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

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A B S T R A C T

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 and 5'-CTTATCGTCATCGTC-3' as BZLF1 reverse primer (5'-CACCC 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 His6 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 MgCl2), 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...
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. OD600 was measured. Samples were adjusted to OD600 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 NaH2PO4, 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 12,000g, 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 (iminoacetic 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 CH3COONa, 0.5 M NaCl, pH 4.5), and charged with 10 ml of cobalt solution (equilibration buffer containing 0.1 M CoCl2). 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-PRISEAN 3 (Bio-Rad, Hercules, CA, USA). Samples were treated with Laemmli sample buffer (62.5 mM Tris–HCl buffer (pH 6.8), 10% (w/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% (w/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 BD506 (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.
EMSA

Fluorescent oligonucleotides containing AP-1, ZIIIB, and ZIIIB mutant (ZIIIBm) and their complementary oligonucleotides (Table 1; Applied Biosystems, Foster City, CA, USA) were reconstituted at 100 μM in sterile water, and equal amounts of oligonucleotides were mixed and annealed by incubation at 94 °C for 10 min and cooled down to 16 °C. Unlabeled competitor containing AP-1 were prepared using the same steps.

BZLF1/AP-1 binding reactions were prepared using EMSA accessory kit (Novagen, San Diego, CA, USA), each at 0.5 × EMSA buffer (50 mM KCl, 10 mM HEPES, 0.2 mM EDTA, 25% (v/v) glycerol, pH 8.0), 0.25 mM dithiothreitol, 12.5 ng/μl sonicated salmon sperm DNA, 0.005 U poly(dI-dC)poly(dI-dC), 0.25 μg/μl BZLF1 and 0.01 μM fluorescent probe AP-1. To estimate the amount of active protein using competition assay, BZLF1/fluorescent probe AP-1 binding reactions were titrated with unlabeled competitor AP-1 (0.05, 0.1, 0.2, 0.4, 0.7, and 1.2 μM). The reaction mixtures were incubated at 25 °C for 30 min. A 6% non-denaturing polyacrylamide gel containing 0.5 × Tris–borate–EDTA (0.5 × TBE: 45 mM Tris, 45 mM boric acid, 1 mM EDTA) and 1% (v/v) glycerol was prepared using Mini-PROTEAN 3 (Bio-Rad, Hercules, CA, USA). The gel was prerun at 4 °C, 80 V for 30 min in 0.5 × TBE. The reaction mixtures (3 μl each) were loaded to the polyacrylamide gel and electrophoresed at 4 °C, 80 V for 1 h 30 min in 0.5 × TBE. The gel was digitized on Typhoon FLA 9500 (GE Healthcare, Piscataway, NJ, USA) using BPG1 (570DF20) filter.

To determine the protein/DNA binding specificity, all BZLF1/DNA binding reactions were prepared at 1 × EMSA buffer (100 mM KCl, 20 mM HEPES, 0.2 mM EDTA, 20% (v/v) glycerol, pH 8.0), 0.5 mM dithiothreitol, 25 ng/μl sonicated salmon sperm DNA, 0.01 U poly(dI-dC)poly(dI-dC), 0.1 μg/μl BZLF1, and 0.1 μM fluorescent probe. The reaction mixtures were incubated at 25 °C for 1 h. To minimize band smearing during electrophoresis, the reaction mixtures were diluted at 1:20 in EMSA sample buffer (0.5 × TBE, 6% (v/v) glycerol, 5 × 10⁻⁴% (w/v) bromophenol blue, and 5 × 10⁻⁴% (w/v) xylene cyanol FF). Low concentrations of loading dye were used to prevent background fluorescence. The samples (5 μl each) were applied to the gel, and electrophoresed at 4 °C, 80 V for 40 min in 0.5 × TBE. The gel was digitized on Typhoon FLA 9500 using BPG1 (570DF20), LPG (O575), and LPB (Y510) filters.

