Molecular Biology

Entry Clone Accession: n/a
Entry Clone Source: Site-directed mutagenesis
SGC Construct ID: ACVR1A-c096
Protein Region: I208-D499
Vector: pFB-LIC-Bse
Tag: N-6HIS;N-TEV
Host: DH10Bac

Sequence (with tag(s)):
MGHHHHHHSSGVDLGTENLYFQSMQRTVARDITLLECVGKGRYGEVWRGSWQGENVA
VKIFSSRDEKSWFRETELYNTVMLRHENILGFIASDMTSRHSSTQLWLITHYHEMGSLYD
YLQLTTLDTVSCLRIVLSIASGLAHLHIEIFGTQGKPAIAHRDLKSKNILVKKNGQCCIADL
GLAVMHSQSTNQLDVGNNPRVGTKRYMAPEVLDETIQVDCFDSYKRVDIWAFGLVLWE
VARRMVSNGIVEDYKPPFYDVVPNDPSFEDMRKVVCVDQQRPNIPNRWFSDPTLTSLAK
LMKECWYQNPSARLTALRIKKTLTKID
Sequence after tag cleavage:
SMQRTVARDITLLECVGKGRYGEVWRGSWQGENVAVKIFSSRDEKSWFRETELYNTVML
RHENILGFIASDMTSRHSSTQLWLITHYHEMGSLYDYLQLTTLDTVSCLRIVLSIASGLAH
LHIEIFGTQGKPAIAHRDLKSKNILVKKNGQCCIADLGLAVMHSQSTNQLDVGNNPRVGT
KRYMAPEVLDETIQVDCFDSYKRVDIWAFGLVLWEVARRMVSNGIVEDYKPPFYDVVPN
DPSFEDMRKVVCVDQQRPNIPNRWFSDPTLTSLAKLMKECWYQNPSARLTALRIKKTLT
KID
DNA Sequence:
CCATGGGCCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTG
TACTTCCAATCCATGCAAAGAACAGTGGCTCGCGATATTACACTGTTGGAGTGTGTCG
GGAAAGGCAGGTATGGTGAGGTGTGGAGGGGCAGCTGGCAAGGGGAAAATGTTGCC
GTGAAGATCTTCTCCTCCCGTGATGAGAAGTCATGGTTCAGGGAAACGGAATTGTACA
ACACTGTGATGCTGAGGCATGAAAATATCTTAGGTTTCATTGCTTCAGACATGACATCA
AGACACTCCAGTACCCAGCTGTGGTTAATTACACATTATCATGAAATGGGATCGTTGTA
CGACTATCTTCAGCTTACTACTCTGGATACAGTTAGCTGCCTTCGAATAGTGCTGTCCA
TAGCTAGTGGTCTTGCACATTTGCACATAGAGATATTTGGGACCCAAGGGAAACCAGC
CATTGCCCATCGAGATTTAAAGAGCAAAAATATTCTGGTTAAGAAGAATGGACAGTGT
TGCATAGCAGATTTGGGCCTGGCAGTCATGCATTCCCAGAGCACCAATCAGCTTGATG
TGGGGAACAATCCCCGTGTGGGCACCAAGCGCTACATGGCCCCCGAAGTTCTAGATG
AAACCATCCAGGTGGATTGTTTCGATTCTTATAAAAGGGTCGATATTTGGGCCTTTGGA
CTTGTTTTGTGGGAAGTGGCCAGGCGGATGGTGAGCAATGGTATAGTGGAGGATTACA
AGCCACCGTTCTACGATGTGGTTCCCAATGACCCAAGTTTTGAAGATATGAGGAAGGT
AGTCTGTGTGGATCAACAAAGGCCAAACATACCCAACAGATGGTTCTCAGACCCGAC
ATTAACCTCTCTGGCCAAGCTAATGAAAGAATGCTGGTATCAAAATCCATCCGCAAGA
CTCACAGCACTGCGTATCAAAAAGACTTTGACCAAAATTGATTGACAGTAAAGGTGG
ATACGGATCCGAATTCGAGCTCCGTCGACAAGCTT

Protein Expression

Medium: Insect Xpress
Antibiotics: Ampicillin
Procedure: Sf9 cells at a density of 2x106/ml were infected with recombinant ACVR1 baculovirus (virus stock P2; 3ml of virus stock/1000 ml of cell culture). Cells were shaken at 110

rpm at 27 °C in an Innova shaker. After 72 hours post-infection the cultures were harvested by centrifugation for 20min at 900rpm. Cell pellets from each 1L flask were resuspended in 15 ml binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole). Calbiochem protease inhibitor SET V was added to the cell suspension at a 1:2000 dilution and transferred to 50 ml tubes, and stored at -20 °C.

