Structure of the carboxy-terminal region of a KCNH channel

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The KCNH family of ion channels, comprising ether-a-go-go (EAG), EAG-related gene (ERG), and EAG-like (ELK) K⁺-channel subfamilies, is crucial for repolarization of the cardiac action potential, regulation of neuronal excitability and proliferation of tumour cells. The carboxy-terminal region of KCNH channels contains a cyclic-nucleotide-binding homology domain (CNBHD) and C-linker that couples the CNBHD to the pore. The C-linker/CNBHD is essential for proper function and trafficking of ion channels in the KCNH family. However, despite the importance of the C-linker/CNBHD for the function of KCNH channels, the structural basis of ion-channel regulation by the C-linker/CNBHD is unknown. Here we report the crystal structure of the C-linker/CNBHD of zebrafish ELK channels at 2.2-Å resolution. Although the overall structure of the C-linker/CNBHD of ELK channels is similar to the cyclic-nucleotide-binding domain (CNBD) structure of the related hyperpolarization-activated cyclic-nucleotide-modulated (HCN) channels, there are marked differences. Unlike the CNBD of HCN, the CNBHD of ELK displays a negatively charged electrostatic profile that explains the lack of binding and regulation of KCNH channels by cyclic nucleotides. Instead of cyclic nucleotide, the binding pocket is occupied by a short β-strand. Mutations of the β-strand shift the voltage dependence of activation to more depolarized voltages, implicating the β-strand as an intrinsic ligand for the CNBHD of ELK channels. In both ELK and HCN channels the C-linker is the site of virtually all of the intersubunit interactions in the C-terminal region. However, in the zebrafish ELK structure there is a reorientation of the C-linker so that the subunits form dimers instead of tetramers, as observed in HCN channels. These results provide a structural framework for understanding the regulation of ion channels in the KCNH family by the C-linker/CNBHD and may guide the design of specific drugs.

KCNH channels are voltage-gated K⁺ channels that regulate the electrical excitability of heart and nerve cells. Similar to other K⁺-selective channels, KCNH channels are composed of four subunits surrounding a centrally located pore. Each subunit contains a voltage-sensor domain (transmembrane segments S1–S4), and a pore domain (transmembrane segments S5–S6 and an intervening pore-forming loop) (Fig. 1a). KCNH channels have a Per-Arnt-Sim (PAS) domain in the amino-terminal region and a C-linker and CNBHD in the C-terminal region (Fig. 1a). Many of the unique gating properties of KCNH channels arise from these intracellular domains. Whereas the structure of the N-terminal region has been solved for ERG channels, there is no structural information on the C-linker/CNBHD for any of the KCNH channels.

KCNH channels are part of a large family of cyclic-nucleotide-regulated channels that includes the HCN and cyclic-nucleotide-gated (CNG) channels (Supplementary Fig. 1a). Unlike HCN and CNG channels, KCNH channels are not regulated by direct binding of cyclic nucleotides. Instead, it has been suggested that the CNBHD of KCNH channels may be an orphan receptor for an as yet unidentified channel regulator. Indeed, it has been recently shown that mouse EAG1 channels are regulated by the direct binding of flavonoids to the C-linker/CNBHD. In addition, the C-linker/CNBHD has been shown to interact with the N-terminal PAS domain and regulate gating. Mutations in both of these regions in human ERG1 channels cause long-QT syndrome (LQTS), a heart arrhythmia that can cause cardiac death. Therefore the C-linker/CNBHD is an important regulatory domain in KCNH channels.

To understand the role of the C-linker/CNBHD in KCNH channel function, we sought to determine the X-ray structure of the C-linker/CNBHD of an ion channel in the KCNH family. Using a screen based on fluorescence-detection size-exclusion chromatography (FSEC), we

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**Figure 1** | Topology and electrophysiological properties of zebrafish ELK channels. a, Cartoon of two of the four subunits of ELK channels. The pore-forming loop and S5–S6 transmembrane domains are grey. The N-terminal α-helix and PAS domain are magenta. The ‘elbow’ and ‘shoulder’ regions of the C-linker are represented by the red and pink cylinders, respectively. The αC-helix, represented by a cylinder, is green and the rest of the CNBHD is blue. b, Currents from zebrafish ELK channels recorded in the inside-out patch configuration.
we identified the C-linker/CNBHD of Danio rerio (zebrafish) ELK as a potential candidate for crystallization. Zebrafish ELK shares substantial sequence similarity with mammalian ELK channels (Supplementary Fig. 1c).

