Material and Methods

Entry Clone Source: FivePrime

Entry Clone Accession:

SGC Construct ID: ACVR1A-c076

Entry clone accession/ sequence:

CCATGGGCCACCATCATCATCATCAT
TCTTCTGGTGTAGATCTGGGTACCGA
GAACCTGTACTTCCAATCCATGACCA
CCAATGTTGGAGACAGCACTTTAGCA
GATTTATTGGATCATTCGTGTACATC
AGGAAGTGGCTCTGGTCTTCCTTTTC
TGGTACAAAGAACAGTGGCTCGCCAG
ATTACACTGTTGGAGTGTGTCGGGAA
AGGCAGGTATGGTGAGGTGTGGAGGG
GCAGCTGGCAAGGGGAAAATGTTGCC
GTGAAGATCTTCTCCTCCCGTGATGA
GAAGTCATGGTTCAGGGAAACGGAAT
TGTACAACACTGTGATGCTGAGGCAT
GAAAATATCTTAGGTTTCATTGCTTC
AGACATGACATCAAGACACTCCAGTA
CCCAGCTGTGGTTAATTACACATTAT
CATGAAATGGGATCGTTGTACGACTA
TCTTCAGCTTACTACTCTGGATACAG
TTAGCTGCCTTCGAATAGTGCTGTCC
ATAGCTAGTGGTCTTGCACATTTGCA
CATAGAGATATTTGGGACCCAAGGGA
AACCAGCCATTGCCCATCGAGATTTA
AAGAGCAAAAATATTCTGGTTAAGAA
GAATGGACAGTGTTGCATAGCAGATT
TGGGCCTGGCAGTCATGCATTCCCAG
AGCACCAATCAGCTTGATGTGGGGAA
CAATCCCCGTGTGGGCACCAAGCGCT
ACATGGCCCCCGAAGTTCTAGATGAA
ACCATCCAGGTGGATTGTTTCGATTC
TTATAAAAGGGTCGATATTTGGGCCT
TTGGACTTGTTTTGTGGGAAGTGGCC
AGGCGGATGGTGAGCAATGGTATAGT
GGAGGATTACAAGCCACCGTTCTACG
ATGTGGTTCCCAATGACCCAAGTTTT
GAAGATATGAGGAAGGTAGTCTGTGT
GGATCAACAAAGGCCAAACATACCCA
ACAGATGGTTCTCAGACCCGACATTA
ACCTCTCTGGCCAAGCTAATGAAAGA
ATGCTGGTATCAAAATCCATCCGCAA
GACTCACAGCACTGCGTATCAAAAAG
ACTTTGACCAAAATTGATTGACAGTA
AAGGTGGATACGGATCCGAATTCGAG
CTCCGTCGACAAGCTT

Expressed protein sequence:

mghhhhhhssgvdlgtenlyfq*smT

TNVGDSTLADLLDHSCTSGSGSGLPF
LVQRTVARQITLLECVGKGRYGEVWR
GSWQGENVAVKIFSSRDEKSWFRETE
LYNTVMLRHENILGFIASDMTSRHSS
TQLWLITHYHEMGSLYDYLQLTTLDT
VSCLRIVLSIASGLAHLHIEIFGTQG
KPAIAHRDLKSKNILVKKNGQCCIAD
LGLAVMHSQSTNQLDVGNNPRVGTKR
YMAPEVLDETIQVDCFDSYKRVDIWA
FGLVLWEVARRMVSNGIVEDYKPPFY
DVVPNDPSFEDMRKVVCVDQQRPNIP
NRWFSDPTLTSLAKLMKECWYQNPSA
RLTALRIKKTLTKID

Vector: pFB-LIC-Bse

Tags and additions: MGHHHHHHSSGVDLGTENLYFQ*SM. cleavable N-terminal hexahistidine tag.

Host: SF9 Spodoptera frugiperda Insect cells

Growth medium, induction protocol:

Sf9 cells at a density of 2x10 ^{6}/ml were infected with recombinant ACVR1 baculovirus (virus stock P2; 1ml of virus stock/100 ml of cell culture). Cells were shaken at 120 rpm at 27°C in an Innova shaker. After 48 hours post-infection the cultures were harvested by centrifugation for 10min at 6000rpm. Cell pellets from each 1L flask were resuspended in 20 ml binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole), transferred to 50 ml tubes, and stored at -20°C. Calbiochem protease inhibitor SET V was added to the cell suspension at a 1:100 dilution

Extraction buffer, extraction method: The frozen cells were thawed and the volume increased to 50 ml with binding buffer. The cells were lysed by ultrasonication over 15 min with the sonicator pulsing ON for 5 sec and OFF for 10. A final concentration of 0.15% PEI was added to the lysate. The cell lysate was spun down by centrifugation at 21K rpm at 4°C for 1 h. The supernatant was recovered for purification.

