

## Domain–domain interactions in ion channels

Jie Zheng

Department of Physiology and Membrane Biology, University of California, Davis, Davis, CA 95616

Ion channels are, with a few exceptions, multi-subunit complexes made of interacting functional domains (Hille, 2001). Combining different functionalities yields ion channels with complexity, allowing them to operate like a biological micro-machine. This general design permits the selective regulation of channel behaviors, such as the single-channel conductance or the activation rate. Cooperativity between interacting domains further allows channel activity to be controlled with high sensitivity and precision. Moreover, the domain-based design principle has promoted the evolution of new ion channels by adding or switching a functional domain. Thus, understanding how domains interact in ion channels is crucial to better appreciate channel function.

New details of a mysterious case of domain–domain interaction in the human ether-à-go-go-related gene (hERG) channel have emerged from a study in this issue by the Trudeau group (Gianulis et al.). The hERG channel plays a critical role in controlling heart beat (Fig. 1 A) (Bers, 2001). The opening of hERG channels generates the delayed-rectifier potassium current,  $I_{Kr}$ , that contributes to repolarization of cardiac action potential. As an outward current,  $I_{Kr}$  helps to bring cardiac membrane potential back to the resting level to terminate ventricular contraction. To fulfill this role, the timing of  $I_{Kr}$  is critical. The hERG channel needs to remain nonconducting during the plateau of the cardiac action potential that lasts over 100 ms. Failing to do so, for example, because of mutations of the channel protein, leads to abnormalities in action potential duration that cause the second most common form of long QT syndrome (LQT2) (Sanguinetti et al., 1995). Previous work has revealed that the channel refuses to produce a current during membrane depolarization by rushing through the conducting open state, O, and tumbling into a nonconducting inactivated state, I (Fig. 1 A) (Trudeau et al., 1995; Smith et al., 1996). At the end of the plateau, membrane repolarization releases the channel from the I state back to the O state to produce  $I_{Kr}$ . Once the channel reaches the O state, the duration of  $I_{Kr}$  depends on the rate of the O→C transition, the deactivation process. It is known that the deactivation rate of hERG channel is tightly controlled by the channel's eag domain, located in the N-terminal intracellular region (Fig. 1 B).

What has been debated is how the eag domain does this important job.

The eag domain is a compact protein domain consisting of the first 135 amino acids of the hERG channel (Morais Cabral et al., 1998). It can control the deactivation rate either as part of the channel's N terminus or as a channel-free peptide (Morais Cabral et al., 1998; Gustina and Trudeau, 2009). It is clear that the eag domain binds to another region of the hERG channel, thereby coaxing the channel to remain in its open state more than 10-fold longer to produce a larger and longer  $I_{Kr}$ . Two channel domains have been considered as the possible eag-interacting partner: the S4–S5 linker and the intracellular C-terminal domain, which contains the C-linker and the cyclic nucleotide-binding homology domain (CNBHD) (Fig. 1 B). Conflicting evidence in favor of one or the other has been gathered with experimental approaches that have been used previously to study domain–domain interactions, each with certain limitations. For example, association of the isolated eag domain with the C-terminal domain was tested in solution with affinity pull-down assays (Gustina and Trudeau, 2011), whereas association with the S4–S5 linker was monitored in solution with nuclear magnetic resonance (Li et al., 2010). False positive results could potentially arise from such assays because regions that could never come into contact in an intact channel in the cell membrane could interact with each other in solution. Functional assays based on mutational perturbation of interacting structures suffer from the possibility of nonspecific effects of mutations on distant channel structures through allosteric coupling. Because earlier efforts failed to resolve the debate on the identity of the eag domain-binding partner, alternative methods are required to approach the problem from a different angle.

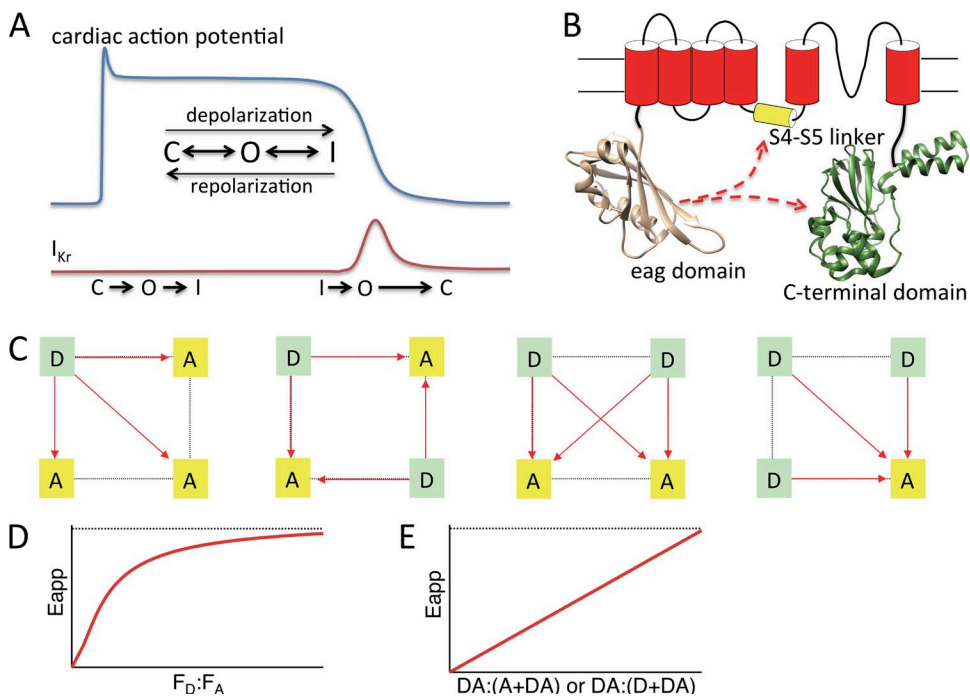
The alternative method that the Trudeau group chose in their present study (Gianulis et al., 2013) is fluorescence resonance energy transfer (FRET; also known as Förster resonance energy transfer) (Förster, 1948), a technique in which energy coupling between two fluorophores is taken as an indication of close proximity of

