Entry Clone Source: Site-directed mutagenesis

Entry Clone Accession: n/a

SGC Construct ID: MAP2K6A-c025

GenBank GI number: gi|14589900

Entry clone source: MGC, site directed mutagenesis

GI number: gi|14589900

Final protein sequence (tag sequence shown in lowercase):

mhhhhhhssgvdlgtenlyfq^SMEVKAD
DLEPIMELGRGAYGVVEKMRHVPSGQIMA
VKRIRATVNSQEQKRLLMDLDISMRTVDC
PFTVTFYGALFREGDVWICMELMDTSLDK
FYKQVIDKGQTIPEDILGKIAVSIVKALE
HLHSKLSVIHRDVKPSNVLINALGQVKMC
DFGISGYLVD**D**VAK**D**IDAGCKPYMAPERI
NPELNQKGYSVKSDIWSLGITMIELAILR
FPYDSWGTPFQQLKQVVEEPSPQLPADKF
SAEFVDFTSQCLKKNSKERPTYPELMQHP
FFTLHESKGTDVASFVKLILGD

^ TEV cleave site
The mutated residues (Ser207/Asp; Thr211Asp are highlighted in red)

Amplified construct sequence:

CATATGCACCATCATCATCATCATTCTTC
TGGTGTAGATCTGGGTACCGAGAACCTGT
ACTTCCAATCCATGGAGGTGAAGGCAGAT
GACCTGGAGCCTATAATGGAACTGGGACG
AGGTGCGTACGGGGTGGTGGAGAAGATGC
GGCACGTGCCCAGCGGGCAGATCATGGCA
GTGAAGCGGATCCGAGCCACAGTAAATAG
CCAGGAACAGAAACGGCTACTGATGGATT
TGGATATTTCCATGAGGACGGTGGACTGT
CCATTCACTGTCACCTTTTATGGCGCACT
GTTTCGGGAGGGTGATGTGTGGATCTGCA
TGGAGCTCATGGATACATCACTAGATAAA
TTCTACAAACAAGTTATTGATAAAGGCCA
GACAATTCCAGAGGACATCTTAGGGAAAA
TAGCAGTTTCTATTGTAAAAGCATTAGAA
CATTTACATAGTAAGCTGTCTGTCATTCA
CAGAGACGTCAAGCCTTCTAATGTACTCA
TCAATGCTCTCGGTCAAGTGAAGATGTGC
GATTTTGGAATCAGTGGCTACTTGGTGGA
CGATGTTGCTAAAGACATTGATGCAGGTT
GCAAACCATACATGGCCCCTGAAAGAATA
AACCCAGAGCTCAACCAGAAGGGATACAG
TGTGAAGTCTGACATTTGGAGTCTGGGCA
TCACGATGATTGAGTTGGCCATCCTTCGA
TTTCCCTATGATTCATGGGGAACTCCATT
TCAGCAGCTCAAACAGGTGGTAGAGGAGC
CATCGCCACAACTCCCAGCAGACAAGTTC
TCTGCAGAGTTTGTTGACTTTACCTCACA
GTGCTTAAAGAAGAATTCCAAAGAACGGC
CTACATACCCAGAGCTAATGCAACATCCA

TTTTTCACCCTACATGAATCCAAAGGAAC
AGATGTGGCATCTTTTGTAAAACTGATTC
TTGGAGACTGACAGTAAAGGTGGATACGG
ATCCGAA

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank]

Tags and additions: Cleavable N-terminal His6 tag.

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

Growth medium, induction protocol: 1ml from a 10 ml overnight culture containing 50 µg/ml kanamycin was used to inoculate 1 litre of LB containing 50 µg/ml kanamycin. Cultures were grown at 37°C until the OD_{600} reached ~0.3 then the temperature was adjusted to 18°C. Expression was induced for overnight using 1 mM IPTG at anOD_{600} of 0.8. 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; 0.5 mM TCEP.

Extraction buffer, extraction method: Frozen pellets were thawed and cells lysed using a high pressure cell disrupter. The lysate was centrifuged at 17,000 rpm for 30 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; 0.5 mM TCEP

Procedure: Supernatant was applied by gravity flow, followed by a wash with 100 ml binding buffer. The column flow-through was collected.

Column 2: Ni-affinity. Ni-NTA (Qiagen), 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, 0.5 mM TCEP
Wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 30 mM Imidazole, 5% glycerol, 0.5 mM TCEP
Elution buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 50 to 250 mM Imidazole , 5% Glycerol, 0.5 mM TCEP

Procedure: The flowthrough from column 1 was loaded by gravity flow on the Ni-NTA column. The column was then washed with 100 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.

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; 250 mM NaCl, 5 mM DTT

Procedure: MAP2K6 was concentrated and applied to an S200 16/60 HiLoad gel filtration column equilibrated in 25 mM HEPES, pH 7.5; 250 mM NaCl, 5 mM DTT using either an ÄKTAprime or ÄKTAexpress system.

Mass spectrometry characterization: LC- ESI -MS TOF gave a measured mass of 32710 for this construct as predicted from the sequence of this protein.

Protein concentration: Protein was concentrated to 9.8 mg/ml using an Amicon 3 kDa cut-off concentrator.

Crystallization: Initial crystals grown in 30% PEG-3350, 0.2M MgCl2, Tris-HCl pH 7.5 were used for seeding. Diffraction quality crystals were grown at 4°C in 150 nl drops from a 1:1 ratio of protein and

reservoir solution (0.05 M Mg formate pH5.9; 10 w/v PEG3350).

Data Collection: Crystals were cryo-protected using 20% glycerol.
X-ray source: Diffraction data were collected at the SLS beamline X10 at a single wavelength of 1.000 Å.
Resolution: 2.3 Å resolution limit.