Expression of zebrafish ELK channels in Xenopus laevis oocytes gave rise to robust voltage-activated K\(_{\text{1}}\) currents with electrophysiological characteristics similar to the ones reported previously for mammalian ELK channels\(^{22–25}\) (Fig. 1b). Zebrafish ELK channels were activated by depolarizing voltage steps and showed inactivation at voltages \(> + 40 \text{ mV}\) (Fig. 1b). The half-maximal activation voltage (\(V_{1/2}\)) was \(-45.3 \pm 3.2 \text{ mV} (n = 18)\) with a slope of e-fold per \(13.8 \pm 0.6 \text{ mV} (n = 18)\) (Supplementary Fig. 1b). In comparison, human ELK2 channels activate with \(V_{1/2}\) of \(-22.8 \pm 0.5 \text{ mV}\) and a slope of \(18.1 \pm 0.4 \text{ mV}\), and inactivate at voltages \(> + 20 \text{ mV}\) (ref. 22). As previously observed for mouse EAG and human ERG channels\(^4,11\), application of cAMP had no effect on the currents through zebrafish ELK channels (Supplementary Fig. 1b).

The C-linker/CNBHD of zebrafish ELK channels crystallized in two space groups, \(C_{2221}\) and \(P_{1211}\), and diffracted X-rays to 2.2- and 2.3-Å resolution, respectively (Supplementary Table 1). The structure of the zebrafish ELK C-linker/CNBHD was solved by the single-wavelength anomalous dispersion (SAD) phasing method using selenomethionine derivative crystals.

The crystal structure revealed that the C-linker of zebrafish ELK channels consists of six \(\alpha\)-helices (\(\alpha_A\)–\(\alpha_F\)) with \(\alpha_A\) and \(\alpha_B\) helices forming an antiparallel helix–turn–helix motif. The CNBHD consists of eight \(\beta\)-strands forming an antiparallel \(\beta\)-roll, three \(\alpha\)-helices (\(\alpha_A\)–\(\alpha_C\)), and a short \(\beta\)-strand (\(\beta_9\)) after the \(\alpha_C\)-helix (Fig. 2a). The general architecture of the CNBHD of ELK is similar to the fold of CNBDs in other proteins\(^{26}\).
Although the overall folds of the CNBHDs of ELK and HCN2 channels are similar, superposition of the two structures reveals remarkable differences (Fig. 2b and Supplementary Fig. 2a). The root mean squared deviation (r.m.s.d.) for the $\alpha$-carbons of the two structures (residues 626–740 of zebrafish ELK and residues 523–635 of mouse HCN2; ref. 10) is 4.7 Å with the largest differences observed for the three $\alpha$-helices in the CNBHD. The $\alpha$A- and $\alpha$B-helices of ELK are moved away from the cavity formed by the $\beta$-roll that serves as the cyclic-nucleotide-binding pocket in other CNBD-containing proteins. The $\alpha$A-helix is moved by about 4.5 Å and the $\alpha$B-helix is moved by about 3.4 Å relative to their positions in the cAMP-bound form of HCN2. The position of the $\alpha$A-helix of ELK is similar to the position of the $\alpha$A-helix in cAMP-bound Mlo1K channels27 and the position of the $\alpha$B-helix is similar to the unliganded Mlo1K channels27-29 (Supplementary Fig. 3). The differences in the $\alpha$C-helix are even more marked. The $\alpha$C-helix of ELK is shorter and bent, and is followed by a short $\beta$-strand, $\beta$9 (Fig. 2b). These differences all reside in the $\alpha$-helices of the CNBD that undergo ligand-dependent conformational changes in HCN and CNG channels.

Out of the ten residues that directly interact with cAMP in HCN2 channels, only three (V667, L677 and G684) are conserved in zebrafish ELK channels (Supplementary Fig. 4). Moreover, most of the residues in the phosphate-binding cassette of HCN2, including R591, are not conserved, and the $\alpha$P-helix is missing in zebrafish ELK. The electrostatic profiles of the CNBD of ELK and HCN2 channels reveal that the putative ligand-binding pocket formed by the $\beta$-roll cavity is negatively charged for ELK but positively charged for HCN2 channels (Fig. 2c, d). The negatively charged electrostatic profile would not be favourable for binding of a negatively charged cyclic nucleotide. Consistent with this, crystallization of zebrafish ELK in the presence of 5 mM cAMP did not reveal any new electron density corresponding to cAMP (Supplementary Table 1 and data not shown). These differences in the CNBD structures probably account, at least in part, for the lack of cyclic nucleotide regulation of KCNH channels4,11.

