Entry Clone Source: MGC

Entry Clone Accession: IMAGE:6143235

SGC Construct ID: ABCB10A-c109

GenBank GI number: gi|9961244

Vector: pFB-CT10HF-LIC. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

ATGGCCGGACTCCCGGAGGCCCGGAA
GCTCCTGGGGCTGGCGTACCCTGAGC
GCCGGAGGCTGGCAGCTGCGGTTGGA
TTTCTCACGATGTCCAGTGTTATCTC
CATGTCTGCCCCTTTCTTCCTGGGGA
AGATCATTGATGTCATCTATACCAAC
CCCACTGTGGACTACAGCGACAACCT
GACCCGCCTCTGCCTAGGGCTCAGTG
CCGTGTTTCTGTGTGGTGCTGCCGCC
AATGCCATTCGTGTCTACCTCATGCA
AACTTCAGGTCAGCGCATTGTGAATA
GGCTGAGAACTTCATTATTCTCCTCC
ATTCTGAGGCAGGAGGTTGCTTTCTT
TGACAAGACTCGCACAGGAGAATTGA
TTAACCGCCTCTCATCAGACACTGCA
CTCCTGGGGCGCTCAGTGACTGAAAA
CCTCTCAGATGGGCTCAGGGCCGGGG
CCCAGGCTTCTGTAGGCATCAGTATG
ATGTTTTTTGTCTCACCTAATCTGGC
CACCTTTGTTTTGAGCGTGGTGCCTC
CAGTGTCAATCATTGCTGTAATTTAT
GGGCGATATCTACGGAAACTGACCAA
AGTCACTCAGGATTCCCTGGCACAAG
CCACTCAGCTAGCTGAGGAACGTATT
GGAAATGTAAGAACTGTTCGAGCTTT
TGGGAAAGAAATGACTGAAATCGAGA
AATATGCCAGCAAAGTGGACCATGTA
ATGCAGTTAGCAAGGAAAGAGGCATT
CGCCCGGGCTGGTTTCTTTGGAGCAA
CTGGGCTCTCCGGAAACCTGATCGTG
CTTTCTGTCCTGTACAAAGGAGGGCT
GCTGATGGGCAGTGCCCACATGACCG
TGGGTGAACTCTCTTCCTTCCTAATG
TATGCTTTCTGGGTTGGAATAAGCAT
TGGAGGTCTGAGCTCTTTCTACTCGG
AGCTGATGAAAGGACTGGGTGCAGGG
GGGCGCCTCTGGGAGCTCCTGGAGAG
AGAGCCCAAGCTGCCTTTTAACGAGG
GGGTCATCTTAAATGAGAAAAGCTTC
CAGGGTGCTTTGGAGTTTAAGAACGT
GCATTTTGCCTATCCAGCTCGCCCAG
AGGTGCCCATATTTCAGGATTTCAGC
CTTTCCATTCCGTCAGGATCTGTCAC

GGCACTGGTTGGCCCAAGTGGTTCTG
GCAAATCAACAGTGCTTTCACTCCTG
CTGAGGTTGTACGACCCTGCTTCTGG
AACTATTAGTCTTGATGGCCATGACA
TCCGTCAGCTAAACCCAGTGTGGCTG
AGATCCAAAATTGGGACAGTGAGTCA
GGAACCCATTTTGTTTTCTTGCTCTA
TTGCTGAGAACATTGCTTATGGTGCT
GATGACCCTTCCTCTGTGACCGCTGA
GGAAATCCAGAGAGTGGCTGAAGTGG
CCAATGCAGTGGCCTTCATCCGGAAT
TTCCCCCAAGGGTTCAACACTGTGGT
TGGAGAAAAGGGTGTTCTCCTCTCAG
GTGGGCAGAAACAGCGGATTGCGATT
GCCCGTGCTCTGCTAAAGAATCCCAA
AATTCTTCTCCTAGATGAAGCAACCA
GTGCGCTGGATGCCGAAAATGAGTAC
CTTGTTCAAGAAGCTCTAGATCGACT
GATGGATGGAAGAACGGTGTTAGTTA
TTGCCCATCGTCTGTCCACCATTAAG
AATGCTAATATGGTTGCTGTTCTTGA
CCAAGGAAAAATTACTGAATATGGAA
AACATGAAGAGCTGCTTTCAAAACCA
AATGGGATATACAGAAAACTAATGAA
CAAACAAAGTTTTATTTCAGCAGCAG
AGAACCTCTACTTCCAATC

Expressed sequence (Tag sequence in lowercase):

MAGLPEARKLLGLAYPERRRLAAAVG
FLTMSSVISMSAPFFLGKIIDVIYTN
PTVDYSDNLTRLCLGLSAVFLCGAAA
NAIRVYLMQTSGQRIVNRLRTSLFSS
ILRQEVAFFDKTRTGELINRLSSDTA
LLGRSVTENLSDGLRAGAQASVGISM
MFFVSPNLATFVLSVVPPVSIIAVIY
GRYLRKLTKVTQDSLAQATQLAEERI
GNVRTVRAFGKEMTEIEKYASKVDHV
MQLARKEAFARAGFFGATGLSGNLIV
LSVLYKGGLLMGSAHMTVGELSSFLM
YAFWVGISIGGLSSFYSELMKGLGAG
GRLWELLEREPKLPFNEGVILNEKSF
QGALEFKNVHFAYPARPEVPIFQDFS
LSIPSGSVTALVGPSGSGKSTVLSLL
LRLYDPASGTISLDGHDIRQLNPVWL
RSKIGTVSQEPILFSCSIAENIAYGA
DDPSSVTAEEIQRVAEVANAVAFIRN
FPQGFNTVVGEKGVLLSGGQKQRIAI
ARALLKNPKILLLDEATSALDAENEY
LVQEALDRLMDGRTVLVIAHRLSTIK
NANMVAVLDQGKITEYGKHEELLSKP
NGIYRKLMNKQSFISAaenlyfq^sh
hhhhhhhhhdykddddk

^ 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 $2x10^6$ per litre of cell culture in SF900 medium (Invitrogen) were infected with recombinant baculovirus (5ml P2 virus per litre cell culture) and incubated for 72 hours. Cells were harvested by centrifugation and the pellet was flash frozen in liquid N_2.

