XX02LIMK1A-d001 : LIMK1 + CFL1 + ATP-Î³-S; Adenosine 5'-[gamma-thio]triphosphate tetralithium salt: human LIM kinase 1 (LIMK1) in complex with CFL1 and ATP-Î³-S

PDB Code: 5L6W

Material and Methods

SGC target ID: LIMK1

Entry Clone Source: TKC

Entry Clone DNA Sequence:

TACTTCCAATCCATGCCACACCGCATCTTCCGGCCGTCGGACCTCATCCACGGGGAGGTGCTGG
GCAAGGGCTGCTTC
GGCCAGGCTATCAAGGTGACACACCGTGAGACAGGTGAGGTGATGGTGATGAAGGAGCTGATCC
GGTTCGACGAGGAG
ACCCAGAGGACGTTCCTCAAGGAGGTGAAGGTCATGCGATGCCTGGAACACCCCAACGTGCTCA
AGTTCATCGGGGTG
CTCTACAAGGACAAGAGGCTCAACTTCATCACTGAGTACATCAAGGGCGGCACGCTCCGGGGCA
TCATCAAGAGCATG
GACAGCCAGTACCCATGGAGCCAGAGAGTGAGCTTTGCCAAGGACATCGCATCAGGGATGGCCT
ACCTCCACTCCATG
AACATCATCCACCGAGACCTCAACTCCCACAACTGCCTGGTCCGCGAGAACAAGAATGTGGTGG
TGGCTGACTTCGGG
CTGGCGCGTCTCATGGTGGACGAGAAGACTCAGCCTGAGGGCCTGCGGAGCCTCAAGAAGCCAG
ACCGCAAGAAGCGC
TACACCGTGGTGGGCAACCCCTACTGGATGGCACCTGAGATGATCAACGGCCGCAGCTATGATG
AGAAGGTGGATGTG
TTCTCCTTTGGGATCGTCCTGTGCGAGATCATCGGGCGGGTGAACGCAGACCCTGACTACCTGC
CCCGCACCATGGAC
TTTGGCCTCAACGTGCGAGGATTCCTGGACCGCTACTGCCCCCCAAACTGCCCCCCGAGCTTCT
TCCCCATCACCGTG
CGCTGTTGCGATCTGGACCCCGAGAAGAGGCCATCCTTTGTGAAGCTGGAACACTGGCTGGAGA
CCCTCCGCATGCAC
CTGGCCGGCCACCTGCCACTGGGCCCACAGCTGGAGCAGCTGGACAGAGGTTTCTGGGAGACCT
ACCGGCGCGGCGAG
AGCTGACAGTAAAGGTGGATA

Vector: pFB-LIC-Bse

Tag: N-terminal His6-TEV

SCG Construct ID : LIMK1A-c056

Construct Protein Sequence:

MGHHHHHHSSGVDLGTENLYFQSMPH
RIFRPSDLIHGEVLGKGCFGQAIKVT
HRETGEVMVMKELIRFDEETQRTFLK
EVKVMRCLEHPNVLKFIGVLYKDKRL
NFITEYIKGGTLRGIIKSMDSQYPWS
QRVSFAKDIASGMAYLHSMNIIHRDL
NSHNCLVRENKNVVVADFGLARLMVD
EKTQPEGLRSLKKPDRKKRYTVVGNP
YWMAPEMINGRSYDEKVDVFSFGIVL

CEIIGRVNADPDYLPRTMDFGLNVRG
FLDRYCPPNCPPSFFPITVRCCDLDP
EKRPSFVKLEHWLETLRMHLAGHLPL
GPQLEQLDRGFWETYRRGES
Residues marked in red are cleaved by TEV protease

Host: Insect cells (Sf9)

Expression & Harvest:

Exponentially growing Sf9 cells (2x 106 cells/mL) were infected with high titre baculovirus stock (1:65) and incubated in shaker flasks (60 hours, 90 rpm, 27°C). Following this, the cell suspensions were centrifuged (15 minutes, 800x g, 4°C), and the cell pellets resuspended in PBS. After another centrifugation (15 minutes, 800x g, 4°C), the cell pellets were stored at -80°C.

Purification:

Lysis Buffer:50 mM Hepes pH 7.4, 200 mM NaCl, 20 mM Imidazole, 0.5 mM TCEP.
Binding Buffer:50 mM Hepes pH 7.4, 500 mM NaCl, 20 mM Imidazole, 5% Glycerol, 0.5 mM TCEP.
Wash Buffer 1: As Binding Buffer except 1 M NaCl and 40 mM imidazole.
Wash Buffer 2: As Binding Buffer except 60 mM imidazole.
Elution Buffer: As Binding Buffer except 300 mM imidazole.
The cell pellet was thawed, resuspended in 100 mL lysis buffer and sonicated (2 minutes, amplitude 35%, on ice). The cell lysate was cleared by centrifugation (60 minutes, 36,000x g, 4°C). The supernatant was combined with 7.5 mL Ni Sepharose and loaded onto a gravity flow column. The column was washed with 100 mL of Binding Buffer and 50 mL each of Wash Buffer 1 and 2. 25 ml of Elute Buffer was passed through to elute the protein. Fractions containing protein were pooled, combined with recombinant TEV protease (mass ratio 1:25) and incubated with rotation (over night, 4°C). Following this, 7.5 mL Ni Sepharose was added, and the suspension was loaded onto a gravity flow column. The flowthrough was collected and concentrated to 5 mL. Finally, the protein was polished by gel filtration using an AKTAxpress system with an S200 16/600 column and GF buffer (20 mM HEPES/NaOH pH 7.4, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP). Fractions containing protein were pooled, concentrated and stored at -80°C.

