The structural mechanism of KCNH-channel regulation by the eag domain

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The KCNH voltage-dependent potassium channels (ether-a`-go-go, EAG; EAG-related gene, ERG; EAG-like channels, ELK) are important regulators of cellular excitability1–3 and have key roles in diseases such as cardiac long QT syndrome type 2 (LQT2)4, epilepsy5, schizophrenia6 and cancer7. The intracellular domains of KCNH channels are structurally distinct from other voltage-gated channels. The amino-terminal region contains an eag domain, which is composed of a Per-Arnt-Sim (PAS) domain and a PAS-cap domain8, whereas the carboxy-terminal region contains a cyclic nucleotide-binding homology domain (CNBHD), which is connected to the pore through a C-linker domain. Many disease-causing mutations localize to these specialized intracellular domains, which underlie the unique gating and regulation of KCNH channels9. It has been suggested that the eag domain may regulate the channel by interacting with either the S4–S5 linker or the CNBHD10,11. Here we present a 2 Å resolution crystal structure of the eag domain–CNBHD complex of the mouse EAG1 (also known as KCNH1) channel. It displays extensive interactions between the eag domain and the CNBHD, indicating that the regulatory mechanism of the eag domain primarily involves the CNBHD. Notably, the structure reveals that a number of LQT2 mutations at homologous positions in human ERG, in addition to cancer-associated mutations in EAG channels, localize to the eag domain–CNBHD interface. Furthermore, mutations at the interface produced marked effects on channel gating, demonstrating the important physiological role of the eag domain–CNBHD interaction. Our structure of the eag domain–CNBHD complex of mouse EAG1 provides unique insights into the physiological and pathophysiological mechanisms of KCNH channels.

KCNH channels have two large intracellular regions that underlie the specialized gating and regulation of this channel family9 (Fig. 1a). The CNBHD in the C-terminal region shares similarity with the cyclic nucleotide-binding domains of cyclic nucleotide-gated (CNG) and hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels12,13. The CNBHD of KCNH channels, however, does not bind cyclic nucleotides and regulates KCNH channel gating in a cyclic nucleotide-independent manner13. The N-terminal region contains an eag domain, not present in CNG and HCN channels, that also has a key role in channel gating. Composed of a 110-amino-acid PAS domain preceded by a highly conserved 25-amino-acid PAS-cap (Fig. 1a and Supplementary Fig. 1), the eag domain regulates activation and inactivation in EAG1, and deactivation in human (h)ERG1 (also known as KCNH2) channels14,15. Moreover, many of the human mutations that cause LQT2 and cancer localize to the conserved eag domain16–17. In addition, high expression of a primate-specific isoform of ERG (KCNH2-3.1), missing most of the eag domain, was shown to correlate with risk for schizophrenia18. The mechanism underlying KCNH channel regulation by the eag domain remains unclear. Two competing hypotheses have emerged. Some evidence suggests that the eag domain interacts with the S4–S5 linker and directly regulates the movement of the S4 voltage sensor19,20. By contrast, other evidence favours the CNBHD as the interaction partner of the eag domain14,16,17. Using fluorescence anisotropy we found that the eag domain interacts directly with the CNBHD with an affinity of 13.2 ± 2.3 μM (Supplementary Fig. 2).

To uncover the mechanism for how the eag domain regulates the channel, we solved the X-ray crystal structure of the eag domain–CNBHD complex to 2.0 Å resolution (Supplementary Table 1). There were eight chains in the asymmetric unit: four eag domains and four CNBHDs. The spatial arrangement of the eag domains and CNBHDs within the asymmetric unit showed three different eag domain–CNBHD interactions, here termed interactions A, B and C (Supplementary Fig. 3). Interaction A, however, had considerably greater buried surface area than the other interactions (Supplementary Table 2) and was confirmed

Figure 1 | Structure of the eag domain–CNBHD complex of mEAG1.

a, Cartoon of a cross section of a KCNH channel. Transmembrane domains are in grey, the N-terminal eag domains are in green, the C-linkers are in orange and the CNBHD domains are in blue. The intrinsic ligand motifs are highlighted in yellow, and the post-CNBHD in red. b, Structure of the eag domain–CNBHD complex. Colour corresponds to panel a. c, Disease-causing mutations at the interface of the eag domain–CNBHD complex. LQT2 mutations are shown in yellow. Cancer-associated mutations are shown in red. Y44 is involved in both LQT2 and cancer and is shown in red.
by mutagenesis experiments in the intact channel (see below). Therefore interaction A was designated as the biological unit.

