Molecular Biology

Entry Clone Accession:
Entry Clone Source: By PCR
SGC Construct ID: ACVR1Z-c041
Protein Region: T172-C509
Vector: pFB-LIC-Bse
Tag: N-6 HIS;N-TEV
Host: DH10Bac

Sequence (with tag(s)):
MGHHHHHHSSGVDLGTENLYFQSMTTNVGDSTLADLLDHSCTSGSGSGLPFLVQRTVAR
EITLLECVGKGRYGEVWRGSWQGENVAVKIFSSRDEKSWFRETELYNTVMLRHENILGFI
ASDMTSRHSSTQLWLITHYHEMGSLYDYLQLTTLDTVSCLRIVLSIASGLAHLHIEIFGTQ
GKPAIAHRDLKSKNILVKKNGQCCIADLGLAVMHSQSTNQLDVGNNPRVGTKRYMAPEV
LDETIQVDCFDSYKRVDIWAFGLVLWEVARRMVSNGIVEDYKPPFYDVVPNDPSFEDMR
KVVCVDQQRPNIPNRWFSDPTLTSLAKLMKECWYQNPSARLTALRIKKTLTKIDNSLDKL
KTDC
Sequence after tag cleavage:
SMTTNVGDSTLADLLDHSCTSGSGSGLPFLVQRTVAREITLLECVGKGRYGEVWRGSWQ
GENVAVKIFSSRDEKSWFRETELYNTVMLRHENILGFIASDMTSRHSSTQLWLITHYHEM
GSLYDYLQLTTLDTVSCLRIVLSIASGLAHLHIEIFGTQGKPAIAHRDLKSKNILVKKNGQC
CIADLGLAVMHSQSTNQLDVGNNPRVGTKRYMAPEVLDETIQVDCFDSYKRVDIWAFGL
VLWEVARRMVSNGIVEDYKPPFYDVVPNDPSFEDMRKVVCVDQQRPNIPNRWFSDPTLT
SLAKLMKECWYQNPSARLTALRIKKTLTKIDNSLDKLKTDC
DNA Sequence:
CCATGGGCCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTG
TACTTCCAATCCATGACCACCAATGTTGGAGACAGCACTTTAGCAGATTTATTGGATCA
TTCGTGTACATCAGGAAGTGGCTCTGGTCTTCCTTTTCTGGTACAAAGAACAGTGGCT
CGCGAGATTACACTGTTGGAGTGTGTCGGGAAAGGCAGGTATGGTGAGGTGTGGAGG
GGCAGCTGGCAAGGGGAAAATGTTGCCGTGAAGATCTTCTCCTCCCGTGATGAGAAG
TCATGGTTCAGGGAAACGGAATTGTACAACACTGTGATGCTGAGGCATGAAAATATCT
TAGGTTTCATTGCTTCAGACATGACATCAAGACACTCCAGTACCCAGCTGTGGTTAATT
ACACATTATCATGAAATGGGATCGTTGTACGACTATCTTCAGCTTACTACTCTGGATAC
AGTTAGCTGCCTTCGAATAGTGCTGTCCATAGCTAGTGGTCTTGCACATTTGCACATAG
AGATATTTGGGACCCAAGGGAAACCAGCCATTGCCCATCGAGATTTAAAGAGCAAAA
ATATTCTGGTTAAGAAGAATGGACAGTGTTGCATAGCAGATTTGGGCCTGGCAGTCAT
GCATTCCCAGAGCACCAATCAGCTTGATGTGGGGAACAATCCCCGTGTGGGCACCAA
GCGCTACATGGCCCCCGAAGTTCTAGATGAAACCATCCAGGTGGATTGTTTCGATTCTT
ATAAAAGGGTCGATATTTGGGCCTTTGGACTTGTTTTGTGGGAAGTGGCCAGGCGGAT
GGTGAGCAATGGTATAGTGGAGGATTACAAGCCACCGTTCTACGATGTGGTTCCCAAT
GACCCAAGTTTTGAAGATATGAGGAAGGTAGTCTGTGTGGATCAACAAAGGCCAAAC
ATACCCAACAGATGGTTCTCAGACCCGACATTAACCTCTCTGGCCAAGCTAATGAAAG
AATGCTGGTATCAAAATCCATCCGCAAGACTCACAGCACTGCGTATCAAAAAGACTTT
GACCAAAATTGATAATTCCCTCGACAAATTGAAAACTGACTGTTGACAGTAAAGGTGG
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 P3; 2ml 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: Extraction buffer, extraction method: The frozen cells were thawed and the volume increased to 40 ml with binding buffer. The cells were lysed by sonication over 9 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 gravity flow onto the Ni-sepharose column. The bound protein was eluted by applying a step gradient of imidazole using 10 ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 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 S200 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 S200 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 S200 16/60 column, and run at a flow-rate of 1 ml/min. The protein was eluted at 90-110 ml. Fractions containing the protein were pooled together.
Columns: Column 1: NiNTA 2ml gravity flow affinity column. Column 2: Size Exclusion Chromatography using S200 HiLoad resin;
Concentration: 10.0 mg/ml
Mass-spec Verification: The purified protein was homogeneous and had an experimental mass of 40.905 kDa, as expected from primary sequences allowing for N-terminal Met removal and subsequent acetylation. Mass was 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% acetonitrile in water with 0.1% formic acid.
Compound Exact Mass: 352.142307

Structure Determination

Crystallization: Protein was buffered in 50 mM HEPES pH 7.5, 300 mM NaCl, 10 mM DTT and 10mM L-arginine, 10 mM L-glutamate. The protein was concentrated to 10 mg/ml (calculated using an extinction co-efficient of 58900) in the presence of the inhibitor K02288a (1 mM end concentration). Crystals were grown at 20°C in 150 nl sitting drops mixing 50 nl protein solution with 100 nl of a reservoir solution containing 0.1M MES pH 6.5 and 12%(w/v) PEG 20000. 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 0.9778 Å. Data could be analysed to a resolution of 2.3

Å.

Data Processing: Data were processed with MOSFLM [1] 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, 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.