The zebrafish ELK CNBHD structure displays another unique feature: the $\beta$9-strand after the $\alpha$C-helix forms direct interactions with the $\beta$-roll cavity in ELK channels (Fig. 2e and Supplementary Figs 2b and 5). Interestingly, the phenyl ring of Y740 on the $\beta$9-strand is positioned in an analogous place to the purine ring of cAMP in HCN2 channels and L742 is positioned in an analogous place to the cyclic phosphate of cAMP15. Virtually all ion channels in the KCNH family have either tyrosine or phenylalanine at the position corresponding to Y740 in zebrafish ELK and a leucine at position corresponding to L742 (Supplementary Fig. 1c). These observations raise an intriguing possibility that the $\beta$9-strand might act as an intrinsic ligand, a portion of the protein that occupies the ligand-binding site whose displacement regulates the channel.

To explore a possible regulatory role of the $\beta$9-strand, we examined the effect of mutations in the $\beta$9-strand on the function of intact zebrafish ELK channels. Zebrafish ELK channels with either a point mutation Y740A or deletion of the $\beta$9-strand ($\Delta$740–742) exhibited robust voltage-activated currents that inactivated at voltages $> + 40$ mV, similar to wild-type ELK channels (Supplementary Fig. 6a). However, for both mutations, the $V_{1/2}$ values for activation were significantly larger ($P < 0.01$, Student’s $t$-test) than the $V_{1/2}$ of wild-type channels (wild type: $-45.3 \pm 3.2$ mV, $n = 18$; Y740A: $-29.8 \pm 4.4$ mV, $n = 19$; $\Delta$740–742: $-28.3 \pm 4.4$ mV, $n = 11$) (Fig. 2f and Supplementary Fig. 6b). This 15 mV shift in the voltage dependence of activation in the mutants is similar in magnitude to the effect of cAMP on HCN channels, lending further support to the possibility that the $\beta$-9-strand may function as an intrinsic ligand for ELK channels. Interestingly, mutations in the region corresponding to the $\beta$-9-strand of human ERG channels are associated with LQTS7,8,10. Additional experiments will need to be done to further test the intrinsic ligand hypothesis.

Similar to HCN channels, the C-linker of zebrafish ELK channels is the primary region of intersubunit interactions in the crystal structure with a buried solvent accessible surface area of about 2,520 Å$^2$ for each subunit (Fig. 3a). The intersubunit interface can be likened to an ‘elbow’ on the shoulder, where the ‘elbow’ formed by the $\alpha$A’- and $\alpha$B’-helices of one subunit is resting on the ‘shoulder’ formed by the $\alpha$D’-helix and the $\alpha$C’–$\alpha$D’ loop of the neighbouring subunit (Figs 2a, 3a, d and Supplementary Fig. 7). The structure of the elbow-on-the-shoulder interface between neighbouring subunits is very similar between ELK and HCN2 channels (Fig. 3d), except that the region before the $\alpha$D’-helix is not $\alpha$-helical in ELK.

Unexpectedly, however, the elbow-on-the-shoulder interface in zebrafish ELK occurs with a two-fold related subunit (rotated by 180°), instead of a four-fold related subunit (rotated by 90°) like in HCN channels (Fig. 3a, b). This difference results from a ~55° rotation in the region after the $\alpha$B’-helix in ELK relative to HCN2 channels (Fig. 3c). The implication of this marked rotation of the ‘elbow’ is a dimeric assembly of the C-linker/CNBHDs in the zebrafish ELK structure (Fig. 3a), as opposed to the tetrameric assembly of the C-linker/CNBHDs in HCN channels10 (Fig. 3b). The intersubunit interface and the dimeric assembly are preserved in both sets of molecules in the asymmetric unit and for both crystal forms of zebrafish ELK (Supplementary Fig. 8), suggesting that they are independent of crystal contacts. FSEC experiments also revealed that the GFP-tagged C-linker/CNBHD of zebrafish ELK channels dimerizes at sufficiently
high concentrations in solution (Supplementary Fig. 9). Additional experiments would be required to determine if the quaternary state of the C-linker/CNBHD in intact channels has a two-fold or four-fold symmetry (Supplementary Discussion and Supplementary Fig. 10).

