RIPK2A

PDB Code: 4C8B

Material and Methods

Entry Clone Source: Mammalian Gene Collection

Entry Clone Accession: GI:33871163

SGC Construct ID: RIPK2A-c033

Amplified DNA sequence:

CCATGGGCCACCATCATCATCATCAT
TCTTCTGGTGTAGATCTGGGTACCGA
GAACCTGTACTTCCAATCCATGAGCG
CCCTGCCCACCATTCCCTACCACAAA
CTCGCCGACCTGCGCTACCTGAGCCG
CGGCGCCTCTGGCACTGTGTCGTCCG
CCCGCCACGCAGACTGGCGCGTCCAG
GTGGCCGTGAAGCACCTGCACATCCA
CACTCCGCTGCTCGACAGTGAAAGAA
AGGATGTCTTAAGAGAAGCTGAAATT
TTACACAAAGCTAGATTTAGTTACAT
TCTTCCAATTTTGGGAATTTGCAATG
AGCCTGAATTTTTGGGAATAGTTACT
GAATACATGCCAAATGGATCATTAAA
TGAACTCCTACATAGGAAAACTGAAT
ATCCTGATGTTGCTTGGCCATTGAGA
TTTCGCATCCTGCATGAAATTGCCCT
TGGTGTAAATTACCTGCACAATATGA
CTCCTCCTTTACTTCATCATGACTTG
AAGACTCAGAATATCTTATTGGACAA
TGAATTTCATGTTAAGATTGCAGATT
TTGGTTTATCAAAGTGGTGCATGATG
TCCCTCTCACAGTCACGAAGTAGCAA
ATCTGCACCAGAAGGAGGGACAATTA
TCTATATGCCACCTGAAAACTATGAA
CCTGGACAAAAATCAAGGGCCAGTAT
CAAGCACGATATATATAGCTATGCAG
TTATCACATGGGAAGTGTTATCCAGA
AAACAGCCTTTTGAAGATGTCACCAA
TCCTTTGCAGATAATGTATAGTGTGT
CACAAGGACATCGACCTGTTATTAAT
GAAGAAAGTTTGCCATATGATATACC
TCACCGAGCACGTATGATCTCTCTAA
TAGAAAGTGGATGGGCACAAAATCCA
GATGAAAGACCATCTTTCTTAAAATG
TTTAATAGAACTTGAACCAGTTTTGA
GAACATTTGAAGAGATAACTTTTCTT
GAAGCTGTTATTCAGCTAAAGAAAAC

AAAGTTACAGAGTGTTTGACAGTAAA
GGTGGATACGGATCCGAATTCGAGCT
CCGTCGACAAGCTT

Expressed protein sequence:

mghhhhhhssgvdlgtenlyfq*sMS
ALPTIPYHKLADLRYLSRGASGTVSS
ARHADWRVQVAVKHLHIHTPLLDSER
KDVLREAEILHKARFSYILPILGICN
EPEFLGIVTEYMPNGSLNELLHRKTE
YPDVAWPLRFRILHEIALGVNYLHNM
TPPLLHHDLKTQNILLDNEFHVKIAD
FGLSKWCMMSLSQSRSSKSAPEGGTI
IYMPPENYEPGQKSRASIKHDIYSYA
VITWEVLSRKQPFEDVTNPLQIMYSV
SQGHRPVINEESLPYDIPHRARMISL
IESGWAQNPDERPSFLKCLIELEPVL
RTFEEITFLEAVIQLKKTKLQSV

Note this construct contains the mutation R171C in the kinase activation loop

Vector:pFB-LIC-Bse

Tags and additions: TEV-cleavable (*) C-terminal hexahistidine tag. (lower case)

Host: SF9 Spodoptera frugiperda Insect cells

Growth medium, induction protocol:

2 L of SF9 cells at a density of 2million/ml were infected with 10ml of Virus/L.Cells were incubated at 27°C in the shaker incubator and harvested after 48 hours. Cells were harvested by centrifugation at 900xG at 4°C for 15 min. Cell pellets from each flask (1l volume) were resuspended in 15ml binding buffer, transferred to 50ml 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 protease inhibitor SET V (Calbiochem) added to the cell suspension at 1:1000 dilution. The cells were lysed by high pressure homogenization in an Emulsiflex C5 cell homogeniser. Polyethyleneimine (PEI) was added to a final concentration of 0.5% to precipitate DNA. The cell lysate was clarified by centrifugation at 21,000 rpm at 4°C for 1 h and filtered using syringe filters with a 1.2µm pore size.

Column 1: Ni-Affinity Chromatography □ 2ml Ni-sepharose slurry applied to a 1.5 x 10 cm column.

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 500mM Imidazole

Procedure:

2ml of 50 % Ni-sepharose slurry (Amersham) was equilibrated in binding buffer and added to the filtered lysate, which was incubated with the Ni-sepharose for 1 hour at 4°C with slow rotation to maximize binding. The lysate was then applied to a 1.5 x 10 cm column by gravity flow. The remaining resin was then washed with 2x50ml binding 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 to 500mM

imidazole). The protein content of collected fractions was visualized using SDS-PAGE and fractions containing RIPK2A were pooled.

Enzymatic treatment: Lambda phosphatase treatment. Pooled fractions were treated with lambda phosphatase overnight at 4°C.

Column 2: Size Exclusion Chromatography S75 HiLoad 26/60 Superdex run on ÄKTAprime.

Buffer:

Gel Filtration buffer: 10mM HEPES pH7.5, 500mM NaCl, 5% Glycerol, 1mM TCEP

Procedure: Prior to applying the protein, the S75 26/60 column was washed and equilibrated with gel filtration buffer. Eluted protein from Ni-sepharose column was concentrated to 2ml using an Amicon Ultra-15 filter with a 10kDa cut-off. The concentrated protein was directly applied onto the equilibrated S75 26/60 column, and run at a flow-rate of 2.5 ml/min. 3ml fractions were collected and visualized using SDS-PAGE. Those containing RIPK2A were pooled.

Concentration: The protein was supplemented with 5 mM L-arginine, 5 mM L-glutamate and 2mM DTT before being concentrated to a final concentration of 3.7mg/ml (measured by OD_{280} based on extinction coefficient 46870) in an Amicon Ultra-15 filter with a 10 kDa cut-off.

Mass spec characterization: The purified protein was homogeneous and had an experimental mass of 38243.7 Da. The theoretical expected mass from the construct sequence is 38385.1 Da. The mass discrepancy correlates to an Arg/Cys substitution, identified as R171C by sequencing the construct DNA sequence, as well as loss of the N-terminal methionine and acetylation of the N-terminus. Masses were determined by LC-MS, using an Agilent LC/MSD QTOF 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 3.7mg/ml was buffered in 10mM HEPES, pH 7.5, 250mM NaCl, 1 mM TCEP, 5 mM L-arginine, 5 mM L-glutamate and 2mM DTT. 3-(2-Imidazo[1,2-b]pyridazin-3-ylethynyl)-4-methyl-N-[4-[(4-methylpiperazin-1-yl)methyl]-3-(trifluoromethyl)phenyl]benzamide (ABL-kinase inhibitor Ponatinib) was added to the final sample. Crystals were grown at 20°C in 150 nl sitting drops mixing 50 nl protein solution with 50 nl of a reservoir solution containing 0.1M ammonium citrate and 16%(w/v) PEG 3350. On mounting crystals were cryo-protected with an additional 25% ethylene glycol.

Data Collection: Resolution: 2.75 Å **X-ray source: Diamond I04**

Crystals of RIPK2A diffracted to a resolution of 2.75 Å (scaled resolution). A full dataset was collected at 100 K on Diamond Light Source beamline I04. Crystals belonged to the Monoclinic space group P212121 with unit-cell parameters a=59 Å b=86 Å c=137 Å, a=90° β=90° γ=90°. Two molecules were present in the asymmetric unit. Data were indexed and integrated using XDS and scaled using AIMLESS. Phases were found using molecular replacement in PHASER. PHENIX.SCULPTOR was used to optimize PDB entry 3PPZ for use as a search model. The structure was built using PHENIX.AUTOBUILD and then refined and modified using alternate rounds of REFMAC5 and COOT. The final model was validated using the PHENIX validation tools.