Pim1 - Human Proviral Integration Site for MuLV in complex with LY333531 (Ruboxistaurin)

PDB:2J2I

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:
Entry Clone Source:TSK
SGC Clone Accession:
Tag:mhhhhhhssgvdlgtenlyfq*s(m) . N-terminal his6 tag, TEV-protease cleavable (*)
Host:BL21(DE3)

Construct

Prelude:
Sequence:

mhhhhhhssgvdlgtenlyfqsMLLSKIN SLAHLRAAPCNDLHATKLAPGKEKEPLES QYQVGPLLGSGGFGSVYSGIRVSDNLP
VA IKHVEKDRISDWGELPNGTRVPMEVVLLK KVSSGFSGVIRLLDWFERPDSFVLILERP EPVQDLFDFITERGALQEELARSF
FWQVL EAVRHCHNCGVLHRDIKDENILIDLNRGE LKLIDFGSGALLKDTVYTDFDGTRVYSPP EWIRYHRYHGRSAAVWSLGIL
LYDMVCGD IPFEHDEEIIRGQVFFRQRVSSECQHLIR WCLALRPSDRPTFEEIQNHPWMQDVLLP QETAEIHLHSLSPGPS

Vector:pLIC- SGC1

Growth

Medium:
Antibiotics:
Procedure:10 ml overnight cultures in LB, 100 µg/ml ampicillin were centrifuged, resuspended in fresh buffer, and used to inoculate 1 litre of LB medium containing 100 µg/ml ampicillin. Cultures were grown at 37°C until they reached an OD600 of 0.3 and then cooled to 18°C. Expression was induced for 4 hours using 1 mM IPTG at an OD600 of 0.8. The cells were collected by centrifugation, transferred to 50 ml tubes, resuspended in 30 ml binding buffer, and frozen. Binding buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 5% Glycerol.

Purification

Procedure
Column 1: Ion exchange - Nucleic acid removal. DEAE cellulose (DE52, Whatmann), 10 gr of resin in 2.5 x 20 cm column. The resin was hydrated in 2.5M NaCl, then washed with 20 ml binding buffer prior to loading the sample.
Procedure: Supernatant was applied at gravity flow, followed by a wash with 20 ml binding buffer. The column flow-through was collected.

Column 2: Ni-affinity. Ni-NTA (Qiagen), 5 ml of 50% slurry in 1.5 x 10 cm column, washed with binding buffer.
Procedure: The flowthrough from column 1 was loaded by gravity flow on the Ni-NTA column. The column was then washed with 3 x 10 ml wash buffer at gravity flow. The protein was eluted by gravity flow by applying 5-ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 250 mM); fractions were collected until essentially all protein was eluted. After elution DTT was added to a final concentration of 10 mM.

Dephosphorylation: Samples containing Pim1 were pooled and alkaline phosphatase was added in the same buffer. Protein sample was treated for 12 h.

Column 3: Ion exchange Mono Q column.
Procedure: Dephosphorylated Pim1 was applied to MonoQ in buffer A and eluted from the column by a linear gradient.

Concentration: Pim1 samples containing unphosphorylated protein were pooled and concentrated in Centricons (10 kDa cut off). Dephosphorylation was monitored using LC- ESI MS-Tof.

Extraction

Procedure
The frozen cells were thawed on ice and lysed using a high pressure cell disruptor. The lysate was centrifuged at 17,000 RPM for 30 minutes. Supernatant was collected and binding buffer was added to 50 ml.
Concentration:
Ligand
MassSpec: The purified protein was homogeneous and had an experimental mass of 35546 Da as expected from its primary structure
Intact Mass: Masses of purified proteins were confirmed 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% acetonitrile in water with 0.1% formic acid.
Crystallization:LY333531 ((S)-13-[(dimethylamino) methyl]-10,11,14,15 -tetrahydro-4,9:16, 21-dimetheno-1H, 13H-dibenzo[e,k] pyrrolo[3,4-h][1,4,13] oxadiazacyclohexa decane-1,3(2H)-d ione, Ruboxistaurin) was purchased from AXXORA and added at a concentration of 1 mM from

a 50 mM DMSO stock solution to the protein. Crystals were grown at 4°C in 3 µl sitting drops mixing 150 nl Pim1 (10 mg/ml in 50mM Hepes pH 7.5, 280mM NaCl, 5% Glycerol, 10mM DTT with 50 nl of a solution containing 20% PEG 1K; 0.20M Li2SO4; 0.1M Citrate/phosphate buffer pH 4.2.

NMR Spectroscopy:

Data Collection: Resolution: 1.9Å, X-ray source: rotating anode (Rigaku FR-E SuperBright), single wavelength.

Data Processing: