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Positive selection-guided mutational analysis revealing two key functional sites of scorpion ERG K^+ channel toxins

Xueli Wang [a,1], Juana Maria Jimenez-Vargas [b,1], Chenqi Xu [c], Lourival D. Possani [b,*], Shunyi Zhu [a,*]

[a] Group of Animal Innate Immunity, State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, PR China

[b] Department of Molecular Medicine and Bioprocesses, Instituto de Biotecnologia, Universidad Nacional Autónoma de México, Av. Universidad, 2001, P.O. Box 510-3, Cuernavaca 62210, Mexico

[c] Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 320 Yue-yang Road, Shanghai 200031, PR China

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ABSTRACT

Scorpion γ-KTx toxins are important molecular tools for studying physiological and pharmacological functions of human ether-á-go-go related gene (hERG) K^+ channels. To pinpoint functional residues of this class of toxins involved in channel binding, we employed a combined approach that integrates evolutionary information and site-directed mutagenesis. Among three positively selected sites (PSSs) identified here, two (Gln18 and Met35) were found to be associated with the toxin's function because their changes significantly decreased the potency of ErgTx1 (also called CnErg1) on hERG1 channel. On the contrary, no potency alteration was observed at the third PSS (Ala42) when the mutation was introduced, which could be due to its location far from the functional surface of the toxin. Our strategy will accelerate the research of structure–function relationship of scorpion K^+ channel toxins.

1. Introduction

The hERG (human ether á-go-go related genes) voltage gated K^+ channels play critical roles in the maintenance of normal electrical activity in the heart [1]. Because they are often targeted by a wide range of drugs, hERG channels are also associated with an increased risk of drug-induced arrhythmias and cardiac death [2]. As high-affinity ligands of hERG channels, scorpion toxins from the γ-KTx subfamily have been proved to be ideal probes in elucidation of physiological and pharmacological roles and structure–function relationship of this class of channels [3]. These toxins contain a cysteine-stabilized α-helix and β-sheet (CSαβ) structural motif with 36–47 amino acids and 3–4 disulfide bridges. Since the discovery of ErgTx1, the first peptide inhibitor specific for hERG channels from the venom of *Centruroides noxius* [4], 28 other members were also identified thus far, e.g. BeKm-1, CsEKerg1, CeErg4 and CeErg5 [5–7]. It has been shown that ErgTx1 and BeKm-1 bind to the outer vestibule of hERG with a 1:1 stoichiometry but may not physically occlude the pore [8–11].

Although the functional importance of several evolutionarily conserved residues (i.e. Lys13, Tyr14, Tyr16, Tyr17, Phe36, Phe37, and Lys38) (all residues numbered according to ErgTx1 in this study) have been recognized [12,13], little is known regarding variable sites of the γ-KTx subfamily members. Given that positive selection-driven accelerated evolution frequently occurs at functional regions of proteins (Table S1), we believe that the recognition of such regions will be helpful in rapid determination of protein functional surfaces through experimental studies. We have previously identified six positively selected sites (PSSs), including Ala12, Gln18, Gln21, Asp22, Met35, and Ala42, which all show high sequence variability within the subfamily [14]. To provide more precise guidance for mutational experiments, we re-analyzed positive selection of the γ-KTx subfamily by using a larger set of data and more robust maximum likelihood models of codon substitutions [15]. Three common amino acid sites (Gln18, Met35 and Ala42) are again identified as PSSs and thus were chosen for mutational analysis. Of the three PSSs, two were found to be involved in the binding of ErgTx1 to hERG channel. Our results demonstrate the usefulness of evolutionary information in rapidly identifying functional sites of animal toxins in combination with site-directed mutagenesis.

2. Methods

2.1. Maximum likelihood analysis

Protein and nucleotide sequences of γ-KTxs were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov) by using ErgTx1 as a query to perform BLASTP search. Multiple sequence

* Corresponding authors.
E-mail addresses: possani@ibt.unam.mx (L.D. Possani), Zhusy@ioz.ac.cn (S. Zhu).
[1] These authors equally contributed to this work.

