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PURIFICATION OF RECOMBINANT EBOLA VIRUS GLYCOPROTEIN AND VP40 FROM A HUMAN CELL LINE

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14. ABSTRACT:
The recent, widespread Ebola virus outbreak that originated in West Africa underscores the need for novel diagnostic and therapeutic tools to treat and contain future outbreaks. In line with this goal, the in-house production of Ebola antigens allows researchers to develop and test novel reagents without the requirement for high-level biological containment. In this report, we describe procedures for the recombinant expression and purification of the Ebola Zaire structural glycoprotein and matrix protein VP40 from a human cell line. Plasmids coding for the expression of these proteins were transiently transfected into human embryonic kidney cells 293 and secreted into the culture medium. Next, purification proceeded using immobilized metal affinity chromatography and anion exchange chromatography. The typical yield for each of these proteins was approximately 1 mg/30 mL of cell culture with >90% purity. The procedures described herein may be useful for research efforts involving antibody discovery and immunoassay development targeting the Ebola virus.

15. SUBJECT TERMS

Ebola virus (EBV)	Glycoprotein (GP)	Protein purification	Antigen
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PREFACE

The work described in this report was authorized under Defense Threat Reduction Agency project number CB3636. The work was started in April 2015 and completed in January 2016.

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1. INTRODUCTION

1.1 Background

The 2014 Ebola epidemic that originated in West Africa underscored the need for vaccines, diagnostics, and readily available therapeutics for curtailing the spread of the lethal Ebola virus (EBV). Currently, no licensed treatments exist for EBV infection, and care is often supportive (i.e., administration of intravenous fluids and oxygen). Although some antibody-based drugs, such as ZMapp (Qiu et al., 2014), are under development, the safety and efficacy associated with the widespread use of such drugs is unknown. To develop additional novel reagents that can be used to prevent, diagnose, and treat EBV infection, it is often necessary to produce recombinant viral proteins for research and development efforts. In this study, we describe procedures for the recombinant expression and purification of two such proteins, the EBV structural glycoprotein (GP) and a viral matrix protein (VP40) from the Zaire viral strain.

1.2 Study Objective

The purpose of this study was to characterize the eukaryotic expression and purification of recombinant EBV GP and VP40.

1.3 EBV GP

Structural GP is believed to be the only viral protein responsible for entry into host cells (Takada et al., 1997; Wool-Lewis and Bates, 1998), and thus, it is a logical target for vaccine and therapeutic development. EBV GP is a large, heavily glycosylated protein. It is generally believed that synthetic peptides do not represent immunologically relevant epitopes for highly glycosylated proteins (Sanchez et al., 2001). As such, development efforts for vaccines, immunoassays, and therapeutics should be directed toward a full-length, mature, and fully modified EBV GP to properly target functionally significant conformational epitopes of the protein. The procedure described in this report produces GP in a human cell line in an effort to maintain native conformation and glycosylation patterns.

1.4 EBV VP40

The EBV VP40 protein is a matrix protein that is essential for viral binding and budding at the host membrane during replication. It has been demonstrated that expression of VP40 in mammalian cells is sufficient to induce the formation of viral-like particles similar to those produced by a wild-type virus (Noda et al., 2002); therefore, VP40 may be a therapeutic target (Stahelin, 2014). As the most abundantly expressed protein of the EBV genome, VP40 also represents a logical target for protein-based viral diagnostics.

2. MATERIALS AND METHODS

2.1 Cells and Plasmids

EBV GP and VP40 were expressed in human cells as secreted proteins. The pCL mammalian expression vector was used to express residues 1–649 of the wild-type EBV Zaire GP sequence with the addition of a C-terminal hexahistidine tag for purification. This represents the full-length wild-type protein with a truncation of the C-terminal transmembrane domain. A pFLAG-CMV mammalian expression vector was used to express residues 1–326 of the EBV Zaire VP40 protein sequence. The recombinant protein contains an N-terminal hexahistidine tag followed by maltose binding protein to assist in protein folding for purification. This is connected to VP40 at the C-terminus by a 14 amino acid linker, which contains an AcTEV protease recognition site.