Here we present the first crystal structure, to our knowledge, of the C-linker/CNBHD of a KCNH channel. The structure reveals a putative ligand-binding pocket that differs markedly from the cyclic-nucleotide-binding pocket of HCN channels and does not appear to bind cyclic nucleotides. Instead of a ligand, the putative ligand-binding pocket of zebrafish ELK channels is occupied by a novel β9-strand after the βC-helix. Mutations of the β9-strand shifted the voltage dependence of activation, suggesting that the β9-strand is a regulatory element for ELK channels that acts as an intrinsic ligand. The crystal structure also displays an unexpected conformation of the C-linker that leads to dimerization of the C-linker/CNBHDs of zebrafish ELK channels. These findings provide a structural framework to understand the regulation of KCNH channels by the C-linker/CNBHD.

**METHODS SUMMARY**

**Protein purification.** The C-linker/CNBHD of zebrafish ELK (amino acids Q543–L790) was subcloned into a modified pMak2T vector (New England Biolabs) containing an N-terminal MBP affinity tag followed by a thrombin cleavage site. The protein was expressed in BL21 (DE3) *Escherichia coli* cells as previously described, purified on an amylose affinity column and then on an ion-exchange column after an overnight cleavage with thrombin at 4°C. The purified protein was concentrated to 20–30 mg ml⁻¹ for crystallization. Selenomethionine derivatives were generated as previously described.

**Crystallography.** Crystals were grown at 20°C using the sitting-drop vapour diffusion method. 150-ml drops of the concentrated protein and reservoir solutions were mixed 1:1 by a Mosquito (TTP LABTECH). The final protein solution contained: −250 mM KC¹, 1 mM TCEP, 30 mM HEPES, 100 mM citric acid, pH 3.5. The reservoir solution contained: 6% w/v 1,5-diaminopentane dihydrochloride, 180 mM ammonium acetate, 22.5% (w/v) PEG 3350, 90 mM Tris pH 8.5 for crystal T41; 1.8 mM non-salt detergent sulphobetaine (NDSB)-211, 180 mM ammonium acetate, 22.5% (w/v) PEG 3350, 90 mM Tris pH 8.5 for crystal T42; 6% (w/v) D- (+) galactose, 180 mM ammonium sulphate, 22.5% (w/v) PEG 3350, 90 mM HEPES, pH 7.5 for crystal T26; and 5 mM CaCl₂, 180 mM ammonium acetate, 22.5% (w/v) PEG 3350, 90 mM Tris pH 8.5 for crystal T84.

The crystallographic data are summarized in Supplementary Table 1 and details of the structure determination are described in Methods.

**Electrophysiology.** The full-length zebrafish ELK channel (GenInfo Identifier (GI) 159570347) with a C-terminal Flag epitope was generated by Bio Basic Inc. 90 mM Tris, pH 8.5 for crystal T84.

**crystal T26; and 5 mM cAMP, 180 mM ammonium acetate, 22.5% (w/v) PEG 3350, 90 mM Tris, pH 8.5 for crystal T42.**

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Received 29 May; accepted 23 November 2011. Published online 9 January 2012.
METHODS

FSEC. The C-linker/CNBHD of zebrafish ELK channels (amino acids Q543–L750) was subcloned into a modified pMALc2T vector (New England Biolabs) containing an N-terminal MBP affinity tag followed by a thrombin cleavage site. The protein was expressed in BL21 (DE3) E. coli cells as previously described. The cells were harvested by centrifugation, resuspended in a lysis buffer (500 mM KCl, 1 mM TCEP, 30 mM HEPES, 1 mM PMSF, 2.5 mg ml⁻¹ DNase; pH 8.0) and sonicated. Insoluble protein was separated by centrifugation and the supernatant was analysed on a Superdex 200 10/300 GL column (GE Healthcare).

Scale-up protein purification. The C-linker/CNBHD of zebrafish ELK was purified on an amylose affinity column and was then loaded on a HitTrap SP FF ion-exchange column following an overnight cleavage with thrombin at 4 °C. The protein was eluted with a linear KCl gradient and 100 mM cGMP, 180 mM ammonium acetate, 22.5% (w/v) PEG 3350, 90 mM Tris, pH 8.5 was used to separate the protein at high concentrations. The protein was concentrated to 20–30 mg ml⁻¹ for crystallization. Selenomethionine derivations were generated as previously described.

