ACVR1A (4DYM) Materials & Methods

Entry Clone Source: Site-directed mutagenesis

Entry Clone Accession: N/A

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

Amplified construct sequence:

CCATGGGCCACCATCATCATCATCAT
TCTTCTGGTGTAGATCTGGGTACCGA
GAACCTGTACTTCCAATCCATGCAAA
GAACAGTGGCT**CAC**CAGATTACACTG
TTGGAGTGTGTCGGGAAAGGCAGGTA
TGGTGAGGTGTGGAGGGGCAGCTGGC
AAGGGGAAAATGTTGCCGTGAAGATC
TTCTCCTCCCGTGATGAGAAGTCATG
GTTCAGGGAAACGGAATTGTACAACA
CTGTGATGCTGAGGCATGAAAATATC
TTAGGTTTCATTGCTTCAGACATGAC
ATCAAGACACTCCAGTACCCAGCTGT
GGTTAATTACACATTATCATGAAATG
GGATCGTTGTACGACTATCTTCAGCT
TACTACTCTGGATACAGTTAGCTGCC
TTCGAATAGTGCTGTCCATAGCTAGT
GGTCTTGCACATTTGCACATAGAGAT
ATTTGGGACCCAAGGGAAACCAGCCA
TTGCCCATCGAGATTTAAAGAGCAAA
AATATTCTGGTTAAGAAGAATGGACA
GTGTTGCATAGCAGATTTGGGCCTGG
CAGTCATGCATTCCCAGAGCACCAAT
CAGCTTGATGTGGGGAACAATCCCCG
TGTGGGCACCAAGCGCTACATGGCCC
CCGAAGTTCTAGATGAAACCATCCAG
GTGGATTGTTTCGATTCTTATAAAAG
GGTCGATATTTGGGCCTTTGGACTTG
TTTTGTGGGAAGTGGCCAGGCGGATG
GTGAGCAATGGTATAGTGGAGGATTA
CAAGCCACCGTTCTACGATGTGGTTC
CCAATGACCCAAGTTTTGAAGATATG
AGGAAGGTAGTCTGTGTGGATCAACA
AAGGCCAAACATACCCAACAGATGGT
TCTCAGACCCGACATTAACCTCTCTG
GCCAAGCTAATGAAAGAATGCTGGTA
TCAAAATCCATCCGCAAGACTCACAG
CACTGCGTATCAAAAAGACTTTGACC
AAAATTGATTGACAGTAAAGGTGGAT
ACGGATCCGAATTCGAGCTCCGTCGA
CAAGCTT

Engineered R206H mutation in bold

Expressed sequence (Tag sequence in lowercase):

mghhhhhhssgvdlgtenlyfq*SMQ
RTVA**H**QITLLECVGKGRYGEVWRGSW
QGENVAVKIFSSRDEKSWFRETELYN
TVMLRHENILGFIASDMTSRHSSTQL
WLITHYHEMGSLYDYLQLTTLDTVSC
LRIVLSIASGLAHLHIEIFGTQGKPA
IAHRDLKSKNILVKKNGQCCIADLGL
AVMHSQSTNQLDVGNNPRVGTKRYMA
PEVLDETIQVDCFDSYKRVDIWAFGL
VLWEVARRMVSNGIVEDYKPPFYDVV
PNDPSFEDMRKVVCVDQQRPNIPNRW
FSDPTLTSLAKLMKECWYQNPSARLT
ALRIKKTLTKID

^ TEV cleavage site

Engineered R206H mutation in bold

Tags and additions: Cleavable N-terminal His6 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 P3; 1ml of virus stock/100 ml of cell culture). Cells were shaken at 110 rpm at 27°C in an Innova shaker. After 48 hours post-infection the cultures were harvested by centrifugation for 20min at 6000rpm. 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.

Extraction buffer, extraction method: The frozen cells were thawed and the volume increased to 90 ml with binding buffer. The cells were lysed using an Emulsiflex C5 homogeniser. The cell lysate was spun down by centrifugation at 21.5K rpm and 4°C for 1 h. The supernatant was recovered for purification.

Column 1: Anion-exchange for Nucleic acid removal with DEAE cellulose (DE52, Whatmann)10 g of resin was suspended in 50 ml 1 M NaCl, and then applied onto a 2.5 x 20 cm column. The resin was then equilibrated with 50 ml binding buffer prior to loading the sample.

Column 1 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

Column 1 Procedure: The supernatant was first applied onto the column by gravity flow, which was followed by a wash with 50 ml wash buffer. The column flow-through and wash was directly applied onto a Ni-IDA column.

Column 2: Ni-Affinity Chromatography. 6 ml of 50 % Ni-IDA slurry was applied onto a 1.5 x 10 cm column. The column was equilibrated with binding buffer (25ml).

Column 2 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 to 250 mM imidazole, 0.1mM TCEP

Column 2 Procedure: The flow-through from column 1 (DE52) was applied by gravity flow onto the Ni-IDA 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 to remove the tag.

Column 3: Size Exclusion Chromatography S200 HiLoad 16/60 Superdex run on ÄKTA-Express

Column 3 Buffers:
Gel filtration buffer: 150 mM NaCl, 25 mM Hepes pH 7.5

Column 3 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.

Mass spec characterization: The purified protein was homogeneous and had an experimental mass of 34487.9 Da, as expected from primary sequences. 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.

Crystallization: Protein was buffered in 25 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM DTT and 10mM L-Arg, 10mM L-Glu. To this 1mM K00135 was added and the protein concentrated to 10 mg/ml (calculated using an extinction co-efficient of 58900). Crystals were grown at 20°C in 150 nl sitting drops mixing 75 nl protein solution with 75 nl of a reservoir solution containing 1.6M $MgSO_4$ and 0.1M MES pH 6.5. On mounting crystals were cryoprotected with mother liquor plus 20% glycerol and flash frozen in liquid nitrogen.

Data Collection:
Resolution: 2.42Å
X-ray source: Diamond Light Source, station I02, using monochromatic radiation at wavelength 0.9795Å