SGC target ID: CFL1

Entry Clone Source: MGC

Entry Clone DNA Sequence :
CATATGCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGA
GAACCTGTACTTCCAATCCATGGCCTGCGGTGTGGCTGTCTCTGATGGTG
TCATCAAGGTGTTCAACGACATGAAGGTGCGTAAGTCTTCAACGCCAGAG
GAGGTGAAGAAGCGCAAGAAGGCGGTGCTCTTCTGCCTGAGTGAGGACAA
GAAGAACATCATCCTGGAGGAGGGCAAGGAGATCCTGGTGGGCGATGTGG
GCCAGACTGTCGACGATCCCTACGCCACCTTTGTCAAGATGCTGCCAGAT
AAGGACTGCCGCTATGCCCTCTATGATGCAACCTATGAGACCAAGGAGAG
CAAGAAGGAGGATCTGGTGTTTATCTTCTGGGCCCCCGAGTCTGCGCCCC
TTAAGAGCAAAATGATTTATGCCAGCTCCAAGGACGCCATCAAGAAGAAG
CTGACAGGGATCAAGCATGAATTGCAAGCAAACTGCTACGAGGAGGTCAA
GGACCGCTGCACCCTGGCAGAGAAGCTGGGGGGCAGTGCCGTCATCTCCC
TGGAGGGCAAGCCTTTGTGACAGTAAAGGTGGATACGGATCCGAA

SCG Construct ID :CFL1A-c002

Construct Protein Sequence :
MHHHHHHSSGVDLGTENLYFQSMACGVAVSDGVIKVFNDMKVRKSSTPEEV
KKRKKAVLFCLSEDKKNIILEEGKEILVGDVGQTVDDPYATFVKMLPDKDC

RYALYDATYETKESKKEDLVFIFWAPESAPLKSKMIYASSKDAIKKKLTGI
KHELQANCYEEVKDRCTLAEKLGGSAVISLEGKPL
This is a variant protein S3C. Cysteine instead of Serine. Residues marked in red are cleaved by TEV protease.

Vector: pNIC28-Bsa4

Tag:N-terminal His6-TEV

Host:BL21

Expression & Harvest Transformation: The CFL1 construct DNA was transformed into homemade competent cells of the expression strain by a standard heat shock procedure. Colonies were used to inoculate 50 mL of LB media containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol, which was placed in a 37°C shaker overnight. The next day 4x 10 mL of this starter culture was used to inoculate 4x 1L of LB media containing 37.5 µg/mL kanamycin in 2L baffled shaker flasks. When the OD600 was approximately 0.45, the temperature was reduced to 20°C and when the OD600 was approximately 0.6 the cells were induced by the addition of 0.5 mM IPTG. The expression was continued overnight. Cells were spun at 5000rpm for 10 mins and the pellets resuspended in Lysis Buffer and then frozen at -80°C.

Purification:

Lysis Buffer:50 mM Hepes pH 7.4, 200 mM NaCl, 20 mM Imidazole, 0.5 mM TCEP.
Binding Buffer:50 mM Hepes pH 7.4, 500 mM NaCl, 20 mM Imidazole, 5% Glycerol, 0.5 mM TCEP.
Wash Buffer 1: As Binding Buffer except 1 M NaCl and 40 mM imidazole.
Wash Buffer 2: As Binding Buffer except 60 mM imidazole.
Elution Buffer: As Binding Buffer except 300 mM imidazole.
The cell pellet was thawed, resuspended in lysis buffer and sonicated (12 minutes, amplitude 35%, on ice). The cell lysate was cleared by centrifugation (60 minutes, 36,000x g, 4°C). The supernatant was combined with 7.5 mL Ni Sepharose and loaded onto a gravity flow column. The column was washed with 100 mL of Binding Buffer and 50 mL each of Wash Buffer 1 and 2. 25 ml of Elute Buffer was passed through to elute the protein. Fractions containing protein were pooled, combined with recombinant TEV protease (mass ratio 1:25) and incubated with rotation (over night, 4°C). Following this, 7.5 mL Ni Sepharose was added, and the suspension was loaded onto a gravity flow column. The flowthrough was collected and concentrated to 5 mL. Finally, the protein was polished by gel filtration using an AKTAxpress system with an S200 16/600 column and GF buffer (20 mM HEPES/NaOH pH 7.4, 500 mM NaCL, 5% Glycerol, 0.5 mM TCEP). Fractions containing protein were pooled, concentrated and stored at -80°C.

LIMK1 and CFL1
The LIMK1 and CFL1 protein were mixed in to 1:1 ratio and incubated for 2 hours on ice. The protein was polished by gel filtration using an AKTAxpress system with an S200 16/600 column and GF buffer (20 mM HEPES/NaOH pH 7.4, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP).

Crystallisation
ATP-Î³-S was added to the protein final concentration of 1 mM and was concentrated to 10 mg/ml. Crystals grew from a 1:2 ratio of protein solution (10 mg/mL) and precipitant solution (0.2M potassium chloride - 35% pentaerythritol propoxylate 5/4 - 0.1M HEPES pH 7.5), using the vapour diffusion method.

Data Collection: Resolution: 2.53Å
X-ray source: Crystals were cryo-protected by equilibration into precipitant solution containing 20% ethylene glycol and flash frozen in liquid nitrogen. Data was collected at Diamond, beamline I02.