The human Expi293 Expression System (ThermoFisher Scientific; Waltham, MA) was used for transient protein expression. Expi293F cells were derived from the line of human embryonic kidney cells 293 (i.e., HEK293 cells), and they were grown in a humidified incubator at 37 °C and 8% CO_2, with shaking at 130 rpm.

2.2 Protein Expression in Expi293 Cells

Transient transfection and expression of EBV GP and VP40 proteins in Expi293 cells were conducted in accordance with the manufacturer's protocol. Essentially, Expi293 cells were grown to a density of 2.5×10^6 cells/mL in a T-75 flask containing 25 mL of Expi293 expression medium. Thirty micrograms of plasmid DNA were added to Opti-MEM medium (ThermoFisher Scientific) to form a final volume of 1.5 mL, and 80 µL of ExpiFectamine 293 reagent (ThermoFisher Scientific) was added to Opti-MEM medium in a separate container to form a final volume of 1.5 mL. These solutions were incubated for 5 min at room temperature, and then, they were combined and incubated at room temperature for an additional 20–30 min. The mixture was added to the cell culture, which was grown, as described in Section 2.1, for 16–18 h. Transfection Enhancers 1 and 2 were then added to the cell culture, and the cells were grown for an additional 3–6 days before harvesting, for a total of 4–7 days of expression.

After transfection, protein was harvested by centrifugation of the cell culture at 500×*g* for 10 min at 4 °C. The resultant supernatant was either used immediately or stored at –20 °C and thawed at room temperature before purification.

2.3 EBV GP Purification

Approximately 240 mL of cell culture supernatant was dialyzed against 4 L of 20 mM HEPES, pH 7.3, and 200 mM NaCl at 4 °C with one buffer change after 4 h to reduce the concentration of metal ion chelators in the cell culture medium. Buffer 1 (20 mM HEPES, pH 7.3, 200 mM NaCl, and 40 mM imidazole) was used to equilibrate 10 mL of bulk HisPur Ni-NTA resin (ThermoFisher Scientific) and then added to the buffer-exchanged cell culture supernatant and stirred gently overnight at 4 °C. The following morning, the resin was captured on a fritted chromatography column and washed with 50 mL of Buffer 2 (20 mM HEPES,

pH 7.3, 75 mM NaCl, and 40 mM imidazole). GP was eluted with 20 mL of Buffer 3 (20 mM HEPES, pH 7.3, 75 mM NaCl, and 400 mM imidazole).

Eluate from the Ni-NTA column was then pumped onto two consecutive 5 mL HiTrap Q HP columns (GE Healthcare; Pittsburgh, PA) that were pre-equilibrated with Buffer 2 using a GE AKTA Fast Protein Liquid Chromatography (FPLC; GE Healthcare; London, UK) system. Buffer 4 (20 mM HEPES, pH 7.3, and 75 mM NaCl) was then washed over the columns to remove the imidazole until the 280 nm absorbance was restored to baseline. Elution was performed with a salt gradient, going from 100% Buffer 4 to 100% Buffer 5 (20 mM HEPES, pH 7.3, and 1 M NaCl) over a volume of 150 mL. Peaks corresponding to the expected molecular weight of EBV GP, as determined by electrophoresis (described in Section 2.5), were pooled, aliquoted, and snap-frozen in liquid nitrogen before being stored at –80 °C.

2.4 EBV VP40 Purification

EBV VP40 was purified from approximately 240 mL of cell supernatant by first dialyzing the sample against 4 L of 20 mM MOPS, pH 7.4, and 400 mM NaCl overnight at 4 °C. After filtration to remove particulates, the sample was loaded onto a 5 mL HisTrap HP column (GE Healthcare) using an AKTA FPLC system in buffer containing 20 mM MOPS, pH 7.4, 400 mM NaCl, and 40 mM imidazole. VP40 was eluted using a gradient from 40 to 400 mM imidazole over 30 min at 3 mL/min.

2.5 Electrophoresis and Western Blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was conducted on protein samples using NuPAGE (LifeTechnologies Corporation, Frederick, MD) 4–12% Bis-Tris 1.0 mm protein gels and NuPAGE LDS sample buffer under reducing conditions in accordance with the manufacturer's instructions.

