phenotype are found scattered over the structural gene and several localize to helix D (supplementary table 1). A representation showing their location on the complex's molecular surface is depicted in figure 5A. Strikingly, these mutations predominantly cluster in the N-terminal third of the assembly and include the loop upstream of the assembly, forming a contiguous surface. Curiously, all of the mutations involve non-a or d coiled-coil position residues. Thus, they either perturb partner specification in assembly (for example, R594H) or formation of protein-protein interaction interfaces or both. Apparently, they do not perturb folding and tetrameric assembly. One mutation, G589D, has been shown to abrogate binding of the AKAP protein votiao, important for sympathetic regulation of the cardiac action potential as mediated by β-adrenergic receptor signaling (36). Other possibilities could involve proteins important for trafficking of the channel complex to the plasma membrane (15).

We thus focused our characterization efforts on five point mutations of helix D that perturbed current expression, in an attempt to define their molecular mechanism. The residues' locations on the helix D coiled-coil are graphically depicted in figure 5B. These mutations should impinge upon the molecular features described above. Specifically, G589D, a LQT mutation, along with A590W, N593G and possibly H620S may affect both intersubunit interfaces and the surface available for proteinprotein interactions. Mutations L602M, H620S, and possibly A590W may affect the core of the complex. current-voltage relations The (I-V curves) determined for the various mutants (figure 5C-D), indicate that some (N593G, L602M, H620S) give rise to moderately impaired current magnitude while others' (G589D and A590W) current magnitude is severely diminished. Previously published reports for G589D conflict; Kass and coworkers published data showing unimpaired K^+ currents and normal plasma membrane location (36) while Roden and coworkers demonstrated complete K^+ current inhibition, as we observe, and intracellular channel retention (15). In order to determine if these mutants' current deficiency was due to problems with targeting, we performed cell surface biotinylation assays on four mutants. The results are shown in figure 5E. The mutants exhibit diminished but variable cell surface expression when compared to WT. To some degree, functional current expression correlates with cell surface expression.

We then asked if the mutations might be causing difficulties in assembly. We addressed this question by expressing and purifying the point mutants in complex with CaM in our recombinant system. These complexes were then studied by sedimentation velocity and/or size-exclusion chromatography. The results are shown in figure 5F-G. The WT Cterminus/CaM complex provides a size and shape standard for the 4:4 tetrameric assembly with c(s)distribution centered around 8 s. We quantitated the fraction of mutant complex that sediments at the peak corresponding to a tetrameric assembly from the derived c(s) distribution of each individual sedimentation velocity experiment. The mutants G589D, A590W, L602M, form 76, 69 and 71 percent tetrameric assemblies, respectively, while the WT forms 88 percent. Generally, the mutants exhibit more polydispersity than the WT. Based on sizeexclusion chromatography, the mutants N593G and H620S behaved almost exactly like the WT. We conclude that all the mutants appear to assemble into proper tetrameric complexes, albeit with slighter lower efficiency than WT. Therefore, their lower functional expression does not stem from flawed assembly but either faulty trafficking or other defective regulation or both.

As noted earlier, the helix D complex is capped by a histidine ring that coordinates a metal ion. This appears to be a novel structural feature not yet observed in structures from natural sequences, although similar structures have been engineered for designer coiled-coils. First, we sought to identify the metal ion. To this end, we purified, desalted and suspended a synthetic peptide of helix D in buffer containing appropriate candidate divalent metal salts (20 μM) or in buffer alone. Then, samples were dialyzed extensively against buffer alone and analyzed by ICP spectrometry (table 4). The data clearly indicate that the preferred metal ion was Cu^{2^+} .

We expect that Zn²⁺ can bind as well (data not shown). Structurally, this identification makes good inorganic chemical sense since Cu²⁺ is well suited for the observed square pyramid 5-coordination as a water molecule is bound above the metal ion. In addition, the coordination lengths are appropriate (2.17 Å). Other copper binding proteins, such as laccase, an oxidoreductase from fungi (38), have similar stereochemistry wherein four histidines chelate the copper.

We asked if metal-binding is necessary for assembly formation by using thermal denaturation as measured with CD. Addition of metal did not change the melting temperature, indicating no stabilizing effect. We then turned to mutagenesis and functional assays to evaluate the significance of metal binding. When we mutated the histidine 620 to alanine, no appreciable effect was seen on current recorded from transfected cells. When the histidine was changed to serine, though not significant, some inhibition (~50 %) of the current was detected (table 3 and figure 5C-D).