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alignment was performed by CLUSTAL X [16]. Signal peptides and C-terminal extra residues were removed and only mature peptides were left for maximum likelihood analysis. Four sequences (CnErg2, CeErg5, CeErg4, and CsEkerg1) were excluded in our analysis due to the lack of corresponding nucleotide sequences. BeKm-1 and BmKKx2 were also ruled out due to rather low sequence similarity. The neighbor-joining method in MEGA 5.1 [17] was used to construct a phylogenetic tree of the aligned nucleotide sequences for further statistical analysis. Codon-substitution models were used to obtain the nonsynonymous-to-synonymous rate ratio (ω = dN/dS) using CODEML [18]. The presence of a positively selected rate class is detected by comparing the likelihood of a neutral model with that of a selection model [19,20]. Model M0 assumes one ω for all sites that does not allow the existence of positive selection [20,21]. Two pairs of models make two likelihood ratio tests (LRTs) by M1a (nearly neutral model) against M2a (positive selection model) and M7 (β distribution model) against M8 (β and ω model). M1a allows for $\omega < 1$ as well as $\omega = 1$. M2a, adding an extra class of sites to M1a, allows for $\omega > 1$ (positive selection). M7 does not allow for positive selection. M8 also allows for $\omega > 1$ (positive selection) by adding an extra class of sites to M7 that assumes a β distribution. Two LRTs that calculated $2\Delta l$ were compared against χ^2 critical value of 9.21 at 1% significance levels (df = 2). Upon detection of the positively selected signals, posterior probabilities for the positive selection class under M2a and M8 were calculated by the Bayes empirical Bayes (BEB) approach [21].

2.2. Recombinant expression and protein purification

The methods for the expression, purification and folding of the ErgTx1 mutants have been described previously [13,22], provided as Supplementary data. Recombinant genes of the mutants Q18A

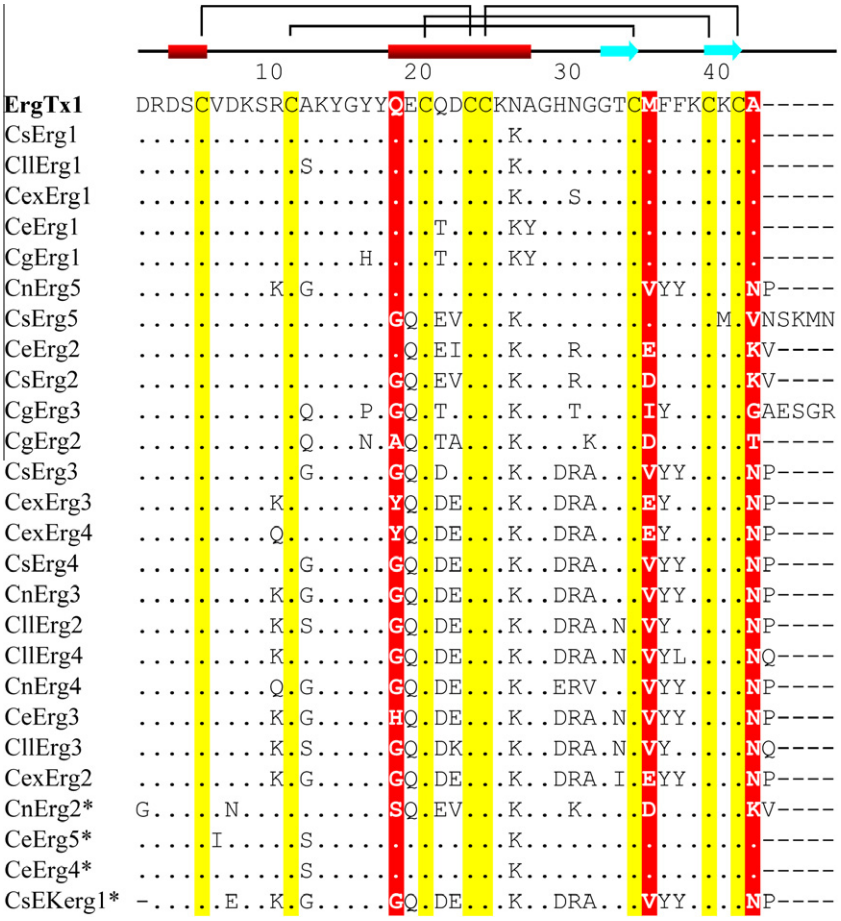

Fig. 1. Multiple sequence alignment of γ-KTx-type scorpion toxins. Cysteines are highlighted in yellow. Three positively selected sites identified here are shadowed in red. Cylinder and arrows represent α-helix and β-strands, respectively, which are extracted from the structural coordinates of ErgTx1 (PDB code: 1NE5). (*) represents toxins excluded in the maximum-likelihood analysis. Names, species and accession numbers of the toxins are listed in Table S2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Parameter estimates and likelihood ratio test (LRT) statistics for the γ-KTx scorpion toxin genes.

