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Overproduction in *Escherichia coli* and purification of Epstein–Barr virus EBNA-1

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Abstract

Epstein–Barr virus nuclear antigen 1 (EBNA-1) is a multi-functional protein of the Epstein–Barr virus (EBV). Due to its low abundance in EBV-transformed cells, overproduction in a foreign host is preferred to obtain purified EBNA-1 protein. The EBNA-1 gene possesses a large number of *Escherichia coli* rare codons (23%). By using *E. coli* BL21(DE3)Rosetta2 cells that augment the low-abundance tRNA genes, the expression level of EBNA-1 in *E. coli* was greatly enhanced. EBNA-1 was then purified by applying the whole cell extract soluble fraction to a Ni–NTA Superflow column and eluting with an imidazole gradient. The improved overexpression in *E. coli* followed by a one-step Ni–NTA purification resulted in a sufficient amount of pure EBNA-1 protein to test DNA binding activity, and prepare and test EBNA-1-specific monoclonal antibodies (mAbs).

Keywords: EBNA-1; Rare codon

Epstein–Barr virus (EBV)[1] nuclear antigen 1 (EBNA-1) is responsible for a variety of functions within EBV-transformed cells. Through site-specific binding to the origin of plasmid replication (*oriP*), EBNA-1 is the only viral factor required in *trans* for efficient latent replication of the EBV genome [1]. EBNA-1 is essential for faithful segregation of the EBV nuclear plasmid to each daughter cell [2]. Additionally, EBNA-1 regulates transcription at multiple viral promoters [3–5] and has been shown to inhibit p53-mediated apoptosis in Burkitt's lymphoma cells [6].

EBNA-1 is not abundant in EBV-transformed cells and previously could not be expressed in *Escherichia coli* cells. The traditional EBNA-1 purification procedure is time-consuming and expensive [7]. In that procedure, Sf-9 insect cells are infected with baculovirus expressing EBNA-1. The nuclear extract is isolated and applied to a heparin–agarose column. The EBNA-1-containing fraction is applied to a DNA affinity column containing oligonucleotides encoding *oriP*. Finally the purified protein is concentrated using MonoQ chromatography.

We show that a derivative of EBNA-1, lacking approximately 225 residues of the Gly-Gly-Ala repeat though wild-type in function, has a large number of rare codons in its sequence. We used this derivative as it is commonly utilized in EBV studies. When the host is provided with tRNAs to the rare codons, His-tagged EBNA-1 can be expressed in *E. coli*. The active, overproduced protein can then be purified using Ni–NTA chromatography by applying the soluble fraction directly to the column and eluting with an imidazole gradient. The purified protein can bind DNA site specifically and was used to generate 11 new murine anti-EBNA-1 mAbs. These experiments indicate that for the first time, this fully functional derivative of EBNA-1 has been expressed in *E. coli*.

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[1] *Abbreviations used:* EBV, Epstein–Barr virus; EBNA-1, Epstein–Barr virus nuclear antigen 1; PCR, polymerase chain reaction; IPTG, isopropylthiogalactoside; PBST, phosphate-buffered saline with 0.01% Tween 20; GuHCl, guanidine hydrochloride; EMSA, electrophoretic mobility shift assay; SQ, subcutaneously; IP, intraperitoneally; ELISA, enzyme-linked immunosorbent assay; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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Materials and methods

Reagents and buffers

All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise specified. All restriction endonucleases were obtained from New England Biolabs (Beverly, MA). Ligations were performed with the Rapid DNA Ligation kit from Roche (Indianapolis, IN).

Oligonucleotides and DNA amplification and sequencing

All oligonucleotides for recombinant DNA construction were synthesized by the University of Wisconsin Biotechnology Center. All recombinant DNA constructs were sequenced at the McArdle Laboratory DNA Sequencing Facility using an ABI PRISM 373 DNA Sequencer (Applied Biosystems, Foster City, CA).