Extraction buffer, extraction method: Frozen pellets were thawed and re-suspended in hypotonic buffer for lysis using a dounce homogeniser and DIAX homogeniser (Heidolph) at 10,000 r.p.m for 2 minutes. Cell membranes were collected by ultracentrifugation at 100,000Xg and the homogenisation repeated. The membranes were further washed with hypertonic buffer twice and the washed membranes were re-suspended in extraction buffer supplemented with 1% DDM, 0.1% CHS and incubated at 4°C with stirring. The extracted protein was separated from insoluble membranes by ultracentrifugation and collected for purification.
Hypotonic buffer: 10 mM HEPES, pH7.5; 0.5 mM EDTA**.**
Hypertonic buffer: 10 mM HEPES, pH7.5; 1M NaCl; 0.5 mM EDTA
Extraction/wash buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl; 20 mM Imidazole; 0.5 mM $MgCl_2$; 0.5 mM TCEP.

Column 1: Co-affinity. Cobalt Talon (Clontech), 4 ml of 50 % slurry in 1.5 x 10 cm column, washed with extraction/ wash buffer.

Column 1 Buffers:
Extraction/wash buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl; 20 mM Imidazole; 0.5 mM

$MgCl_2$ and 0.5 mM TCEP; 0.02% DDM; 0.002% CHS or 0.0001% Cardiolipin.
Elution buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl; 300 mM Imidazole; 0.5 mM $MgCl_2$; 0.5 mM TCEP; 0.02% DDM; 0.002% CHS.

Column 1 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 25 CV of wash buffer followed by elution in 1 CV fractions until all the protein was eluted.

Enzymatic treatment: The protein was cleaved by the addition of 1mg of TEV protease followed by incubation overnight at 4°C to purified protein with concurrent buffer exchange by dialysis using a 3kDa cut off dialysis membrane into gel filtration buffer. The protease was removed and tag cleaved protein was collected by binding to Cobalt talon and collecting the flow-through.

Column 2: Size Exclusion Chromatography. Superose6 (GE Healthcare).

Dialysis/Gel filtration buffer: 20 mM HEPES, pH 7.5; 200 mM NaCl; 0.5 mM TCEP; 0.5 mM $MgCl_2$; 0.02% DDM; 0.002% CHS.

Column 2 Procedure: The protein was concentrated and applied to a Superose6 gel filtration column equilibrated with gel filtration buffer using an ÄKTA Prime system.

Mass spectrometry characterization: The purified protein was homogeneous and had an experimental mass of 64664Da, as expected from the primary sequence with a post translational modification of loss of methionine and acetylation. Masses were determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% methanol in water with 0.1% formic acid.

Protein concentration: Protein was concentrated to >8mg/ml using a 100kDa cut-off concentrator and back diluted to 5-8mg/ml with the addition of 2 mM AMP-PNP. For the cardiolipin crystal form (Form B), cholesteryl hemisuccinate (CHS) was replaced with cardiolipin (CDL) at 0.05% for extraction and 0.001% for purification. The nucleotide was replaced with 2 mM AMP-PCP.

Crystallisation: Crystals were grown in sitting drops at 20°C.
Form A: Drops (200nl) comprising protein solution (5mg/ml; ABCB10 DDM/CHS/AMP-PNP) and reservoir solution (0.1-0.2 M NaCl, 5-7% (v/v) jeffamine M600, 30-40% (v/v) PEG400, 0.1 M glycine pH 9.5) in protein:reservoir ratios of 2:1 or 1.5:1 were equilibrated against 20µl of the same reservoir solution. The Form A crystals were obtained from a longer construct than the one described above.
Form B: Drops comprising 100nl protein solution (8mg/ml; ABCB10 DDM/CDL/AMP-PCP) and 100nl of reservoir solution (0.1 M NaCl, 0.1 M glycine pH 9.5, 30-36% (v/v) PEG300) were equilibrated against 20µl of the same reservoir solution. Crystal plates were transferred to 6°C prior to directly flash cooling crystals in liquid nitrogen.

Data collection: Data were collected at 100°K using a spiral scan collection strategy and a 10x50µm beamsize on I24 microfocus beamline (Diamond Light Source, UK).
Form A: Native data were collected to 3.4Å from a single crystal (λ=0.9686Å). Data for a mercury derivative, prepared by soaking native crystals overnight with 1 mM EMTS, were collected to 4Å (λ=0.9779Å). A higher resolution native dataset (3.15Å) was collected from a crystal soaked for 5mins in mother liquor containing 10 mM lutetium chloride.

Form B: Data were collected to 2.85Å using a single 10µmx10µm crystal volume (λ=0.9686Å).

Structure Solution: Initial phases were calculated for Form A using SIRAS. Two Hg sites were located with SHELXD and phase refinement was carried out with SHARP / SOLVE. Phase extension / density modification to 3.4Å yielded an interpretable map and allowed an initial backbone trace to be built. Molecular replacement was used to position this initial model in the Form B unit cell. The resultant electron density maps showed clear density for the missing regions and enabled unambiguous sequence assignment and model completion. Refinement was carried out with REFMAC and autoBUSTER.

Resolution: 2.85Å.