No. XX
XX05CRK7A
CRK7 + CCNK: Human cyclin-dependent kinase 12 (CDK12) in complex with cyclin K (CCNK), and the covalent inhibitor THZ531
PDB-code: 5ACB

CDK12

Entry Clone Source: Gregg Morin, University of British Columbia

Entry Clone Accession: N/A

SGC Construct ID: CRK7A-c021

Amplified DNA sequence:

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TACTTCCAATCCATGACAGAAAGCGA
CTGGGGGAAACGCTGTGTGGACAAGT
TTGACATTATTGGGATTATTGGAGAA
GGAACCTATGGCCAAGTATATAAAGC
CAAGGACAAAGACACAGGAGAACTAG
TGGCTCTGAAGAAGGTGAGACTAGAC
AATGAGAAAGAGGGCTTCCCAATCAC
AGCCATTCGTGAAATCAAAATCCTTC
GTCAGTTAATCCACCGAAGTGTTGTT
AACATGAAGGAAATTGTCACAGATAA
ACAAGATGCACTGGATTTCAAGAAGG
ACAAAGGTGCCTTTTACCTTGTATTT
GAGTATATGGACCATGACTTAATGGG
ACTGCTAGAATCTGGTTTGGTGCACT
TTTCTGAGGACCATATCAAGTCGTTC
ATGAAACAGCTAATGGAAGGATTGGA
ATACTGTCACAAAAAGAATTTCCTGC
ATCGGGATATTAAGTGTTCTAACATT
TTGCTGAATAACAGTGGGCAAATCAA
ACTAGCAGATTTTGGACTTGCTCGGC
TCTATAACTCTGAAGAGAGTCGCCCT
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GTACCGACCTCCAGAACTACTGCTAG
GAGAGGAACGTTACACACCAGCCATA
GATGTTTGGAGCTGTGGATGTATTCT
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CAGCTAGAACTGATCAGCCGACTTTG
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TCGAAGGCGTCTACGAGAAGAATTCT
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AGACCCTACAGAGCGACTTCCTTAAA
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GATGTCGAACTCAGCAAAATGGCTCC
TCCAGACCTCCCCCACTGGCAGGATT
GACAGTAAAGGTGGATA

Expressed protein sequence:
MGHHHHHHSSGVDLGTENLYFQSMTE
SDWGKRCVDKFDIIGIIGEGTYGQVY
KAKDKDTGELVALKKVRLDNEKEGFP
ITAIREIKILRQLIHRSVVNMKEIVT
DKQDALDFKKDKGAFYLVFEYMDHDL
MGLLESGLVHFSEDHIKSFMKQLMEG
LEYCHKKNFLHRDIKCSNILLNNSGQ
IKLADFGLARLYNSEESRPYTNKVIT
LWYRPPELLLGEERYTPAIDVWSCGC
ILGELFTKKPIFQANLELAQLELISR
LCGSPCPAVWPDVIKLPYFNTMKPKK
QYRRRLREEFSFIPSAALDLLDHMLT
LDPSKRCTAEQTLQSDFLKDVELSKM
APPDLPHWQD

Vector: pFB-LIC-Bse

Tags and additions: MGHHHHHHSSGVDLGTENLYFQ*SM. cleavable N-terminal hexahistidine tag.

Cyclin K

Entry Clone Source: MGC

Entry Clone Accession: BC015935

SGC Construct ID: CCNKA-c001

Amplified DNA sequence:
TACTTCCAATCCATGTCAGTAACTTC
AGCAAACCTGGACCACACAAAGCCAT
GTTGGTACTGGGATAAGAAAGACTTG
GCTCATACACCCTCACAACTTGAAGG
ACTTGATCCAGCCACCGAGGCCCGGT
ACCGCCGAGAGGGCGCTCGGTTCATC
TTTGATGTGGGCACACGTTTGGGGCT
ACACTATGATACCCTGGCAACTGGAA
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TTTCATTCCTTCAAGCAATTCCCAAG
ATATGTGACAGGAGCCTGTTGCCTCT
TTCTGGCTGGGAAAGTAGAAGAAACA
CCAAAAAAATGTAAAGATATCATCAA
AACAGCTCGTAGTTTATTAAATGATG
TACAATTTGGCCAGTTTGGAGATGAC
CCAAAGGAGGAAGTAATGGTTCTGGA
GAGAATCTTACTGCAGACCATCAAGT
TTGATTTACAGGTAGAACATCCATAC
CAGTTCCTACTAAAATATGCAAAGCA
ACTCAAAGGTGATAAAAACAAAATTC
AAAAGTTGGTTCAAATGGCATGGACA

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CTTGTCACTGCAGTGGGAACCAGAGA
TCATAGCAGTAGCAGTGATGTATCTC
GCAGGACGTTTGTGCAAATTTGAAAT
ACAAGAATGGACCTCCAAACCCATGT
ATAGGAGATGGTGGGAGCAGTTTGTT
CAAGATGTCCCGGTCGACGTTTTGGA
AGACATCTGCCACCAAATCCTGGATC
TTTACTCACAAGGAAAACAACAGATG
CCTCATTGACAGTAAAGGTGGATA