Figure 1b shows the structure of the eag domain–CNBHD complex in the biological unit. The structure of the CNBHD (residues 552–720) includes the last 35 amino acids of the C-linker, the CNBHD, and 22 amino acids of the post-CNBHD region (Fig. 1b and Supplementary Fig. 1). The structure of the eag domain (residues 16–136) includes the last 35 amino acids of the post-CNBHD region (Fig. 1b and Supplementary Fig. 4). These mutations alter channel gating as well as trafficking to the membrane and were shown to regulate channel activity, shifting the voltage dependence and kinetics of activation and deactivation. Interestingly, mutations in the eag domain of KCNH channels result in similar alterations9,14,16. The interaction between the eag domain and CNBHD (Fig. 1c) was therefore called the ‘intrinsic ligand’. Mutations in this intrinsic ligand motif of the CNBHD were particularly surprising (Fig. 2a, b). Intrinsic ligand and post-CNBHD regions of the eag domain form an interaction with the post-CNBHD segment of the CNBHD; and (3) an amphipathic helix (zCAP) in the PAS-cap domain forms an interaction with the β-rol of the CNBHD (Fig. 1a, b).

The direct interaction of the PAS domain with the intrinsic ligand motif of the CNBHD was particularly surprising (Fig. 2a, b). Previous structures of isolated CNBHD from meEAG1 (ref. 12) and zebrafish ELK11 showed that a short loop (residues 697–701) following the εC helix occupies the space filled by cyclic nucleotides in canonical cyclic nucleotide-binding domains (Supplementary Fig. 1). This short loop was therefore called the ‘intrinsic ligand’. Mutations in this intrinsic ligand were shown to regulate channel activity, shifting the voltage dependence and kinetics of activation and deactivation. Interestingly, mutations in the eag domain of KCNH channels result in similar alterations9,14,16. The interaction between the eag domain and the intrinsic ligand, and the functional similarities between mutations in these domains, suggest that the intrinsic ligand motif may be a critical determinant of the regulation of KCNH channels by the eag domain.

Another surprising site of interaction of the eag domain is the post-CNBHD region (beginning at residue 702). This region, immediately C-terminal to the intrinsic ligand, has been shown to mediate the regulation

Figure 2 | The various interfaces between the eag domain and CNBHD, and different complex types in the asymmetric unit. a–c, Cartoon and surface representation of the eag domain–CNBHD complex (a). Interaction and surface representation of the eag domain and CNBHD is shown in green and blue, respectively. The intrinsic ligand and post-CNBHD region are in yellow and red, respectively. The non-interacting parts of the structure are in grey. Red and purple rectangles define regions detailed in b (intrinsic ligand) and c (post-CNBHD region), respectively. d, Cartoon and surface representation of the asymmetric unit complexes related by non-crystallographic symmetry in the same conformation. Two-fold symmetry axis indicated. e, Comparison of the structures of the two complex types, colour coded as in d. Dashed red lines show distances between D47 of the eag domain and E578 of the CNBHD.
of KCNH channel function by a variety of cellular signalling events, including phosphorylation, and interaction with kinases, integrins and Ca2+-calmodulin24–26. The eag domain–CNBHD structure shows that the post-CNBHD region interacts with the βα–ββ loop of the eag domain (Fig. 2a, c and Supplementary Fig. 1). The interaction between the eag domain and the post-CNBHD region suggests that KCNH channel regulation through the post-CNBHD region may involve its interaction interface with the eag domain.

The crystal contains two different conformations of the eag domain–CNBHD complex. The asymmetric unit is composed of four copies of the complex, related by a two-fold non-crystallographic symmetry (Fig. 2d). This results in two pairs of the complex (‘type I’ and ‘type II’ complexes) that are in a similar conformation within each pair (average r.m.s.d. = 0.16 Å), but a different conformation and B-factor distribution between pairs (r.m.s.d. = 0.96 Å) (Fig. 2d and Supplementary Fig. 5). Alignment of the two conformations reveals that the angle between the eag domain and the CNBHD differs by 5° and that the distance between the two domains differs by 1.8 Å (between D47 of the eag domain and E578 of the CNBHD) (Fig. 2e and Supplementary Video 1). In addition, the αP helix (residues 649–651; Supplementary Fig. 1) undergoes a substantial rearrangement between the two conformations, possibly due, at least in part, to variation in the local crystal contacts (Fig. 2e and Supplementary Video 1). These two conformations, if present in the intact channel, suggest that the eag domain–CNBHD complex is not static and represent a possible rearrangement associated with the gating regulation of the channel by the eag domain.

