RAC + Cl

PDB:2G0N
Entry Clone Accession:RAC3A-s001
Entry Clone Source:MGC
Tag:N-terminal hexahistidine tag with a TEV cleavage site
Host:BL-21(DE3)R3

Construct

Prelude: This is a mutant form of RAC 3 with the Cys178 replace by Gly. The Cys178Gly mutation is indicated by *.
Sequence:

SMQAIKCVVVGDGAVGKTCLLISYTTNAFPGEYIPTVFDNYSANVMVDGKPVNLGLWDTAGQEDYDRLRPLSYPQTDVFLICFSLVS
PASFENVRAKWYPEVRHHCPHTPILLVGTKLDLRDDKDTIERLRDKKLAPITYPQGLA MAREIGSVKYLECSALTQRGLKTVFDEA
I RAVLG*

Vector:pNIC28-Bsa4

Growth

Procedure: Freshly transformed E. coli cells was used to inoculate 1 litre of TB plus 100 mg/mL ampicillin. When OD_{600} reached ~0.5 the temperature was shifted down from 37 degC to 25 degC for 1 hour before induction with the addition of 1 mM IPTG. Protein expression was allowed to carry on for a further 4 hours before harvest.

Purification

Buffers
Wash buffer I (WBI): 50 mM Tris pH 8.0, 150 mM NaCl, 5% Glycerol, 10mM MgCl2, 10mM Imidazole pH 8.0.
Wash buffer II (WBII): 50 mM Tris pH 8.0, 150mM NaCl, 5% Glycerol, 10mM MgCl2, 30mM Imidazole pH 8.0.
Elution buffer (EB): 50 mM Tris pH 8.0, 150 mM NaCl, 5% Glycerol, 10mM MgCl2, 250mM Imidazole pH 8.0.
GF buffer: 50 mM Tris pH 8.0, 500 mM NaCl, 0.5mM TCEP
Procedure
Low pressure chromatography using Bio-Rad Econo column (2.5 cm x 13 cm).

Total volume of Ni-NTA added to BioRad drip column: 4 mL (50%). Resin washed with 12.5 mL of WB1. The supernatant was applied to a column using 5 mL pipette and allowed to pass over the resin. The flow through was collected in a 50 mL Falcon tube and applied once more to the column. Two wash steps followed. Wash with 12.5 ml of WBI. Wash with 12.5 ml column vols of WBII. Elute with 14 mL of EB into 7x2 mL fractions.

Nucleotide Exchange and TEV cleavage: After elution the protein was concentrated to a final volume of 5 mL for nucleotide exchange and the protein concetration measured. To this was

added a 25-fold excess of Gpp(NH)p, 5 mM EDTA, 1 mM DTT 100 µL of TEV protease and 14 microlitres of calf alkaline phosphatase (10,000U/mL), mixed and left overnight at 4 degC.

The next day $MgCl_2$ was added to a final concentration of 10 mM before gel filtration.

Any remaining TEV protease and uncleaved RAC3 was removed by placing 200 mL of 50% Ni-NTA agarose in a 1.5 mL eppendorf tubes, add 1 mL of GF buffer mix, spin down and remove buffer. Repeat this resin wash step once.

Add the TEV treated protein sample to the resin and mix for 30 min. Finally spin down resin and collect the supernatant which contains the cleaved RAC3A.

Extraction

Buffers
EX: 50 mM Hepes pH 8.0, 150 mM NaCl, 5% Glycerol, 10 mM Imidazole pH 8.0, 10 mM $MgCl_2$.
Procedure
1 tablet protein inhibitor in 10ml EX buffer was added to the 1L growth pellet. Total vol: 45 mL (estimate).
Cell breakage: 5 passes through the Emulsiflex C5 high pressure homogeniser. Total vol: 50 mL (estimate).
Centrifuge for 40 mins at 16000 rpm and 4degC to remove cell debris. Discard pellet.

All extraction steps were carried out at 4degC.
Concentration:RAC3A was concentrated to 22 mg/ml before aliquoting into 50 microl volumes and freezing in the -80°CC freezer.
MassSpec: Native Mass Spec for detection of non-covalently bound complexes indicated:

8+ charge state	exp mass	9+ charge state	exp mass	abs diff*	rel diff
A 2558.2	20 458	A 2274.0	20 457	+545	
B 2560.8	20 478	B 2276.4	20 479		$+Na^+$
C 2563.4	20 499	C 2278.4	20 497		$+2Na^+$

*rel. to 19 912
Peaks A/B/C correspond to RAC3A plus Na-GppNHp or Na-GTP (+545) with one or two additional Na ions.

Crystallization: Crystals grew from a 1:2 ratio mix of RAC3A(GppNHp)-to-reservoir (0.1M BIS-TRIS pH 5.5; 25 % PEG3350).
Data Collection: Resolution: 1.9 angstrom; X-ray source: Rotating anode, Rigaku FR-E superbright