Target ID: GBE1A
Entry Clone ID: GBE1A-s001
Allele ID: GBE1A-a106
Construct ID GBE1A-c106
Clone ID GBE1A-k106
Expression ID GBE1A-e118
Purification ID: GBE1A-p004
Entry clone source: MGC
Entry clone accession: gi| 4574938
Vector: pFB-LIC-Bse
Cell line: DH10Bac
Tags and additions: N-terminal, TEV protease cleavable hexahistidine tag

Coding DNA sequence

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CCATGGGCCACCATCATCATCATCAT
TCTTCTGGTGTAGATCTGGGTACCGA
GAACCTGTACTTCCAATCCATGTTGA
AGCCCTACGCCGTGGACTTCCAGCGC
AGGTATAAGCAGTTTAGCCAAATTTT
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AAGATGGTGGTTGGAAGAATATCGCT
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GAGGCTTTTGAACATAATGGGCGTCC
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Final protein sequence

mhhhhhhssgvdlgtenlyfq*smLK
PYAVDFQRRYKQFSQILKNIGENEGG
IDKFSRGYESFGVHRCADGGLYCKEW
APGAEGVFLTGDFNGWNPFSYPYKKL
DYGKWELYIPPKQNKSVLVPHGSKLK
VVITSKSGEILYRISPWAKYVVREGD

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SFGYQITSFFAASSRYGSPEELQELV
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CPDSITIAEDVSGMPALCSPISQGGG
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NMGDIVYTLTNRRYLEKCIAYAESHD
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GYLNFMGNEFGHPEWLDFPRKGNNES
YHYARRQFHLTDDDLLRYKFLNNFDR
DMNRLEERYGWLAAPQAYVSEKHEGN
KIIAFERAGLLFIFNFHPSKSYTDYR
VGTALPGKFKIVLDSDAAEYGGHQRL
DHSTDFFSEAFEHNGRPYSLLVYIPS
RVALILQNVDL

Sequence MHHHHHHSSGVDLGTENLYFQ*SM is the purification tag plus TEV protease recognition site (*) (lower case in sequence).

Expression

High five cells were grown in Sf9 medium at 27°C. Cells were infected at a density of 2x106/ml with recombinant baculovirus (virus stock P2; 1ml of virus stock/1l of cell culture) Culture was supplemented with FCS to final concentration 1%. 120 hours post infection the cultures were collected and centrifuged for 20min at 1500 xg. Cellular pellets were discarded and supernatants were used as a source of a protein.

Purification

Cell Lysis

Cell pellets were dissolved in approximately 50ml lysis buffer and broken by sonication for 10min @ 35% amplitude followed by homogenization by 2 passes at 12,000 psi. The cell debris was pelleted at 40,000 x g and the supernatant used for further purification.

Lysis Buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 7.4, 0.5 mM TCEP, 1 tablet per 50 ml protease inhibitor cocktail EDTA-free (Roche)

Column 1: Ni-NTA (1.5 ml volume in a gravity-flow column).

The clarified cell extract was added to 1.5 ml of Ni-NTA pre-equilibrated with lysis buffer and passed through a glass column. The column was then washed with Binding Buffer (2 x 15 ml) and Wash Buffer (2 x 15 ml). The protein was eluted with 25 ml of Elution Buffer in 5 x 2.5 ml fractions.

Binding Buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 7.4, 0.5 mM TCEP

Wash Buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 40 mM Imidazole pH 7.4, 0.5 mM TCEP

Elution Buffer: 50 mM Hepes pH 7.4, 500 mM NaCl, 5% Glycerol, 250 mM Imidazole pH 7.4, 0.5 mM TCEP

Column 2: Superdex 200 16/60 Gel Filtration.

The first three elution fractions from column 1 were pooled and concentrated to 5 ml with a 30 kDa mwco spin concentrator and injected into an s200 16/60 column (preequilibrated in GF Buffer) at 1.0 ml/min. 1.0 ml fractions were collected.

The protein eluted at between 80 ml and 90 ml volume.

GF Buffer: 10 mM Hepes pH 7.4, 500 mM NaCl, 0.5 mM TCEP, 5% Glycerol.

Concentration

Pooled protein fractions were concentrated to 16.5 mg/ml using a 30 kDa mwco concentrator.

Mass spectrometry characterisation

After TEV protease digestion:

Measured mass: 78939.2 Da

Expected mass: 79023.3 Da

Crystallization

Crystals were grown by vapour diffusion in sitting drop at 4°C. A sitting drop consisting of 50 nl protein and 100 nl well solution was equilibrated against well solution containing 0.05 M sodium-succinate and 22% PEG 3350 supplemented with 4% formamide (Hampton additive screen). Crystals were mounted in the presence of 25% (v/v) ethylene glycol and flash-cooled in liquid nitrogen.

Data collection

Resolution: 2.70 Å

X-ray source: Diamond I04