Preparative SDS-PAGE Electrophoresis of a Recombinant Epstein-Barr Virus Encoded Protein and Its Application in Serodiagnostic Test Systems

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Abstract

Serodiagnosis of Epstein-Barr virus (EBV) infection is currently based on the detection of antibodies to distinct EBV antigens. In the past few years the specificity and sensitivity of serodiagnostic assay systems have been considerably improved by the use of purified recombinant EBV antigens. After cloning and high-level expression of a particular viral protein as DHFR fusion protein in *Escherichia coli*, we purified the recombinant antigen to near homogeneity with the help of continuous elution electrophoresis. Sera from both EBV-positive and -negative donors were screened by immunoblot analysis and enzyme-linked immunosorbent assay for IgM and IgG antibodies against the EBV-encoded protein p23. The recombinant antigen seems to be a useful diagnostic marker for EBV infection, since antibodies were not detectable in 30 of 30 EBV-negative sera, and 294 of 302 EBV-positive sera contained either IgM or IgG anti-p23 antibodies (or both).

Introduction

In the field of clinical diagnosis the determination of specific antibodies against distinct structural or functional antigenic proteins of a given pathogen is the most commonly used diagnostic tool for the detection of viral infections. Although most of the established test systems still use natural antigen from different sources, the advent of genetic engineering opens up the possibility of producing proteins of limited natural availability. Recombinant technology has already proven to be an excellent alternative for the production of specific antigens, which are able to improve sensitivity as well as specificity of derivative test systems. In general, the production of recombinant antigens for diagnostic purposes is inexpensive compared to the use of purified natural antigens. The major problems associated with such test systems are identification of viral antigens that guarantee a certain serological diagnosis, their expression, and, most importantly, the subsequent purification of the recombinant proteins to near homogeneity.

Epstein-Barr virus (EBV) is an ubiquitous human B-lymphotropic herpesvirus. It is the causative agent of infectious mononucleosis, a mostly self-limiting lymphoproliferative disease, and is closely associated with an increasing number of severe human malignancies. Therefore, an early and accurate diagnosis as well as the follow-up of severe infections are of vital importance.

Previous experiments by our group led to identification of a 23 kD viral protein recognized by antibodies from more than 96% of EBV carriers tested. After identification of the coding region, the corresponding fragment of the viral genome was cloned into a suitable prokaryotic expression vector and, following a careful optimization process, the recombinant protein was expressed in *E. coli* cells at a very high level.

Here we report the large-scale purification of the EBV-encoded 23 kD protein to near homogeneity by a combination of urea-solubilization and preparative continuous elution SDS-PAGE electrophoresis using the Bio-Rad Model 491 prep cell device. Following prep cell purification, the recombinant antigen was ready to use in serodiagnostic test systems. The presented purification strategy can be adapted to a variety of recombinant proteins.

Methods

Cloning and Expression of the Viral Protein in *E. coli*

Since we were interested in both cloning and expressing the entire coding region of the protein, and no appropriate restriction sites were present at the genomic level, PCR was used to modify the ends of the target gene. With this approach, it was possible to construct a recombinant pQE-40 expression vector (QIAGEN GmbH, Hilden, Germany) that harbors the coding region in a proper reading frame downstream of the murine dihydrofolate-reductase (DHFR) gene. The plasmid was introduced into a suitable *E. coli* strain and protein production was induced with IPTG (isopropyl-β-D-thiogalactopyranoside). Bacterial cells were harvested by centrifugation, resuspended in three volumes of sonication buffer (50 mM Na-phosphate, pH 7.8, 300 mM NaCl, 0.1 mM phenylmethylsulfonylfluoride), containing 5 µg/ml DNase I and 1 mg/ml lysozyme, and

incubated for 10 min at room temperature. Subsequently, the suspension was sonicated on ice (150 W, 5 x 30 sec) and centrifuged at 4,000 x g for 10 min, and aliquots were analyzed by SDS-PAGE to monitor expression of the recombinant protein (Mini-PROTEAN® II electrophoresis system).

The recombinant p23-DHFR fusion protein was expressed at a high level (constituting up to 50% of the total cellular protein), and a preferential localization in inclusion bodies was observed. As demonstrated in Figure 1, the supernatant contained little p23-DHFR (lane 1), and most of the recombinant protein was located in the insoluble fraction (lane 2). Since this insoluble fraction consists of some *E. coli* proteins, bacterial cell wall debris, and p23-DHFR, inclusion body-directed expression represents a convenient purification step for this recombinant antigen.