Correspondence to Jie Zheng: jzheng@ucdavis.edu

the domains carrying the fluorophores. Aided by the availability of green fluorescent protein mutants that emit fluorescence in a wide range of colors (thus making nice FRET pairs), and the ease of tagging these fluorophores to cloned proteins, FRET has been widely adopted by biomedical researchers. FRET provides a handy tool for biomedical investigations at the molecular level. The distance over which FRET occurs—roughly 100 Å—matches nicely the dimension of many biological molecules. FRET efficiency varies inversely with the sixth power of the distance between the FRET pair, dropping to virtually zero when the distance is beyond 100 Å. This means that two fluorescently tagged proteins or domains must be in extremely close proximity to produce a strong FRET signal, making FRET an excellent indicator of domain–domain interactions in intact proteins in their native environment, and indeed a natural choice to address the eag domain–binding partner question. FRET has, in fact, been used previously for exactly this purpose (Fernández-Trillo et al., 2011). Surprisingly, however, the conclusions drawn from the two FRET studies are once again contradictory. It is not the first time that the FRET method has led to apparently contradictory results (Zheng, 2010). Given the intrinsic high sensitivity of FRET, and the rapid development of fluorescence detection technology and analysis methodologies, why are FRET measurements still prone to mistakes?

An important premise for the preceding discussion on FRET is that one is dealing with a simple homogenous

population of FRET donor–acceptor pairs. Biological samples are more often than not heterogeneous, which has apparently led many FRET endeavors astray. The multi-subunit design of ion channels presents the first challenge to FRET analysis, because multiple FRET pairs will coexist if each subunit is fluorescently tagged (Fig. 1 C). At low levels of excitation, when fluorophores reside mostly in the quiescent state, the system may be approximated as a mixture of various independent donor–acceptor pairs. Simple modeling approaches based on the constraints of a symmetrical subunit arrangement have been shown to adequately handle this type of complexity (Cheng et al., 2007). When FRET experiments are done with a cell-based expression system, as was the case for both hERG channel FRET studies (Fernández-Trillo et al., 2011; Gianulis et al., 2013), additional complexities arise. Different combinations of donor and acceptor fluorophores occur when subunits can assemble freely (Fig. 1 C), with the probability of having each combination dependent on the relative abundance of donor- and acceptor-tagged subunits. This can again be handled with appropriate modeling (Fig. 1 D) (Cheng et al., 2007). (In cases in which a preferred subunit stoichiometry exists, specific FRET designs can be devised to solve the stoichiometry problem; Zheng et al., 2002.) When FRET is used to detect association between a channel subunit and an ancillary binding partner, one faces another type of stoichiometric uncertainty. It has been demonstrated previously, using voltage-gated



**Figure 1.** Detecting a hERG channel domain–domain interaction with FRET. (A) Schematic drawing of the relationship between the cardiac action potential (top),  $I_{Kr}$  (bottom), and hERG channel gating. (B) Location of the eag domain (brown; Protein Data Bank accession no. 4HQ4), C-linker/CNBHD domain (green; 3UKV), and S4–S5 linker (yellow cylinder). Dashed lines indicate the potential interactions examined in the present and previous FRET studies. (C) Multiple FRET pathways within a four-fold symmetrical ion channel complex when each subunit is tagged with a donor or acceptor fluorophore. (D and E) The apparent FRET efficiency of an experimental system ( $E_{app}$ ) is a function of the distribution of donor-only (D), acceptor-only (A), and donor-acceptor (DA) species. Dotted lines indicate the saturated level of FRET. D represents

a case of randomly assembled tetrameric channel subunits when  $E_{app}$  is quantified from acceptor fluorescence (Cheng et al., 2007).  $F_D$  and  $F_A$  are total donor and acceptor fluorescence, respectively. E represents a case of channel-ancillary binding protein association (Erickson et al., 2001).

calcium channels that bind calmodulin, that the FRET signal follows a titration curve, increasing with the fraction of calmodulin-bound channels (Fig. 1 E) (Erickson et al., 2001). To accurately measure FRET, both free calmodulin molecules and unbound channels must be excluded from the analysis.