To determine the functional role of the eag domain–CNBHD interaction, we mutated the interaction interface between the eag domain and the CNBHD in intact mEAG1 channels. The interface contains a salt bridge between R57 in the αP helix of the PAS domain and D642 in the β6 strand of the CNBHD (Fig. 3a and Supplementary Fig. 4). This salt bridge is present in both conformations of the biological unit and is not present in the other eag domain–CNBHD interactions in the crystal. Notably, R57 and D642 are conserved throughout the KCNH family (Supplementary Fig. 1) and has been shown to be crucial for proper activation and inactivation gating in KCNH channels9,27. Notably, our structure of the eag domain–CNBHD complex reveals that an amphipathic helix (αCAP) of the PAS-cap domain of mEAG1 interacts directly with the CNBHD (Fig. 4a). The structure shows that αCAP is nestled between a hydrophobic patch of the PAS domain and the β4–β5 strands and β8–β9 loop of the CNBHD. The structure of the PAS-cap domain was previously assessed using NMR of isolated eag domain from hERG1 (refs 10, 19, 22). Structural alignment reveals that the PAS-cap domain in our eag domain–CNBHD complex is in a very different orientation from the NMR structures (Fig. 4b). The orientation, however, is very similar to recent X-ray crystallography structures of isolated eag domains from hERG1 and ELK21. We propose that the spatial organization of the PAS-cap domain within the eag domain–CNBHD complex recapitulates the native conformation of this domain in the intact channel, and that the PAS-cap domain exerts its functional effects through its interaction with the CNBHD.

Although the PAS-cap domain itself does not contain any known LQT2 mutations and only one cancer-associated mutation (hEAG1(E19D)), its surroundings are rich in pathological loci, including hERG1 LQT2 mutation E788D29 (E627 in mEAG1) in the β4 strand of the CNBHD30,31. These mutations may alter the gating properties of the channel by destabilizing the PAS-cap domain interaction with the CNBHD. To test this hypothesis, we measured the currents from mEAG1 channels mutated in the PAS-cap domain (R7A–R8A and R7E–R8E) and in the CNBHD region where the PAS-cap is bound (E627A and E627R). These positions are highly conserved within the KCNH family (Supplementary Fig. 1). The voltage dependence of activation of the PAS-cap mutations R7A–R8A and R7E–R8E were significantly shifted to more depolarized potentials, compared to wild-type channels (Fig. 4c and Supplementary Table 4). The CNBHD mutations also showed a robust depolarizing shift, similar to that of the R7E–R8E mutant (Fig. 4c).

**Figure 3 | A salt bridge between the eag domain and the CNBHD.**

a. Cartoon and surface representation of the primary interaction interface (interaction A) of the eag domain–CNBHD complex (left). Eag domain is shown in green, CNBHD in blue and interface residues in red. A detailed illustration of the R57–D642 salt bridge in shown on the right, overlaid with 2Fo–Fc composite omit map contoured at 1σ (grey mesh).

b. Summary of time to 90% maximum current amplitude for wild-type (WT) and mutants (error bars indicate ± s.e.m.; n = 6–10 cells; P < 0.005, Student’s t-test).
neighbouring subunit. In which the eag domain of one subunit interacts with the CNBHD of a channels suggest that this arrangement may exhibit domain swapping through sites in the post-CNBHD. Mutagenesis studies in hERG1 indicate that the interaction interface between the two domains serves as a hot-spot for many of these mutations and provides a framework to better understand the mechanisms of gating and regulation of KCNH channels. Furthermore, understanding the molecular interactions within the KCNH channel complex, and their disruption in disease, may enable design of novel therapies for these devastating conditions.