Model	l	Estimates of parameters	LRT	Positively selected sites
M0 (one ratio)	-839.49	$\omega = 0.46$		None
M1a (nearly neutral)	-780.13	$p_0 = 0.59$, $\omega_0 = 0.007$, $p_1 = 0.41$, $\omega_1 = 1.00$		Not allowed
M2a (positive selection)	-768.23	$p_0 = 0.57$, $\omega_0 = 0.003$, $p_1 = 0.33$, $\omega_1 = 1.00$, $p_2 = 0.10$, $\omega_2 = 4.37$	23.8 (9.21)	**18Q**, **35M**, 42A
M7 (β)	-779.19	$p = 0.02$, $q = 0.026$		Not allowed
M8 (β & ω)	-768.53	$p_1 = 0.15$, $\omega = 3.64$, $p_0 = 0.85$, $p = 0.016$, $q = 0.047$	21.32 (9.21)	**18Q**, **35M**, 42A

Note: Numbers in parentheses represent the critical values of $\chi^2_{\%}$ with df = 2. PSSs with posterior probabilities $P \geqslant 0.95$ are shown here and those with $P > 0.99$ are boldfaced. l is the log likelihood. Residues are numbered according to ErgTx1.

and A42T were synthesized by a two-step protocol described by Ke and Madison [23]. The nucleotide sequence of the forward primer (*Bam*H1-EntERG) is: 5′-GGCTCTGGATCCGGTGATGACGATGACAAG-GACAGAGATAGCTGCGTC-3′, which contains the *Bam*HI restriction enzyme site and the coding-regions of the enterokinase cleavage site and the amino-terminal residues 1–6 of ErgTx1. The reverse primer (*Not*-Erg) was: 5′-TTTTTCAAGTGTAAATGTGCGTAATCACGT GCGGCCGCA=AGTCGAGAATGACTC-3′.The nucleotide sequences of the two mutagenic primers are: Q18A: 5′-CTGACACTCTGCGTAG-TATCC-3′; and A42T: 5′-ACGTGATTACGTACATTTACA-3′.

2.3. Electrophysiological experiments

The method for culturing Chinese hamster ovary cells (CHO) and the solutions and drugs used in the assays have been described previously [13,22]. Currents were recorded at room temperature by patch clamp recording technique. Pipette resistance was about 1.5–2.2 MΩ. In order to reduce voltage errors to <5% of the protocol pulse, we compensated the cell capacitance and series resistance by 85–90% before each run of voltage clamp protocols. pClamp10 (Axon Instruments, USA) and Origin 7 (Microcal Inc. USA) were used for data acquisition and analysis, respectively. All data recordings were obtained with stimulation at −120 mV for 500 ms, preceded by a 500 ms at 60 mV at frequency of 0.1 Hz. Holding potential was clamped at −80 mV and data are shown as mean ± -SEM (n = 9).

Peak currents, measured after 100 s of application of the toxin at different concentrations, were normalized by comparing with the currents obtained in control conditions, and were further used to plot dose-response curves of ErgTx1 and its mutants to hERG1. The experimental data were fitted with a Hill equation: $I_T/I_{max} = \{1 + ([T]/K_D)^n\}^{-1}$, where I_{max} is the maximal current; $[T]$ is the toxin concentration; I_T is the current at a certain $[T]$; K_D is the dissociation constant, corresponding to the $[T]$ that produces 50% inhibition of the hERG current; and n is the Hill coefficient.

