

ABCB10A (4AYW) Materials & Methods

Entry Clone Source: MGC

Entry Clone Accession: IMAGE: 6143235

SGC Construct ID: ABCB10A-c007

GI Number: 9961244

Vector: pFB-LIC-Bse. Details [[PDF](#)] ; Sequence [[FASTA](#)] or [[GenBank](#)]

DNA sequence:

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ATGCGAGGCCCTGCCTGGCAGG
GGACGAGGCCTGGCGCGGGCGG
CGCGCCTCCGGGACAAGGGCGG
CTGCGCCCGCAGCGGCCGGACTCCC
GGAGGCCCGGAAGCTCCTGGGCTGG
CGTACCTGAGCGCCGGAGGCTGGCA
GCTCGGGTGGATTCTCACGATGTC
CAGTGTATCTCCATGTCTGCCCTT
TCTTCCTGGGAAGATCATTGATGTC
ATCTATACCAACCCCAGTGGACTA
CAGCGACAACCTGACCCGCCTTGCC
TAGGGCTCAGTGCCGTGTTCTGTGT
GGTGCTGCCGCCAATGCCATTCTGTGT
CTACCTCATGCAAACCTCAGGTCAAGC
GCATTGTGAATAGGCTGAGAACTTCA
TTATTCTCCTCCATTCTGAGGCAGGA
GGTTGCTTCTTGACAAGACTCGCA
CAGGAGAATTGATTAACCGCCTCTCA
TCAGACACTGCACTCCTGGGCGCTC
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TCAGGGCCGGGCCAGGCTCTGTA
GGCATCAGTATGATGTTTTGTCTC
ACCTAATCTGCCACCTTGTGTTGA
GCGTGGTGCCTCCAGTGTCAATCATT
GCTGTAATTATGGCGATATCTACG
GAAACTGACCAAAGTCACTCAGGATT
CCCTGGCACAAGCCACTCAGCTAGCT
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TGTCGAGCTTTGGAAAGAAATGA
CTGAAATCGAGAAATATGCCAGCAA
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AACCTGATCGTGTCTGTCCTGTA
CAAAGGAGGGCTGCTGATGGCAGTG
CCCACATGACCGTGGGTGAACCTCTC
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CTGGGTGCAGGGGGCGCCTCTGGGA
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GCTCCTGGAGAGAGAGGCCAAGCTGC
CTTTAACGAGGGGGTCATCTTAAAT
GAGAAAAGCTCCAGGGTCTTGG
GTTTAAGAACGTGCATTTGCCTATC
CAGCTGCCAGAGGTGCCATATT
CAGGATTTCAGCCTTCCATTCCGTC
AGGATCTGTCACGGCACTGGTGGCC
CAAGTGGTTCTGGCAAATCAACAGTG
CTTCACTCCTGCTGAGGTGTACGA
CCCTGCTCTGGAACTATTAGTCTTG
ATGGCCATGACATCCGTAGCTAAC
CCAGTGTGGCTGAGATCCAAAATTGG
GACAGTGAGTCAGGAACCCATTTGT
TTCTTGCTCTATTGCTGAGAACATT
GCTTATGGTGCATGACCCCTCCTC
TGTGACCGCTGAGGAAATCAGAGAG
TGGCTGAAGTGGCCAATGCAGTGGCC
TTCATCCGAATTCCCCAAGGGTT
CAACACTGTGGTGGAGAAAAGGGTG
TTCTCCTCTCAGGTGGGCAGAAACAG
CGGATTGCGATTGCCGTGCTCTGCT
AAAGAATCCAAAATTCTCTCCTAG
ATGAAGCAACCAGTGCCTGGATGCC
GAAAATGAGTACCTGTTCAAGAAC
TCTAGATCGACTGATGGATGGAAGAA
CGGTGTTAGTTATTGCCATCATCTG
TCCACCATTAAGAATGCTAATATGGT
TGCTGTTCTGACCAAGGAAAATTA
CTGAATATGGAAAACATGAAGAGCTG
CTTCAAAACCAAATGGGATATACAG
AAAACATAATGAACAAACAAAGTTTA
TTTCAGCATGA

