Molecular Biology

Entry Clone Accession: BC105015
Entry Clone Source: MGC
SGC Construct ID: PIP3EA-c002
Protein Region: A42-Q142
Vector: pNIC28-Bsa4, N-terminal His tag followed by a TEV cleavage site.
Host: BL21(DE3)-R3-pRARE2

Sequence (with tag(s)):
MHHHHHHSSGVDLGTENLYFQSMADCQGWLYKKKEKGSFLSNKWKKFWVILKGSSLY
WYSNQMAEKADGFVNLPDFTVERASECKKKHAFKISHPQIKTFYFAAENVQEMNVWLN
KLGSAVIHQ
Sequence after tag cleavage:
SMADCQGWLYKKKEKGSFLSNKWKKFWVILKGSSLYWYSNQMAEKADGFVNLPDFTV
ERASECKKKHAFKISHPQIKTFYFAAENVQEMNVWLNKLGSAVIHQ
DNA Sequence:
CATATGCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTGTA
CTTCCAATCCATGGCTGACTGCCAAGGGTGGCTGTATAAGAAAAAGGAAAAGGGAAG
TTTCCTAAGCAACAAATGGAAAAAGTTCTGGGTGATACTGAAGGGGTCGTCACTGTA
CTGGTATAGCAATCAAATGGCAGAGAAAGCTGATGGATTTGTCAACCTGCCTGATTTC
ACTGTGGAAAGAGCATCTGAATGCAAGAAAAAGCATGCTTTTAAGATCAGCCATCCA
CAGATCAAGACCTTTTATTTTGCAGCTGAGAATGTGCAGGAAATGAACGTGTGGTTAA
ATAAACTTGGATCGGCTGTAATCCATCAGTGACAGTAAAGGTGGATACGGATCCGAA

Protein Expression

Medium: Terrific Broth
Antibiotics: Kanamycin
Procedure: Recombinant protein expression was induced by the addition of 0.3 mM isopropyl 1-thio-- D-galactopyranoside to bacterial cultures grown in TB (Terrific Broth) containing 50 mg/ml kanamycin at an OD600 of 2.0 at 37 °C in UltraYield baffled flasks. Cultures were further incubated at 18 °C overnight.

Protein Purification

Procedure: Cell pellets were thawed and resuspended in buffer A (50 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 10 mM imidazole, 1 mM tris(2-carboxyethyl)phosphine (TCEP)**.** Cells were lysed using sonication and cell debris was removed by centrifugation at 40,000 g for 1 h at 4 °C. Clarified lysates were applied to a 2.5-ml Ni-sepharose immobilized metal ion affinity chromatography gravity flow column, washed with 10 column volumes (CV) of buffer A, followed by 10 CV of wash buffer (buffer A with 45 mM imidazole). Proteins were eluted by addition of 5CV of buffer A containing 300 mM imidazole and analyzed by SDS-PAGE. Fractions containing PIP3E were pooled and cleaved with the addition of His6-tagged TEV protease (1:20 mass ratio) overnight at 8 °C. Imidazole was removed by concurrent dialysis during cleavage, using a 3.5-kDa MWCO snakeskin membrane in buffer B (20 mM HEPES, pH 7.5, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP). TEV protease was removed using a Ni-sepharose gravity flow column with cleaved FOXN1 being present in the flow through and 10 mM wash fractions. Cleaved protein was pooled and concentrated with a 3-kDa MWCO centrifugal concentrator. Final separation was by size exclusion chromatography, using a HiLoad 16/60 Superdex S75 column equilibrated in buffer B, and run at 1.2 ml/min.

Protein identity was confirmed by LC/ESI-TOF mass spectrometry with a single peak of mass 12401.62 corresponding to an expected mass of 12040.92.

Structure Determination

Crystallization: For crystallization PIP3E was concentrated to 20 mg/ml using a 10,000 mwco centrifugal concentrator. Crystallization was performed by sitting drop vapor diffusion at 20°C in 150nl sitting drops with a 1:2 ratio of protein to precipitant. Crystals grew from conditions containing 0.2M lithium sulfate, 25% PEG3350 and 0.1M bis-tris pH 5.5. Crystals were cryo-protected by transferring to a solution of mother liquor supplemented with 25 % ethylene glycol and flash-cooled in liquid nitrogen.

Data Collection: Diffraction data were collected on beamline I04-1 at Diamond Light Source, at a wavelength of 0.92Å to a maximum resolution of 1.19Å. The data was processing using the program XDS.
Data Processing: The structure was solved by molecular replacement using the program PHASER and the structure of the PH domain of PEPP1 (PDBid 1UPQ) as a search model. The structure was refined using PHENIX REFINE to a final Rfactor=16.7%, Rfree=18.8%.