Expressed protein sequence:
MGHHHHHHSSGVDLGTENLYFQSMSV
TSANLDHTKPCWYWDKKDLAHTPSQL
EGLDPATEARYRREGARFIFDVGTRL
GLHYDTLATGIIYFHRFYMFHSFKQF
PRYVTGACCLFLAGKVEETPKKCKDI
IKTARSLLNDVQFGQFGDDPKEEVMV
LERILLQTIKFDLQVEHPYQFLLKYA
KQLKGDKNKIQKLVQMAWTFVNDSLC
TTLSLQWEPEIIAVAVMYLAGRLCKF
EIQEWTSKPMYRRWWEQFVQDVPVDV
LEDICHQILDLYSQGKQQMPH

Vector: pFB-LIC-Bse

Tags and additions: MGHHHHHHSSGVDLGTENLYFQ*SM. cleavable N-terminal hexahistidine tag.

S. cerevisiae CAK

Entry Clone Source: MRC PPU, University of Dundee (plasmid DU5172)

Entry Clone Accession: U60192

SGC Construct ID: CAK1SCA-s002

Construct DNA sequence:
ggatccAAACTGGATAGTATAGACAT
TACACACTGTCAGTTGGTCAAATCTA
CTAGAACTGCTAGGATTTATAGGTCG
GATACATATGCCATTAAATGTCTAGC
ACTAGATTTCGATATCCCGCCACATA
ACGCCAAATTCGAAGTATCGATA TT
AAACAAACTGGGCAACAAATGTAAGC
ACATCTTACCTCTTCTAGAGTC TAA
GGCTACCGATAATAATGACCTATTGT
TGTTGTTTCCCTTTGAAGAGA TGAA
CCTTTATGAGTTCATGCAAATGCACT
ATAAAAGAGATAGAAGAAAA AAAAA
TCCCTATTACGATTTGCTAAATCCCA
GTATCCCAATTGTTGCGGA CCCCCC
CGTTCAGAAATATACTAATCAATTGG
ACGTCAATCGGTATTCTT TGTCCTT
TTTCCGGCAAATGGTTGAAGGGATTG

CATTCTTACATGAGAAC AAGATCAT
TCACCGCGACATCAAACCGCAAAATA
TCATGCTAACAAACAA TACCAGCAC
CGTATCCCCAAAGTTGTACATAATTG
ATTTTGGCATCTCTT ATGACATGGC
AAATAACTCACAAACAAGTGCGGAAC
CCATGGATAGCAAG GTGACGGATAT
AAGCACAGGAATTTACAAGGCCCCAG
AAGTGCTTTTTGG AGTAAAATGCTA
TGATGGTGGCGTGGACGTGTGGTCGT
TGTTGATAATTA TTTCTCAGTGGTT
CCAGAGAGAAACAAGCCGTATGGGGC
ACGTTCCGGCC ATGATTGATGACGG
CAGCGACGACATGAACTCAGATGGAA
GCGATTTCAG ACTGATTTGCTCAAT
ATTCGAAAAGTTGGGCATACCGTCCA
TTCAGAAAT GGGAAGAGGTTGCGCA
ACACGGCTCGGTTGATGCATTTGTTG
GTATGTTT GGTGCAGATGGCGATGG
CAAGTATGTACTGGACCAGGAGAAAG
ATGTACA GATTAGCATTGTTGAGAG
GAATATGCCTCGACTGGACGAGATTG
CGGATG TCAAAGTCAAGCAGAAGTT
CATTAATTGTATCCTGGGGATGGTTT
CATTT TCACCAAACGAAAGATGGAG
CTGTCAAAGAATCTTGCAAGAATTAG
AAAA GCCATAAgaattc

Expressed protein sequence:
MSYYHHHHHHDYDIPTTENLYFQG
AMGSKLDSIDITHCQLVKSTRTAR
IY RSDTYAIKCLALDFDIPPHNA
KFEVSILNKLGNKCKHILPLLESK
ATDNN DLLLLFPFEEMNLYEFMQ
MHYKRDRRKKNPYYDLLNPSIPIV
ADPPVQKY TNQLDVNRYSLSFFR
QMVEGIAFLHENKIIHRDIKPQNI
MLTNNTSTVSP KLYIIDFGISYD
MANNSQTSAEPMDSKVTDISTGIY
KAPEVLFGVKCYDG GVDVWSLLI
IISQWFQRETSRMGHVPAMIDDGS
DDMNSDGSDFRLICSIF EKLGIP
SIQKWEEVAQHGSVDAFVGMFGAD
GDGKYVLDQEKDVQISIVER NMP
RLDEIADVKVKQKFINCILGMVSF
SPNERWSCQRILQELEKP*

Vector: pFASTBAC.HTb

Tags and additions: MSYYHHHHHHDYDIPTTENLYFQ*G cleavable N-terminal hexahistidine tag.

