CRK7

PDB:4CXA

Revision

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Entry Clone Accession:N/A
Entry Clone Source: Gregg Morin, University of British Columbia
SGC Clone Accession:
Tag:MGHHHHHHSSGVDLGTENLYFQ*SM. cleavable N-terminal hexahistidine tag.
Host:SF9 Spodoptera frugiperda Insect cells

Construct

Prelude:CRK7A-c020
Sequence:

MGHHHHHHSSGVDLGTENLYFQSMTESDWGKRCVDKFDIIGIIGEGTYGQVYKAKDKDTGELVALKKVRLDNEKEGFPITAIREIKI
LRQLIHRSVVNMKEIVTDKQDALDFKKDKGAFYLVFEYMDHDLMGLLESGLVHFSEDHIKSFMKQLMEGLEYCHKKNFLHRDIKCSN
ILLNNSGQIKLADFGLARLYNSEESRPYTNKVITLWYRPPELLLGEERYTPAIDVWSCGCILGELFTKKPIFQANLELAQLELISRL
CGSPCPAVWPDVIKLPYFNTMKPKKQYRRRLREEFSFIPSAALDLLDHMLTLDPSKRCTAEQTLQSDFLKDVELSKMAPPDLPHWQD
CHELWSKKRRRQRQ

Vector:pFB-LIC-Bse

Growth

Medium:
Antibiotics:
Procedure:

Purification

Buffers
Procedure

Extraction

Buffers
Procedure
Co-expression of CDK12 and CCNK:

Sf9 cells were grown in Insect-Xpress media (Lonza), to a density of 2x106cells/ml and were infected with recombinant baculoviruses for both CDK12 and CCNK (P2 virus stocks; 2 ml of CDK12 virus stock and 1 ml of CCNK virus stock, per 1L of cell culture). Cells were shaken at 95 rpm at 27°C in an Innova shaker. After 72 hours post-infection the cultures were harvested by centrifugation for 25min at 900xg at 4°C. A cell pellet from 2L culture was made up to 50 ml in binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole), transferred to 50 ml falcon tubes, and stored at -20°C. Calbiochem protease inhibitor cocktail set III was added to the cell suspension at a 1:5000 dilution.

Extraction method: The frozen cells were thawed and the volume increased to 80 ml with binding buffer. Calbiochem protease inhibitor cocktail set III was added to the cell suspension at a 1:5000 dilution. The cells were lysed by ultrasonication (Sonic, Vibra Cell) over 12 min at 35% amplitude, with the sonicator pulsing ON for 5 sec and OFF for 10 sec. Polyethylenimine (PEI) was added to a final concentration of 0.5% to precipitate DNA and the cell lysate clarified by centrifugation at 21,000 RPM for 1 hour at 4°C.The supernatant was recovered for purification.

Column 1:
Ni-Affinity Chromatography. 5 ml of 50 % nickel-sepharose resin slurry (GE Healthcare) was applied onto a 1.5 x 10 cm column. The column was washed with ultra-pure water (1CV), then pre-equilibrated with binding buffer (1CV).

Buffers:
Binding Buffer: 500mM NaCl, 50mM HEPES pH 7.5, 5% Glycerol, 5mM Imidazole, 0.5mM TCEP
Wash Buffer: 500mM NaCl, 50mM HEPES pH 7.5, 5% Glycerol, 30mM Imidazole, 0.5mM TCEP
Elution Buffer I: 500mM NaCl, 50mM HEPES pH 7.5, 5% Glycerol, 50mM Imidazole, 0.5mM TCEP
Elution Buffer II: 500mM NaCl, 50mM HEPES pH 7.5, 5% Glycerol, 100mM Imidazole, 0.5mM TCEP
Elution Buffer III: 500mM NaCl, 50mM HEPES pH 7.5, 5% Glycerol, 150mM Imidazole, 0.5mM TCEP
Elution Buffer IV: 500mM NaCl, 50mM HEPES pH 7.5, 5% Glycerol, 250mM Imidazole, 0.5mM TCEP

Immobilised metal affinity chromatography procedure:
The supernatant, following centrifugation, was filtered and applied by gravity flow onto the Ni-sepharose column using. The bound protein was then washed with 100ml binding buffer and subsequently with 60 ml wash buffer. CDK12/CCNK protein was then eluted by applying a step gradient of imidazole - using 10 ml fractions of elution buffer with increasing concentration of imidazole (50 mM, 100mM, 150mM and 250 mM). Elution fractions were analyzed by SDS PAGE and the 250 mM imidazole fractions was kept for subsequent steps. 10 mM DTT was added for overnight storage at 4□C.Enzymatic treatment: 0.1mg of TEV protease was added to the Ni-eluted protein to remove the tag. Incubation was overnight at 4°C.