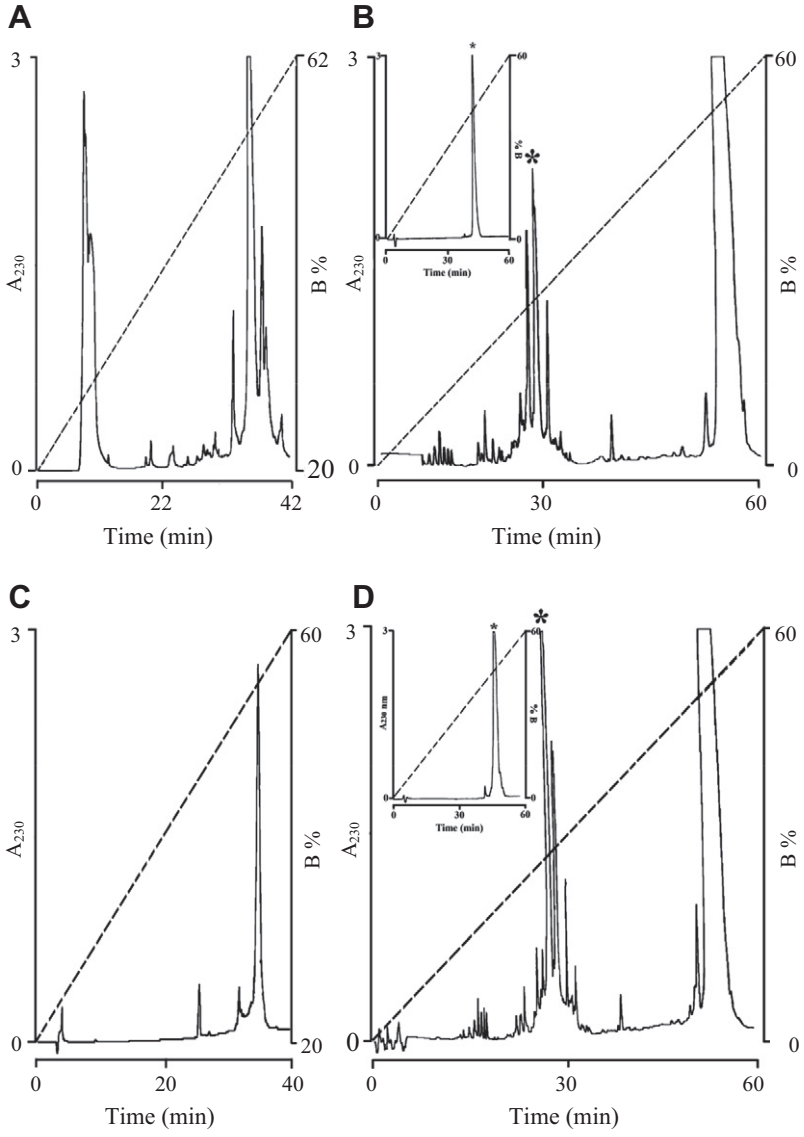

Fig. 2. Purification of the mutants Q18A and A42T. (A) The major peak eluted around 36.6 min corresponds to ThioEKQ18A; (B) The component eluted at about 28% of buffer B corresponds to Q18A (labeled with an asterisk), which was further separated by a C_{18} reverse-phase analytical column, as indicated in the inset; (C) the major peak eluted at around 38 min corresponds to ThioEKA42T; (D) the component eluted at about 27% of buffer B corresponds to A42T (labeled with an asterisk), which was further separated by a C_{18} reverse-phase analytical column, as indicated in the inset.

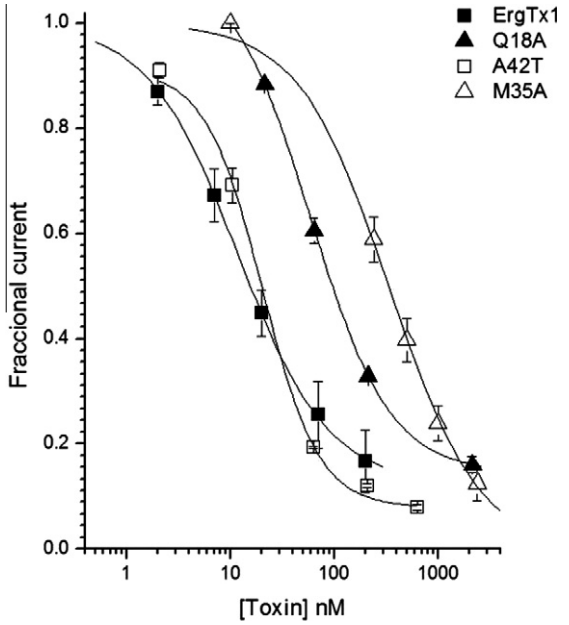

Fig. 3. Dose-response curves of Q18A and A42T of ErgTx1 on hERG1 channel, M35A and ErgTx1 are alao presented here as a control [22].

