Molecular Biology

Entry Clone Accession:
Entry Clone Source: Tony Pawson
SGC Construct ID: WNK2A-c003
Protein Region: A454-A549
Vector: pNIC28-Bsa4
Tag: N-6HIS;N-TEV
Host: BL21(DE3)-R3-pRARE2

Sequence (with tag(s)):
MHHHHHHSSGVDLGTENLYFQSMAEDTGVRVELAEEDHGRKSTIALRLWVEDPKKLKG
KPKDNGAIEFTFDLEKETPDEVAQEMIESGFFHESDVKIVAKSIRDRVALIQWRRERIWPA
Sequence after tag cleavage:
SMAEDTGVRVELAEEDHGRKSTIALRLWVEDPKKLKGKPKDNGAIEFTFDLEKETPDEV
AQEMIESGFFHESDVKIVAKSIRDRVALIQWRRERIWPA
DNA Sequence:
CATATGCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTGTA
CTTCCAATCCATGGCAGAGGACACAGGCGTGAGGGTGGAGCTCGCGGAGGAGGACC
ACGGCAGGAAGTCCACCATCGCCCTGAGGCTCTGGGTGGAAGACCCCAAGAAACTG
AAGGGAAAGCCCAAGGACAATGGAGCCATAGAGTTCACCTTCGACCTGGAGAAGGA
GACGCCGGATGAGGTGGCCCAAGAGATGATTGAGTCTGGATTCTTCCACGAGAGTGA
CGTCAAGATCGTGGCCAAGTCCATCCGTGACCGCGTGGCCTTGATCCAGTGGCGGCG
GGAGAGGATCTGGCCCGCGTGACAGTAAAGGTGGATACGGATCCGAA

Protein Expression

Medium: LB
Antibiotics: Kanamycin and Chloramphenicol
Procedure: A glycerol stock was used to inoculate a 15ml starter culture of LB with 50ug/ml Kanamycin and 34 ug/ml Chloramphenicol. The starter culture was grown overnight at 37ºC, shaking at 220rpm. The starter culture was used to inoculate 1l cultures supplemented with Kanamycin and Chloramphenicol at 5ml/l which were then grown at 37ºC, shaking at 180rpm, until reaching an optical density of 0.6-0.8. Cultures were induced with 0.4mM Isopropyl β-D-1-thiogalactopyranoside and cooled to 18ºC and left shaking overnight at 180rpm. Cultures were harvested by centrifugation at 5000g for 10 mins. Cell pellets were resuspended in 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5 % glycerol plus Merck Set III protease inhibitor and mixed by inversion before storage at -20°C.

Protein Purification

Procedure: Cells were lysed by sonication and lysates clarified by centrifugation at 21,000rpm following the addition of 0.125% polyethyleneimine. The resulting supernatant was incubated with Ni-IMAC resin (equilibrated in 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5 % glycerol), mixing by inversion, at 4ºC for 1 hour. This was centrifuged at 750g for 5mins, the

supernatant removed and resin resuspended in 50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5 % glycerol. This was applied to a gravity column and washed and eluted with 50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, and 30-250mM imidazole. Fractions containing protein (as seen by SDS-PAGE) were treated with TEV protease and 10mM DTT overnight at 4ºC prior to gel filtration on an S75 gel filtration column using 50 mM HEPES pH 7.5, 300 mM NaCl and 1mM TCEP as the running buffer. Additional purification was carried out by loading fractions from gel filtration onto a Ni-IMAC gravity column equilibrated in 50 mM HEPES pH 7.5, 300 mM NaCl and 1mM TCEP and collecting the flow through. Fractions containing purified protein (verified by SDS-PAGE) were concentrated to 18.25 mg/ml using a centrifugal concentrator.

Columns: Column 1: Ni Batch; Column 2: S75; Column 3: Ni rebind.
Concentration: 18.25 mg/ml
Mass-spec Verification: Intact mass verified by LC-MS as 11321.7 Da

Structure Determination

Crystallization: Protein was incubated with the peptide LTQVVHSAGRRFIVSPVPESRLR ("WNK1-RFXV-23-Ext1") at 2mM in 50mM HEPES pH 7.5 before filtration through a 0.22um spin filtration unit. Crystals were grown using the sitting drop vapor diffusion method at 20ºC. Crystals were grown in 150 nl drops consisting of 1:1 mother liquor (20% PEG3350 , 10% ethylene glycol, 0.2M sodium fluoride) to protein (12.0 mg/ml) with the peptide at 2mM. 25% ethylene glycol was added to the crystal as a cryoprotectant and the crystal was mounted and flash frozen in liquid nitrogen.
Data Collection: Data was collected at beamline I04 at 100K.