Column 2:
Reverse Ni-Affinity Chromatography. 0.5 ml of 50 % nickel-sepharose resin slurry (GE Healthcare) was applied onto a Bio-Rad Poly-Prep drip column. The column was washed with ultra-pure water, then pre-equilibrated with gel filtration buffer.

Buffer:

Gel Filtration buffer: 300 mM NaCl, 50 mM Hepes pH 7.5, 0.5mM TCEPBinding Buffer:

500mM NaCl, 50mM HEPES pH 7.5, 5% Glycerol, 5mM Imidazole, 0.5mM TCEP
Wash Buffer: 500mM NaCl, 50mM HEPES pH 7.5, 5% Glycerol, 30mM Imidazole, 0.5mM TCEPElution Buffer IV: 500mM NaCl, 50mM HEPES pH 7.5, 5% Glycerol, 250mM Imidazole, 0.5mM TCEP

Reverse Ni-Affinity Chromatography:
Protein-containing fractions were pooled and buffer exchanged into gel filtration buffer, then reverse nickel-affinity purification applied to further purify the protein.
The concentrated sample from immobilised metal affinity chromatography was applied by gravity flow onto the Ni-sepharose column. The column was then washed sequentially twice with 10 ml gel filtration buffer, then 10 ml binding buffer, 10 ml wash buffer and finally 10 ml elution buffer (containing 250mM imidazole). Fractions were analyzed by SDS PAGE and showed the CDK12 complex has eluted in the first gel filtration buffer fraction. 10 mM DTT was added to the protein and the sample was concentrated for gel filtration.

Column 3: Size Exclusion Chromatography - S75 HiLoad 26/60 Superdex column (GE Healthcare) run on ÄKTA-Express
Buffer:

Gel Filtration buffer: 300 mM NaCl, 50 mM HEPES pH 7.5, 0.5mM TCEP
Gel filtration procedure: Prior to applying the protein, the S75 HiLoad 26/60 Superdex column was washed and equilibrated with gel filtration buffer. The concentrated protein was diluted in gel filtration buffer, to around 3ml and directly applied onto the equilibrated S75 HiLoad 26/60 Superdex column, and run at a flow-rate of 1 ml/min. Fractions (1.8 ml each) containing the protein were pooled together. The eluted protein was supplemented with L-arginine (1 mM), L-glutamate (1 mM) and dithiothreitol (DTT) (10 mM).
Concentration: The protein complex was concentrated to a final concentration of 6.3 mg/ml .
Ligand
MassSpec: The intact mass of the proteins was confirmed by Electrospray Ionisation/Time-of-Flight Mass Spectrometry (ESI-MS, Agilent Technologies). Purified CKD12 was sub-stoichiometrically phosphorylated with experimental masses of 39579 (unphosphorylated) and 39659 (phosphorylated). CCNK had an experimental mass of 30.403 kDa. All masses agreed with the known primary sequences of CDK12 and CCNK, respectively. Masses were determinedusing an Agilent LC/MSD TOF system. 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 6.3 mg/ml was buffered in 50 mM HEPES, pH 7.5, 300 mM NaCl, 0.5 mM TCEP, 1 mM L-arginine, 1mM L-glutamate, and 10 mM DTT. 1 mM AMP-PNP and 5 mM MgCl2 were added to the final sample. Crystals were grown at 20°C in 150 nl sitting drops mixing 50 nl protein solution with 100 nl of a reservoir solution containing 0.15M DL-malic acid, 20% PEG3350. On mounting crystals were cryo-protected with an additional 15% ethylene glycol.
NMR Spectroscopy:
Data Collection: Resolution: 3.15 Å resolution
Data Processing: