Molecular Biology

Entry Clone Accession:
Entry Clone Source: Alexei Degterev
SGC Construct ID: RIPK2A-c036
Protein Region: G3-V317
Vector: pFB-LIC-Bse
Tag: N-6HIS;N-TEV
Host: DH10Bac

Sequence (with tag(s)):
MGHHHHHHSSGVDLGTENLYFQSMGEAICSALPTIPYHKLADLRYLSRGASGTVSSARH
ADWRVQVAVKHLHIHTPLLDSERKDVLREAEILHKARFSYILPILGICNEPEFLGIVTEYMP
NGSLNELLHRKTEYPDVAWPLRFRILHEIALGVNYLHNMTPPLLHHDLKTQNILLDNEFH
VKIADFGLSKWRMMSLSQSRSSKSAPEGGTIIYMPPENYEPGQKSRASIKHDIYSYAVITW
EVLSRKQPFEDVTNPLQIMYSVSQGHRPVINEESLPYDIPHRARMISLIESGWAQNPDERP
SFLKCLIELEPVLRTFEEITFLEAVIQLKKTKLQSV
Sequence after tag cleavage:
SMGEAICSALPTIPYHKLADLRYLSRGASGTVSSARHADWRVQVAVKHLHIHTPLLDSER
KDVLREAEILHKARFSYILPILGICNEPEFLGIVTEYMPNGSLNELLHRKTEYPDVAWPLRF
RILHEIALGVNYLHNMTPPLLHHDLKTQNILLDNEFHVKIADFGLSKWRMMSLSQSRSSK
SAPEGGTIIYMPPENYEPGQKSRASIKHDIYSYAVITWEVLSRKQPFEDVTNPLQIMYSVS
QGHRPVINEESLPYDIPHRARMISLIESGWAQNPDERPSFLKCLIELEPVLRTFEEITFLEAVI
QLKKTKLQSV
DNA Sequence:
CCATGGGCCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTG
TACTTCCAATCCATGGGGGAGGCCATCTGCAGCGCCCTGCCCACCATTCCCTACCACA
AACTCGCCGACCTGCGCTACCTGAGCCGCGGCGCCTCTGGCACTGTGTCGTCCGCCC
GCCACGCAGACTGGCGCGTCCAGGTGGCCGTGAAGCACCTGCACATCCACACTCCGC
TGCTCGACAGTGAAAGAAAGGATGTCTTAAGAGAAGCTGAAATTTTACACAAAGCTA
GATTTAGTTACATTCTTCCAATTTTGGGAATTTGCAATGAGCCTGAATTTTTGGGAATA
GTTACTGAATACATGCCAAATGGATCATTAAATGAACTCCTACATAGGAAAACTGAATA
TCCTGATGTTGCTTGGCCATTGAGATTTCGCATCCTGCATGAAATTGCCCTTGGTGTAA
ATTACCTGCACAATATGACTCCTCCTTTACTTCATCATGACTTGAAGACTCAGAATATCT
TATTGGACAATGAATTTCATGTTAAGATTGCAGATTTTGGTTTATCAAAGTGGCGCATG
ATGTCCCTCTCACAGTCACGAAGTAGCAAATCTGCACCAGAAGGAGGGACAATTATCT
ATATGCCACCTGAAAACTATGAACCTGGACAAAAATCAAGGGCCAGTATCAAGCACG
ATATATATAGCTATGCAGTTATCACATGGGAAGTGTTATCCAGAAAACAGCCTTTTGAA
GATGTCACCAATCCTTTGCAGATAATGTATAGTGTGTCACAAGGACATCGACCTGTTAT
TAATGAAGAAAGTTTGCCATATGATATACCTCACCGAGCACGTATGATCTCTCTAATAG
AAAGTGGATGGGCACAAAATCCAGATGAAAGACCATCTTTCTTAAAATGTTTAATAGA
ACTTGAACCAGTTTTGAGAACATTTGAAGAGATAACTTTTCTTGAAGCTGTTATTCAG
CTAAAGAAAACAAAGTTACAGAGTGTTTGACAGTAAAGGTGGATACGGATCCGAATT
CGAGCTCCGTCGACAAGCTT

Protein Expression

Medium: Insect Xpress
Antibiotics: Ampicillin
Procedure: Sf9 cells at a density of $2x10^6$/ml were infected with recombinant RIPK2

baculovirus (virus stock P2; 3ml of virus stock per 1000ml cell culture). Cells were shaken at 110rpm at 27°C in an Infors shaker with a radii of 25mm. After 72 hours since infection the cultures were harvested by centrifugation at 900g for 20 mins. Cell pellets were resuspended in 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5 % glycerol plus Merck Set III protease inhibitor and stored at -20°C.

Protein Purification

Procedure: Cells were lysed by sonication and lysates clarified by centrifugation at 21,000rpm following the addition of 0.125% polyethyleneimine. The resulting supernatant was incubated with Ni-sepharose resin (equilibrated in 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5 % glycerol), mixing by inversion, at 4ºC for 1 hour. This was centrifuged at 700g for 5mins, the supernatant removed and resin resuspended in 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5 % glycerol. This was applied to a gravity column and washed and eluted with 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, and 30-250mM imidazole. Fractions containing protein (as seen by SDS-PAGE) were treated with TEV protease overnight at 4ºC prior to gel filtration on an S200 gel filtration column using 50 mM HEPES pH 7.5, 300 mM NaCl and 1mM TCEP as the running buffer. Protein containing fractions, as observed by SDS-PAGE, were pooled and concentrated to 13.6 mg/ml using a centrifugal concentrator.

Columns: Column 1: Ni-NTA Batch bind; Column 2: S200.
Concentration: 13.6 mg/ml
Mass-spec Verification: Intact mass with 2-5 phosphorylations confirmed by LC-MS as 36496.8, 36576.7, 36656.6 and 36736.6.

Structure Determination

Crystallization: Protein was incubated with the compound PK010729a (CNC(=O)c1cc(Oc2ccc(NC(=O)Nc3ccc(SCC(=O)O)c(F)c3)c(F)c2)ccn1) dissolved in DMSO at a final concentration of 2mM for 10 mins on ice prior to 0.22um spin filtration and the setting up of crystal plates. Crystals were grown using the sitting drop vapor diffusion method at 20ºC. Crystals were grown in 150nl drops consisting of 1:2 mother liquor (25% PEG Smear Low , 0.1M MES pH 6.5, 0.05M magnesium acetate , 0.05M magnesium chloride) to protein (10.0 mg/ml) with compound at 2mM. 25% ethylene glycol was added to the crystal as a cryoprotectant and the crystal was mounted and flash frozen in liquid nitrogen.

Data Collection: Data was collected at beamline I04-1 at 100K.
Data Processing: Data was processed to a resolution of approximately 2.38 Å using XDS and either xia2 or autoPROC, or DIALS.