Crystallization. Crystals were grown at 20 °C using the sitting-drop vapour diffusion method. 150-nl drops of the concentrated protein and reservoir solution were mixed 1:1 by a Mosquito (TTP LABTECH). The final protein solution contained: ~250 mM KCl, 1 mM TCEP, 30 mM HEPES, 100 mM cGMP, 180 mM ammonium acetate, pH 3.5. The reservoir solution contained: 6% w/v 1,5-diamino pentane dihydrochloride, 180 mM ammonium acetate, 22.5% (w/v) PEG 3350, 90 mM Tris, pH 8.5 for crystal T141; 1.8 M non-detergent sulphobetaine (NDSB)-211, 180 mM ammonium acetate, 22.5% (w/v) PEG 3350, 90 mM Tris, pH 8.5 for crystal T26; and 5 mM cAMP, 180 mM ammonium acetate, 22.5% (w/v) PEG 3350, 90 mM Tris, pH 8.5 for crystal T84. The crystals were cryoprotected in reservoir solution supplemented with 25% glycerol before being flash frozen in liquid nitrogen.

Data collection and structure determination. Diffraction data sets were collected at the Advanced Light Source (beamline 8.2.1) at Lawrence Berkeley National Laboratory in Berkeley, California. Data were analysed with Mosfilm 32 and HKL2000 33 software. Molecular replacement using the structure of the C-linker/CNBHD of zebrafish ELK was performed as a search model followed by numerous cycles of refinement in PHENIX and the rest of the data sets were solved by molecular replacement using this structure as a search model followed by numerous cycles of refinement in PHENIX and manual model building in Coot 34. The molecular replacement was carried out using Phaser in PHENIX. The asymmetric unit contained three molecules in the C222₁ space group and four in the P12₁ space group. The structures of different ELK molecules in the asymmetric unit and molecules in the two different space groups were very similar, with r.m.s.d. values for the α-carbons calculated for the entire sequence of the resolved C-linker/CNBHD ranging from 0.4–0.7 Å (Supplementary Fig. 8). The crystallographic data and refinement statistics are summarized in Supplementary Table 1. Electron density was visible for all but several terminal residues in molecules A and B of the native structure in the C222₁ space group. Molecules A and B of the native C222₁ structure were used for analysis in this paper. Analysis with Molprobity 36 of the final models indicated no Ramachandran outliers for T42 and T26 structures, and 0.59 (%) for T84. Figures were made using PyMOL. The topology of the C-linker/CNBHD of zebrafish ELK was defined by PROCHECK (http://www.wwpdb.org/). The electrostatic potential surface calculations were carried out using the APBS 39 plugin for PyMOL and the PARSE force field, and coloured from red (−3 kT/e) to blue (+3 kT/e) where k is Boltzmann’s constant, T is absolute temperature, and e is the charge on an electron.

Electrophysiology. The full-length zebrafish ELK channel (GI: 159570347) with a C-terminal Flag epitope was generated by Bio Basic Inc. and subcloned into the pGEMHE-oocyte expression vector. The cRNA was transcribed using the T7 mMessage mMachine Ultra kit (Ambion). Expression of the wild-type and mutant (Y740A and Δ740–742) zebrafish ELK channels in Xenopus oocytes and current recordings from inside-out patches allowing 10 min for run up following excision were done as described below 40. Both pipette and bath solutions contained 130 mM KCl, 10 mM HEPES, 0.2 mM EDTA, pH 7.2. 1 mM cAMP was added to the bath solution as indicated. Zebrafish ELK currents were elicited by applying a series of 100-ms voltage pulses (ranging from −140 to +160 mV in 20 mV increments) from a pre-pulse potential of −140 mV, followed by a 150 ms tail pulse to −100 mV. Currents were leak-subtracted with P/4 protocol. To obtain conductance versus voltage curves, peak tail current amplitudes at −100 mV were normalized to the largest peak conductance amplitude, which followed a step to +40 mV. These normalized data were then plotted against the test voltage, and were fit with a Boltzmann equation:

\[
\frac{G}{G_{\text{max}}} = \frac{1}{1 + e^{\left(\frac{V - V_{1/2}}{s}\right)}}
\]

where V represents the test voltage, V₁/₂ is the midpoint activation voltage, and s is the slope of the relation.

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