A heme-binding domain in neuronal voltage-gated potassium channels

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EXPERIMENTAL PROCEDURES

Heme-agarose affinity purification and mass spectrometry of neuronal lysates. Primary cortical neurons were prepared either from male and female 14-day-old foetuses of the BALB/c mouse strain bred in-house. The isolated neocortex of embryos were gently dissociated to release the neurons, which were washed twice in Neurobasal medium (GIBCO, Carlsbad, CA) supplemented with 10% foetal calf serum. Cell suspensions were plated on poly-L-lysine-coated 35-mm plates at a density of 1 x 10^6 cells per dish as previously (1). After attachment of the cells, the plating medium was changed to serum-free culture medium containing 96% (v/v) Neurobasal medium (GIBCO), GlutaMAX (2 mM), 2% B-27 supplement (GIBCO/Invitrogen, Paisley, UK), streptomycin (100 µg/ml) and penicillin (100 U/ml). Viability of the cells was estimated by the trypan blue exclusion assay and typically was around 85%. After 5 days, cytosine arabinoside (10 µM) was added to the culture medium for 3 days to stop proliferation of glial cells or fibroblasts. The cells were grown in a humidified incubator at 37° C (95% room air/5% CO_2). After 12 days in culture, neurons displayed fully developed network of projections.

Neuronal lysates were screened for possible heme-binding proteins using a modification of a previously published heme-agarose affinity (2). Binding of target proteins to heme-agarose was performed as previously (3), with modifications, Fig. S1B, that minimise pulling down proteins that bind to the heme-agarose resin non-specifically. The affinity chromatography method utilizes an agarose matrix with immobilized heme (immobilization is accomplished through the propionic acid group of heme (4), Fig. S1A) to identify and purify proteins that bind to the beads. However, heme can bind non-specifically to proteins. Thus, an additional step was included where a solution of hemin was added as a competitor (Fig. S1B). Briefly, 100 µl of heme-agarose (Sigma-Aldrich) was washed three times in 1 ml of 100 mM Na/K phosphate (pH 6.0) and 200 mM NaCl, and centrifuged at 750 × g for 5 min. Heme-agarose was incubated with 200 µl of cell lysate at room temperature for 2 hours with gentle mixing. To remove unbound proteins the beads were washed three times with the same buffer used for the binding step, pellets containing the beads were harvested by centrifugation (750×*g*, 5 min) and the supernatant discarded. The agarose-heme beads with proteins bound after the first incubation were re-suspended in binding buffer and 100 nM hemin (a competitor) was added to the suspension. The second incubation was performed for 2 hours at room temperature with gentle mixing. During this stage, proteins were displaced from the heme-agarose beads as they bind to hemin in solution. Finally, the beads were removed by centrifugation and the supernatant containing candidate heme-bound proteins was analyzed by SDS-PAGE followed by mass spectrometry. Using this approach, only proteins that were displaced from the agarose beads to bind specifically to the (heme) competitor (*i.e.* more likely to be *bona fide* heme binding proteins) were subjected to proteomics analysis, Fig. S1C. Sepharose 4B (Sigma-Aldrich), which is an agarose bead without heme attached, was used as a non-specific binding control.

For mass spectrometry analyses, SDS-PAGE gels were sliced, de-stained, dehydrated, and digested with trypsin (Promega) either overnight at 30 °C or for 3 hours at 37 °C. Tryptic peptides were extracted using 0.2% trifluoroacetic acid, solubilized in 5% formic acid, and analyzed by LC-MS/MS using a nanoLC system (CapLC, Waters) interfaced to a QTof hybrid mass spectrometer (Waters). Mass spectrometry data were acquired using Masslynx 4.0 with automatic precursor ion selection for double and highly charged ions. Peptide spectra were processed by Proteinlynx software identified though comparison to the nonredundant SwissProt database (ftp://us.expasy.org/databases/) using the MASCOT program (Matrix Science). Protein and peptide identification was validated using Scaffold (Proteome Software Inc.). Proteins were identified using the following settings: 50% peptide and 50% protein probabilities with convincing spectra (stringency settings were low due to the expected low abundance of proteins in the sample from the binding assay with free heme as a competitor).
Data Availability. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (5) partner repository with the dataset identifier PXD019887 and 10.6019/PXD019887.