Protein Purification

Procedure: The frozen cells were thawed and the volume increased to 80 ml with binding buffer. The cells were lysed by sonication over 18 min with the sonicator pulsing ON for 5 sec and OFF for 10 sec. The DNA was precipitated using 0.15% PEI (polyethyleneimine) pH 8. The cell lysate was spun down by centrifugation at 21.5K rpm at 4°C for 1 h. The supernatant was recovered for purification. Column 1: Ni-Affinity Chromatography. 4 ml of 50 % Ni-sepharose slurry was applied onto a 1.5 x 10 cm column. The column was equilibrated with binding buffer (25ml). Buffers: Binding buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole, 0.1mM TCEP Wash buffer: 50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 25 mM imidazole, 0.1mM TCEPElution buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% Glycerol; 50 to 250 mM imidazole, 0.1mM TCEP Procedure: The supernatant was applied by batch binding incubation to the resin for 30 min at 4 °C before the resin was recovered by centrifugation at 700g for 5 minutes. The supernatant was discarded and the resin resuspended in 50ml binding buffer before being applied to a 1.5 x 10cm column. The bound protein was eluted by applying a step gradient of imidazole using 7 ml portions of elution buffer with increasing concentration of imidazole (1 x 50 mM, 3 x 250 mM). Enzymatic treatment: 0.1mg of TEV protease was added to the Ni-eluted protein (Overnight incubation at 4 °C) to remove the tag. Column 2: Size Exclusion Chromatography S75 HiLoad 16/60 Superdex run on ÄKTA-Express Buffer: Gel Filtration buffer: 300 mM NaCl, 50 mM Hepes pH 7.5, 0.5mM TCEP Procedure: Prior to applying the protein, the S75 16/60 column was washed and equilibrated with gel filtration buffer. The protein was concentrated to 5 ml using an Amicon Ultra-15 filter with a 10 kDa cut-off. The concentrated protein was directly applied onto the equilibrated S75 16/60 column, and run at a flow-rate of 1 ml/min. The protein was eluted at 65 - 80 ml. Fractions containing the protein were pooled together and stored with 10mM DTT. The protein was concentrated down to 21mg/ml before being flash frozen and stored at -80 °C. Column 3: Upon thawing for crystallization, the protein was passed down a Size Exclusion Chromatography S75 HiLoad 16/60 Superdex run on ÄKTA-Express Buffer: Gel Filtration buffer: 300 mM NaCl, 50 mM Hepes pH 7.5, 0.5mM TCEP Procedure: Prior to applying the protein, the S75 16/60 column was washed and equilibrated with gel filtration buffer. The concentrated protein was directly applied onto the equilibrated S75 16/60 column, and run at a flow-rate of 1 ml/min. The protein was eluted at 65 - 80 ml and fractions collected before reconcentrating down to 17.5mg/ml prior to crystallization.

Columns: Column 1: Ni NTA; Column 2: GF75; Column 3: GF75;
Concentration: 17.5 mg/ml
Mass-spec Verification: Confirmed

Structure Determination

Crystallization: Protein was buffered in 50 mM HEPES pH 7.5, 300 mM NaCl and 10 mM DTT. The protein was concentrated to 17.5 mg/ml (calculated using an extinction co-efficient of 58900) in the presence of the inhibitor K06803a (LH-2244-252) (1 mM end concentration). Crystals were grown at 20 °C in 300 nl sitting drops mixing 200 nl protein solution with 100 nl of a reservoir solution containing 1.5M ammonium sulphate, 0.1M tris pH 8.5, 4% glycerol. On mounting crystals were cryoprotected with mother liquor plus 25% ethylene glycol before transfer to liquid nitrogen.

Data Collection: Diffraction data were collected at the Diamond Light Source, station I03 using monochromatic radiation at wavelength 0.9763 Å. Data could be analysed to a resolution of 2.17 Å.

Data Processing: Data were processed with MOSFLM and subsequently scaled using the program AIMLESS from the CCP4 suite. Initial phases were obtained by molecular replacement using the program PHASER and the structure of ALK2 (Protein Data Bank code 3H9R) as a search model. The resulting structure solution was refined using REFMAC5 from the CCP4 suite, Phenix Refine and manually rebuilt with COOT. Appropriate TLS restrained refinement using the tls tensor files calculated from the program TLSMD was applied at the final round of refinement. The complete structure was verified for geometric correctness with MolProbity.