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RFP-tagged Hongotoxin 1 and Its Interactions with KscA-Kv1.1 Hybrid Channels

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Potassium voltage-gated channels of Kv1 subfamily that regulate many cellular processes are now in the focus of research. Inhibition of Kv1-channel activity by high-affinity blockers is one of the most efficient ways to implement structural and functional studies of these channels, as well as to reveal therapeutic potential of the channels in the case of Kv1-mediated pathologies. Fluorescently labeled peptide blockers are widely used in these studies including pharmacological characterization of highly specific Kv1-channel blockers from animal venoms [1].

A number of fluorescent probes were generated by chemical labeling of hongotoxin 1 (HgTx1), a potent 39-aa peptide blocker from scorpion *Centruroides limbatus* with a wide affinity range for Kv1 channels (Fig. 1, A, C). Among them are Cy3-, Cy5- or Alexa-labeled mutant HgTX1-A19C [2] and N-terminally labeled Atto488-HgTx1 [3]. However, the procedures of chemical labeling of peptides with fluorescent dyes have a number of limitations that often result in the low yield, as well as high cost of the labeled product. An efficient alternative to chemical labeling was proposed by bioengineering of chimeric proteins that comprise peptide blockers fused to fluorescent proteins [4]. Recently, eGFP-HgTx1 chimera was obtained, that preserved high activity of HgTx1 for the target channels [5].

To expand the spectral diversity of HgTx1-based fluorescent probes, RFP-tagged protein chimeras were obtained with N- or C-terminal location of the red fluorescent protein TagRFP (Evrogen, RF). In RFP-HgTx1 chimera (Fig.1, B), N-terminal RFP was separated from HgTx1 by the 45-aa L1 linker, which contained His6-tag for Ni-affinity purification. RFP-HgTx1 was expressed in *E. coli* Rosetta-gami pLysS using plasmid pET23d and purified from bacteria with a high yield (~120 mg per 1 l of culture). In HgTx1-RFP chimera (Fig.1, B), C-terminal RFP was separated from HgTx1 by the 20-aa L3 linker, and the chimera was expressed in *E.coli* in the form of a fusion with maltose-binding protein (MBP). The target HgTx1-RFP was obtained after digestion of MBP fusion protein by TEV protease with the yield about 30 mg per 1 l of culture. The L3 linker of HgTx1-RFP contained no His6-tag.

Binding activities of RFP-tagged HgTx1 chimeras were studied using hybrid potassium channel KcsA-Kv1.1, which is a close structural homolog of the pore domain of eukaryotic Kv1.1 channel [6]. The hybrid channels were expressed in the plasma membrane of *E.coli*, and binding of RFP-tagged HgTx1 to these channels was detected at the surface of *E.coli* spheroplasts as described earlier [7] using laser scanning confocal fluorescent microscopy (Fig.1, D, E).

Both RFP-HgTx1 and HgTx1-RFP were shown to interact with KcsA-Kv1.1 channels in a concentration-dependent and saturable mode with the measured Kd values of 35±7 and 19 ± 3 nM, respectively, indicating that C-terminal location of RFP is preferable in the studied case (Fig. 1, F). It should be noted that Kd of RFP-HgTx1 is lower than that of previously studied eGFP-HgTx1 (3.2±1.4 nM for KcsA-Kv1.1 [5]), suggesting that a fluorescent protein tag is able to affect noticeably the blocker affinity to the target channel.

To demonstrate the potential of RFP-tagged HgTx1 chimeras as fluorescent probes, competitive binding studies were carried out using several Kv1-channel blockers, namely, charybdotoxin (ChTx, α-KTx 1.1),

HgTx1 (α-KTx 2.5), and agitoxin 2 (AgTx2, α-KTx 3.2). As shown in Fig. 1, G, the peptides displaced RFP-tagged HgTx1 chimeras from the complexes with the hybrid channels, suggesting that binding of both RFP-tagged chimeras to the target KcsA-Kv1.1 is specific and reversible. The peptides competed with RFP-tagged HgTx1 proteins at nanomolar concentrations that corresponded well to their known affinities [8].

In conclusion, RFP-HgTx1 and HgTx1-RFP can be used as prospective red fluorescent probes for characterization of binding affinity of Kv1.1-channel blockers, as well as for screening purposes [9].