DISCUSSION

We outline our findings with a working model for the KCNQ1 C-terminus structure and its functional correlates. A structural model is drawn in figure 6. The C-terminus structure may be best summarized by dividing it into two halves, proximal and distal to the membrane. The proximal half binds CaM. The configuration of CaM association that best agrees with our data has one CaM binding by sandwiching helices A and B of a single subunit. Accordingly, CaM does not play a direct role in oligomerization but apparently participates in forming a module, pivotal for regulating channel gating (8,11). Nevertheless, apoCaM does play a critical role in correct C-terminal folding since its absence results in no functional channels and no soluble C-terminus (8,11), while Ca2+-CaM is not required, as seen from experiments using CaM₁₂₃₄ (figure 1F and (11)). Also, calcium does not induce dimerization of the proximal C-terminus as observed in our sedimentation velocity experiments with Δ helices C-D/CaM, unlike the SK channel structure (39). Helices A and B are connected by a long stretch of polypeptide, dubbed above the intervening loop, that has little sequence conservation, regions of lowcomplexity, and little predicted secondary structure.

This intervening loop is not required for formation of the tetrameric C-terminus/CaM complex (figure 1C).

distal C-terminal half contains two tandemly-arranged coiled-coils. This half directs oligomerization due to two structural components. namely the helix C module, the first coiled-coil that is dimeric and subsequently the helix D module, the second coiled-coil that is tetrameric. These two components are linked by a proteolytically susceptible linker, strongly suggesting that this 22 residue span is mobile and not well structured. Two independent observations support that conclusion: one, adventitious proteolysis in this region enabled crystallization of the helix D complex from the Cterminus Δ loop/CaM complex and two, residues 574 through 586, while present in the protein are not discernible in the electron density map.

The two coiled-coils are intrinsically different since the helix C module fundamental unit appears to be dimeric, probably parallel due to structural considerations. This unit undergoes concentrationdependent self-association into a dimer of dimers. This concentration-dependent self-association may explain findings by Ghosh et al that a fraction of Cterminus (truncated at residue 555)/CaM complex. tetramerizes, as found by chemical cross-linking performed at 1 mg/ml protein concentration (11). Indeed, this truncation leaves intact a significant portion of the helix C module. Similarly, Schwake et al found that mutants disrupting the helix D module in KCNO2 or 3 still afforded functional current, albeit significantly diminished, suggesting that the helix C module is sufficient for tetramerization (14). In contrast, the helix D module fundamental unit is a stable tetramer, even at lower concentrations. Despite the difference in quaternary structure, their individual thermal stabilities are about the same. Thermodynamically, both of the specific modules are less stable than engineered coiled-coils, not unlike some natural coiled-coils that have quite low melting points (40). These Tm results may have implications for the dynamics of KCNQ1 biosynthesis and assembly. However, when they fold/unfold in their native tandem arrangement, there is increased thermal stabilization caused either by structural or kinetic reasons. Thus, the distal C-terminus assembles by a concerted action of its particular modules.

Inspection of sequence conservation (supplementary figure 2) in the distal C-terminus reveals that the helix C module is strongly conserved

within the Kv7 subfamily, at both buried and surfaceaccessible residues. The buried residue conservation may emphasize a general role in promoting oligomerization, while the conservation of surface residues may hint at its role as surface for associating proteins involved in trafficking and regulation. The helix D module differs in its conservation characteristics, exhibiting markedly lower overall levels within the Kv7 subfamily with the aminoterminal third more conserved including solventexposed residues (non a and d positions). We propose three non-exclusive possibilities for the significance of this conservation. First, the lesser Kv7 subfamily conservation is in line with a role that specifies the type of oligomerization i.e. homotypic and/or heterotypic. Second, coiled-coils undergo folding, which may be initiated by so-called "trigger" sequences. Examples of this phenomenon have been isolated in certain coiled-coils and a consensus sequence has been formulated (41). Other studies have shown that trigger sequences are not found in all coiled-coils (42). The KCNQ1 sequence conforms to this trigger sequence except for the first two positions (L to I588 and E to G589). Thus, the conserved N-terminal end may comprise a trigger sequence for helix D assembly. When we perturbed this sequence region (588-594), removing the entire helix D sequence in our attempt to substitute helix D with GCN4-LI, as described above, the Cterminus/CaM complex aggregated in a non-specific manner (data not shown). However, when we preserved this region (588-594), we were able to construct a viable chimeric C-terminus-GCN4-LI 3B-D), emphasizing the sequence's importance for formation of the helix D complex. Third, this region generates a surface for essential interacting proteins, conservation being a hallmark of such interfaces (36). Finally, we note the relative sequence conservation of the linker between the helix C and D modules, discerned also through the prism of LQT syndrome mutations in this region (supplementary table 1). This feature may be due to specific requirements for folding, as just described for the KCNQ1-GCN4 chimeras. Alternatively, these conserved sequences are required for other proteins to bind.