Expressed sequence (small letters refer to tag sequence):

mghhhhhssgvdlgtenlyfqs^MR
GPPAWAGDEAWRRGPAAPPGDKGRLR
PAAAGLPEARKLLGLAYPERRLAAA
VGFLTMSSVISMSAPFFLGKIIDVIY
TNPTVDYSDNLTRLCLGLSAVFLCGA
AANAIRVYLMQTSGQRIVNRLRTSLF
SSILRQEVAFFDKTRTGELEINRLSSD
TALLGRSVTENLSDGLRAGAQASVGI
SM MFFVSPNLATFVLSVPPPVI
VIYGRYLRKLTKVTQDSLQATQLAE
ERIGNVRTVRAFGKEMTEIEKYASKV
DHVMQLARKEAFARAGFFGATGLSGN
LIVLSVLYKGGLLMSAHMTVGE
FLMYAFWVGISIGGLSSFYSELMKGL
GAGGRLWELLEREPKLPFNEGVLNE
KSFQGALEFKNVHFAYPARPEVPIFQ
DFSL SIPSGVTALVGPSGSGKSTVL
SLLRLYDPASGTISLDGHDIRQLNP
VWLRSKIGTVSQEPILFSCSIAENIA
YGADDPSSVTAEEIQRVAEVANAVAF

IRNFPQGFNTVVGEKGVLSSGGQKQR
IAIARALLKNPKILLDEATSALDAE
NEYLVQEALDRLMDGRTVLVIAHLS
TIKNANMVAVLDQGKITEYGKHEELL
SKPNGIYRKLNMNKQSFISA

Tags and additions: N-terminal, TEV cleavable hexahistidine tag. (^ cleavage site)

Host: *Spodoptera frugiperda* (SF9) insect cells

Growth Medium & Induction Protocol: Insect cells with a density of 2×10^6 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 N₂.

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: 10mM HEPES pH7.5, 0.5 mM EDTA

Hypertonic buffer: 10mM HEPES pH7.5, 1M NaCl, 0.5mM EDTA

Extraction/wash buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl; 20 mM imidazole, 0.5mM MgCl₂ and 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, 0.5mM MgCl₂ and 0.5 mM TCEP ,0.02%DDM, 0.002%CHS

Elution buffer: 50 mM HEPES, pH 7.5; 200 mM NaCl; 300 mM imidazole, 0.5mM MgCl₂ and 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 25CV of wash buffer followed by elution in 1CV fractions until all the protein was eluted.

Enzymatic Treatment: 1mg TEV protease was added 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)

Column 2 Buffer:

Dialysis/ Gel Filtration buffer: 20 mM HEPES pH 7.5, 200 mM NaCl, 0.5 mM TCEP, 0.5mM MgCl₂, 0.02% DDM+0.001%Cardiolipin

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

Mass spec characterization:

The purified protein was homogeneous and had an experimental mass of 67075 Da. This represents an unexplained, average mass of +91Da when compared to the calculated mass (66984 Da) based on the known sequence. 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 2mM Mg-AMP-PNP.

Crystallization: Crystals were grown in sitting drops at 20°C.

Drops (200nl) comprising protein solution (5mg/ml; ABCB10A 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.

Crystal plates were transferred to 6°C prior to directly flash cooling crystals in liquid nitrogen.

Data Collection:

Resolution: 3.3Å

Data were collected at 100°K using a spiral scan collection strategy and a 10x50μm beamsize on I24 microfocus beamline (Diamond Light Source, UK).

Native data were collected to 3.4Å from a single crystal ($\Lambda=0.9686\text{\AA}$). Data for a mercury derivative, prepared by soaking native crystals overnight with 1mM EMTS, were collected to 4Å ($\mu=0.9779\text{\AA}$). A higher resolution native dataset (nominally 3.15Å) was collected from a crystal soaked for 5mins in mother liquor containing 10mM lutetium chloride.

Structure Solution: Initial phases were calculated 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. However, both data anisotropy and disorder of the NBD hindered model completion using the plate form crystals. The structure was finally solved using the refined, higher resolution rod-form A structure. Molecular replacement was used to position the separate transmembrane and nucleotide-binding domains. Refinement was carried out with autoBUSTER using LSSR restraints to the higher resolution rod-form A structure.