METHODS SUMMARY

The mouse EAG1 eag domain (residues 6–136) and CNBHD (residues 552–724) were subcloned into a PETM11 vector containing an N-terminal hexa-histidine affinity tag followed by a tobacco etch virus (TEV) cleavage site. The proteins were expressed in BL21(DE3) Escherichia coli cells as described previously, purified on NMR (hERG1) and then on an ion-exchange (CNBHD) or size-exclusion (eag domain) column, following an overnight cleavage with TEV at 4 °C. The proteins were concentrated to about 475 μM, flash-frozen and stored at −80 °C until use. The eag domain and the CNBHD were mixed in a 1:1 molar ratio before experimentation. Crystals were grown at 20 °C using sitting-drop vapour diffusion by mixing a 2:1 (v/v) ratio of protein mixture and a reservoir solution containing 18% (w/v) PEG 3350, 1.8% (v/v) tacsimate, 10 mM MnCl₂ and 90 mM HEPES, pH 7.5. The crystals diffracted to 1.995 Å and belong to space group P3₁2₁2₁ with unit cell parameters a = b = 71.4 Å, c = 151.1 Å and β = 90°. The crystals were flash-frozen in liquid nitrogen.

Electrophysiology recordings were performed using the inside-out configuration of the patch-clamp technique on Xenopus oocytes expressing wild-type and mutant channels as described previously.

Full Methods and any associated references are available in the online version of the paper.

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Author Information Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data Bank under accession code 4LLO. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to W.N.Z. (zagotta@uw.edu).
METHODS
Cloning, expression and purification. Mouse EAG1 (Genbank accession codes NP034730.1 and GQ6754422) eg domain (residues 6–136) and CNBHD (residues 552–712, 552–724 and 552–752) were subcloned using 5’ Ncol and 3’ HindIII sites into a pETM11 vector containing an N-terminal hexa-histidine affinity tag followed by a tobacco etch virus protease (TEV) cleavage site. The internal Ncol restriction site in the CNBHD was removed via a silent mutation. Final CNBHD boundaries were selected based on small-scale expression and purification experiments (residues 552–724). All constructs had a GAG(M) cloning artefact sequence introduced at the N terminus. Full-length complementary DNA of mEAG1 was subcloned into the high-expression pGHi9 vector for expression in *Xenopus* oocytes. The proteins were expressed in BL21(DE3) *Escherichia coli* cells as previously described.11 Bacterial cultures were grown to mid-log phase and induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) overnight at 18 °C. The cultures were spun down and re-suspended in 150 mM NaCl, 20 mM Tris-HCl and 1 mM tris(2-carboxyethyl)phosphine (TCEP), pH 8.0, containing 1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride (AEBSF), 2.5 µg ml⁻¹ DNAse and cComplete protease inhibitor tablets (Roche). Cells were lysed by an EmulsiFlex C-5 homogenizer (Avestin) and the lysate was cleared by centrifugation at 131,000g for 45 min at 4 °C. The proteins were then purified on a 4% affinity resin column (HisTrap HP, GE Healthcare). The hexa-histidine tag was removed by TEV cleavage overnight at 4 °C. The CNBHD was further purified by anion exchange chromatography (Hit Trap Q HP, GE Healthcare) after tenfold dilution with buffer containing 20 mM Tris-HCl, 1 mM TCEP, pH 8.0. The protein eluted with a shallow linear NaCl gradient as one well-separated peak. The TEV-digested eg domain was concentrated and further purified by gel filtration (Superdex 200, GE Healthcare) with buffer containing 150 mM NaCl, 20 mM Tris-HCl, 5 mM dithiothreitol (DTT), pH 8.0. Both proteins were concentrated to about 475 mM dithiothreitol (DTT). The labelled protein was separated from unincorporated dye by gel filtration and concentrated to 50 µM.

Fluorescence size-exclusion chromatography (FSEC)14 and fluorescence anisotropy. For FSEC experiments, FL-eag was mixed in a 1:1 molar ratio with purified CNBHD and the mixture was loaded on a Superdex 75 10/300 GL column (GE Healthcare) mounted on a high-performance liquid chromatography system (Shimadzu) with a fluorescence detector set for detection of fluorescein fluorescence. Fluorescence anisotropy was recorded using a Fluorolog 3 spectrophotometer (HORIBA, Jobin Yvon). In total, 100 nM FL-eag was placed in a quartz cuvette, and anisotropy was measured with increasing concentrations of CNBHD. Anisotropy experiments with 492 nm excitation and 514 nm emission were performed as described previously.12 To estimate binding affinity, plots of the anisotropy versus total CNBHD concentration were fit using the following first-order reaction scheme:

\[
R + L \rightarrow RL
\]

Anisotropy = \( \frac{\left(R + K_a + L\right) - \sqrt{\left(R + K_a + L \right)^2 - 4 \\times R \times L}}{2} + \beta \)

in which \( R \) and \( L \) are concentrations of the free receptor, free ligand, and receptor–ligand complex, respectively, \( R \) and \( L \) are total receptor and total ligand concentrations, respectively, \( K_a \) is the ligand-binding affinity, and \( \alpha \) and \( \beta \) are a scaling factor and an offset factor, respectively.

