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Recombinant production of Epstein-Barr virus BZLF1 trans-activator and characterization of its DNA-binding specificity

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ABSTRACT

This paper describes the recombinant production of a biologically active Epstein-Barr virus BZLF1 trans-activator, i.e., Z-encoded broadly reactive activator (ZEBRA), that recognized specific DNA motifs. We used auto-induction for histidine-tagged BZLF1 expression in *Escherichia coli* and immobilized cobalt affinity membrane chromatography for protein purification under native conditions. We obtained the purified BZLF1 at a yield of 5.4 mg per gram of wet weight cells at 75% purity, in which 27% of the recombinant BZLF1 remained biologically active. The recombinant BZLF1 bound to oligonucleotides containing ZEBRA response elements, either AP-1 or ZIIIB, but not a ZIIIB mutant. The recombinant BZLF1 showed a specific DNA-binding activity which could be useful for functional studies.

Introduction

Epstein-Barr virus (EBV)[1] BZLF1, also known as Z-encoded broadly reactive activator (ZEBRA), is a trans-activator which can switch EBV from a latent to a lytic life cycle. BZLF1 can bind to ZEBRA response elements (ZREs), including AP-1, ZIIIA and ZIIIB [1,2]. Previous studies have shown that recombinant BZLF1 proteins could not bind to a ZIIIB mutant (ZIIIBm) [1,2]. They have tested the DNA-binding specificity for recombinant BZLF1 proteins in whole cell extracts of *Escherichia coli* but not a purified form of BZLF1.

Because EBV infects more than 90% of adult population and involves in pathogeneses of a wide range of diseases, it is our interest to express and purify recombinant BZLF1 retaining specific DNA-binding properties for various functional studies, such as pull-down assays and cellular transduction assays. We used auto-induction media for protein expression in *E. coli* [3], and cobalt chelate membrane absorbers for His-tagged protein purification under native conditions. The purified BZLF1 was studied using electrophoretic mobility shift assay (EMSA) and showed to retain the specific DNA-binding activity.

Materials and methods

Construction of prokaryotic expression vector

Full length *BZLF1* (738 bp) of EBV, strain B95–8 (GenBank: V01555) was subcloned from a recombinant yeast expression vector, pYES2.1 (a generous gift from Prof. Sam Choon Kook) into a prokaryotic expression vector, pET102/TOPO-D (Invitrogen, Carlsbad, CA, USA), using the following procedures.

PCR was done using the recombinant pYES2.1 plasmid as DNA template, 5′-CACCATGATGGACCCAAACTCGAC-3′ as *BZLF1* forward primer (5′-CACC is required for TOPO directional ligation) and 5′-CTTATCGTCATCGTCGAAATTTAAGAGATC–3′ as *BZLF1* reverse primer (5′-CTTATCGTCATCGTC introduces an enterokinase recognition site to the fusion protein at the upstream of the V5 epitope and His_6 tags). The PCR product was purified. TOPO ligation reaction was prepared by mixing 4 μl of purified PCR product, 1 μl of pET102 vector (15–20 ng/μl), and 1 μl of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$), and incubated at room temperature for 15 min.

TOP10 *E. coli* competent cells (Invitrogen, Carlsbad, CA, USA) were transformed with the ligation reaction mixture by heat shock method. In brief, 1 μl of the ligation reaction mixture was added to 50 μl of the competent cells, gently mixed, and incubated on ice for 5 min. The cells were heat-shocked at 42 °C for 30 s, returned to ice

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[1] *Abbreviations used:* ZEBRA, Z-encoded broadly reactive activator; EBV, Epstein-Barr virus; ZREs, ZEBRA response elements; EMSA, electrophoretic mobility shift assay; IPTG, isopropyl-β-D-thiogalactopyranoside; OD, optical density; IDA, iminodiacetic acid; SDS, sodium dodecyl sulfate; PVDF, poly(vinylidene difluoride); TBS-T, Tris-buffered saline-Tween-20; BCIP/NBT, 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium; AP, alkaline phosphatase; TBE, Tris-borate-EDTA; EDTA, ethylenediaminetetraacetic acid; CAI, codon adaptation index; IMAC, immobilized metal affinity chromatography; K_d, dissociation constant; Rdbd, DNA-binding domain of RTA; RTA, replication and transcription activator; Ni-NTA, nickel-nitrilotriacetic acid.

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immediately, and grown in 250 μl of super optimal broth with catabolite repression media at 37 °C for 1 h with moderate shaking (250 rpm).

To select transformants, the bacterial cultures were grown in LB agar plate containing 100 μg/ml ampicillin at 37 °C overnight. Positive clones were identified by colony PCR, using *BZLF1* forward primer and T7 reverse primer (a pET102 vector primer). Plasmids from positive clones were purified, followed by DNA sequencing. Molecular Evolutionary Genetics Analysis 4.0 [4] was used to analyze the sequencing results.

IPTG (isopropyl-β-D-thiogalactopyranoside) induction of protein expression

Prokaryotic expression hosts, BL21Star (DE3) (Invitrogen, Carlsbad, CA, USA) and Rosetta-gami 2 (DE3) (Novagen, San Diego, CA, USA), were evaluated using IPTG induction. The competent cells were transformed with the recombinant pET102 using heat shock method. The starter cultures were grown in LB broth containing 100 μg/ml ampicillin, 37 °C overnight, 250 rpm.

