CDKL1A (4AGU) Materials & Methods

Entry Clone Source: Site-directed mutagenesis

Entry Clone Accession: GI:37596296

SGC Construct ID: CDKL1A-c024

Vector: pNIC-CTHF. Details [PDF]; Sequence [FASTA] or [GenBank]

Amplified construct sequence:

TACTTCCAATCCATGATGGAGAAGTA
TGAAAAAATTGGGAAAATTGGAGAAG
GCTCCTATGGAGTTGTTTTCAAATGT
AGAAACAGGGACACGGGTCAGATTGT
GGCCATCAAGAAGTTTCTGGAATCAG
AAGATGACCCTGTCATAAAGAAAATT
GCCCTTCGGGAAATCCGAATGCTCAA
GCAACTCAAGCATCCCAACCTTGTTA
ACCTCCTGGAAGTCTTCAGGAGGAAA
CGGAGGCTTCACCTGGTGTTTGAATA
TTGTGACCACACAGTTCTCCATGAGT
TGGACAGATACCAAAGAGGGGTACCA
GAACATCTCGTGAAGAGCATAACTTG
GCAGACACTGCAAGCTGTAAATTTTT
GCCATAAACACAATTGCATACATAGA
GACGTGAAGCCAGAAAATATCCTCAT
CACGAAACATTCCGTGATTAAGCTTT
GTGACTTTGGATTTGCTCGGCTTTTG
ACTGGACCGAGTGACTACTATGACGA
CGAAGTGGCTACCAGGTGGTACCGCT
CCCCTGAGCTGCTGGTGGGGGACACG
CAGTACGGCCCCCCGGTGGATGTTTG
GGCAATTGGCTGTGTCTTTGCTGAGC
TGCTGTCAGGAGTGCCTCTGTGGCCA
GGAAAATCGGATGTGGATCAGCTGTA
TCTGATTAGGAAGACCTTGGGGGATC
TCATTCCTAGGCACCAGCAAGTGTTT
AGCACGAATCAGTACTTCAGTGGAGT
GAAAATTCCAGACCCTGAAGATATGG
AACCACTTGAATTAAAATTCCCAAAC
ATCTCTTATCCTGCCCTGGGGCTCCT
AAAGGGCTGTCTCCACATGGACCCTA
CTGAAAGGCTGACATGTGAACAGCTG
TTGCATCACCCATATTTTGAAAACAT
CAGAGAAATAGAGGATTTGGCAAAAG
AACACGACAAACCAACAAGGAAGACC
CTAAGAAAGAGCCGAAAGCACCACTG
CTTTACAGAAACATCCAAGTTGCAGT
ACCTACCCCAGCTAACTGGCAGCAGC
ATCCTTCCAGCTTTGGATAATAAGAA
GTACTACTGTGATACCAAGAAACTTA

ACTACCGTTTTCCAAACATTTGACAG
TAAAGGTGGATA

Final protein sequence (Tag sequence in lowercase):
MMEKYEKIGKIGEGSYGVVFKCRNRD
TGQIVAIKKFLESEDDPVIKKIALRE
IRMLKQLKHPNLVNLLEVFRRKRRLH
LVFEYCDHTVLHELDRYQRGVPEHLV
KSITWQTLQAVNFCHKHNCIHRDVKP
ENILITKHSVIKLCDFGFARLLTGPS
DYYDDEVATRWYRSPELLVGDTQYGP
PVDVWAIGCVFAELLSGVPLWPGKSD
VDQLYLIRKTLGDLIPRHQQVFSTNQ
YFSGVKIPDPEDMEPLELKFPNISYP
ALGLLKGCLHMDPTERLTCEQLLHHP
YFENIREIEDLAKEHDKPAenlyfq^
shhhhhhdykddddk

^ TEV cleavage site

Tags and additions: TEV-cleavable C-terminal hexahistidine and FLAG tag

Host: BL21(DE3)-R3-pRARE2

Growth Medium & Induction Protocol: A glycerol stock was used to inoculate a 10ml starter culture containing 2xYT (Yeast Extract Tryptone) media with 50µg/ml Kanamycin and 34 µg/ml chloramphenicol. The starter culture was grown overnight at 37°C with shaking at 200 rpm. The following morning, five flasks containing 1 L 2xYT/kanamycin/chloramphenicol were each inoculated with 1 ml of the starter culture. Cultures were incubated at 37°C with shaking at 180 rpm until an OD_{600}nm ≥ 0.6 was reached. The flasks were then cooled down to 18°C. Protein expression was induced by addition of IPTG to a final concentration of 0.4mM and expression carried out overnight. Cells were harvested by centrifugation at 6000 rpm at 4°C for 15 min. Cell pellets from each flask were combined and resuspended in 100ml binding buffer (50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole), transferred to 50 ml tubes, and stored at -20°C.

Binding buffer: 50 mM HEPES, pH 7.5; 500 mM NaCl; 5 mM Imidazole; 5% Glycerol.

Extraction buffer, extraction method: The frozen cells were thawed and supplemented with protease inhibitor SET V (Calbiochem) at 1:2000 dilution, 0.5mM TCEP, 12.5 U/ml benzonase and 10mM arginine/glutamine mix. The cells were lysed by ultrasonication over 8 min with the sonicator pulsing ON for 5 sec and OFF for 10 sec. The cell lysate was spun down by centrifugation at 15,500 rpm at 4°C for 1 h. The clarified cell extract was filtered using syringe filters with a 1.2µm pore size.

Column 1: Ni-Affinity Chromatography - 3ml Ni-sepharose slurry applied to a 1.5 x 10 cm column.

Column 1 Buffers:
Binding buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole
Wash buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 30mM Imidazole
Elution buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 50 to 400mM Imidazole

Column 1 Procedure: 3ml of 50 % Ni-sepharose slurry (Amersham) was added to a 1.5 x 10 cm column and equilibrated in binding buffer with 0.5mM TCEP. The clarified cell extract

was applied by gravity flow to the column. The resin was then washed with 100ml binding buffer and 30ml wash buffer to remove nonspecifically binding proteins. The bound target protein was eluted by applying a step gradient of imidazole (5 ml fractions of elution buffer supplemented with 50mM, 100mM, 150mM and 400mM imidazole). The protein content of collected fractions was visualized using SDS-PAGE and fractions containing CDKL1A were pooled.

Enzymatic Treatment TEV protease cleavage. Pooled fractions were treated with TEV protease overnight at 4°C.

Column 2: Size Exclusion Chromatography - S200 HiLoad 16/60 Superdex run on ÄKTAxpress

Column 2 Buffers:
Gel Filtration buffer: 50mM HEPES pH7.5, 250mM NaCl, 50mM Arg/Glu, 0.5mM TCEP

Column 2 Procedure: Prior to applying the protein, the S200 16/60 column was washed and equilibrated with gel filtration buffer. Eluted protein from Ni-sepharose column was concentrated to 5ml using an Amicon Ultra-15 filter with a 10kDa cut-off. The concentrated protein was applied onto the equilibrated S200 16/60 column via a syringe filter and run at a flow-rate of 1 ml/min. 1.5ml fractions were collected and visualized using SDS-PAGE. Those containing CDKL1A were pooled.

Column 3: Ni-Affinity rebind â□□ 1ml Ni-sepharose slurry applied to a 1.5x10cm column.

Column 3 Buffers:
Binding buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 5mM Imidazole
Wash buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 30mM Imidazole
Elution buffer: 50mM HEPES, pH 7.5; 500mM NaCl; 5% Glycerol; 50 / 250mM Imidazole

Column 3 Procedure: 1ml of 50 % Ni-sepharose slurry (Amersham) was added to a 1.5 x 10 cm column and equilibrated in binding buffer. Pooled fractions from gel filtration were supplemented with 10mM imidazole and applied by gravity flow to the column. The column was washed with 3ml binding buffer, 3ml wash buffer, 3ml 50mM imidazole elution buffer and 5ml 250mM imidazole elution buffer. The protein content of collected fractions was visualized using SDS-PAGE. Purified CDKL1A was located in the flow-through from the column, which was retained for crystallization.

Concentration: The protein was concentrated to a final concentration of 10mg/ml (measured by OD_{280} based on extinction coefficient 41370) in an Amicon Ultra-15 filter with a 10 kDa cut-off.

Mass spectrometry characterization:
Observed mass: 36253.07 Da
Expected mass: 36252 Da

Mass spec procedure: The purified protein was homogeneous and had an experimental mass of 36253.07 Da. The theoretical expected mass from the construct sequence is 36252 Da. Masses were determined by LC-MS, using an Agilent LC/MSD TOF system with reversed-phase HPLC coupled to electrospray ionisation and an orthogonal time-of-flight mass analyser. Proteins were desalted prior to mass spectrometry by rapid elution off a C3 column with a gradient of 5-95% methanol in water with 0.1% formic acid.

Crystallization: Protein at 10mg/ml was buffered in 50mM HEPES, pH 7.8, 180mM NaCl, 5 mM DTT, 0.8% glycerol. Inhibitor K00611 (N-(5-{[(2S)-4-amino-2-(3-

chlorophenyl)butanoyl]amino}-1H-indazol-3-yl)benzamide) was added to the final sample at a concentration of approximately 0.83mM. The peptide HHASPRK was added at a final concentration of 2 mM. Crystals were grown at 20°C in 500 nl sitting drops mixing 400 nl protein solution with 100 nl of a reservoir solution containing 17.5% PEG 3350, 0.1M ammonium chloride pH 6.3 and 0.05M magnesium formate. On mounting crystals were cryo-protected with an additional 20% glycerol.

Data Collection:
Resolution: 2.4 Å **X-ray source**: Diamond I24

Crystals of CDKL1A diffracted to a resolution of 2.4 Å(scaled resolution). A full dataset was collected at 100 K on Diamond Light Source beamline I24. Crystals belonged to the Trigonal space group P32 with unit-cell parameters a=124 Å b=124 Å c=49 Å, α=90° β= 90° γ= 120°. Three protein molecules were present in the asymmetric unit. Data were indexed and integrated using XDS and scaled using SCALA. Phases were found using molecular replacement in PHENIX.AUTOMR. PHENIX.SCULPTOR was used to optimize the PDB entry 4AAA for use as a search model. The structure was refined and modified using alternate rounds of BUSTER and COOT, and the final model validated using the JCSG Quality Control Server. There was insufficient density to model the HHASPRK peptide.