4MR5 (complex with RVX208)

PDB Code: 4MR5

Material and Methods

Entry Clone Source: Synthetic

GI number: gi|4826806

Expressed sequence:

```
MHHHHHHSSGVDLGTENLYFQSMEQLKHCNGILKELLSKKHAAYAWPFYKPVDASALGLHD
YHDIIKHPMDLSTVKRKMENRDYRDAQEFAADVRLMFSNCYKYNPPDHDVVAMARKLQDVFEFR
YAKMPD
```

^ TEV cleave site

Amplified construct sequence:

```
CATATGCACCATCATCATCATCATTCTTCTGGTGTAGATCTGGGTACCGAGAACCTGTA
CTTCCAATCCATGGAACAACTGAAGCATTGCAATGGCATTCTTAAAGAGCTCCTGAGTA
AAAAACACGCCGCCTATGCGTGGCCATTTTATAAACCGGTGGATGCCTCTGCGCTGGGT
CTGCATGATTATCACGATATCATTAAACATCCGATGGATCTCTCAACCGTTAAACGTAA
AATGGAAAATCGCGATTATCGTGATGCCCAGGAATTTGCGGCGGATGTACGCCTCATGT
TTTCGAACTGCTACAAATATAACCCTCCAGATCACGATGTTGTGGCAATGGCACGAAAG
CTACAGGATGTATTTGAGTTCCGTTATGCCAAGATGCCAGATTGACAGTAAAGGTGGAT
ACGGATCCGAA
```

Vector: pNIC28-Bsa4

Tags and additions: Cleavable N-terminal His6 tag.

Host: BL21 (DE3)R3-pRARE2 (Phage resistant strain)

Growth medium, induction protocol: 10 ml from a 50 ml overnight culture containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol were used to inoculate each of two 1 liter cultures of TB containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. Cultures were grown at 37 oC until the OD600 reached ~2.5 then the temperature was adjusted to 18 oC. Expression was induced overnight using 0.1 mM IPTG at an OD600 of 3.0. The cells were collected by centrifugation and the pellet re-suspended in binding buffer and frozen.

Binding buffer: 50 mM HEPES pH 7.5; 500 mM NaCl; 10 mM imidazole, 5% glycerol.

Extraction buffer, extraction method: Frozen pellets were thawed and fresh 0.5 mM TCEP, 1 mM PMSF added to the lysate. Cells were lysed using sonication. The lysate was centrifuged at 17,000 rpm for 60 minutes and the supernatant collected for purification.

Column 1: Ni-affinity. Ni-sepharose (Amersham), 5 ml of 50% slurry in 1.5 x 10 cm column, washed with binding buffer.

Buffers:

Binding Buffer:50 mM HEPES pH 7.5, 500 mM NaCl, 5 mM imidazole, 5% glycerol.
Wash Buffer:50 mM HEPES pH 7.5, 500 mM NaCl, 30 mM Imidazole, 5% glycerol.
Elution Buffer:50 mM HEPES pH 7.5, 500 mM NaCl, 5% glycerol, 50 to 250 mM Imidazole (step elution)..

Procedure: The supernatant was loaded by gravity flow on the Ni-sepharose column. The column was then washed with 30 ml wash buffer at gravity flow. The protein was eluted by gravity flow by applying 5-ml portions of elution buffer with increasing concentration of imidazole (50 mM, 100 mM, 150 mM, 200 and 250 mM); fractions were collected until essentially all protein was eluted.

Enzymatic treatment: The N-terminal His tag was cleaved by treatment with TEV protease, overnight.

Column 2 : Size Exclusion Chromatography. Superdex S75 16/60 HiLoad

Buffers :10 mM HEPES, pH 7.5; 500 mM NaCl, 5% glycerol

Procedure : The protein was concentrated and applied to an S75 16/60 HiLoad gel filtration column equilibrated in 10 mM HEPES, pH 7.5; 500mM NaCl, 5% glycerol using an Ã□KTAexpress system.

Mass spec characterization: LC- ESI -MS TOF gave a measured mass of 12966 Da for this construct as predicted from the sequence of this protein.

Protein concentration: Protein was concentrated to 10 mg/ml using an Amicon 3 kDa cut-off concentrator

Crystallisation Crystals were grown at 4 oC in 150 nl sitting drops from a 1:2 ratio of a protein solution (6.7 mg/ml) to reservoir solution containing 0.20M Na(form), 0.1M BTProp pH 8.5, 20.0% PEG 3350 and 10.0% EtGly

Data Collection: Resolution: Crystals were cryo-protected using the well solution supplemented by 20 % ethylene glycol and flash frozen in liquid nitrogen.
X-ray source: Diffraction data were collected from a single crystal on a Rigaku FRE-E at a single wavelength of 1.54 Å and the structure was refined to 1.63 Å.
Phasing: The structure was solved by molecular replacement using an ensemble of known bromodomain structures as a starting model.