PIK3C2G

PDB:2WWE

Revision

Revision Type:created
Revised by:created
Revision Date:created
Entry Clone Accession:gi|194353959, BC130277
Entry Clone Source: Mammalian Gene Collection
SGC Clone Accession:PIK3C2GA-k023
Tag:N-terminal hexahistidine tag with integrated TEV protease cleavage site: mhhhhhhssgvdlgtenlyfq*sm
Host:*E. coli* BL21(DE3) R3 pRARE

Construct

Prelude:
Sequence:

MHHHHHHSSGVDLGTENLYFQSMSIERATILGFSKKSSNLYLIQVTHSNNETSLTEKSFEQFSKLHSQLQKQFASLTLPEFPHWWHL
PFTNSDHRRFRDLNHYMEQILNVSHEVTNSDCVLSFFLSE

Vector:pNIC-Bsa4

Growth

Medium:
Antibiotics:
Procedure: Cells from a glycerol stock were used to inoculate 40 mL TB supplemented with 100 µg/mL kanamycin and 34 µg/mL chloramphenicol, and grown at 30 °C overnight. 25 mL of the overnight culture was used to inoculate 1.5 L TB supplemented with 8 g/L glycerol, 50 µg/mL kanamycin and approximately 5-10 drops of Dow Corning Antifoam. The culture was grown in a LEX bioreactor system (Harbinger Biotechnology) at 37 °C until OD600 reached ~2.4. The culture was down-tempered to 18 °C over a period of one 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 (39 g wet cell weight) was resuspended in lysis buffer (1.5 mL/g cell pellet) supplemented with one tablets of Complete EDTA-free protease inhibitor (Roche Applied Science) and 2000 U Benzonase (Merck) per 100 mL lysis buffer, and stored at -80 °C.

Purification

Procedure
Columns

IMAC: Ni-charged 2 x 1 ml HiTrap Chelating HP (GE Healthcare)
Gel filtration column: HiLoad 16/60 Superdex 75 Prep Grade (GE Healthcare)

Procedure
Purification of the native 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 the Ni-charged HiTrap Chelating column 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 subsequently concentrated using an Amicon Ultra-15 centrifugal filter device, 5 000 NMWL (Millipore). The concentration was measured to 26.4 mg/mL in a volume of 1.2 ml and the samples were stored at -80 ºC. The identity of the protein was confirmed by mass spectrometry.

Extraction

Procedure
The cell suspension was quickly thawed in water. Cells were disrupted by sonication (VibraCell, Sonics) at 80% amplitude for 3 min effective time (puls 4s on, 4s off) and cell debris was removed by centrifugation (49,000 x *g*, 30 min, 4 ºC). The supernatant was decanted and filtered through a 0.45 µm flask filter.
Concentration:
Ligand
MassSpec:
Crystallization: Native crystals were obtained by the sitting drop vapour diffusion method in a 96-well plate. 0.2 µl of the protein solution (26.4 mg/ml) was mixed with 0.1 µl of well solution consisting of 0.1M Tri-sodium citrate dihydrate pH 5.5, 20% PEG3000. The plate was incubated at 4 ºC. Crystals of dimensions 75x75 µm had appeared after 5 days and grew to a final size of 125x125 µm within two weeks. The crystals were transferred to cryo solution containing 0.1M Tri-sodium citrate dihydrate pH 6.5, 22% PEG3000, 20% Glycerol, 0.3M NaCl, and flash cooled in liquid nitrogen prior to data collection.
NMR Spectroscopy:
Data Collection: Native data was collected to 1.25 Å at DIAMOND (I04). The crystal belonged to space group P 32 2 1 with cell parameters of a=53.42 Å b=53.42 Å and c=75.13 Å.
Data Processing: Data were integrated in iMosflm and scaled with Scala. The structure was solved by molecular replacement in Phaser using a search model consisting of an ensemble of the superpositioned coordinates from Phox domains 2AR5, 2IWL, 1O7K, 2V6V. ArpWarp could be used for automatic model building of the one molecule present in the asymmetric unit. Manual rebuilding was performed in Coot and cycled with restrained refinement in REFMAC5, which resulted in the final model where R= 16.5% and Rfree=18.4%. The coordinates and structure factors were deposited in the PDB with accession code 2WWE.