Two salient structural features may explain the observation that human KCNQ1 homo-tetramerizes exclusively i.e. it is partner-specific, while other members can heterotetramerize or homotetramerize (13,16). First, the KCNQ1 helix D should be about

two helical turns longer than the other family members. This additional heptad repeat will prefer assembly with a partner of equal length and sequence in order to realize most fully its folding potential. Indeed, the recent report of the the KCNQ4 helix D complex structure supports this notion (43). Second, human KCNQ1 contains a metal coordination site capping its assembly.

Regarding this latter feature, our findings lead us to posit that since the histidines are positioned at the a heptad repeat position, pointing into the interior of the coiled-coil and alanine is found frequently at the a position, the assembly or folding of tetrameric helix D is maintained. However, when serine is introduced, a hydrophilic residue infrequently found at that position and incapable of coordinating a metal ion due to its short length, assembly is affected. Therefore, the metal coordination found in helix D of human KCNQ1 may have physiological relevance by providing an additional mechanism for assembly partner specificity. A possible structural precedent for this type of mechanism is found in Kv channels that contain the tetramerization domain, T1. In that case, certain subtypes encode a Zn²⁺ binding motif that determines partner subtype specificity (44). Further experimentation will be required to test whether metal coordination may indeed be a KCNQ1 specificity determinant.

Both of the helix C and D modules in KCNQ1 are non-redundant at the functional level, even though structurally there may be a degree of overlap. Our cellular studies show that helix C's function is complex, since a construct like Δ helix C, despite its apparent biochemical viability, failed to reach the plasma membrane, implying that helix C plays a role in channel trafficking (figure 2G-I). Hence, we assign it as a module for proper assembly and trafficking to the plasma membrane. Our experiments with helix D clearly demonstrate that it encodes a complex function also. Thus, the tetramerization functionality could be replaced in part on the biochemical level with GCN4-LI, but is insufficient for functional expression, suggesting a role in trafficking, implicitly by association of proteins to this region (15). Our analysis of helix D point mutants e.g. A590W, that demonstrate an ability to assemble to levels approaching that of WT. nonetheless shows impaired functional expression, attributable in part to faulty trafficking. We speculate that such mutations may perturb binding surfaces required for association of trafficking or regulatory proteins, particularly in the amino-terminal region of the helix D module. Such an interpretation may also explain the mechanism for LQT mutations e.g. G589D in this region.

What does the C-terminus do? T1. the intracellular domain amino-terminal to the membrane domain in several Kv channel subfamilies, is not essential for Kv1 and Shaker channel formation. Jan and coworkers suggested that it enhances channel assembly by increasing the local concentration of subunits in a multistep process (30). Inherent to this hypothesis, is the premise that another oligomerization element exists which resides in the membrane domain of the protein. T1, then improves quaternary structure formation efficiency. In contrast, KCNQ1 channel formation cannot be coaxed out of truncations lacking its C-terminus. Once again, the membrane domain must contribute considerably to channel assembly but is not sufficient. The Cterminus must provide the necessary energy deficit for assembly. We propose that the helix C module may contribute adequate free energy for this deficit at some level, thereby creating a dimer of dimers, although this may vary from Kv7 subfamily member to member (14). While this role might explain its

outstanding conservation, this functionality is not sufficient for full maturation of the channel. To fully specify the correct partners for the tetramer and also stabilize oligomerization, the tetrameric helix D module is required. A similar functional architecture has been shown for the related EAG family of potassium channels (45). Previous and present results intimate that it provides a protein-protein interaction platform for trafficking and regulation, in some measure analogous to T1. Consequently, the whole C-terminus constitutes an elongated, non-globular, multi-tiered scaffold for associating proteins like CaM and others central to the signaling process (46).

Finally. the layering, physically, oligomerization predicts a staged channel assembly. whose temporal sequence at this point remains unknown. The use of coiled-coils in channel assembly perforce couples folding i.e. tertiary structure formation with oligomerization quaternary structure formation, parallel conclusions drawn by Deutsch and Robinson regarding the biosynthesis of Kv channels that employ T1 (47).

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FOOTNOTES

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Abbreviations used: AKAP - A kinase-anchoring protein, β -ME - beta-mercaptoethanol, CaM - calmodulin, CD - circular dichroism, DTT - dithiothreitol, Kv - voltage-gated K+ channels, LQT(S) - long QT syndrome, PBS - phosphate buffered saline, SAD - single wavelength anomalous diffraction.

FIGURE LEGENDS

<u>Figure 1:</u> KCNQ1 C-terminus/CaM complex structure. A. Cartoon of KCNQ1 primary and secondary structure organization as based on sequence analysis, showing the association of CaM to helices A and B. B. Schematic depiction of recombinant constructs. All of the constructs were coexpressed with CaM. C. SDS-PAGE of relevant proteins. Samples shown are composite from different gels. Samples run in the presence of 1 mM EDTA are denoted with an asterisk. The Δ loop C-terminus/CaM sample was electrophoresed in the presence of 1 mM CaCl₂ so as to facilitate separation of the two bands. D. Sedimentation equilibrium gradient of the C-terminus/CaM complex. The solid and dotted lines represent the gradient expected for a 4:4 and a 4:2 complex, respectively, while the grey circles represent experimental data. E. Sedimentation velocity c(s) distributions. The different distributions are indicated in the legend. Both the C-terminus and Δ helices C-D are in complex with CaM. Experiments were performed at 50,000 rpm and 10° C. The *s*-values have been corrected to standard conditions. F. Elution profiles from analytical size-exclusion chromatography of C-terminus/CaM complexes using a Superose 6 column. CaM₁₂₃₄ is a calmodulin mutated at all four Ca²⁺ binding sites, thereby abrogated Ca²⁺ binding. Absorbance units (AU) measured at 280 nm were normalized on a 0 to 1 scale for each individual sample.

<u>Figure 2:</u> Structure and function of helix C module. A. Schematic depiction of recombinant constructs. Both the C-terminus and Δ helix D constructs were coexpressed with CaM, whereas the GST-helix C was not. B. SDS-PAGE of relevant proteins. Samples shown are composite from different gels. Samples run in the presence of 1 mM EDTA are denoted with an asterisk. C. CD spectrum of helix C, measured in 5 mM phosphate buffer, pH 7.5. Inset, melting curve of helix C. The $T_m = 35^\circ$ C and was determined by the inflection point for the thermal transition using numerical differentiation. D. Sedimentation equilibrium gradient of helix C peptide. The solid lines show the best fit gradients for three different concentrations, corresponding to a dimer/tetramer association model as fit by global analysis. E. Sedimentation equilibrium gradient of the Δ helix D/CaM complex. Data shown is at a protein concentration of 0.6 mg/ml. The solid and dashed lines represent the gradient expected for a 2:2 and a 4:4 complex, respectively. F. Sedimentation velocity c(s) distributions. Both the C-terminus and Δ helix D are in complex with CaM. Experiments were performed at 50,000 rpm and



10° C. The *s*-values have been corrected to standard conditions. G. Representative current traces of WT KCNQ1 and KCNQ1 Δ helix C mutant . From a holding potential of -90 mV, CHO cells were stepped for 3 sec from -70 mV to +60 mV in 10 mV increments and repolarized for 1 sec at -60 mV. H. Immunocytochemical mapping of COS cells transfected with WT KCNQ1 and KCNQ1 Δ helix C mutant plasmids and detected with rabbit anti-KCNQ1 antibodies. Results clearly show that while the KCNQ1 Δ helix C mutant exhibits essentially intracellular localization with strong accumulation in the ER, the WT channel protein display both intracellular but also significant plasma membrane distribution. Images were taken using a Zeiss 510 meta confocal microscope with a 458 nm excitation argon laser line. I. Cell surface biotinylation of KCNQ1 Δ helix C. FLAG-tagged WT KCNQ1 and Δ helix C were expressed in HEK 293 cells, labeled with NHS-LC-Biotin, pulled down with streptavidin beads, and probed with anti-FLAG antibodies. Signals were corrected to channel protein input, normalized to WT, and expressed as ratios (n=3, *** denotes P < 0.001 two-tailed *t*-test).