Analysis of the association of the free eag domain with the hERG channel presents a similar problem to that of calmodulin–calcium channel association. To detect potential association, the Trudeau group controlled the expression ratio between the eag domain and the channel subunit so that they operated at the right-hand side of the binding curve (Fig. 1 E). FRET signal was measured using the “spectra FRET” method designed upon a combined system of fluorescence microscope and spectrograph (Zheng et al., 2002; Takanishi et al., 2006). By positioning the spectrograph’s input slit over the edge of each cell, the measured fluorescence signal was predominately from the plasma membrane, which contains properly folded and assembled channel proteins as well as eag domains associated with channels. Furthermore, FRET was measured from the acceptor fluorophore (Citricine, which is an improved version of the yellow fluorescence protein) that was attached to the channel subunit. This design is critical because it ensures that a FRET signal is detected only when the eag domain is bound to the channel, i.e., operating on the right-hand side of the binding curve (Fig. 1 E). The donor fluorescence from eag-tagged cyan fluorescence protein (CFP) was subtracted during FRET analysis; thus, even though the area of the cell covered by the spectrograph slit might contain some unbound eag domains, it would not affect FRET detection. The earlier FRET study (Fernández-Trillo et al., 2011) quantified FRET from the donor fluorophore CFP that was attached to the free eag domain. Should a significant fraction of unbound eag domains be included in the analysis (that is, operating on the left-hand side of the binding curve; Fig. 1 E), the FRET signal from bound eag domains would be covered. (If there was no association of the eag domain to the channel, measurements from the plasma membrane area with a TIRF system would only collect the channel-tagged acceptor fluorescence but not the free eag-tagged donor fluorescence; hence, there would be no donor fluorescence to quantify FRET from.)

Results from the study by Gianulis et al. (2013) support the idea that the eag domain interacts with the hERG channel CNBHD (Fig. 1 B). Disruption of the S4–S5 linker by mutations did not prevent the interaction or FRET, whereas deleting the C-terminal domain prevented both the interaction and FRET. Using a live cell-based FRET hybridization assay (Erickson et al., 2003), the Trudeau group further confirmed that the eag domain could bind to the isolated C-terminal domain. With the FRET method, they also provided evidence that such interaction occurs between subunits in

intact channels instead of within the same subunit. The picture that emerges from this and previous studies is that, in the hERG channel, the four subunits form a network through eag–CNBHD interactions, resembling four people standing in a circle all holding hands. Interestingly, this picture is very similar to that presented by CNG channels (Trudeau and Zagotta, 2002; Zheng et al., 2003).

Indeed, hERG channels resemble CNG channels and hyperpolarization-activated cyclic nucleotide–modulated (HCN) channels in many ways. For example, their C-terminal structures are closely related. Both CNG and HCN channels have a functional cyclic nucleotide–binding domain in the intracellular C terminus. The hERG C terminus also contains a CNBHD that is structurally similar to the cyclic nucleotide–binding domain of CNG and HCN channels (Fig. 1 B) (Zagotta et al., 2003; Brelidze et al., 2012). An important difference is that, for CNBHD of the hERG channel, the position for the ligand is occupied by a peptide segment of the CNBHD, whereas in the cyclic nucleotide–binding domain of CNG and HCN channels, the equivalent segment is next to the bound cyclic nucleotide, helping to hold the ligand in place. The CNBHD structure resembles a baby sucking his thumb, thus preventing food from being put in his mouth. Indeed, the hERG channel is not regulated by cyclic nucleotides. Furthermore, in CNG channels the N terminus of one subunit interacts with the C terminus of its neighboring subunit in a hand-in-hand pattern (Trudeau and Zagotta, 2002; Zheng et al., 2003). The N–C domain–domain interaction of CNG channels exhibits a strong auto-excitatory effect on channel activity and provides a mechanism for calmodulin modulation (Varnum and Zagotta, 1997) as well as for controlling channel trafficking (Trudeau and Zagotta, 2002).

This study by Gianulis et al. (2013) establishes that the hERG channel has an N–C domain–domain interaction between the eag domain and the C-terminal domain, providing a basis for addressing many intriguing questions. It underlines the need to determine at the atomic level how the eag domain interacts with the C terminus. Can binding of the eag domain relieve the thumb-in-mouth conformation and allow ligand binding to CNBHD? Can the “thumb” peptide come out when a ligand is present? It is also important to understand how the domain–domain interaction between the eag domain and the C-terminal domain slows down the deactivation process of the hERG channel and prolongs  $I_{Kr}$ . Does it stabilize the open conformation of the channel, hinder the closing transition, or affect another process? Furthermore, what is the role of the S4–S5 linker in determining hERG channel deactivation? Does it play any role in the process controlled by the eag domain/CNBHD? Alternatively, does the S4–S5 linker regulate deactivation through a separate mechanism, say, by

affecting the channel's activation process (which is mediated by another important domain–domain interaction between the voltage-sensing domain and the pore domain)? As always, the answer to an old question leads to many new ones.

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