Table S1. Axial ligation (light blue), Soret and Q-band absorption maxima and high-frequency vibrational modes (grey) found in various heme-binding PAS domain proteins.

Protein	**Axial ligands**	**Ferric**		**Fe(II)-CO**					**Ref.**
		Soret	*Q bands*	*Soret*	*Q bands*	ν_4	ν_3	ν_2	
hERG3(PAS)	Cys/His	420, 370	517, 550, 652	421	540, 569	1373	1502 1585	1553	This work
Clock PAS-A	Unknown	412	535,565	420	540, 570	1373	1502	1583	(6)
NPAS2-PASA	Cys/His	412	538	420	530, 568	1373	1474 1490 1504		(7,8)
NPAS2 PAS-B	His/?	419	536	420 426	536, 571 530, 561	1373	1468 1502	1550 1581	(9)
mPer2 PAS-A	Cys/?	421	536	420	539 565	1372	1470 1501	1553 1585	(10)
mPer2 PAS-B	Unknown	412	536, 564, 630	-		-			(11)
*Ec*DOS PAS	His/H_2O	416	530, 564	423	540, 570	1370	1496	1581	(12-14)
FixLN	His	412	543, 579, 604	425	545, 576	1372	1490		(15)

Table S2. Crystallographic data and refinement statistics, values in parentheses are for the outer shell.

Data collection	**hERG3 PAS domain**
Space group	$P4_3\ 2_1\ 2$
Cell dimension a, b, c (Å) α β γ (°)	32.78, 32.78, 200.68, 90.0, 90.0, 90.0
Resolution (Å)	66.9-1.39 (1.43-1.39)
CC(1/2)	0.999 (0.766)
I/σI	13.93 (1.05)
Completeness (%)	100.0 (100.0)
Multiplicity	12.0 (12.3)
Refinement	
No. reflections (free)	23378 (1208)
R_{work}/R_{free} (%)	18.2 / 23.4
Wilson B-factor (A^2)	11.6
Overall *B*-factors	35.0
R.m.s deviations	
Bond lengths (Å)	0.0197
Bond angles (°)	2.234
Ramachandran	
Most favoured (%)	99.0
Allowed (%)	1.0

Table S3. Final cycle refinement geometry restraints and weights in Refmac5 (16).

Sigma	
Bonding distances	1.0
Bond Angles	1.0
Planar Groups	1.0 (all) 1.0(Main Chain)
Chiral Centres	1.0
Non-bonded contacts (Å)	1.0 (overall)
Sigma for simple VdW	0.2
Sigma for VdW through torsion	0.2
Sigma for H-bond	0.2
Sigma for metal-ion	0.2
Sigma for DUMMY and other atom	0.3
Distance for donor-acceptor + (vdW1 +vdW2)	-0.3
Distance for acceptor H + (vdw1)	0.1
VDW distance through torsion + (vdW1+vdW2)	-0.3
Distance for DUMMY-others +(vdW1+vdW2)	-0.7
Torsion angles weight	1.0
Thermal Factors weight	1.0
Main chain bond (1-2 neighbour)	1.5Å^2
Main chain angle (1-3 neighbour)	2.0Å^2
Side chain bond	3.0Å^2
Side chain angle	4.5Å^2