3. Results and discussion

To perform evolutionary analysis, we collected a total of 26 ErgTx1-related sequences from the GenBank database (Fig. 1; Table S1, provided as Supplementary data), which display 60%–97% sequence identity among the members. All the γ-KTx peptides consist of eight conserved cysteines with identical arrangement pattern (C. . .C. . .CXXCC. . .C. . .CXC) (X, any amino acids) to form four disulfide bridges. They possess a nearly identical N-terminal sequence as well as four conserved lysines (sites 8, 13, 25, and 38), two conserved tyrosines (sites 14 and 17), and three conserved glycines (sites 15, 28, and 32). Moreover, sites 36 and 37 are frequently occupied by an aromatic amino acid.

To study whether positive selection has promoted adaptive evolution of the γ-KTx subfamily, we performed maximum-likelihood (ML) analysis of codon substitution models. A phylogenetic tree used in this analysis is shown in Fig. S1. The log-likelihood values and parameter estimates of the γ-KTx toxins under various site models are shown in Table 1. The ω value under M0 was estimated to be 0.46 with the lowest log-likelihood score ($l = -839.49$) among all the models used here, implying its unreliability. To test positive selection of the toxins, we selected four models (M1a, M2a, M7, and M8) to construct two LRTs (M1a/M2a and M7/M8). In M1a, the proportion of conserved sites (p_0) is 0.59, with $\omega_0 = 0.007$ and a proportion of $p_1 = 0.41$ with $\omega_1 = 1$, the value of l is −780.13, while in M2a, the value of l is −768.23 and there is an additional proportion of $p_2 = 0.10$ with $\omega_2 = 4.37$. The LRT ($2\Delta l$)