Figure 3: Oligomeric states and functional data of helix D module. A. Sedimentation equilibrium gradient of helix D peptide. The solid line shows the best fit gradient corresponding to a tetramer while the other lines denote trimer, dimer, and monomer gradients, as indicated, B. Schematic depiction of recombinant constructs, All of the constructs were coexpressed with CaM. C. SDS-PAGE of relevant proteins. Samples shown are composite from different gels. All samples were run in the presence of 1 mM EDTA. D. Sedimentation velocity c(s) distributions. All proteins are in complex with CaM. Experiments were done at 50000 rpm and 10° C. The s-values have been corrected to standard conditions. The estimated molecular weights for the 7.6, 12.4, 16.3 s peaks are 170, 350, and 530 kDa, respectively. E. Representative current traces of WT KCNQ1 and the KCNQ1-GCN4LI mutant. From a holding potential of -90 mV, CHO cells were stepped for 3 sec from -70 mV to +60 mV in 10 mV increments and repolarized for 1 sec at -60 mV. F. Immunocytochemical mapping of COS cells transfected with WT KCNQ1 and KCNQ1-GCN4LI mutant plasmids and detected with rabbit anti-KCNO1 antibodies. Results clearly show that while the KCNO1-GCN4LI mutant exhibits essentially intracellular localization with strong accumulation in the ER, the WT channel protein displays both intracellular but also significant plasma membrane distribution, denoted by arrowheads. Images were taken using a Zeiss 510 meta confocal microscope with a 458 nm excitation argon laser line. G. Cell surface biotinylation of KCNO1-GCN4LI, FLAG-tagged WT KCNO1 and KCNO1-GCN4LI fusion were expressed in HEK 293 cells, labeled with NHS-LC-Biotin, pulled down with streptavidin beads, and probed with anti-FLAG antibodies. Signals were corrected to channel protein input, normalized to WT, and expressed as ratios (n=3, *** denotes P < 0.001 two-tailed *t*-test).

Figure 4: Structure of the tetrameric coiled-coil helix D complex. A. Ribbon depiction of the assembly with each chain colored differently from a side perspective (N-terminus, bottom; C-terminus, top). The metal ion coordinated by the histidine ring at the C-terminal end is shown as an orange sphere. B. Top view (C-terminal) of the complex. C. A helical wheel diagram showing the crystallographic dimer of dimer organization in the tetrameric complex and the relative orientation of the heptad repeat positions. D. Schematic diagram showing interhelical interactions in the coiled-coil. Shaded boxes indicate LQTS mutations. Arrows denote H-bond or van der Waals interactions. Relative ASA (accessible surface area) values (in percent) are shown for each residue as calculated by Naccess (http://wolf.bms.umist.ac.uk/naccess/). E. Melting curve of the helix D module. The $T_m = 35^{\circ}$ C and was determined as before in figure 3C. The buffer used was PBS, pH 7.4. F. Schematic depiction of recombinant constructs. G . SDS-PAGE of the helices C-D protein (residues 535-622). H. CD spectrum of helices C-D measured in 5 mM phosphate buffer, pH 7.5. Inset, melting curve of helices C-D. The $T_m \sim 45^{\circ}$ C by determined by inspection since numerical differentiation was not possible due to the curve's complexity.

<u>Figure 5:</u> Structure-based functional studies. A. LQTS mutations mapped and labeled onto the assembly's surface, colored in various shades of red. B. Structure-based mutants mapped and labeled onto a ribbon depiction of the complex with each mutation colored differently. C. Representative current traces of WT



KCNO1, KCNO1-A590W, KCNO1-H620S and KCNO1-G589D mutants. From a holding potential of -90 mV, CHO cells were stepped for 3 sec from -70 mV to +60 mV in 10 mV increments and repolarized for 1 sec at -60 mV. D. Current densities were plotted as a function of membrane potential for WT KCNQ1, A590W, G589D, N593G, L602M and H620S (n = 2-8 cells) (various symbols as listed). E. Cell surface biotinylation of KCNQ1 point mutatns. FLAG tagged WT KCNQ1 and helix D mutants were expressed in HEK 293 cells, labeled with NHS-LC-Biotin, pulled down with streptavidin beads, and probed with anti-FLAG antibodies. Signals were corrected to channel protein input, normalized to WT, and expressed as ratios (n=3, ** denotes P < 0.01 2-tailed t-test). On the right, a representative cell surface biotinylation immunoblot of FLAG tagged WT KCNQ1 and helix D mutants is shown (upper panel). Whole cell expression levels were also determined for each condition (lower panel). F. Sedimentation velocity c(s) distributions. All proteins are in complex with CaM as determined by SDS-PAGE (not shown). Experiments were done at 50,000 rpm and 10° C. The svalues have been corrected to standard conditions. Both WT and mutants have approximately the same sedimentation coefficient (around 8) where the differences lie within the range of uncertainty of the numerical analysis used in calculating the c(s) distribution. G. Elution profiles from analytical size-exclusion chromatography of various C-terminal point mutant/CaM complexes using a Superose 6 column. Absorbance units (AU) measured at 280 nm were normalized on a 0 to 1 scale for each individual sample.