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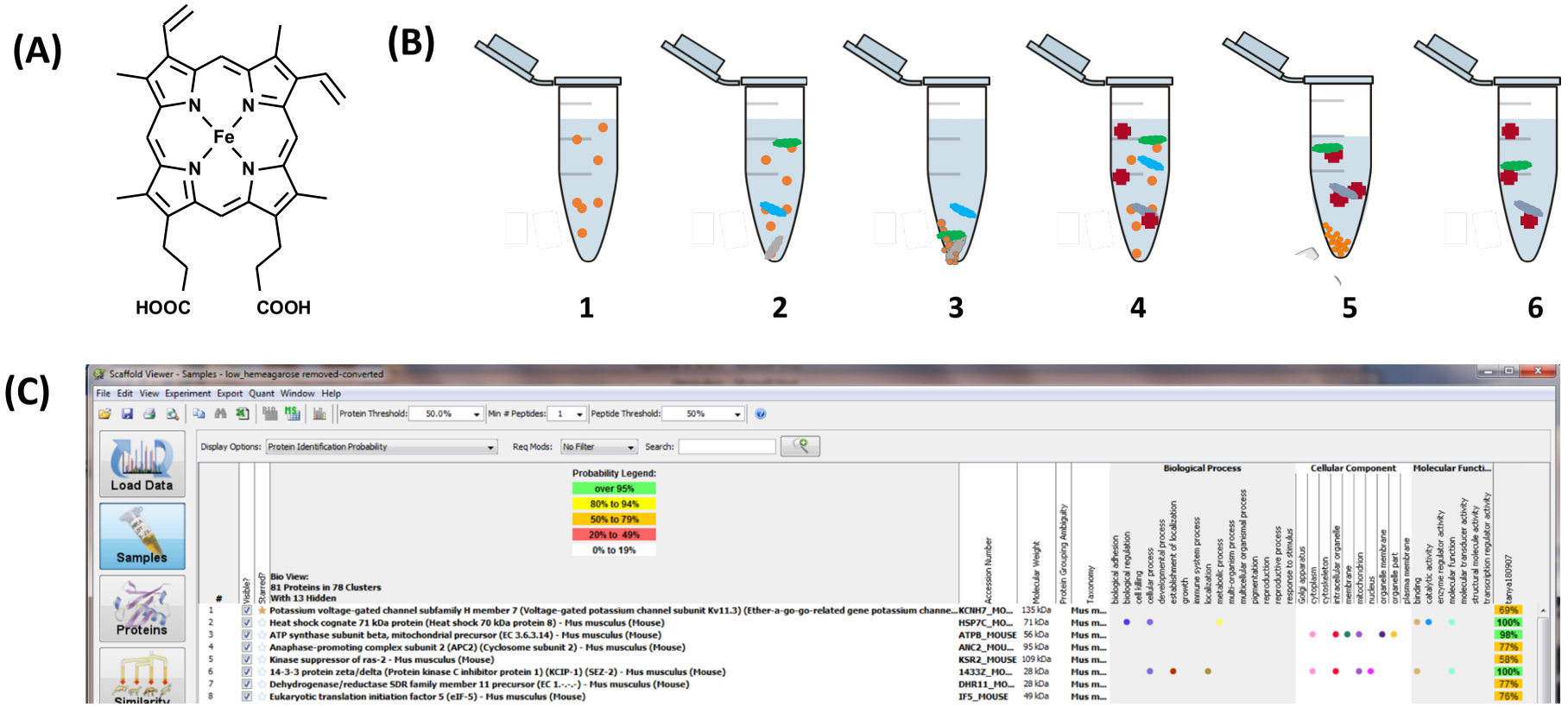
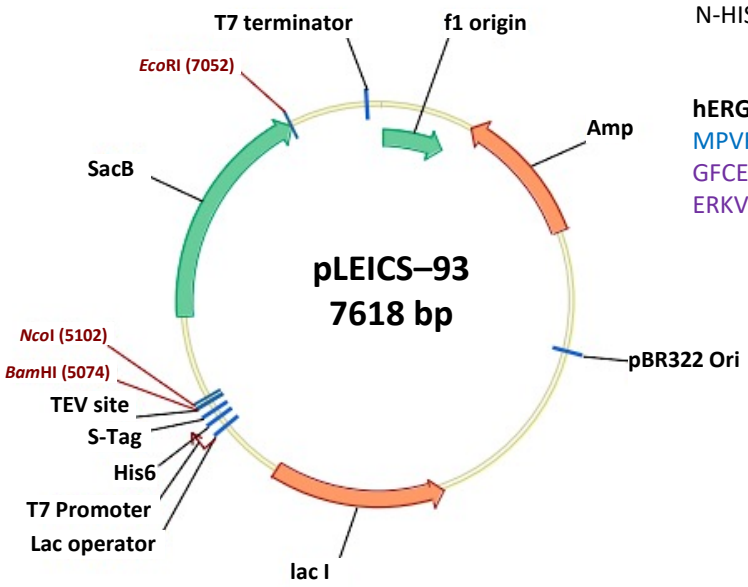


Figure S1. (A) The structure of iron protophyrin IX (heme). **(B)** Schematic representation of the affinity-chromatography approach. (1) Neuronal lysate is added to heme-agarose beads (shown in orange); (2) Proteins from the lysate (shown as coloured disks) bind to heme-agarose during the first incubation; (3) The beads are washed and the supernatant, containing proteins that are not bound to the agarose, is removed; (4) The beads are re-suspended in binding buffer and a solution of hemin (100 nM, shown as red cross) is added to compete for proteins bound to the agarose; (5) The beads are removed by centrifugation; (6) The supernatant, containing heme-bound proteins, is used for proteomics analysis by mass spectrometry. **(C)** Screenshot of the mass spectrometry data obtained, showing hERG3 identified among the proteins that bound free heme (competitor) in the affinity-chromatography assay described in (A).

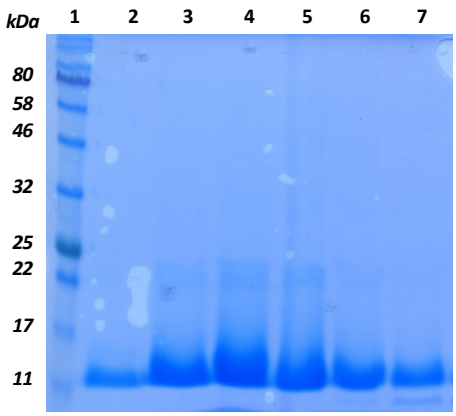
(A)



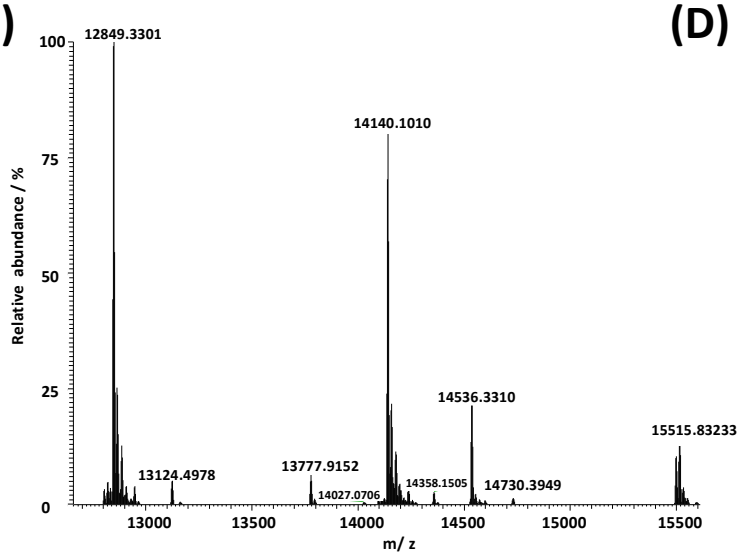
N-terminal tag:
N-HIS$_6$/S: HHHHHH-SSGLVPRGSGM-S-PDLGTGS-ENLYFQ/GA

hERG3(Cap + PAS):
MPVRRGHVAPQNTFLGTIIRKFEGQNKKFIIANARVQNCAIIYCND
GFCEMTGFSRPDVMQKPCTCDFLHGPETKRHDIAQIAQALLGSE
ERKVEVTYYHKNGSTFICNTHIIPVKNQEGVAMMFIINFEYVTDN

(B)



(C)



(D)

Observed MW of hERG3(PAS)	Missing residues from the hERG3(PAS) crystal	Calculated MW of fragments
15515.8323	None	
14730.3949	GAMPVRR	14729.8633
14536.3310	GAMPVRRGH	14535.6700
14358.1505	GAMPVRRGHVA	14365.4587
14140.1010	GAMPVRRGHVAPQ	14140.2112
14027.0706	GAMPVRRGHVAPQN	14026.1073
13777.9152	GAMPVRRGHVAPQNTF	13777.8256
13124.4978	GAMPVRRGHVAPQNTFLG TIIR	13124.0023
12849.3301	GAMPVRRGHVAPQNTFLG TIIRKF	12848.6515

Figure S2. (A) Map of pLEICS-93 vector used for the expression of hERG3 PAS domain (residues 1-135) in *E. coli* BL21(DE3). The sequences of the N-terminal tags and hERG3 are indicated on the right. (B) SDS–PAGE of the fractions (lanes 2-7) from a 10/300 Superdex 200 gel filtration column, after elution of hERG3(PAS). Lanes 1 shows molecular-mass markers (in kDa). The target protein is located around 15 kDa. (C) LC-MS spectrum of a hERG3(PAS) solubilised hERG3(PAS) crystal used for collection of X-ray data, showing the presence of several components (in (D)). These components arise from different lengths of the N-terminal Cap domain (see Fig. 1).