Results and discussion

Previous studies have shown that recombinant BZLF1 proteins in whole cell extracts of E. coli are biologically active with specific DNA-binding properties [1,2]. In this study, we are interested in producing purified recombinant BZLF1 with the same DNA recognition specificity for functional studies.

We chose pET102 expression system because the recombinant BZLF1 proteins are biologically active with specific efficiency and solubility of the target protein, and (ii) C-terminal V5 epitope and His6 tags that enable the immunodetection of the target protein [5,6]. Because His patch thioredoxin is a modified thioredoxin that possesses a metal binding domain, His patch thioredoxin and His6 tags allow immobilized metal affinity chromatography (IMAC)-based purification [5,6].

We tried BL21Star (DE3) and Rosetta-gami 2 (DE3) for BZLF1 expression. Despite Rosetta-gami 2 (DE3) contains pRARE2 plasmid supplying tRNAs for 7 rare codons (AUA, AGG, AGA, CUA, CCC, GGA, and CGG), BL21Star (DE3) expressed a higher level of BZLF1 (Fig. 1). The reason(s) remains unclear to us because (i) codon adaptation index (CAI) for native BZLF1 is 0.61, whereas 1.0 is the ideal CAI (Rare Codon Analysis Tools: http://www.gen-script.com/cgi-bin/tools/rare_codon_analysis/), and (ii) BZLF1 contains the 7 rare codons: 2 AUA, 1 AGG, 2 AGA, 3 CUA, 2 CCC, 4 GGA, 4 CGG (CodonW: http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::codonw).

We adopted auto-induction for recombinant BZLF1 expression because it is a “set and forget” approach and less laborious than conventional IPTG induction. We used MDG non-inducing media to prepare the starter culture, and ZYM-5052 media to grow and to auto-induce BL21Star (DE3) for protein expression. MDG media prevent the leakage of T7 lac promoter of the harbored vector, while ZYM-5052 media provide rich nutrients that are necessary for the expression of certain recombinant proteins in E. coli [3]. In addition, the cell densities obtained using auto-induction (6–8 OD600) were higher than that of IPTG overnight induction (3–4 OD600), which can maximize the yield of the target protein [3]. A prominent ~53-kDa band in the whole cell extract of BL21Star (DE3) transformant was shown in SDS–PAGE gel but not for the negative control (Fig. 2A, left panel), suggesting that the auto-induction was achieved.

To purify the His-tagged BZLF1, we employed membrane adsorbers, i.e., scale-down units that have 15 layers in each unit to carry metal-chelating groups of iminodiacetic acid. Membrane adsorber can be easily customized for IMAC-based purification because (i) its capacity can be multiplied by connecting to additional unit(s), (ii) it can be charged with the metal ion of choice, (iii) it is reusable by stripping and recharging of metal ion, and (iv) its flow rate can be controlled either by a syringe plunger or a peristaltic pump [7]. For our application, 2 connected membrane adsorbers and a syringe were sufficient. Despite cobalt ion has the least binding strength toward His-tagged protein among the commonly used metal ions, such as copper, nickel and zinc ions, we used cobalt ion to charge the purification device because it provides the greatest selectivity towards His-tagged protein [8]. Under native conditions, recombinant BZLF1 was eluted from the cobalt-charged membrane adsorbers using imidazole. A 53–54-kDa doublet in the pooled fraction was visualized in SDS–PAGE gel (Fig. 2A, right panel), showing that the recombinant BZLF1 is of 75% purity. The doublet was identified by both antigen-specific monoclonal antibody and tag-specific anti-V5-AP antibody in Western blotting (Fig. 2B), suggesting that the full length recombinant BZLF1 have been expressed and purified from E. coli. The yield of purified BZLF1 was

