ETV1A + dsDNA (4B06) Materials & Methods

Entry clone source: MGC (Y329S & P427S mutations induced by PCR primers)

Entry clone accession: IMAGE:30345383

SGC Construct ID: ETV1A-c014

Vector: pNIC28-Bsa4. Details [PDF]; Sequence [FASTA] or [GenBank].

DNA sequence:

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CATATGCACCATCATCATCATCATTC
TTCTGGTGTAGATCTGGGTACCGAGA
ACCTGTACTTCCAATCCATGGGACCC
ACATCCCAACGGCGAGGATCACTTCA
GCTCTGGCAGTTTTTGGTAGCTCTTC
TGGATGACCCTTCAAATTCTCATTTT
ATTGCCTGGACTGGTCGAGGCATGGA
ATTTAAACTGATTGAGCCTGAAGAGG
TGGCCCGACGTTGGGGCATTCAGAAA
AACAGGCCAGCTATGAACTATGATAA
ACTTAGCCGTTCACTCCGCTATTACT
ATGAGAAAGGAATTATGCAAAAGGTG
GCTGGAGAGAGATATGTCTACAAGTT
TGTGTGTGATCCAGAAGCCCTTTTCT
CCATGGCCTTTTCAGATAATTGACAG
TAAAGGTGGATACGGATCCGAA
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Final protein sequence (His$_6$ affinity Tag sequence in lowercase):

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mhhhhhhssgvdlgtenlyfq^smGP
T**S**QRRGSLQLWQFLVALLDDPSNSHF
IAWTGRGMEFKLIEPEEVARRWGIQK
NRPAMNYDKLSRSLRYYYEKGIMQKV
AGERYVYKFVCDPEALFSMAF**S**DN

^ TEV protease recognition site

**Please note that there are two primer-induced mutations
present in comparison to the MGC sequence (Y329S & P427S) -
highlighted in bold and underlined above**
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Tags and additions: Cleavable N-terminal His6 tag

Host: BL21(DE3)-R3-pRARE2.

Growth Medium, Induction Protocol:
For the native protein: The expression plasmid was transformed into the host strain and plated on LB-agar containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol. Several colonies were combined to inoculate a 1ml culture in TB (+ 50 µg/ml kanamycin, 34 µg/ml chloramphenicol). The culture was grown overnight, glycerol was added to 15% v/v (from a 60% stock), and the resulting glycerol stock was frozen at -80°C in 100 µl aliquots. A loopful of cells from the glycerol stock was inoculated into 20-ml of TB medium containing 50 µg/ml kanamycin and 34 µg/ml chloramphenicol and grown overnight at 37°C. 2x 1L TB medium

(containing 50 µg/ml kanamycin) were each inoculated with 10 ml of the overnight culture and grown in 2.5L UltraYield baffled flasks until OD_{600} of 3.0. Cells were cooled to 18°C, IPTG added to 0.1mM and growth continued at 18°C overnight. The cells were collected by centrifugation then the pellets were scraped out and transferred to 50-ml Falcon tubes and frozen at -80°C.
For selenomethionine derivitised protein: The expression plasmid was transformed into the host strain and plated on LB-agar containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol. Several colonies were combined to inoculate a 1-ml culture in TB (+ 50 µg/ml kanamycin, 35 µg/ml chloramphenicol). The culture was grown overnight, glycerol was added to 15% v/v (from a 60% stock), and the resulting glycerol stock was frozen at -80°C in 100 µl aliquots. A loopful of cells from the glycerol stock was inoculated into 6x 10-ml of LB medium containing 50 µg/ml kanamycin and 35 µg/ml chloramphenicol and grown overnight at 37°C. Cultures were harvested by centrifugation and washed twice with M9 minimal medium and resuspended in 10 ml M9 minimal medium. 6x 1L M9 minimal medium (containing 0.4% glucose, 2mM $MgSO_4$, 0.1mM $CaCl_2$, 50 µg/ml kanamycin) were each inoculated with 10 ml resuspended cells and grown in 2.5L UltraYield baffled flasks until OD_{600} of 0.80. Selenomethioine was added to 25mg/L along with leucine, isoleucine and valine to 50mg/L and lysine, threonine, and phenylalanine to 100mg/L (all amino acids dissolved in 0.2M HEPES pH 7.5). Cultures were grown for a further 1.5 hours until OD_{600} of 1.2 and then cooled to 18°C for 1 hour. Additional selenomethioine was added (final total concentration of 75mg/L) and IPTG was added to 0.1 mM, and growth continued at 18°C overnight. The cells were collected by centrifugation then the pellets were scraped out and transferred to 50-ml Falcon tubes and frozen at -80°C.

Cell Extraction: Frozen cell pellets (approx 50g) were thawed briefly in a bath of warm water (20 - 37°C) then transferred to ice. Three volumes (i.e. 3 ml for every gram of cells) of lysis buffer was added. The cells were resuspended by agitating and disrupted sonication for 20 minutes on ice. Nucleic acids and cell debris were removed by adding 0.15% PEI (polyethyleneimine) from a 5% (w/v, pH 7.5) stock, stirring for 15 minutes, then centrifugation for 60 minutes at 25,000 x g.
Lysis buffer: 50 mM Na-phosphate buffer, pH 7.5, 500 mM NaCl, 10 mM imidazole, 5% glycerol, 0.5 mM TCEP, 1x Protease Inhibitors Cocktail Set VII (Calbiochem, 1/1000 dilution), and 15 units/ml Benzonase.