<u>Figure 6:</u> Quaternary structure model of the KCNQ1 C-terminus/CaM complex. CaM molecules have been drawn as grey surface representations, binding to helices A and B in an anti-parallel manner (labels indicate amino-terminal end of the helix). It is not known if helices A and B are in contact with each other. The two subunits depicted are colored yellow and green. The membranous protein surfaces have been modeled using the mammalian Shaker structure (4). Only two subunits related by a dyad are depicted for clarity and no intent is made to suggest that those subunits will actually form the dimer at helix C. Rather, it seems more likely that neighboring subunits would form a dimer at helix C. The helix C complex has been drawn to undergo dimerization of its dimeric coiled-coil while the helix D complex is depicted as a stable, tetrameric parallel coiled-coil, as seen in the crystal structure. The complete blue and red chains have been omitted for clarity.

Table 1. Crystallographic statistics

Data collection and phasing statistics	Native	SeMet	Re
Wavelength (Å)	0.9792	0.9788	0.9788
Space group	P3 ₁ 21	P3 ₁ 21	P3 ₁ 21
Unit cell parameters (Å)	a = b = 51.3	a = b = 51.6	a = b = 51.4
	c = 71.1	c = 72.1	c = 71.0
Total reflections	67168	131874	257557
Unique reflections	7656	7195	7214
Completeness (%) ^a	99.2 (99.5)	96.3 (99.5)	99.5 (100)
$R_{\text{merge}}(\%)^{a,b}$	6.5 (33.7)	5.0 (12.6)	5.7 (13.2)
I/σ^a	19.6 (3.36)	47.4 (21.0)	52.9 (26.0)
Resolution range (Å)	50 - 2.0	50 - 2.5	50 - 2.5
f'/f''		-7.6/4.9	-7.1/12.1
Phasing power (anomalous)		1.88	0.743
Phasing power (isomorphous)		0.743	0.145
Figure of merit		0.3	
Beamline (ESRF)	ID-29	BM-16	ID-14-4

Refinement statistics

No. of reflections (working/test)	7,283/354
d_{\min} (Å)	2.0
$R_{\text{work}}/R_{\text{free}}$ (%)	22.3/25.5
Rms deviation from ideality	
Bond lengths	0.008
Bond angles	1.36
B factors (\mathring{A}^2) (rmsd of bonded	1.8/5.0
atoms-main/side chain)	
Average B factor (Å ²)	32.3
No. of protein atoms/solvent	642/52



a Values of the highest resolution shell are given in parentheses ${}^{b}R_{merge} = \Sigma_{hkl}\Sigma_{i}\left|I_{hkl,i}\right| - \langle I\rangle_{hkl}\left|/\right. \\ \Sigma_{hkl}\Sigma_{i}|I_{hkl,i}| \text{ where }I_{hkl}\text{ is the intensity of a reflection and } \\ <I\rangle_{hkl}\text{ is the average of all observations of this reflection}$

 Table 2. Coiled-coil parameters

	cc radius r_0 (Å)	cc pitch (Å)	Residue phase (°)	Residues per turn	Rise per residue (Å)
KCNQ1 Helix D	7.54	186.9	a: 19.34 d: -29.41	3.61	1.5
GCN4-LI	7.14	187.9	a: 20.65 d: -28.69	3.60	1.52



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Table 3. Electrophysiological characterization of KCNQ1 mutants

