Entry Clone Source: Protein from Stephan Feller group

Entry Clone Accession: gi|9257197

SGC Construct ID: BAIAP2A-c001

GenBank GI number: gi|9257197

Vector: pGEX-6P-2.

Amplified construct sequence:

ATGTCCCCTATACTAGGTTATTGGAA
AATTAAGGGCCTTGTGCAACCCACTC
GACTTCTTTTGGAATATCTTGAAGAA
AAATATGAAGAGCATTTGTATGAGCG
CGATGAAGGTGATAAATGGCGAAACA
AAAAGTTTGAATTGGGTTTGGAGTTT
CCCAATCTTCCTTATTATATTGATGG
TGATGTTAAATTAACACAGTCTATGG
CCATCATACGTTATATAGCTGACAAG
CACAACATGTTGGGTGGTTGTCCAAA
AGAGCGTGCAGAGATTTCAATGCTTG
AAGGAGCGGTTTTGGATATTAGATAC
GGTGTTTCGAGAATTGCATATAGTAA
AGACTTTGAAACTCTCAAAGTTGATT
TTCTTAGCAAGCTACCTGAAATGCTG
AAAATGTTCGAAGATCGTTTATGTCA
TAAAACATATTTAAATGGTGATCATG
TAACCCATCCTGACTTCATGTTGTAT
GACGCTCTTGATGTTGTTTTATACAT
GGACCCAATGTGCCTGGATGCGTTCC
CAAAATTAGTTTGTTTTAAAAAACGT
ATTGAAGCTATCCCACAAATTGATAA
GTACTTGAAATCCAGCAAGTATATAG
CATGGCCTTTGCAGGGCTGGCAAGCC
ACGTTTGGTGGTGGCGACCATCCTCC
AAAATCGGATCTGGAAGTTCTGTTCC
AGGGGCCCCTGGGATCCGGCCGTATG
CGGGTGAAGGCCATCTTCTCCCACGC
TGCTGGGGACAACAGCACCCTCCTGA
GCTTCAAGGAGGGTGACCTCATTACC
CTGCTGGTGCCTGAGGCCCGCGATGG
CTGGCACTACGGAGAGAGTGAGAAGA
CCAAGATGCGGGGCTGGTTTCCCTTC
TCCTACACCCGGGTCTTGGACTGACC
GGAATTCCCGGGTCGACTCGAGCGGC
CGCATCGTGACTGA

Final protein sequence (Tag sequence in lowercase):

mspilgywkikglvqptrllleylee
kyeehlyerdegdkwrnkkfelglef
pnlpyyidgdvkltqsmaiiryiadk
hnmlggcpkeraeismlegavldiry
gvsriayskdfetlkvdflsklpeml

kmfedrlchktylngdhvthpdfmly
daldvvlymdpmcldafpklvcfkkr
ieaipqidkylksskyiawplqgwqa
tfgggdhppksdlevlfq^GPLGSGR
MRVKAIFSHAAGDNSTLLSFKEGDLI
TLLVPEARDGWHYGESEKTKMRGWFP
FSYTRVLD

^ TEV cleavage site

Tags and additions: Cleavable N-terminal GST tag.

Host: BL21 (DE3)R3-pRARE2 (Phage resistant strain).

Growth medium, induction protocol: 50ml from a 0.4L overnight culture containing 75µg/ml carbenicillin were used to inoculate each of six 1L cultures of TB containing 75µg/ml carbencillin. Cultures were grown at 37°C until the OD_{600} reached ~1.5 then cooled before inducing expressioni overnight with 0.05 mM IPTG at 20°C. The cells were collected by centrifugation and the pellet re-suspended in binding buffer and frozen.
Lysis buffer: PBS, 1% Triton-X100; 100 mM + protease inibitor cocktail.
Extraction buffer, extraction method: Re-suspended cells were lysed by sonication. The lysate was centrifuged at 48,000g for 60 minutes and the supernatant collected and then snap-frozen in liquid nitrogen.

Column 1: Glutathione-affinity beads; GSH-sepharose (GE Healthcare), 7 ml of a 50% slurry in 2.5 x 15 cm Econo-column (Bio-Rad), washed with binding buffer.

Column 1 Buffers:
Binding buffer: PBS, 1% Triton-X100, 100 mM EDTA.
Wash buffer: 50 mM Tris, pH 7.5; 100 mM EDTA; 0.1% Tween20.
Elution buffer: 100 mM reduced glutathione (pH adjusted to 8.0 with Tris-HCl pH 8.8).

Column 1 Procedure: The supernatant was thawed and then glutathione-sepharose beads were added before gentle mixing overnight at 4°C. The beads were then washed with 3 x 30ml wash buffer using gravity flow through the column. The protein was eluted by gentle mixing overnight with elution buffer and the solution recovered by gravity flow. Additional fractions were collected by eluting with 3 x 1 column volumes of fresh elution buffer.

Enzymatic treatment: The N-terminal GST tag was cleaved by treatment with PreScission protease, overnight.

Column 2: Size Exclusion Chromatography. Superdex 75 16/60 HiLoad.

Column 2 Buffer: 20 mM Tris, pH 7.5; 150 mM NaCl.

Column 2 Procedure: The protein was concentrated and applied to an S75 16/60 HiLoad gel filtration column equilibrated with 20 mM Tris pH 7.5, 150 mM NaCl using an ÄKTA FPLC system. Column buffer was exchanged to 5 mM Tris pH 8.5 by dialysis.

Column 3: etc.

Protein concentration: Protein was concentrated to 14mg/ml using an Vivaspin 3kDa cut-off concentrator.

Mass spectrometry characterization: LC- ESI -MS TOF gave a measured mass of 7502Da for this construct as predicted from the sequence of this protein.

Crystallisation: Crystals grown at 20°C in in 150nl sitting drops from a 1:1 ratio of protein to reservoir solution containing 0.2 M ammonium acetate, 0.1 M Tris-HCl pH 8.5, 30% isopropanol.

Data collection: Crystals were cryo-protected using the well solution supplemented by 25% ethylene glycol and flash frozen in liquid nitrogen.
X-ray source: Diffraction data were collected from a single crystal on Diamond beamline I02 at a single wavelength of 0.9795Å and the structures was refined to 1.5Å.
Phasing: The structure was solved by molecular replacement using an ensemble of known SH3 domain structure as starting model.