Molecular Biology

Entry Clone Accession:
Entry Clone Source: Tony Pawson
SGC Construct ID: WNK3B-c016
Protein Region: A133-E414
Vector: pNIC28-Bsa4
Tag: N-6HIS;N-TEV
Host: BL21(DE3)-R3-pRARE2

Sequence (with tag(s)):
MHHHHHHSSGVDLGTENLYFQSMAEMKAVATSPSGRFLKFDIELGRGAFKTVYKGLDTE
TWVEVAWCELQDRKLTKAEQQRFKEEAEMLKGLQHPNIVRFYDSWESILKGKKCIVLVT
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YSECQNAAQIYRKVTSGIKPASFNKVTDPEVKEIIEGCIRQNKSERLSIRDLLNHAFFAEDT
GLRVE
Sequence after tag cleavage:
SMAEMKAVATSPSGRFLKFDIELGRGAFKTVYKGLDTETWVEVAWCELQDRKLTKAEQQ
RFKEEAEMLKGLQHPNIVRFYDSWESILKGKKCIVLVTELMTSGTLKTYLKRFKVMKPK
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DNA Sequence:
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GCCACAATCCCTCCTGGTCCTAAATGA

Protein Expression

Medium: LB
Antibiotics: Kanamycin/Chloramphenicol
Procedure: A glycerol stock was used to inoculate a starter culture which was supplemented with Kanamycin (50 µg/ml)/ Chloramphenicol (34 µg/ml). These were then grown overnight at 37 °C with shaking at 240 RPM. The starter culture was used to inoculate LB flasks supplemented with Kanamycin/Chloramphenicol. The cultures were then grown at 37 °C with shaking at 160 RPM to an OD_{600} of between 0.6-0.8. The cultures were then cooled to 18 °C, induced with 0.3 mM IPTG and the protein was expressed overnight at 18 °C /160 RPM. Cultures were harvested, pellets made up to 40 ml with Nickel affinity binding buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5% Glycerol) and 40 ul of SET III protease inhibitors (Merck) added before storage at -20 °C.

Protein Purification

Procedure: Cells were lysed by sonication, 0.125% polyethyleneimine added, and lysates were clarified by centrifugation at 50,000G. The supernatant was then applied to a Nickel-NTA gravity column and washed and eluted with 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, and 10-300 mM imidazole. Protein containing fractions were then treated with TEV protease overnight at 4 °C prior to gel filtration on an S200 gel filtration column using low salt 50 mM HEPES pH 7.5, 50 mM NaCl, 1 mM TCEP as the running buffer. Fractions containing the protein were pooled, concentrated and loaded onto a 5ml HiTrap S IEX column, and eluted with 50 mM HEPES pH 6.5, 1mM TCEP, 0-1M NaCl. The protein was then buffer exchanged into 50 mM HEPES pH 7.5, 50 mM NaCl, 1 mM TCEP, and concentrated to 12 mg/ml using a spin concentrator.

Columns: Column 1: Ni-NTA Drip ; Column 2: S200 Gel Filtration; Column 3: Hi-trap Q, 5 ml
Concentration: 12 mg/ml
Mass-spec Verification: Intact mass was correct and verified that the protein was diphosphorylated

Structure Determination

Crystallization: Crystals were grown using the sitting drop vapor diffusion method at 20˚C. 4 mM AMP-PNP (Adenosine 5'-(beta,gamma-imido)triphosphate; beta, gamma-Imidoadenosine 5'-triphosphate), 10 mM $MgCl_2$, and 10 mM DTT was added to the protein. Crystals were grown in 150 nl drops consisting of 1:2 mother liquor (25% PEG3350 -- 0.1M bis-tris pH 5.5) to protein (12 mg/ml). 20% Ethylene glycol was added and the crystal was then mounted and flash frozen in liquid nitrogen.

Data Collection: Data was collected at beamline I04 at 100K.
Data Processing: Data was processed to a resolution of approximately 2.4 Å using XDS and either xia2 or autoPROC, or DIALS.