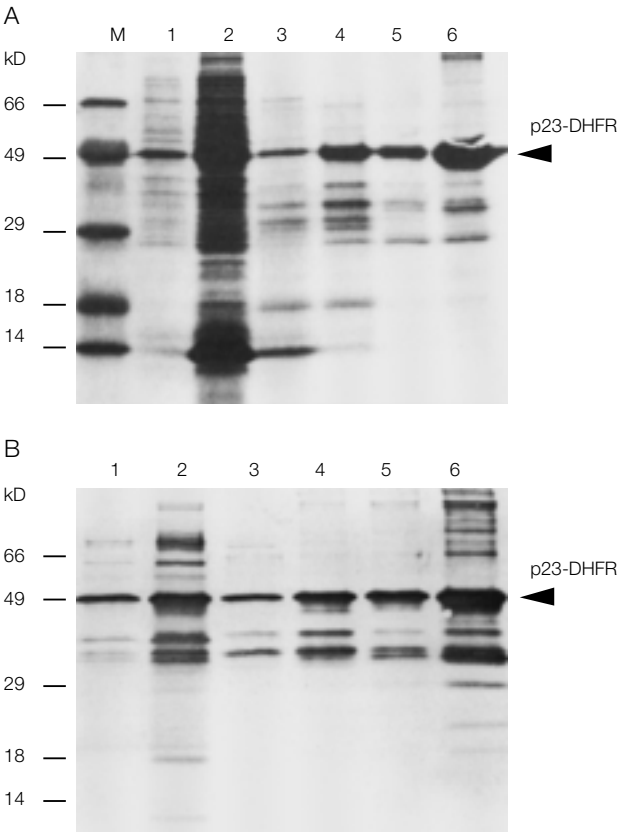

Fig. 1. Purification of the recombinant p23-DHFR protein analyzed by Coomassie Blue-stained SDS-PAGE (A) and immunoblotting with a pool of EBV-positive sera (B). Lane 1, soluble fraction of a crude sonication extract after IPTG induction; lane 2, insoluble fraction; lanes 3–6, supernatants after incubation of the insoluble fraction with suspension buffer containing 2 M urea, 4 M urea, 6 M urea, and 8 M urea, respectively. Based on the marker proteins (lane M), the position of the p23-DHFR protein is indicated.

Fractionated Solubilization of the Inclusion Body Preparation

To convert p23-DHFR into a soluble form, the inclusion body preparation was successively incubated with suspension buffer (0.1 M Na-phosphate, pH 8.0, 10 mM Tris-HCl, pH 8.0) containing 2 M, 4 M, 6 M, and 8 M urea, respectively. After centrifugation, each step of this solubilization procedure was monitored by analyzing the soluble and insoluble fractions on a Coomassie Blue-stained SDS-PAGE gel. Most of the contaminating *E. coli* proteins were solubilized at 2–6 M urea (Figure 1, lanes 3–5) and were discarded with the supernatants, whereas most of the 49 kD recombinant antigen (23 kD p23 + 26 kD DHFR) was solubilized at 8 M urea and thus separated from bacterial cell debris, which remains insoluble. The corresponding immunoblot, developed with a pool of EBV-positive sera, demonstrates the immunological reactivity of the expressed protein (Figure 1B). Nevertheless, additional purification steps were necessary to obtain the recombinant protein in such a purity that it could be used as an antigen in immunological test systems.

Usually, metal chelate affinity chromatography (MCAC) is recommended for the purification of His-tagged proteins. In the presence of 8 M urea, which was essential to keep the protein in solution, no satisfactory purification could be observed during MCAC procedures (data not shown). Therefore, continuous elution electrophoresis was applied for a further purification of the recombinant protein solubilized in 8 M urea.

Preparative SDS-PAGE

To reduce the sample volume, the *E. coli* inclusion body preparation (suspension buffer, 8 M urea) was dialyzed extensively against Tris-HCl (50 mM, pH 8.0). The white precipitate was collected and boiled in 1 ml sample buffer for 5 min. The sample was loaded onto a 12% cylindrical SDS-PAGE gel and electrophoresed as indicated in Table 1. Collection of 2 ml fractions was started when the Bromophenol Blue band passed the gel matrix.