Column 1:

Ni-Affinity Chromatography. 5 ml of 50 % Ni-IDA 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 TCEP

Elution buffer : 50 mM HEPES, pH 7.5; 500 mM NaCl; 5% Glycerol; 50 □ 250 mM imidazole, 0.1mM TCEP

Procedure:

The supernatant following centrifugation was applied by gravity flow onto the Ni-sepharose column. The bound protein was then washed with 50ml binding buffer and subsequently with 30 ml wash buffer. ACVR1 protein was then eluted by applying a step gradient of imidazole □

using 5 ml fractions of elution buffer with increasing concentration of imidazole (1 x 50 mM, 3 x 250 mM). Elution fractions were analyzed by SDS PAGE and the 3 x 250 mM imidazole fractions were kept and pooled. 10 mM DTT was added for overnight storage at 4°C.

Enzymatic treatment: 0.1mg of TEV protease was added to the Ni-eluted protein to remove the tag. Incubation was overnight at 4°C

Complex Assembly:

3mg of ACVR1A and 5mg of FKBP12.6 (see below for FKBP12.6 methods) were incubated at 4°C for 30 minutes.

Column 3: 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, 05mM TCEP

Procedure: Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. The two proteins were mixed and concentrated to 3 ml using an Amicon Ultra-15 filter with a 3 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 80 95 ml. Fractions containing the protein were pooled together.

Mass spec characterization: The purified protein was homogeneous and had an experimental mass of 37.352 and 11.869 kDa, as expected from primary sequences. 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% acetonitrile in water with 0.1% formic acid.

MATERIALS & METHODS FOR FKBP12.6 PRIOR TO COMPLEX FORMATION

Entry Clone Accession:

SGC Construct ID: FKBP1BA-c001

Entry clone accession/ sequence:

ATGGGCGTGGAGATCGAGACCATCTC
CCCCGGAGACGGAAGGACATTCCCCA
AGAAGGGCCAAACGTGTGTGGTGCAC
TACACAGGAATGCTCCAAAATGGGAA
GAAGTTTGATTCATCCAGAGACAGAA
ACAAACCTTTCAAGTTCAGAATTGGC
AAACAGGAAGTCATCAAAGGTTTTGA
AGAGGGTGCAGCCCAGATGAGCTTGG
GGCAGAGGGCGAAGCTGACCTGCACC
CCTGATGTGGCATATGGAGCCACGGG
CCACCCCGGTGTCATCCCTCCCAATG
CCACCCTCATCTTTGACGTGGAGCTG
CTCAACTTAGAGTGA

Expressed protein sequence:

mhhhhhhssgvdlgtenlyfq*smGV
EIETISPGDGRTFPKKGQTCVVHYTG
MLQNGKKFDSSRDRNKPFKFRIGKQE
VIKGFEEGAAQMSLGQRAKLTCTPDV
AYGATGHPGVIPPNATLIFDVELLNL
E

Vector: pNIC28-Bsa4

Tags and additions: MHHHHHHSSGVDLGTENLYFQ*SM. cleavable N-terminal hexahistidine tag.

Host: BL21(DE3)-R3-pRARE2

Growth medium, induction protocol:

A glycerol stock was used to inoculate a 50 ml starter culture containing LB media and 34 µg/ml chloramphenicol and 50 µg/ml kanamycin. The starter culture was grown overnight at 37°C with shaking at 250 rpm. A flask containing 1L LB media with 34 µg/ml chloramphenicol and 50 µg/ml kanamycin was inoculated with 10 ml of the starter culture. The 1L culture was incubated at 37°C with shaking at 160 rpm until an OD600nm e 0.5 was reached. The flasks were then cooled down to 21°C and 0.4 mM IPTG added to induce protein expression overnight. Cells were harvested by centrifugation at 5000 rpm at 4°C for 15 min. The cell pellet was resuspended in 30 ml binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole), transferred to a 50 ml tube, and stored at -20°C.

Extraction buffer, extraction method:

The frozen cells were thawed. The cells were lysed by ultrasonication over 15 min with the sonicator pulsing ON for 5 sec and OFF for 10. A final concentration of 0.15% PEI was added to the lysate. The cell lysate was spun down by centrifugation at 21K rpm at 4°C for 1 h. The supernatant was recovered for purification..

Columns 1 and 2:

FKBP12.6 was purified from the supernatant using the same column 1/column 2 protocol as shown above for ACVR1. The two proteins were mixed as described above before further purification as described above.

Enzymatic treatment: 0.1mg of TEV protease was added to the Ni-eluted protein to remove the tag. Incubation was overnight at 4°C

Crystallisation of the ACVR1-FKBP12 complex

Protein was buffered in 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM DTT. To this 1mM dorosomorphin was added and the protein concentrated to 7.5 mg/ml (calculated using an extinction co-efficient of 61880). Crystals were grown at 4°C in 150 nl sitting drops mixing 100 nl protein solution with 50 nl of a reservoir solution containing 1.8M ammonium citrate. On mounting crystals were cryoprotected with mother liquor plus 25% ethylene glycol and flash frozen in liquid nitrogen.

Data Collection: Resolution: 2.17 Å resolution

X-ray source: Diamond Light Source, station I04, using monochromatic radiation at wavelength 0.9686 Å