2.6 Protein Concentration Determination

The concentrations of EBV GP and VP40 were determined using the Beer–Lambert Law and measuring the absorbance of the proteins at 280 nm. The theoretical extinction coefficient of each protein was used in the calculation (1.30 L/g-cm for recombinant GP and 1.14 L/g-cm for recombinant VP40).

3. RESULTS

3.1 EBV GP Purification and Yields

EBV GP was expressed in the human Expi293 cell line for up to 7 days; aliquots of medium were collected periodically during the course of expression. Western blotting of these cell culture supernatant samples revealed that optimal protein expression with minimal degradation occurred after 4 days of induction (Figure 1), and GP was harvested at this time.

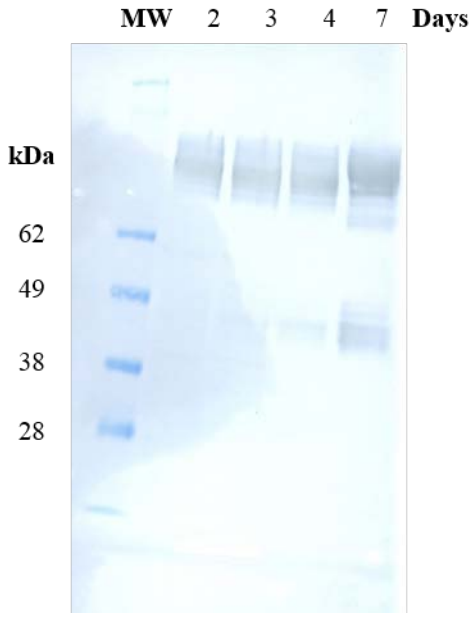

Figure 1. Time course of EBV GP expression in Expi293 cells. Western blot analysis revealed that after 4 days of induction, protein production appeared to be maximal with negligible degradation; the lower molecular weight species that appeared at approximately 40 kDa in the samples for days 4 and 7 was a degradation product. MW is the molecular weight marker.

Purification of EBV GP was first conducted by immobilized metal ion affinity chromatography (IMAC) anion exchange chromatography. Representative chromatogram and SDS–PAGE images of anion exchange chromatography samples are shown in Figure 2. Typically, 1 mg of purified protein was obtained from every 30 mL of cell culture medium, with >90% purity.

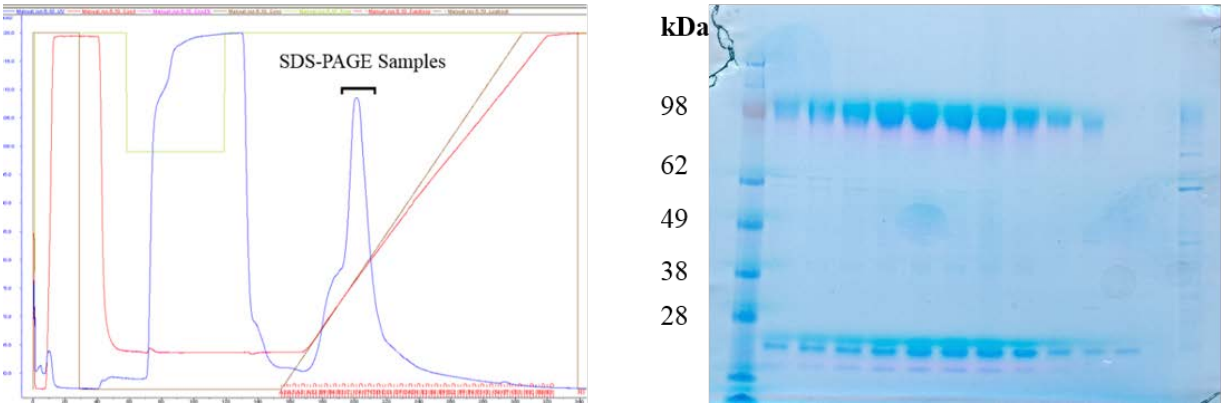

Figure 2. (Left) Chromatogram of EBV GP elutions from two consecutive Q columns and (right) SDS–PAGE image of resultant fractions. The expected molecular weight of recombinant EBV GP, based solely on amino acid composition, was 73 kDa. Because of its high level of glycosylation, our predominant purified product had an approximate molecular weight of 100 kDa, with a lower molecular weight species present.