Fresh LB broth (10 ml) was seeded with 1500 μl of a starter culture, and incubated at 37 °C, shaking at 250 rpm. At mid-log phase, the fresh culture was divided into half to assign IPTG-induced and uninduced cultures. IPTG solution (0.1 M) was added to the designated culture at a final concentration of 1 mM.

Once the inducer was added, 500 μl was withdrawn from each culture. The samples were centrifuged at maximum speed for 3 min. Supernatants were discarded. Pellets were labeled as 0-h time point and kept at −20 °C. Samplings were repeated at 1 h intervals until 4-h time point. The remaining cultures were incubated overnight, followed by final samplings for overnight time point.

Auto-induction of protein expression

A starter culture was prepared by growing the freshly transformed host cells in 10 ml of MDG non-inducing minimum media [3] containing 100 μg/ml ampicillin, at 37 °C overnight, 250 rpm. A negative control culture with mock-transformed expression host was prepared in media without antibiotic.

Auto-induction was done by growing the host cells (at 1000-fold dilutions of starter cultures) in 25 ml of ZYM-5052 media [3] containing 100 μg/ml ampicillin (in a 250 ml shake flask), at 28 °C overnight, 250 rpm. OD_{600} was measured. Samples were adjusted to OD_{600} of 1 and then harvested for SDS–PAGE analysis. The remaining auto-induced culture was harvested for protein purification.

Immobilized cobalt affinity membrane chromatography

The cell pellet (0.36 g, estimated from 5.19 ± 0.19 g of wet weight cells per liter culture) was resuspended in 15 ml of native purification buffer (50 mM NaH_2PO_4, 0.5 M NaCl, pH 8.0), and sonicated at 12 × 10 s with 10 s pauses at 250 W. The sonication cycle was repeated for another 3 times. The suspension was incubated on ice for 15 min between the sonication cycles to prevent overheating. The lysate was clarified by centrifugation at 12,000*g*, 4 °C for 1 h.

A membrane chromatography device was assembled and charged. In brief, a 0.45 μm syringe filter (25 mm diameter) was attached to the inlet (female Luer Lock) of 2 connected units of Sartobind IDA (iminodiacetic acid) 75 membrane adsorbers (Sartorius, Goettingen, Germany) to prevent clogging. The bed volume of the device is about 4.2 ml. Using a Luer Lock syringe, the device was equilibrated with 10 ml of equilibration buffer (0.1 M CH_3COONa, 0.5 M NaCl, pH 4.5), and charged with 10 ml of cobalt solution (equilibration buffer containing 0.1 M $CoCl_2$). To minimize the leaching of cobalt ion, the device was flushed with 10 bed volumes of equilibration buffer, followed by 10 bed volumes of native purification buffer.

The clarified lysate was loaded. About 1-ml fractions were collected continuously until the end of elution. Unbound materials were washed away by 10 bed volumes of native wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 8.0), followed by elution at 250 mM imidazole by native elution buffers (20 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole, pH 8.0).

SDS–PAGE

Discontinuous Tris–glycine gels (4% stacking and 10% separating polyacrylamide gels) were prepared using Mini-PROTEAN 3 (Bio-Rad, Hercules, CA, USA). Samples were treated with Laemmli sample buffer (62.5 mM Tris–HCl buffer (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue and 200 mM dithiothreitol), boiled for 10 min, and clarified by centrifugation at maximum speed for 5 min.

Supernatants of the samples (5 or 10 μl each) were applied to the vertical gels, and electrophoresed at 120 V for 90 min in running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3). The gels were stained with staining solution (0.2% (w/v) Coomassie brilliant blue R-250, 40% (v/v) methanol and 10% (v/v) acetic acid) overnight. Excessive dye was removed by placing the gel in destaining solution (5% (v/v) methanol, 7% (v/v) acetic acid). The gels were then digitized on a flatbed scanner.

Western blotting

Using the pooled, eluted fraction of BZLF1, 2 blots were prepared and probed with (i) antigen-specific primary antibody, mouse anti-EBV Bam HI Z, clone BDI506 (Abcam, Cambridge, MA, USA), and secondary antibody, goat anti-mouse IgG, alkaline phosphatase (AP) conjugate (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and with (ii) tag-specific anti-V5-AP antibody (Invitrogen, Carlsbad, CA, USA).

In brief, protein bands in SDS–PAGE gels were transferred to 0.45 μm poly(vinylidene difluoride), PVDF membranes (Millipore, Billerica, MA, USA) by Mini Trans-Blot cell (Bio-Rad, Hercules, CA, USA) at 50 V for 1 h 30 min in Towbin buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol, pH 8.3). The blots were blocked with milk blocking solution (KPL, USA) at 1:10 in sterile water for 1 h. Tris-buffered saline-Tween-20 buffer (TBS-T: 50 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.6) were used for washing and antibody dilution. One of the blots was washed 3 times with TBS-T buffer, and incubated with the antigen-specific primary antibody at 1:200 for 1 h. The blot was washed 3 times and probed with secondary antibody at 1:10,000. After another 3 wash steps, the blot was developed with BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) one-component phosphatase substrate (KPL, Gaithersburg, MD, USA). The color development was halted by rinsing the blot with sterile water, and air-drying. The blot was digitized on a flatbed scanner. The procedures were repeated to probe the other blot with anti-V5-AP antibody at 1:5000.

Protein determination

The pooled fraction of recombinant BZLF1 was buffer-exchanged to TBS buffer using Vivaspin 15R (Sartorius, Goettingen, Germany). The protein solution was diluted at 1:10 and assayed with Qubit protein assay kits (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.