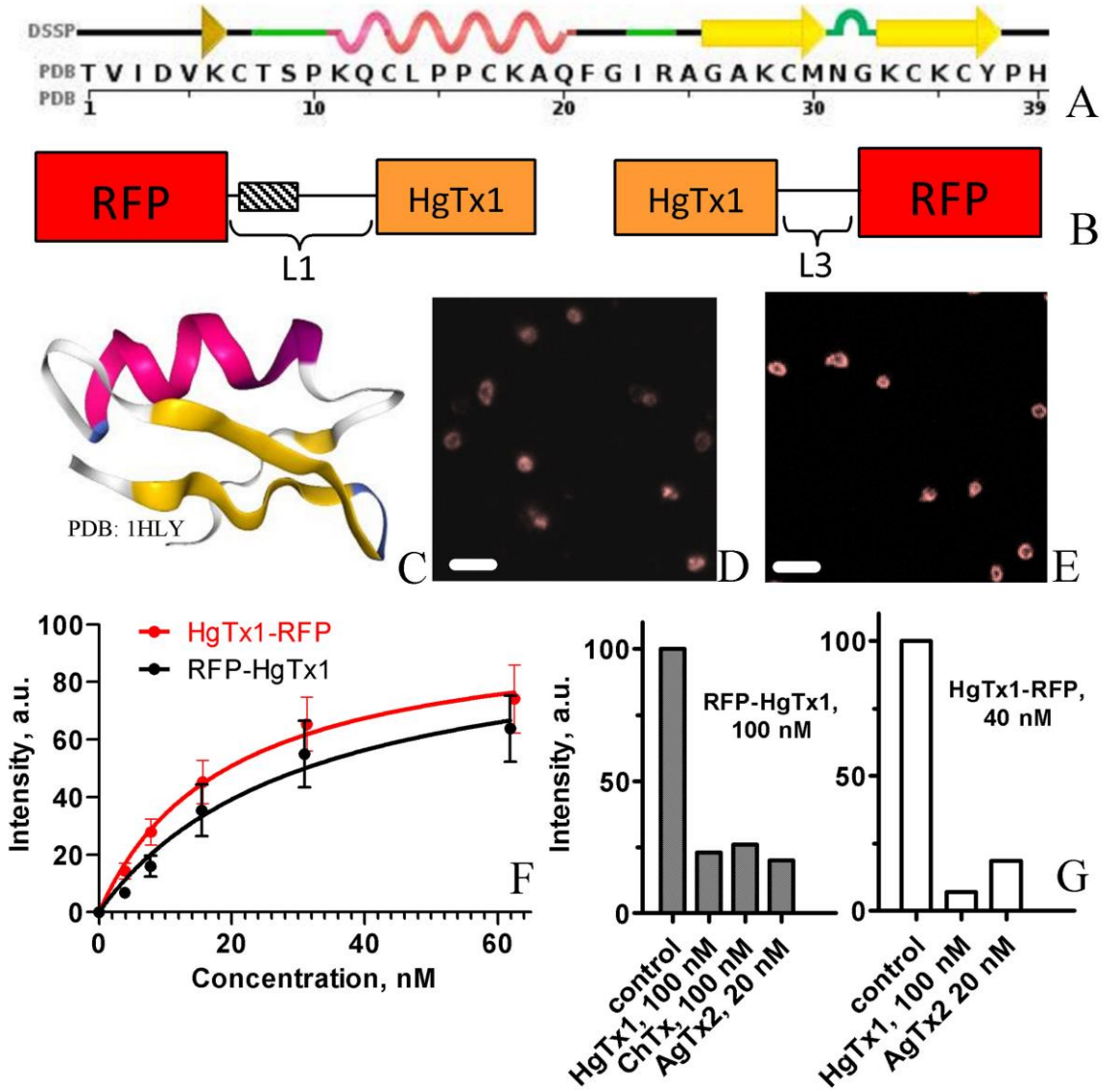

Figure 1. (A) Primary and secondary structure of HgTx1. (B) Design of RFP-HgTx1 and HgTx1-RFP bioengineered constructs. His6-tag is denoted as a hatched rectangle. (C) 3D structure of HgTx1. (D, E) Confocal fluorescence images showing binding of RFP-HgTx1 (D) and HgTx1-RFP (E) with KcsA-Kv1.1 at the membrane of spheroplasts. Bar is 3 μm. (F) Concentration dependent binding of RFP-HgTx1 and HgTx1-RFP to KcsA-Kv1.1. (G) Competition between RFP-HgTx1 (HgTx1-RFP) and different peptide ligands for the binding to KcsA-Kv1.1.

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