In our experience with this construct, recombinant EBV GP purification has always resulted in two species: one of approximately 100 kDa and the other roughly at 20 kDa (Figure 2). In vivo, the protein is cleaved into two subunits (GP_1 and GP_2) by a host protease, and each subunit has different roles in the viral life cycle (Lee et al. 2010). GP_1 is the larger, N-terminal portion of the protein, and GP_2 is the smaller, C-terminal piece. To verify that the two species present after purification were in fact GP_1 and GP_2, as opposed to GP and an unspecified contaminant, we performed two sets of western blots using (1) a primary antibody from the ECBC Critical Reagents Program that is known to target the Ebola GP protein and (2) a primary antibody targeting the polyhistidine tag found on the C-terminus of our recombinant protein. The anti-GP antibody was found to bind to the larger species but not to the smaller one. The anti-polyhistidine antibody bound exclusively to the smaller species (Figure 3), which confirmed that the smaller molecular weight species was the C-terminal portion of the full length GP protein. This is consistent with the two purified species being GP_1 and GP_2 and inconsistent with either species being an unspecified contaminant.

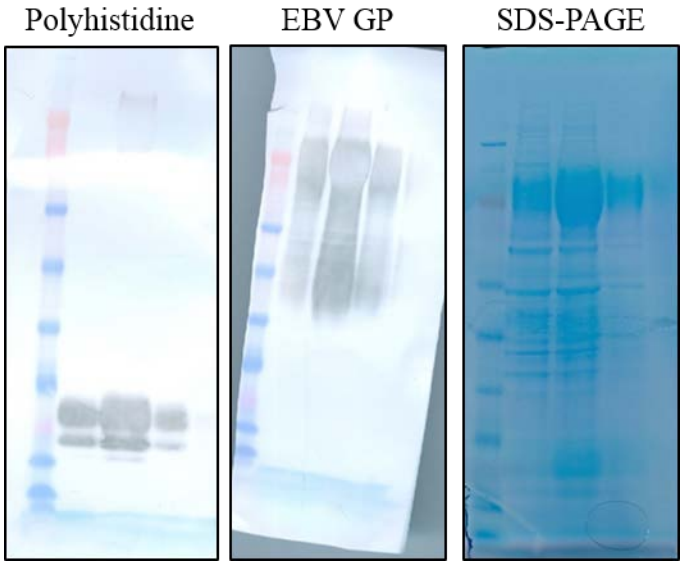

Figure 3. Western blots of crude cell supernatant from cells expressing EBV GP from three separate transfections (one per lane). (Left and middle) Primary antibodies targeting the C-terminal polyhistidine tag and EBV GP were used to identify protein components. (Right) SDS–PAGE gel of these samples is shown for reference.

3.2 EBV VP40 Purification and Yields

VP40 was expressed in Expi293 cells for 7 days before the cell supernatant containing the recombinant protein was harvested. The supernatant was then dialyzed overnight against a neutral buffer to reduce the concentration of nickel-chelating agents in the medium, after which IMAC was used to purify VP40. A typical chromatogram and SDS–PAGE gel of the purified recombinant VP40 are shown in Figure 4. The resultant protein was >90% pure, and typical yields were approximately 1 mg/30 mL of cell supernatant.