Table 1. Model 491 Prep Cell Running Conditions

Resolving gel	12% acrylamide/2.6% C (cross-linker)
Resolving gel length	5.5 cm in a 28 mm gel tube
Resolving gel buffer	0.375 M Tris-HCl, pH 8.8
Stacking gel	4% acrylamide/2.6% C
Stacking gel buffer	125 mM Tris-HCl, pH 6.8
Running buffer	25 mM Tris-HCl, 0.192 mM glycine, 0.1% SDS
Elution buffer	25 mM Tris-HCl, 0.192 mM glycine, 0.1% SDS
Sample buffer	62 mM Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 1 mM EDTA, 0.005% Bromophenol Blue
Sample	The inclusion body preparation (solubilized in 6 M urea) was precipitated and resuspended in 1 ml sample buffer
Elution rate	0.2 ml/min
Fraction size	2 ml
Power	Constant current, 40 mA

Following electrophoresis, 200 µl aliquots of every fifth eluted fraction were TCA-precipitated and analyzed by Coomassie Blue-stained SDS-PAGE and immunoblotting for the presence of p23-DHFR (Figure 2). As confirmed by high-sensitivity immunoblot analysis, the protein preparation was purified to near homogeneity in the course of the electroelution procedure. Only a single protein band, corresponding to the 49 kD recombinant p23-DHFR, was visible in lanes 7–9 (Figure 2B).

Estimation of protein concentration demonstrated that the p23-DHFR-positive fractions contained approximately 10% of total applied protein. As urea was removed during the electrophoresis procedure, only renatured recombinant protein could be eluted in a soluble form. The decrease in yield is probably due to precipitation of insoluble protein within the PAGE column.

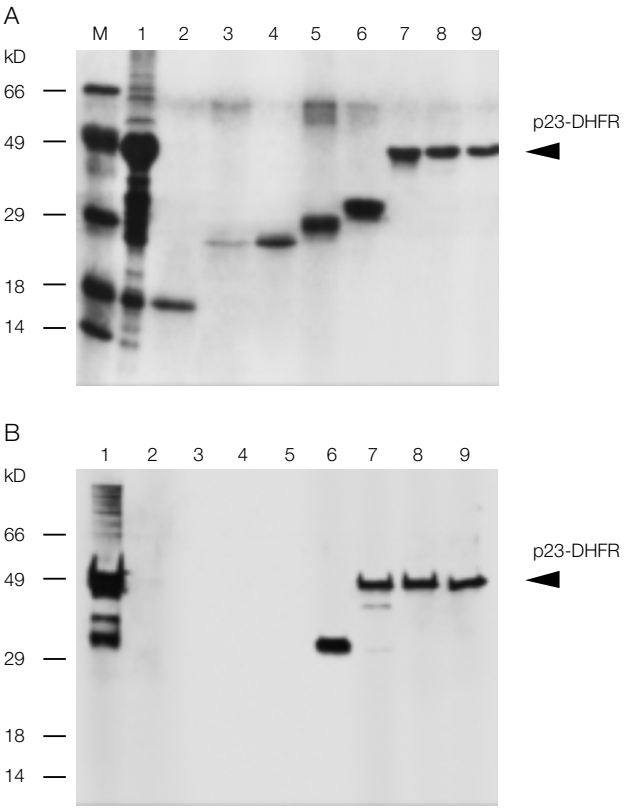

Fig. 2. Purification of p23-DHFR protein by preparative continuous electroelution using the prep cell device. Eluted fractions were analyzed both by Coomassie Blue-stained SDS-PAGE (A) and immunoblotting with a pool of EBV-positive sera (B). Lane 1, 8 M urea supernatant before application onto the electrophoresis device; lanes 2–9, fractions eluted during electrophoresis. Based on the marker proteins (lane M), the position of the p23-DHFR protein is indicated.

Immunoblot Analysis

To evaluate the diagnostic potential of the EBV-encoded recombinant protein in a more systematic manner, immunoblot and ELISA tests were carried out to screen a representative panel of EBV-positive and -negative human sera for anti-p23 IgM- and IgG-specific antibodies.