CHO cells	n	pA*pF ⁻¹ [at +60mV]	V ₅₀	Slope	
WT	12	37.3 ± 5.4	-23.6 ± 1.3	15.7 ± 1	
Δ delta C	4	$2.4 \pm 0.4^{**}$	-	-	
G589M	7	33.2 ± 3.6	-32.5 ± 2.0	15.9 ± 2.7	
G589D	12	$2.45 \pm 0.78**$	-	-	
A590W	2	9.6	-	-	
N593G	6	$20.8 \pm 2.5^{\#}$	-11.9 ± 8.9	21.3 ± 3.9	
D597K	2	28.9	-24.0 ± 1.6	14.0 ± 0.3	
T600L	5	31.5 ± 8.3	-21.9 ± 5.1	28.8 ± 4.3	
L602I	5	32.6 ± 4.3	-28.6 ± 2.2	21.2 ± 2.3	
L602M	5	21.1 ± 4.4	-25.3 ± 3.4	26.1 ± 8.3	
D603G	5	33.1 ± 7.1	$-39.3 \pm 6.2^*$	41.1 ± 9.9**	
H620A	6	39.3 ± 12.5	-21.2 ± 2.7	31.9 ± 9.1	
H620S	5	21.9 ± 6.8	-13.1 ± 1.9	8.5 ± 2.7	
HEK cells					
WT	17	97.5 ± 9.1	-14.3 ± 3.0	18.1 ± 1.3	
D597A	8	101.4 ± 11.5	-14.9 ± 4.8	22.6 ± 2.3	
Q601A	4	139.0 ± 24.8	-23.1 ± 5.4	23.6 ± 3.9	
Q604A	5	127.7 ± 17.3	-19.0 ± 3.2	21.9 ± 0.9	
L608A	4	147.5 ± 24.6	-31.0 ± 9.2	20.8 ± 0.9	
1609L	8	104.1 ± 11.8	-6.8 ± 2.5	15.9 ± 1.9	
D611A	7	81.7 ± 5.1	-15.1 ± 4.8	19.3 ± 2.5	
Q615A	8	109.6 ± 7.4	-30.5 ± 5.6*	20.8 ± 1.9	

Activation curves were fit to a Boltzmann distribution, $G/G_{max} = 1/\{1 + exp[(V_{50}-V)/s]\}$, where V_{50} is the voltage at which the current is half-activated and s is the slope factor. *, P < 0.05, **, P < 0.01 vs. WT, (one way ANOVA followed by Dunnett's Multiple Comparison Test); *, P < 0.05, unpaired two-tailed *t*-test.



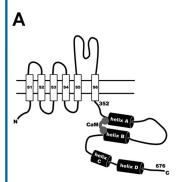
Table 4. ICP spectrometry of Helix D peptide metal binding

	Dialysis buffer ^a	Helix D peptide suspended in metal cocktail ^a	Helix D peptide suspended in buffer ^a	Control peptide suspended in metal cocktail ^a	Metal cocktail ^a
Ca	<0.6	<0.6	<0.6	<0.6	1.5
Cu	<0.02	0.24	< 0.02	<0.02	1.76
Mg	<0.05	<0.05	<0.05	<0.05	0.6
Mn	<0.01	<0.01	<0.01	<0.01	0.12
Zn	<0.03	<0.03	0.06	<0.03	2.4

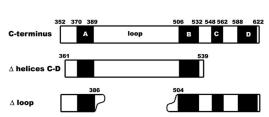
^a in units of mg/ml

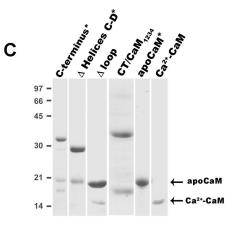


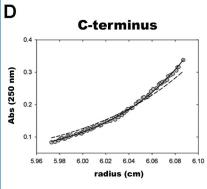


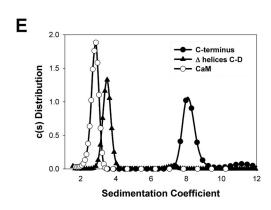


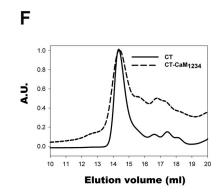




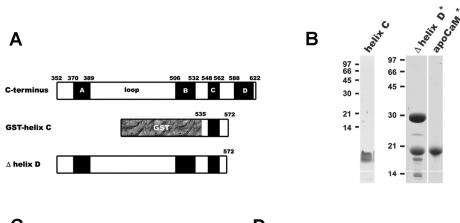


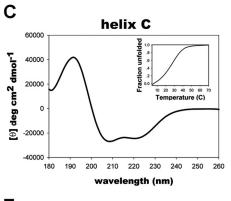


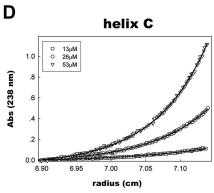


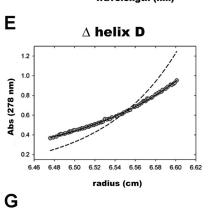


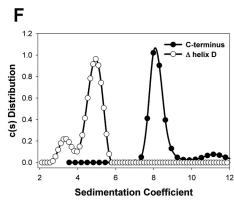
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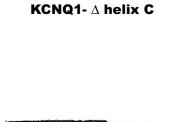


