Entry Clone Source: TKC

Entry Clone Accession: n/a

SGC Construct ID: PAK4A-c010

GenBank GI number: gi|5031975

Final protein sequence (PAK4 GST-fusion):
mspilgywkikglvqptrllleyleekye
ehlyerdegdkwrnkkfelglefpnlpyy
idgdvkltqsmaiiryiadkhnmlggcpk
eraeismlegavldirygvsriayskdfe
tlkvdflsklpemlkmfedrlchktylng
dhvthpdfmlydaldvvlymdpmcldafp
klvcfkkrieaipqidkylksskyiawpl
qgwqatfgggdhppksdlevlfq*****gplgs
SPQREPQRVSHEQFRAALQLVVDPGDPRS
YLDNFIKIGEGSTGIVCIATVRSSGKLVA
VKKMDLRKQQRRELLFNEVVIMRDYQHEN
VVEMYNSYLVGDELWVVMEFLEGGALTDI
VTHTRMNEEQIAAVCLAVLQALSVLHAQG
VIHRDIKSDSILLTHDGRVKLSDFGFCAQ
VSKEVPRRKSLVGTPYWMAPELISRLPYG
PEVDIWSLGIMVIEMVDGEPPYFNEPPLK
AMKMIRDNLPPRLKNLHKVSPSLKGFLDR
LLVRDPAQRATAAELLKHPFLAKAGPPAS
VPLMRQNRTR

PreScission(TM) (rhinovirus 3C)- protease cleavable (*) GST tag

After cleavage:
gplgs SPQREPQRVSHEQFRAALQLVVDP
GDPRSYLDNFIKIGEGSTGIVCIATVRSS
GKLVAVKKMDLRKQQRRELLFNEVVIMRD
YQHENVVEMYNSYLVGDELWVVMEFLEGG
ALTDIVTHTRMNEEQIAAVCLAVLQALSV
LHAQGVIHRDIKSDSILLTHDGRVKLSDF
GFCAQVSKEVPRRKSLVGTPYWMAPELIS
RLPYGPEVDIWSLGIMVIEMVDGEPPYFN
EPPLKAMKMIRDNLPPRLKNLHKVSPSLK
GFLDRLLVRDPAQRATAAELLKHPFLAKA
GPPASIVPLMRQNRTR

Vector: pGEX-6P2. Details [PDF]; Sequence [FASTA] or [GenBank]

Host : BL21 (DE3)

Growth medium, induction protocol: Starter cultures from freshly transformed colonies in 10 ml LB, 0.1 mg/ml ampicillin were grown overnight. This was diluted 1:1000 in fresh media (6L) and was grown at 37°C to an OD_{600} of 0.3 and than transferred to 18°C. Expression was induced at an OD_{600} of 0.8 using 1 mM IPTG. Cells were harvested after 12h by centrifugation, transferred to 50-ml tubes, and frozen in liquid nitrogen.

Extraction buffer, extraction method: Extraction buffer: 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 1mM PMSF. The cell pellets (20 g wet wt) were re-suspended in 50 ml extraction buffer, and lysed by a high pressure cell disrupter. Supernatant was centrifuged for 30 minutes at 60,000 rpm.

Column 1 : Glutathione Sepharose 4B affinity, 5 ml of 50% slurry in 1.5 x 10 cm column, washed with binding buffer.

Buffers: Binding buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM DTT .

Procedure: Supernatant was applied at gravity flow, followed by a wash with 30 ml binding buffer. The GST-fusion was cleaved while bound to the column by addition of PreScission protease. The column was gently rotated overnight a 4°C. The protein was subsequently eluted with 3 bed volumes of binding buffer.

Column 2 : SEC (S75 or S200)

SEC-Buffers: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM DTT .

Procedure: Fractions containing PAK4 were concentrated and applied to a S75 gel fitration column equilibrated in SEC buffer. PAK4 eluted with a retention time corresponding to a monomeric protein of 40 kDa.

Protein concentration and characterization: Centricons 10 kDa cut off in same buffer. The mass of the recombinant Pak4 corresponded to the expected mass and one PO_4 moiety. The protein was >95% pure as judged by SDS PAGE. ESI-MS spectra showed that the recombinant protein had a molecular weight of 34456- which corresponds to the mono-phosphorylated protein, in agreement with the theoretical mass of 34376 Da

Crystallization:

Pak4 - CGP74514A complex: Pak4 was concentrated in the presence of 1 mM CGP74514A; N-(cis-2-Aminocyclohexyl)-N-(3-chlorophenyl)-9-ethyl-9H-purine-2,6-diamine to a final concentration of 10 mg/ml. The protein was crystallized is 96 well sitting drop Greiner plates using the vapour diffusion method at 4°C mixing 100 nl protein solution with a well solution containing 1.2 M ammonium sulfate, 15% PEG200 and 100 mM Tris pH 8.0. The crystals grew within 3 days and were frozen in a buffer containing the well solution and 25% ethylene glycole.

Pak4 apo hexagonal crystal form: Pak4 was concentrated to 10 mg/ml. The protein was crystallized is 96 well sitting drop Greiner plates using the vapour diffusion method at 4°C mixing 100 nl protein solution with a well solution containing 1.5M NaCl and 10% (v/v) ethanol. Crystals were frozen in the crystallization buffer containing 25% ethylene glycole.

Pak4 apo tetragonal crystal form: Pak4 was concentrated to 10 mg/ml. The protein was crystallized is 96 well sitting drop Greiner plates using the vapour diffusion method at 4°C mixing 100 nl protein solution with a well solution containing 0.20M K 3 (cit); 0.1M BTProp pH 6.5; 20.0% PEG 3350; 10.0% EtGly. Crystals were frozen in the crystallization buffer containing 15% ethylene glycole.

Pak4 substrate complex Pak4 was concentrated to 9.3 mg/ml. The protein was crystallized is 96 well sitting drop Greiner plates using the vapour diffusion method at 4°C mixing 100 nl protein solution with a well solution containing 1.7M ammonium sulfate, 15% PEG400, 0.1M Tris pH 8.0. Crystals were frozen in the crystallization buffer containing 20% ethylene glycole.

Data Collection & Resolution:

74 (2BVA) - 2.3Å ; **X-ray source:** SLS-X10

74a (2CDZ) - 2.3Å ; **X-ray source:** Rigaku FRE rotating anode generator

273 (2J0I) - 1.6 Å ; **X-ray source:** SLS-X10

74b (2QON) 1.75 Å ; **X-ray source:** SLS-X10