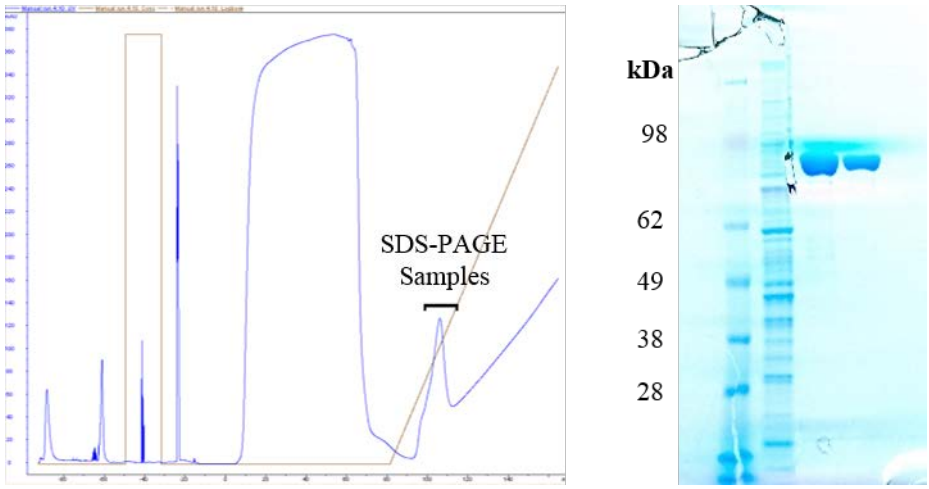

Figure 4. (Left) Chromatogram of a typical VP40 elution profile from a Ni-NTA column. The peak representing eluted VP40 is clearly visible above the 280 nm absorbance baseline, which is increasing as a result of the increasing imidazole concentration. (Right) An SDS–PAGE gel image. From left to right, the lanes are as follows: molecular weight marker, column flow through, and two samples from the elution peak. The recombinant VP40 protein has a molecular weight of approximately 78.2 kDa.

4. CONCLUSIONS

The methodology described in this report details the recombinant expression and purification of intact EBV Zaire GP and VP40 proteins from mammalian cells, with yields of approximately 1 mg/30 mL culture for each protein. GP was purified by an initial round of IMAC followed by two consecutive Q columns. The two molecular weight species that resulted from this procedure are likely GP_1 and GP_2. These occurred in vivo after GP was processed by native host enzymes. VP40 was purified by a single IMAC procedure. Using the procedures described in this study, researchers can generate GP and VP40 EBV antigens to support various research programs, such as antibody discovery or immunoassay development.

LITERATURE CITED

Lee, J.; Saphire, E.O. Ebolavirus Glycoprotein Structure and Mechanism of Entry. *Future Virol*. **2009**, *4*, 621–635.

Noda, T.; Sagara, H.; Suzuki, E.; Takada, A.; Kida, H.; Kawaoka, Y. Ebola Virus VP40 Drives the Formation of Virus-Like Filamentous Particles along with GP. *J. Virol*. **2002**, *76*, 4855–4865.

Qiu, X.; Wong, G.; Audet, J.; Bello, A.; Fernando, L.; Alimonti, J.B.; Fausther-Bovendo, H.; Wei, H.; Aviles, J.; Hiatt, E.; Johnson, A.; Morton, J.; Swope, K.; Bohorov, O.; Bohorova, N.; Goodman, C.; Kim, D.; Pauly, M.H.; Velasco, J.; Pettitt, J.; Olinger, G.G.; Whaley, K.; Xu, B.; Strong, J.E.; Zeitlin, L.; Kobinger, G.P. Reversion of Advanced Ebola Virus Disease in Nonhuman Primates with ZMapp. *Nature* **2014**, *514*, 47–53.

Sanchez, A.; Khan, A.S.; Zaki, S.R.; Nabel, G.J.; Ksiazek, T.G.; Peters, C.J. Filoviridae: Marburg and Ebola Viruses. In *Fields Virology*, 4th ed.; Knipe, D.M., Howley, P.M., Griffen, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E., Eds.; Lippincott-Raven Publishers: Philadelphia, PA, 2001, pp 1279–1304.

Stahelin, R. Could the Ebola Virus Matrix Protein VP40 Be a Drug Target? *Expert Opin. Ther. Targets* **2014**, *18*, 115–120.

Takada, A.; Robison, C.; Goto, H.; Sanchez, A.; Murti, K.G.; Whitt, M.A.; Kawaoka, Y. A System for Function Analysis of Ebola Virus Glycoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14764–14769.

Wool-Lewis, R.J.; Bates, P. Characterization of Ebola Virus Entry by Using Pseudotyped Viruses: Identification of Receptor-Deficient Cell Lines. *J. Virol*. **1998**, *72*, 3155–3160.

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ACRONYMS AND ABBREVIATIONS

EBV	Ebola virus
FPLC	fast protein liquid chromatography
GP	glycoprotein
IMAC	immobilized metal affinity chromatography
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
VP40	viral matrix protein

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