Crystallization and structure determination. Crystals of the eg domain–CNBHD complex were grown at 20 °C using sitting-drop vapour diffusion by mixing a 2:1 ratio (Mosquito, TTP LARTECH) of protein mixture and a reservoir solution, containing 18% (w/v) PEG 3350, 1.8% (v/v) Tascimate, 10 mM NaCl and 90 mM HEPES, pH 7.5. This condition produced crystals within 3 days, which grow to maximum size of about 300 µm \( \times \) 80 µm \( \times \) 80 µm after 21 days. For diffraction data collection, crystals were immersed in liquid nitrogen after cryoprotection in 20% glycerol. Data were collected at 110 K on beamline 8.2.1 at the Advanced Light Source (Lawrence Berkeley National Laboratory). Integration, scaling and merging of the diffraction data were done with the Moslim program13. The crystals belonged to space group P6₃ and the unit cell had dimensions of \( a = b = 162.38 \) Å and \( c = 100.44 \) Å with \( \alpha = \beta = 90° \) and \( \gamma = 120° \). The structure was solved by molecular replacement using the programs Phaser14 and Phenix15 with the mEAG1 CNBHD (PDB accession 4F8A) as the search model. Data collection and refinement statistics are summarized in Supplementary Table 1. Each asymmetric unit contained eight protein subunits forming four eg domain–CNBHD complexes. Electron density was visible for most of the protein chains except for a few disordered terminal residues and the initial 10 and 23 residues of the eg domains of ‘type I’ and ‘type II’ dimers, respectively. Iterative model building and refinement were carried out in Phenix with manual adjustments using COOT16. The final model was refined to a resolution of 1.99 Å with \( R_{work} = 0.167 \) and \( R_{free} = 0.197 \). The majority (98.2%) of the residues are in the most favoured region in the Ramachandran plot. All structural illustrations were prepared with PYMOL (http://www.pymol.org).

Electrophysiology. The cDNA encoding the mEAG1 channel in the pGHi9 vector was kindly provided by G. Robertson (University of Wisconsin-Madison). The cDNA was transcribed using the T7 MEGAscript kit (Ambion). *Xenopus laevis* oocytes were de-folliculated and injected with the cDNA as described previously.17 After manual removal of the vitelline membrane, currents were recorded in the inside-out patch configuration18 with an EPC-10 patch-clamp amplifier (HEKA Elektronik). Patch pipettes were pulled from borosilicate glass and had resistances of 0.4–0.8 MΩ2 after fire polishing. The intracellular (bath) and extracellular (pipette) solutions contained 130 mM KCl, 10 mM HEPES and 0.2 mM EDTA, pH 7.2. For the experiments examining the eg domain–CNBHD salt bridge (with the R57D and D642R mutations) the mEAG1 currents were elicited by applying a series of 1–2 s test pulses to voltages ranging from −120 to +50 mV in 10-mV increments from a holding potential of −100 mV, followed by a 0.5-s voltage pulse to −90 mV. For experiments examining an interaction between the PAS-cap and CNBHD (with mutations in residues R7–R8 and E627), the mEAG1 currents were elicited by a series of 50-ms test pulses to voltages ranging from −140 mV to +200 mV in 10-mV increments, from a holding potential of −100 mV, followed by a 200-ms voltage pulse to −100 mV. Currents were not leak subtracted. Data were acquired with Pulse software (HEKA Elektronik) and analysed with Igor (WaveMetrics).

To measure the conductance–voltage relationships for all electrophysiology experiments, peak tail current amplitudes at −90 or −100 mV, were normalized to the largest peak conductance amplitude. Plots of the normalized conductance versus the test voltage were fit with a Boltzmann function:

\[
G \left(1 + e^{-\frac{V - V_{0.5}}{s}}\right)
\]

in which \( V \) represents the test voltage (mV), \( V_{0.5} \) is the midpoint activation voltage (mV) and \( s \) is the slope of the relation (mV).