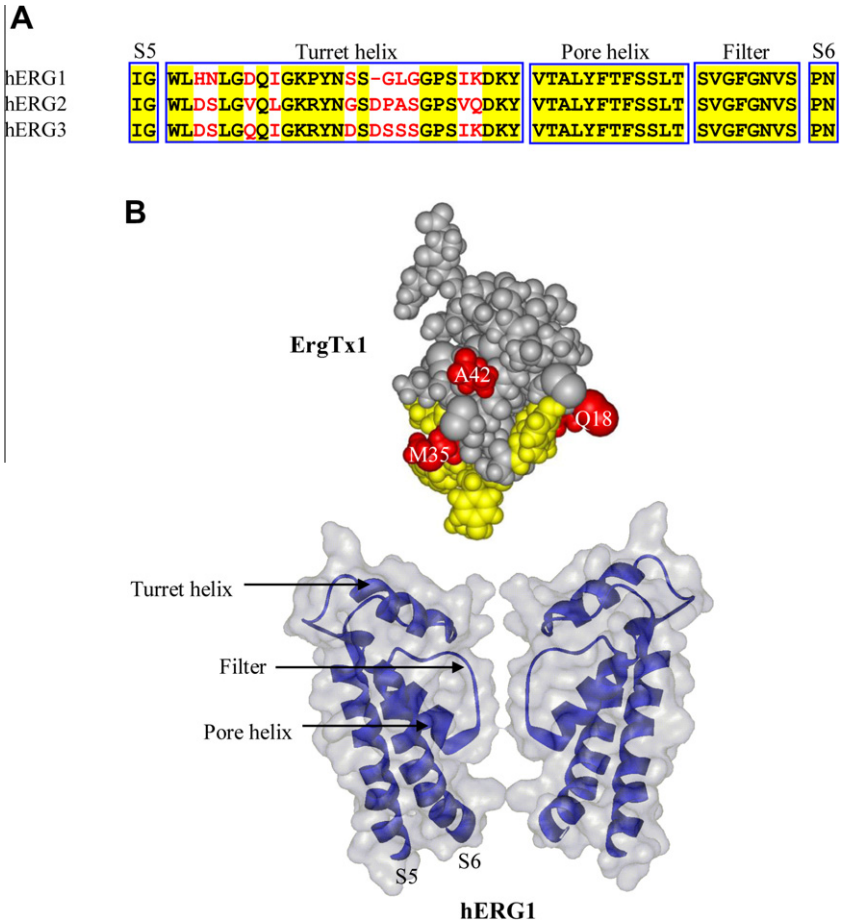

Fig. 4. Proposed model of the interaction between ErgTx1 and hERG1 channel based on experimental data. (A) Sequence comparison highlighting variability of the pore region in three hERG channel isoforms. Identical residues are shadowed in yellow and non-identical residues are indicated in red; (B) The variable PSSs described here (in red) and the conserved functional sites previously identified [12,13,22] (in yellow) are mapped on the structure of ErgTx1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of these two models is 23.8, which is much larger than the χ^2 critical value (df = 2) at the 0.01 statistical level. These results indicate that M2a fits the data better than M1a and thus provide statistical evidence in favor of the existence of positive selection in scorpion γ-KTx toxins. Models M7 and M8 led to the consistent results ($2\Delta l = 21.3$). Because the LRTs suggested positive selection, BEB was used to calculate the posterior probabilities and identified positive selection sites under M2a and M8. Significantly, two selection models identified identical sites evolved by positive selection, which are sites 18, 35 and 42. The posterior probabilities of all the three sites are up to 95%.

To confirm possible functional significance of these PSSs, we designed two mutants (Q18A and A42T) and carried out their expression, purification and functional evaluation. The mutant M35A had previously been described [22]. The expression of the mutants was analyzed by Tricine–SDS–PAGE gel (Fig. S2 provided as Supplementary data). The hybrid proteins of ThioEKQ18A and ThioEKA42T were separated by the IMAC column followed by HPLC using C_{18} semi-preparative reverse-phase column (Fig. 2A and C). As shown in Supplementary Fig. S2, small contaminants were eliminated after digestion with enterokinase and repurification by an analytical C_{18} reverse-phase column (Fig. 2B and D, respectively). The Tricine–SDS–PAGE gel (Fig. S2) and mass spectrometry determination showed a molecular weight of 17410.8 Da for the hybrid protein ThioEKQ18A and 17497.8 Da for ThioEKA42T. After the analytical column separation, the pure mutants had experimental molecular masses of 4673.3 Da for Q18A and 4760.3 Da for A42T. These values are in agreement with the theoretical molecular masses of the two peptides. Electrophysiological effects of the mutants Q18A and A42T on hERG1 channel were observed in CHO cells. The dose-response curves of the peptides were plotted by applying different peptide concentrations ranging from 2 to 2000 nM (Fig. 3) and the experimental data were fitted with a Hill equation, which gave K_D values of 72.9 ± 6.1 nM for Q18A and 14.2 ± 0.8 nM for A42T. Although A42T exhibits a similar activity to the wide-type toxin, Q18A reduced the affinity by over 6-folds. A similar case was also observed in M35A [22].

The functional importance of Q18A and M35A provides a new clue for understanding the interaction between ErgTx1 and hERG1. It has been suggested that the turret helix is a main region involved in the interaction of hERG channels with γ-KTxs [3,8,9,12]. We found that the amino acid sequences of this region are highly variable among different channel isoforms (Fig. 4A), which is presumably a consequence of co-evolution for adaptation to the changes of the variable PSSs of the toxins. In this case, the affinity and selectivity of these toxins could depend on the binding of PSSs to the variable turret helix, while the conserved region of the toxins maintains toxicity due to its binding to the conserved pore helix and filter region of the channels. Supported by these analyses, a rational interaction between ErgTx1 and hERG1 is established (Fig. 4B), which might improve the previous model proposed only based on evolutionary trace analysis [3,11,12,24]. In this model, A42 is far from the toxin-channel interface, in line with the mutational data described here (Fig. 3). In fact, assembly of functional surfaces located in variable and constant regions represents a common strategy observed in many different protein families. A well-known example is the class I MHC family involved in vertebrate immune response, in which antigen recognition site (ARS) is high-frequently driven by positive selection in order to bind different antigens, but the non-ARS region is conserved because of the requirement to bind the conserved effectors [25]. The functional surface of scorpion α-toxins is also composed of two distinct domains: a variable "NC-domain" driven by positive selection and a conserved "core-domain" [26,27].

Prior studies have shown that animal toxins are frequently subjected to positive selection due to co-evolution with their targets [27–30]. Moreover, the presence of PSSs in most multigene families of animal toxins had been also reported [31,32] (Table S2). However, little information is available in terms of their functional roles. We now show that mutational experiments at predicted PSSs identify interacting surfaces between ErgTx1 and hERG1.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.065.

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