PIK3KC3

PDB:3IHY

Revision

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Entry Clone Accession:gi|31657191, BC033004
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:PIK3C3A-k026
Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
Host:*E.coli* BL21(DE3) R3 pRARE, where R3 denotes a derivative of BL21(DE3) resistant to a strain of T1 bacteriophage (SGC Oxford) and the pRARE plasmid originating from the Rosetta strain (Novagen) supplies tRNAs for rare codons.

Construct

Prelude:
Sequence:

MHHHHHHSSGVDLGTENLYFQSMSDHDLKPNAATRDQLNIIVSYPPTKQLTYEEQDLVWKFRYYLTNQEKALTKFLKCVNWDLPQEA
KQALELLGKWKPMDVEDSLELLSSHYTNPTVRRYAVARLRQADDEDLLMYLLQLVQALKYENFDDIKNGLEPTKKDSQSSVSENVSN
SGINSAEIDSSQIITSPLPSVSSPPPASKTKEVPDGENLEQDLCTFLISRACKNSTLANYLYWYVIVECEDQDTQQRDPKTHEMYLN
VMRRFSQALLKGDKSVRVMRSLLAAQQTFVDRLVHLMKAVQRESGNRKKKNERLQALLGDNEKMNLSDVELIPLPLEPQVKIRGIIP
ETATLFKSALMPAQLFFKTEDGGKYPVIFKHGDDLRQDQLILQIISLMDKLLRKENLDLKLTPYKVLATSTKHGFMQFIQSVPVAEV
LDTEGSIQNFFRKYAPSENGPNGISAEVMDTYVKSCAGYCVITYILGVGDRHLDNLLLTKTGKLFHIDFGYILGRDPKPLPPPMKLN
KEMVEGMGGTQSEQYQEFRKQCYTAFLHLRRYSNLILNLFSLMVDANIPDIALEPDKTVKKVQDKFRLDLSDEEAVHYMQSLIDESV
HALFAAVVEQIH

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were grown in 15 ml TB supplemented with 8 g/l glycerol, 100 µg/ml kanamycin and 34 µg/ml chloramphenicol at 30 °C overnight. The overnight culture (15 ml) was used to inoculate 1.5 l TB supplemented with 8 g/l glycerol, 50 µg/ml kanamycin and approximately 200 µl 204 Antifoam A6426 (Sigma). The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~2. The culture was down-tempered to 18 °C over a period of 1 hour before target expression was induced by addition of 0.5 mM IPTG. Expression was allowed to continue overnight and cells were harvested the following morning by centrifugation (4,400 x *g*, 10 min, 4 °C). The resulting cell pellet (42 g wet cell weight) was resuspended in lysis buffer (1.5 ml/g cell pellet), supplemented

with 2000 U Benzonase (Merck) and one tablet of Complete EDTA-free protease inhibitor (Roche Applied Science). The cell suspension was stored at -80 °C.

Purification

Procedure
Columns
IMAC: Ni-charged 1 ml HiTrap Chelating HP (GE Healthcare)
Gel filtration column: HiLoad 16/60 Superdex 200 Prep Grade (GE Healthcare)

Procedure
Purification of the protein was performed as a two step process on an ÄKTAxpress system (GE Healthcare). Prior to purification, columns were equilibrated with IMAC wash1 buffer and gel filtration buffer, respectively. The filtered lysate was loaded onto two Ni-charged HiTrap Chelating columns connected in series and washed with IMAC wash1 buffer followed by IMAC wash2 buffer. Bound protein was eluted from the IMAC column with IMAC elution buffer and automatically loaded onto the gel filtration column. Fractions containing the target protein were pooled and fresh TCEP was added to a final concentration of 2 mM. The total volume of the pooled fractions was 10 ml and the concentration of the protein 0.90 mg/ml. The identity of the protein was confirmed by mass spectrometry.

Tag removal
The N-terminal histidine tag was proteolytically removed by incubating the target protein with His-tagged TEV protease in a molar ratio of 50:1 at 8 ºC overnight. The proteolytic reaction went to completion, as judged by SDS-PAGE. Target protein was purified from tag and protease by passing the reaction mixture over a Ni-charged 1 ml HisTrap HP column (GE Healthcare) pre-equilibrated with IMAC wash1 buffer. The protein was concentrated and the buffer was changed to GF buffer with 2 mM TCEP using a Vivaspin 20 centrifugal filter device with 30,000 MWCO (Sartorius). The final protein concentration was determined to 18.6 mg/ml in a volume of 0.3 ml The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water bath. Cells were disrupted by sonication (Vibra-Cell, Sonics) at 80% amplitude for 3 min effective time (pulsed 4s on, 4s off) and cell debris was removed by centrifugation (49,000 x *g*, 20 min, 4 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
MassSpec:
Crystallization: Crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.1 µl protein solution (18.6 mg/ml) supplemented with AMP-PNP (2.5 mM final solution) was mixed with 0.1 µl of well solution consisting of 0.2 M ammonium acetate, 0.1 M tris pH 8.5 and 25% PEG 3350. The plate was incubated at 20 ºC and crystals appeared in less than 14 days. The crystals were quickly transferred to a cryo solution consisting of well solution with PEG 3350 concentration increased to 26% and complemented with 20% glycerol and 0.3 M NaCl, and flash frozen in liquid nitrogen.
NMR Spectroscopy:
Data Collection: Diffraction data to 2.8 Å resolution was collected at ESRF beamline ID 14-2.
Data Processing: Data was integrated using XDS and scaled using SCALA. The structure was

solved by molecular replacement using PHASER with the phosphatidylinositol 3-kinase catalytic subunit as template (PDB: 1E7U). The space group was P1 with cell dimensions a= 62.30 Å b=96.49 Å c=150.68 Å, α=107.77° β=91.75° γ=92.41°. Five monomers were located in the asymmetric unit. The initial model was improved using PHENIX.AUTOBUILD. The first cycles of refinement were performed with PHENIX.REFINE, while REFMAC5 was used for final refinement and Coot for manual model building. Data in the interval 19.55-2.80 Å resolution was used and at the end of the refinement the R values were: R=18.5% and Rfree=25.2%. Coordinates for the crystal structure were deposited in the Protein Data Bank, accession code 3IHY.