KCNK10C

PDB Code: 4BW5

Material and Methods

Entry Clone Source: 4BW5

Entry Clone Accession: IMAGE: 30915621

SGC Construct ID: KCNK10C-c011

GI number: 54207

Vector: pFB-CT10HF-LIC

Amplified construct sequence:

ATGGGCTTGCAGACCGTCATGAAGTG
GAAGACGGTGGTTGCCATCTTTGTGG
TTGTGGTGGTCTACCTTGTCACTGGC
GGTCTTGTCTTCCGGGCATTGGAGCA
GCCCTTTGAGAGCAGCCAGAAGAATA
CCATCGCCTTGGAGAAGGCGGAATTC
CTGCGGGATCATGTCTGTGTGAGCCC
CCAGGAGCTGGAGACGTTGATCCAGC
ATGCTCTTGATGCTGACAATGCGGGA
GTCAGTCCAATAGGAAACTCTTCCAA
CAACAGCAGCCACTGGGACCTCGGCA
GTGCCTTTTTCTTTGCTGGAACTGTC
ATTACGACCATAGGGTATGGGAATAT
TGCTCCGAGCACTGAAGGAGGCAAAA
TCTTTTGTATTTTATATGCCATCTTT
GGAATTCCACTCTTTGGTTTCTTATT
GGCTGGAATTGGAGACCAACTTGGAA
CCATCTTTGGGAAAAGCATTGCAAGA
GTGGAGAAGGTCTTTCGAAAAAAGCA
AGTGAGTCAGACCAAGATCCGGGTCA
TCTCAACCATCCTGTTCATCTTGGCC
GGCTGCATTGTGTTTGTGACGATCCC
TGCTGTCATCTTTAAGTACATCGAGG
GCTGGACGGCCTTGGAGTCCATTTAC
TTTGTGGTGGTCACTCTGACCACGGT
GGGCTTTGGTGATTTTGTGGCAGGGG
GAAACGCTGGCATCAATTATCGGGAG
TGGTATAAGCCCCTAGTGTGGTTTTG
GATCCTTGTTGGCCTTGCCTACTTTG
CAGCTGTCCTCAGTATGATCGGAGAT
TGGCTACGGGTTCTGTCCAAAAAGAC
AAAAGAAGAGGTGGGTGAAGCAGAGA
ACCTCTACTTCCAATCGCACCATCAT
CACCATCACCATCACCACCATGATTA
CAAGGATGACGACGATAAGTGA

Expressed sequence (small letters refer to tag sequence):

MGLQTVMKWKTVVAIFVVVVVYLVTG
GLVFRALEQPFESSQKNTIALEKAEF
LRDHVCVSPQELETLIQHALDADNAG
VSPIGNSSNNSSHWDLGSAFFFAGTV
ITTIGYGNIAPSTEGGKIFCILYAIF
GIPLFGFLLAGIGDQLGTIFGKSIAR
VEKVFRKKQVSQTKIRVISTILFILA
GCIVFVTIPAVIFKYIEGWTALESIY
FVVVTLTTVGFGDFVAGGNAGINYRE
WYKPLVWFWILVGLAYFAAVLSMIGD
WLRVLSKKTKEEVGEAENLYFQ^SHH
HHHHHHHHDYKDDDDK ^ TEV cleavage site

Tags and additions: C-terminal, TEV cleavable decahistidine / FLAG tag.

Host: Spodoptera frugiperda (SF9) insect cells

Growth medium, induction protocol:

Insect cells with a density of 2x106 per litre of cell culture in SF900 medium (Invitrogen) were infected with recombinant baculovirus (5ml P2 virus per litre cell culture) and incubated for 65 hours. Cells were harvested by centrifugation and the pellet was flash frozen in liquid N2.

Extraction method: Frozen pellets were thawed and re-suspended in extraction buffer supplemented with 1% OGNG/ 0.1% CHS and incubated at 4°C on a tube rotator. The extracted protein was separated from insoluble membranes by centrifugation and collected for purification.

Extraction buffer:

50 mM HEPES, pH 7.5; 200 mM KCl;

Column 1: Co-affinity. Cobalt Talon (Clontech), 1 ml of 50 % slurry/ 1L of cells in 1.5 x 10 cm column, washed with extraction/ wash buffer.

Buffers:

Wash buffer: 50 mM HEPES, pH 7.5; 200 mM KCl; 20 mM imidazole; detergent at 3x CMC.
Elution buffer: 50 mM HEPES, pH 7.5; 200 mM KCl; 250 mM imidazole; detergent at 3xCMC

Procedure:

The solubilised membrane protein was batch bound to Cobalt Talon resin equilibrated with extraction buffer at 4°C for one hour and subsequently poured into a gravity flow glass column. The column was then washed with 30CV of wash buffer followed by elution in 1CV fractions until all the protein was eluted.

Desalting:

IMAC purified protein was loaded onto PD10 columns (GE healthcare) pre- equilibrated in extraction buffer supplemented with detergent at 3x CMC, and eluted with the same buffer.

Enzymatic treatment: TEV protease (1:10, TEV:protein) and PNGaseF (1:20, PNGaseF:protein) was added overnight at 4°C to desalted protein. The enzymes were removed and tag cleaved protein was collected by binding to Cobalt talon and collecting the flow-through.

Column 2: Size Exclusion Chromatography. Superose 6 (GE healthcare).

SEC Buffer: 20 mM HEPES pH 7.5, 200 mM KCl, detergent at 2xCMC

Procedure: The protein was concentrated and applied to a Superose 6 gel filtration column equilibrated with gel filtration buffer using an AKTA purifier system.
Protein concentration: Protein was concentrated to ~20mg/ml using a 30kDa cut-off concentrator and back diluted to 9-11mg/ml.
Crystallization: Crystals grew at 4°C from sitting drops (195nl) comprising 130nl of concentrated protein and 65nl of reservoir equilibrated against 20?l of reservoir solution (0.1M HEPES pH 8.0, 1mM cadmium chloride, 2% (w/v) benzamidine, 31% (v/v) PEG400).
Cryocooling: Crystals were cryo-cooled by slow transfer into artificial mother liquor containing 0.2 M potassium chloride, 2 % (w/v) benzamidine, 0.2 % OGNG / 0.02 % CHS, 0.1 M HEPES, pH 8.0, 35 % (v/v) PEG400 followed by plunging into liquid nitrogen. Diffraction of the crystals was improved by slowly increasing the concentration of PEG400 to above 35 % (v/v) which resulted in a 10 % shrinkage in unit cell volume compared to untreated crystals.
Data Collection: Data were collected at 100K on the microfocus beamline I24 at Diamond Light Source using helical / straight line scans with a beamsize of 10?m x 10?m (wxh). Diffraction was anisotropic with spots to 3.2Å in the best direction and 4.1Å in the worst direction.
Structure Solution: Structure was phased by molecular replacement using the coordinates for the transmembrane regions of TRAAK (3UM7) and TWIK1 (3UKM). The spacegroup is P21 with two channel homodimers per asymmetric unit. Refinement was carried out using BUSTER using NCS and TLS restraints using all data to 3.2Å.
Resolution: 3.4Å