Entry Clone Source: MGC

Entry Clone Accession: IMAGE:30336561

SGC Construct ID: PCAFA-c011

GenBank GI number: gi|40805843

Vector: pNIC28-Bsa4

Amplified construct sequence:
TACTTCCAATCCATGGGAAAAGAGAAAAG
TAAAGAGCCCAGAGACCCTGACCAGCTTT
ACAGCACGCTCAAGAGCATCCTCCAGCAG
GTGAAGAGCCATCAAAGCGCTTGGCCCTT
CATGGAACCTGTGAAGAGAACAGAAGCTC
CAGGATATTATGAAGTTATAAGGTTCCCC
ATGGATCTGAAAACCATGAGTGAACGCCT
CAAGAATAGGTACTACGTGTCTAAGAAAT
TATTCATGGCAGACTTACAGCGAGTCTTT
ACCAATTGCAAAGAGTACAACCCCCCTGA
GAGTGAATACTACAAATGTGCCAATATCC
TGGAGAAATTCTTCTTCAGTAAAATTAAG
GAAGCTGGATTAATTGACTGACAGTAAAG
GTGGATA

Tags and additions: Cleavable N-terminal His6 tag.

Final protein sequence (tag sequence in lowercase):
mhhhhhhssgvdlgtenlyfq^sMGKEKS
KEPRDPDQLYSTLKSILQQVKSHQSAWPF
MEPVKRTEAPGYYEVIRFPMDLKTMSERL
KNRYYVSKKLFMADLQRVFTNCKEYNPPE
SEYYKCANILEKFFFSKIKEAGLID

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

Growth medium, induction protocol: 5ml from a 50 ml overnight culture containing 50mg/ml kanamycin/34mg/ml chloramphenicol was used to inoculate each of two 1 litre cultures of LB containing 50 µg/ml kanamycin/ 34 µg/ml chloramphenicol. Cultures were grown at 37°C until the OD_{600} reached ~0.5 then the temperature was adjusted to 18oC. Expression was induced overnight using 0.5 mM IPTG at an OD_{600} of 0.9. The cells were collected by centrifugation and the pellet resuspended in binding buffer and frozen. **Binding buffer**: 50mM HEPES pH 7.5; 500 mM NaCl; 5 mM imidazole, 5% glycerol.

Extraction buffer, extraction method: Frozen pellets were thawed and fresh 0.5mM TCEP, 1mM PMSF added to the lysate. Cells were lysed using sonication. The lysate was centrifuged at 21,000 rpm for 60 minutes and the supernatant collected for purification.

Column 1: Ion exchange - Nucleic acid removal. DEAE cellulose (DE52, Whatman), 10 g of resin in 2.5 x 20 cm column. The resin was hydrated in 2.5M NaCl, then washed with 20 ml binding buffer prior to loading the sample.

Buffers: 50mM HEPES pH 7.5; 500 mM NaCl; 5 mM imidazole, 5% glycerol

Procedure: Supernatant was applied by gravity flow, followed by a wash with 50 ml binding buffer. The column flow-through was collected.

Column 2: Ni-affinity. Ni-sepharose (Amersham), 5 ml of 50% slurry in 1.5 x 10 cm column, washed with binding buffer.

Buffers : **Binding buffer:** 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol **Wash buffer:** 50 mM HEPES pH 7.5, 500 mM NaCl, 30 mM Imidazole, 5% glycerol **Elution buffer:** 50 mM HEPES pH 7.5, 500 mM NaCl, 50 to 250 mM Imidazole , 5% Glycerol (step elution).
Procedure: The flowthrough from column 1 was loaded by gravity flow on the Ni-sepharose column. The column was then washed with 30 ml wash buffer at gravity flow. The protein was eluted by gravity flow by applying 5-ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 and 250 mM); fractions were collected until essentially all protein was eluted. 10 mM DTT was added for overnight storage.
Enzymatic treatment : The N-terminal His tag was cleaved by treatment with TEV protease
Column 3: Size Exclusion Chromatography. Superdex S200 16/60 HiLoad
Buffers: 25 mM HEPES, pH 7.5; 300 mM NaCl, 0.5 mM TCEP
Procedure: PCAF was concentrated and applied to an S200 16/60 HiLoad gel filtration column equilibrated in 25 mM HEPES, pH 7.5; 300 mM NaCl, 0.5 mM TCEP using an ÄKTAexpress system.
Mass spec characterization: LC- ESI -MS TOF gave a measured mass of 14150.7 for the TEV cleaved protein as predicted from the sequence of this protein.
Protein concentration: Protein was concentrated to 15.75 mg/ml using an Amicon 3 kDa cut-off concentrator.
Crystallization: Crystals were grown at 4°C in 300nl sitting drops from a 2:1 & 1:1 ratio of protein to reservoir solution 20% PEG10K, 4% EtGly and 0.1M HEPES pH 8.2.
Data Collection: Crystals were cryo-protected using the well solution supplemented with 25% ethylene glycol and flash frozen in liquid nitrogen. **X-ray source:** Diffraction data were collected from a single crystal on a Rigaku FR-E SuperBright at a single wavelength of 2.3 Å and the structure was refined to 2.3 Å. **Phasing:** The structure was solved by molecular replacement using an ensemble of known bromodomain structures as a starting model.