Table 1

<table>
<thead>
<tr>
<th>Probe (5′–3′)a</th>
<th>Dye</th>
<th>λmax/nm (absorption)</th>
<th>λmax/nm (emission)</th>
<th>Filterb</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-1 (NED-TCTTATGCTAGTGCTTC)</td>
<td>NED (yellow)</td>
<td>546</td>
<td>575</td>
<td>BPG1</td>
</tr>
<tr>
<td>ZIIIB (PET-CTAGGCGATGGTACGGATC)</td>
<td>PET (red)</td>
<td>556</td>
<td>595</td>
<td>LPG</td>
</tr>
<tr>
<td>ZIIIBm (6FAM-CTAGGCGATGGTACGGATC)</td>
<td>6FAM (blue)</td>
<td>494</td>
<td>518</td>
<td>LPB</td>
</tr>
</tbody>
</table>

a NED, PET and 6FAM fluorescent dyes are propriety to Applied Biosystems. Underlined sequences are ZREs; italicized sequence is a mutant that abolishes BZLF1 binding [1].
b Filters used in Typhoon FLA 9500 (GE Healthcare, USA).
28 mg per liter of the auto-induced culture, or 5.4 mg per gram of wet weight cells.

It should be noted that the calculated molecular weight for the recombinant BZLF1 is 44.3 kDa (26.9 kDa BZLF1 and 17.5 kDa fusion tags), whereas the apparent molecular weights for the recombinant BZLF1 are 53–54-kDa doublet (36–38-kDa doublet of BZLF1 [9–11] and 17.5 kDa fusion tags). The discrepancy in molecular weights may be due to a deviation from theoretical SDS/protein binding ratio [12]. However, the reason(s) for BZLF1 migrates as a doublet in SDS–PAGE, regardless of BZLF1 produced in cell lines (e.g., EBV-infected marmoset cell line B95–8, and superinducible P3HR1-derived cell line HH514.c16) or recombinant BZLF1 produced in E. coli [9–11], is unclear to us.

We did competition EMSA to estimate the amount of active BZLF1. The concentration of the purified BZLF1 used in the competition EMSA was 0.25 μg/µl. Because the purified BZLF1 had a purity of 75% and BZLF1 dimerizes to bind an oligonucleotide [13], we estimated that 0.25 μg/µl of the purified BZLF1 can form up to 2 μM of BZLF1 homodimer. Because the concentration of the BZLF1 homodimer was well above the dissociation constant, K_d (the concentration of active protein needed for 50% occupancy) of a DNA-binding protein with specific site (10–8 –10–10 M) [14] and the concentration of the fluorescent probe AP–1 was limited (10–8 M) (Fig. 3A, first lane), we can estimate the amount of active BZLF1 homodimer using competition EMSA. When the concentration of the unlabeled competitor was higher than the concentration of the active BZLF1 homodimer, we assumed that the unlabeled competitor interacts stoichiometrically with the active BZLF1 homodimer and starts to displace the bound fluorescent probe [15]. We observed a linear relationship between the percent unbound fluorescent probe and the concentration of the unlabeled competitor (Fig. 3B). At 50% of the displacement, one-half of the total concentrations of the oligonucleotides was equivalent to the concentration of the active BZLF1 homodimer, i.e., 0.55 μM, which accounted for 27% of the recombinant BZLF1. The percentage of active protein in the purified BZLF1 was lower than that of the purified Rdbd (DNA-binding
domain of RTA (replication and transcription activator) reported elsewhere, which is 70% [16]. Because purification causes loss of DNA-binding activity of a protein (partial denaturation and/or oxidation of important residues such as cysteines) [15] and the RdBD was purified using nickel-nitrilotriacetic acid, Ni-NTA agarose beads, the amounts of active protein remained using different cell lysis and purification methods warrant further investigations.

We did EMSA to examine the DNA-binding specificity for the recombinant BZLF1 (Fig. 4). Shifted bands were shown in the EMSA reaction mixtures for BZLF1/AP-1 and BZLF1/ZIIIB but not for BZLF1/ZIIIBm, which are in agreement with previous studies [1,2], suggesting that (i) the DNA-binding specificity for the recombinant BZLF1 remains intact, and (ii) the thioredoxin, V5 and His6 fusion partners neither provide steric hindrance to the DNA recognition domain of BZLF1 nor bind non-specifically to DNA. We successfully purified a biologically active BZLF1 trans-activator produced in E. coli.

Acknowledgments

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References