Plasmids

wtEBNA-1 (p1553) and Dom2 (p3015) were previously described [8,9]. wtEBNA-1 is a fully functional EBNA-1 variant that contains 15 residues of the Gly-Gly-Ala repeat present between amino acids 90 and 238 of EBNA-1 encoded by the B95-8 strain of EBV. It is widely used in EBV studies and although the sequence differs from EBNA-1 expressed by the B95-8 strain, the C-terminal His-tagged derivative of this protein will be referred to as wtEBNA-1 in this paper as it is wildtype in function. Dom2 additionally lacks 25 amino acids, 64–89, and inhibits transcriptional activation by EBNA-1. Polymerase chain reaction (PCR) was performed to amplify the wtEBNA-1 and Dom2 DNA fragments and add restriction enzyme sites; *Nde*I at the N-terminus and *Xho*I at the C-terminus, using the following primers: 5′ primer, 5′-ATCATATGTCTGA CGAGGGGCCAGGTAC and 3′ primer, 5′-TACTCGA GCTCCTGCCCTTCCTCACCCTC. The DNA fragment was gel purified using Qiagen Gel Extraction kit (Valencia, CA) and ligated into the pT7Blue-2 perfectly blunt cloning vector (Novagen, Madison, WI). The vector was digested with *Nde*I and *Xho*I, and ligated into the pET22b expression vector (Novagen). Sequencing confirmed the DNA constructs. wtEBNA-1-His-tag recombinant protein contains the plasmid 1553 EBNA-1 coding sequence, Leu-Glu from the *Xho*I sequence, and six His residues at the C-terminus. This protein has 417 amino acids and the monomer has an estimated molecular weight of 42,900 Da. Dom2-His-tag also contains Leu-Glu-His_6 at the C-terminus. It has 392 amino acids and the monomer has an estimated molecular weight of 40,150 Da.

Protein expression

Plasmids were transformed into *E. coli* BL21(DE3) Rosetta2 (Novagen). wtEBNA-1 was additionally cloned into *E. coli* BL21(DE3)pLysS (Novagen) for comparison. Transformants were cultured in LB broth containing 100 μg ampicillin/ml and 35 μg chloramphenicol/ml. Cultures were grown at 37 °C and induced at an OD (600 nm) of 0.6 with 1 mM isopropylthiogalactoside (IPTG) for 4 h.

SDS–PAGE and Western blotting

Proteins were separated by electrophoresis by SDS–PAGE using 4–12% Bis/Tris NuPAGE polyacrylamide gels (Invitrogen, Carlsbad, CA). Western blots were prepared by probing a 0.45 μm nitrocellulose membrane with anti-His-tag mAb (Novagen) or mAb 1EB14 (to be described elsewhere). After washing with PBST (phosphate-buffered saline with 0.01% Tween 20), a secondary antibody (goat anti-mouse IgG, Chemicon International, Temecula, CA) conjugated to alkaline phosphatase was added and the 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) reagent (Fisher Scientific, Fair Lawn, NJ) was used as substrate. Prestained molecular weight markers (Multimark, Invitrogen) were included on all gels.

Purification of His-tagged proteins

A frozen cell pellet (1 g wet weight) from 1 L of induced culture was resuspended in 20 ml low imidazole buffer (50 mM Tris–HCl, pH 7.9, 500 mM NaCl, 0.1% Tween 20, 5% glycerol, and 5 mM imidazole). Cells were lysed by sonication (3 rounds of 30 s bursts on ice). Cell debris and inclusion bodies were removed by centrifugation (27,000*g* for 20 min at 4 °C). The soluble fraction was incubated with 0.5 M guanidine hydrochloride (GuHCl) for 20 min on ice. Low GuHCl helps break up dimers without completely denaturing the protein. A 2 ml Ni–NTA Superflow column (Qiagen) was equilibrated with low imidazole buffer. After loading the soluble fraction, the column was washed with a buffer containing approximately 45 mM imidazole by using 95% low imidazole buffer and 5% high imidazole buffer (50 mM Tris–HCl, pH 7.9, 500 mM NaCl, 0.1% Tween 20, 5% glycerol, and 800 mM imidazole) for five column volumes. His-tagged proteins were eluted using a 45–522 mM imidazole gradient (5–65% high imidazole buffer) over a 30 min period with a flow rate of 1 ml/min. The column was washed with high imidazole buffer to remove any remaining bound proteins. One milliliter fractions were collected. The procedure was carried out on an AKTA purifier 10 (GE Healthcare, Piscataway, NJ). After combining the peak fractions, the purified protein was dialyzed into storage buffer (50 mM Tris–HCl, pH 7.9, 50% glycerol, 0.1 mM EDTA, 0.1 mM DTT, and 0.1 M NaCl) and stored at −20 °C.

Protein determinations

Protein concentration was determined by a modified Bradford assay [10] using Coomassie blue reagent and bovine serum albumin (Pierce, Rockford, IL) as a protein standard.