For immunoblot analysis, the purified p23-DHFR protein preparation (Figure 2B, lane 9) was electrophoresed and transferred to a 0.45 µm nitrocellulose membrane via blotting (Trans-Blot® SD semi-dry transfer cell). Following Poinceau Red staining, membranes were washed twice with phosphate buffered saline (PBS, pH 7.2), and nonspecific binding of antibodies to the membrane was blocked by incubation with 5% nonfat milk in PBS for at least 1 hr at room temperature. After being washed three times with PBS/Tween (PBS containing 0.05% [v/v] Tween 20), the membranes were subsequently incubated with diluted human sera (1:100 in 5% nonfat milk/PBS) for 1 hr. Following serum incubation, blots were washed three times with PBS/Tween and incubated with horseradish peroxidase-conjugated goat anti-human IgG antibody. After a 2 hr incubation at room temperature, blots were washed three times with PBS/Tween, and color was developed with HRP color development reagent DAB.

As illustrated in Figure 3, p23-DHFR was highly antigenic with both NPC (n2267, n243) and other EBV-positive sera (1731 to 1624), and showed no reactivity with EBV-negative sera (1626, 1197). Again, the purity of the protein preparation was demonstrated with these individual sera, as not even marginal reactivities were observed to any proteins different from the 49 kD recombinant antigen.

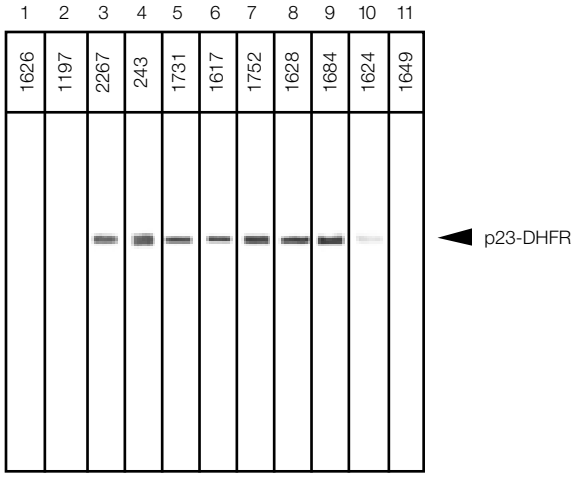

Fig. 3. Immunoblot analysis of the IgG-reactivity of a selected panel of EBV-positive and EBV-negative human sera to p23-DHFR. Blot strips contain electroelution-purified recombinant antigen electrophoresed on 12% SDS-PAGE. Strips 1 and 2 were incubated with EBV-negative sera, strips 3–11 were incubated with sera from EBV-positive individuals. According to a mixture of marker proteins, the position of the p23-DHFR is indicated.

Conclusions

This report describes the purification of a recombinant antigen that was expressed in *E. coli* cells at such a high level that it constituted up to at least 50% of total cellular protein. After sonication, most of the recombinant protein was located in the insoluble fraction. Initial purification of the recombinant protein was performed by fractioned solubilization in buffers containing rising concentrations of urea. Optimization of this technique can overcome, in some cases, the application of tedious and yield-reducing standard protein purification procedures. Although the urea solubilization procedure is capable of separating most of the contaminating bacterial proteins, additional purification steps were necessary to obtain the recombinant protein in such a purity that it could be used as antigen in immunological assay systems. As confirmed by extensive immunoblot analysis, size-dependent fractionation of the protein mixture by continuous elution electrophoresis turned out to be the method of choice for an efficient one-step purification of recombinant antigens to near homogeneity at a preparative scale.

As the urea within the applied sample is slowly removed during the electrophoresis procedure, the recovered protein is, in contrast to the applied protein, renatured and completely soluble in standard electrophoresis buffer. Due to incorrect refolding and precipitation within the electrophoresis column, however, a reduction in the overall yield may be observed. We have purified more than 30 recombinant antigens by continuous elution electrophoresis so far, and, following the presented protocol, the observed yields are ranging from 5 to 40% of total applied protein. Like most of our remaining recombinant antigens, expression of the recombinant p23-DHFR protein from a 250 ml bacterial culture and subsequent purification via the Model 491 prep cell provided pure antigen sufficient for coating more than 400 standard 96-well ELISA plates for optimal results.

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