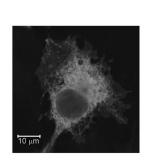


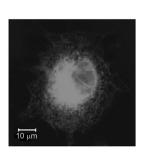


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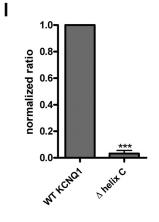
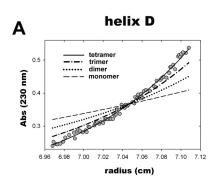
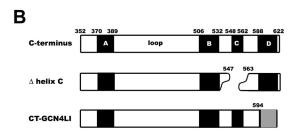
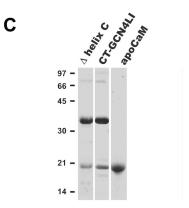


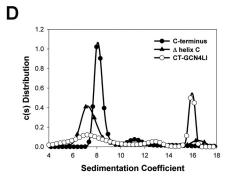
Figure 3







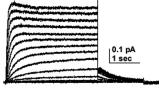


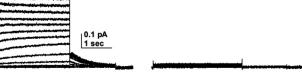


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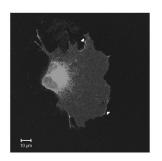
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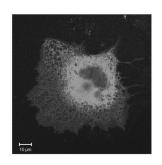
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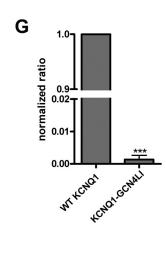




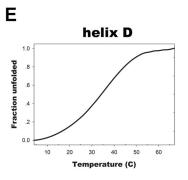
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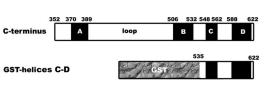


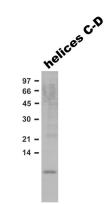




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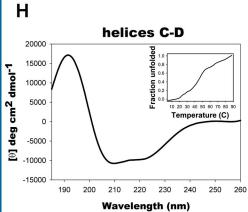




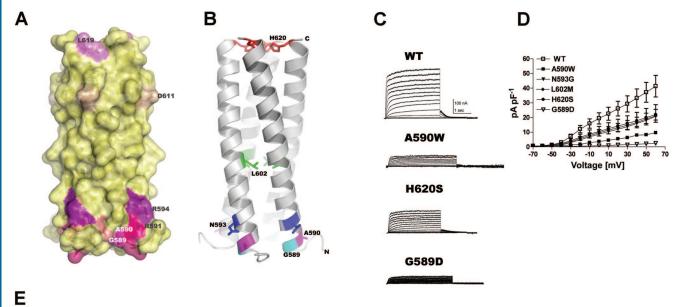


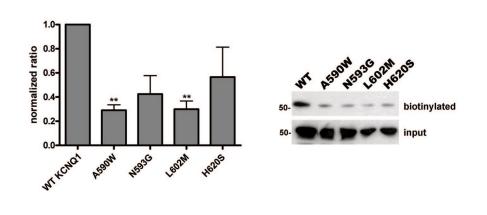


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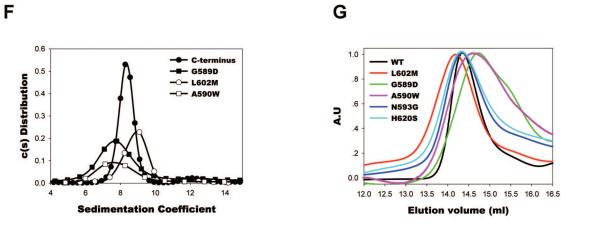
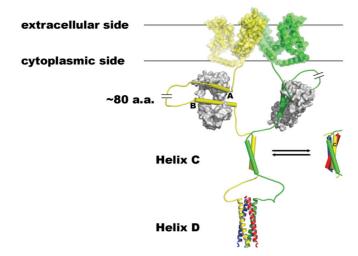


Figure 6





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