Column 1: Ni-affinity, HisTrap Crude FF, 5 ml (GE Healthcare)

Column 1 Buffers:
Affinity buffer: 50 mM Na-phosphate buffer, pH 7.5, 500 mM NaCl, 10 mM imidazole, 5% Glycerol, 0.5 mM TCEP
Wash buffer: 50 mM Na-phosphate buffer, pH 7.5, 500 mM NaCl, 20 mM imidazole, 5% Glycerol, 0.5 mM TCEP
Elution buffer: 50 mM Na-phosphate buffer, pH 7.5, 500 mM NaCl, 300 mM imidazole, 5% Glycerol, 0.5 mM TCEP

Column 1 Procedure: The cell extract was loaded on the column at 4 ml/minute on an AKTA-express system (GE Healthcare). The column was washed with 10 volumes of lysis buffer, 10 volumes of wash buffer, and then eluted with elution buffer at 4 ml/min. The eluted peak of A280 was automatically collected.

Column 2: Gel filtration, Hiload 16/60 Superdex S75 prep grade, 120 ml (GE Healthcare)

Column 2 Buffers:
Gel Filtration buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP

Column 2 Procedure: The eluted fractions from the Ni-affinity Histrap column was loaded on the gel filtration column in GF buffer at 1.2 ml/min. Eluted proteins were collected in 2-ml fractions and analyzed on SDS-PAGE

Enzymatic treatment and Column 3: The N-terminal His6-tag was cleaved by incubating the protein overnight with TEV protease (at 8°C). Cleaved protein was purified by passing over a 2 ml pre-equilibriated 50% Ni-IDA bead solution. Elution was done in GF buffer supplemented with step gradient of 20mM, 40mM or 60mM imidazole, with flowthorough and fractions collected.

Column 4: Gel filtration, Hiload 16/60 Superdex S75 prep grade, 120 ml (GE Healthcare)

Column 4 Buffers:
Gel Filtration buffer: 10 mM HEPES, pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP

Column 4 Procedure: Collected fractions from the Ni-IDA IMAC column was loaded on the gel filtration column in GF buffer at 1.2 ml/min. Eluted proteins were collected in 2-ml fractions and analyzed on SDS-PAGE.

Concentration: The cleaved purified protein was concentrated in a VivaSpin4 (3 K MWCO) to 16.1 mg/ml and stored at 4°C. The protein concentration was determined spectrophotometrically using e280 = 34380.

Mass spec characterization:
Observed mass (taking into account the primer-induced mutations) was 12421.1 (calculated mass was 12421.0 without histidine tag). Observed mass of selenomethionine-derivitised protein was 12656.5 (calculated mass was 12656.0 with selenomethionines with no histidine tag, again taking into account the mutations).

dsDNA substrate preparation:
Oligonucleotides used in crystallization:
5'-ACCGGAAGTG
5'-CACTTCCGGT

Oligonucleotides were resuspended to 1000uM in 10mM Tris-HCl -pH 8.0 and 250ul of each mixed with 50ul 10x annealing buffer (1x is 10mM Tris pH 7.5, 50mM NaCl) by heating to 95°C for 5 min & cooling to RT overnight in a heating block. Thus dsDNA oligo was at 450uM and salt was 50mM NaCl.

ETV1A-dsDNA complex formation:
Frozen protein (at 16mg/ml = 1.2mM) was rapidly & span down hard. 16mg/ml = 1.2mM in GF buffer (10 mM HEPES, pH 7.5, 500 mM NaCl, 5% Glycerol, 0.5 mM TCEP). Diluted 1/3 in GF buffer but without NaCl, thus 166.6mM NaCl & 0.4mM protein (400 uM). Mixed with equal volume of dsDNA, thus ~protein:DNA was ~1:1.1. Kept on ice for complex to bind. Thus complex should now be at 0.2mM (equivalent to 2.7mg/ml protein) in ~130mM NaCl. Concentrated 5-fold in 4ml MWCO 3kDa concentrator to ~200ul. Concentration of protein was 12.5mg/ml (1mM).

Crystallization: The native crystal was grown by vapor diffusion at 4°C in 150nl sitting drops by mixing 50nl of protein solution (12mg/ml) and 100nl of precipitant consisting of 5.6% PEG 300, 5.6% PEG 400, 5.6% PEG 550 MME, 5.6% PEG 600, 5.6% PEG 1000, 200mM NaCl, 0.1M Tris pH 8.5 and 5% glycerol.
The selenomet labelled crystal was grown by vapor diffusion at 4°C in 150nl sitting drops by mixing 75nl of protein solution (14.4mg/ml) and 75nl of precipitant consisting of 20%(w/v) PEG 3350 & 0.2M potassium citrate.

Crystals were cryo-protected by bathing the crystal in mother liquor supplemented with 25% Ethylene glycol and flash-freezing in liquid nitrogen.

Data Collection: Diffraction data for the native crystals was collected at the Diamond synchrotron beamline I04, at a wavelength of 0.9795Å and the selenomet labelled crystal was collected at Diamond synchrotron beamline I03 at a wavelength of 0.9763Å.
The derivatised crystal dataset was used with the native dataset in a SIRAS experiment using SHARP, with solvent flattening using SOLOMAN, resulting in an electron density map clearly showing electron density for both the protein and DNA. The model was built using AutoBuild and manually in COOT. The structure was refined using autoBuster to final Rwork/RFree of 0.203/0.215. Deposited as PDB code 4B06.