CDK12/Cyclin K Complex with THZ531 bound

Host: SF9 Spodoptera frugiperda Insect cells

Materials & Methods

Co-expression of CDK12, CCNK and CAK: Sf9 cells were grown in Insect-Xpress media (Lonza), to a density of 2x106cells/mL and were co-infected with recombinant CDK12, CCNK and S. cerevisiae CAK (CAKSC1) baculoviruses (P2 virus stocks; 2 mL of CDK12 virus stock, 1 mL of CCNK virus stock and 2mL CAK 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. Cell pellet from 1L flasks were made up to 50 mL in binding buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5% Glycerol; 5 mM imidazole). Calbiochem protease inhibitor cocktail set III was added to the cell suspension at a 1:1000 dilution. This was transferred to 50 mL falcon tubes, and stored at -20°C.

Extraction method: The frozen cells were thawed and lysed by ultrasonication (Sonic, Vibra Cell) on ice over 10.5 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 21,500 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 split and applied onto two 1.5 x 10 cm column. The column was washed with ultra-pure water, then pre-equilibrated with binding buffer.

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: Following centrifugation at 21,5000 rpm for 1 hour at 4°C, the supernatant was filtered and applied by gravity flow onto the Ni-sepharose column. The bound protein was then washed with 100 mL binding buffer and subsequently with 60 mL wash buffer. CDK12/CCNK and CAK 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.

Enzymatic treatment: 0.1mg of TEV protease was added to the Ni-eluted protein to remove the tag. Incubation was overnight at 4°C with 10mM DTT and 10mM Arg/Glu.

Column 2: Ion-exchange chromatography - 5mL Hitrap SP column (GE Healthcare) run on ÄKTA-purifier

Buffer:
Buffer A: 50mM MES, pH 6.5; 0.5mM TCEP

Buffer B: 50mM MES, pH 6.5; 0.5mM TCEP; 1M NaCl

Ion-exchange chromatography: CDK12/CCNK protein from Ni-Affinity Chromatography was buffer exchanged into 50mM MES, pH 6.5; 0.5mM TCEP and loaded onto a 5 mL Hitrap SP column column equilibrated in the same buffer. A linear elution gradient was run from 0-500 mM NaCl over 150 mL, followed by 500 mM-1M NaCl over 20 mL. This stage separated any remaining CAK. CDK12/CCNK protein containing fractions were pooled and the buffer adjusted to 300 mM NaCl, 50 mM HEPES pH 7.5, 0.5mM TCEP during concentration in a 10 kD MWCO Amicon Ultra concentrator.

Treatment of CDK12/CCNK protein with THZ531: The CDK12/CCNK complex was then buffer exchanged in a 10 kDa MWCO Amicon Ultra spin concentrator, into a 50 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM TCEP buffer (gel filtration buffer), to raise the pH of the buffer for the subsequent step and to remove any trace of the DTT added before ion-exchange chromatography, as this sulfhydryl-containing reducing agent may interfere with the covalent inhibitor binding to the CDK12 cysteine residue. Incubation of THZ531 with purified mono-phosphorylated CDK12/CCNK complex resulted in near complete covalent binding, as determined by intact mass spectrometry. CDK12/CCNK protein was concentrated to 3 mL volume and 17 μM concentration. THZ531 was then added at 25 μM. The sample was spun at 13,000rpm for 10 minutes to remove any precipitate. The sample was then kept at 4°C overnight. Binding of the compound to CDK12 was monitored by changes to CDK12 intact mass. The intact mass of the protein was confirmed by Electrospray Ionisation/Time-of-Flight Mass Spectrometry (ESI-MS, Agilent Technologies). The purified protein complex had an experimental mass of 37,685 kDa, as expected from the primary sequence of CDK12. Following THZ531 treatment the CDK12 mass shifted to 40,218 kDa consistent with a +559 Da addition corresponding to the covalent binding of THZ531.

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 3 mL 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.

Mass spec characterization: The intact mass of the protein was confirmed by Electrospray Ionisation/Time-of-Flight Mass Spectrometry (ESI-MS, Agilent Technologies). The purified protein complex had an experimental mass of 39,659 and 30,403 kDa, as expected from primary sequences of a monophosphorylated CDK12 and CCNK, respectively. 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.

Crystallisation of the CDK12/CCNK complex: For crystallisation trials, the CDK12/CycK/THZ531 complex was concentrated to 5.5 mg/mL buffered in 50 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM TCEP. Viable diffraction quality crystals were grown at 20°C in 195 nL sitting drops mixing 75 nL protein solution and 20 nL crystal seed stock with 100 nL of a reservoir solution comprising 0.1 M HEPES pH 7.0, 10% PEG8000, 0.2 M magnesium chloride. Crystals were cryo-protected with mother liquor supplemented with an additional 15% ethylene glycol and vitrified in liquid nitrogen after mounting.

Data Collection:2.7 Å resolution
X-ray source: Diffraction data were collected at 100 K on Diamond Light Source beamline I03.