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Purification of Recombinant Vaccinia Virus-expressed Monomeric HIV-1 gp120 to Apparent Homogeneity

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Abstract

Vaccinia virus (VV) has been used to express a variety of heterologous proteins, including HIV envelope (Env) glycoproteins. The Env protein is synthesized as a precursor molecule, gp160, which is cleaved into the surface antigen gp120 and the transmembrane protein gp41. Even though production of gp160 by the VV expression system has been described, its use for gp120 production is not well documented. Here we report a new procedure for the purification of gp120 from serum-containing culture supernatant of VV infected cells. The gp120 protein was enriched to a purity better than 60% on a snowdrop (*Galanthus nivalis*) lectin affinity column in the presence of 0.25% zwitterionic detergent Empigen BB. After additional DEAE anion exchange and Superdex size exclusion chromatography steps, the gp120 monomer was purified free of contamination as determined by SDS-PAGE. The retention of structural integrity was confirmed by determining the affinity constant of purified gp120s to soluble CD4 and a monoclonal antibody IgG1b12, using surface plasmon resonance analysis. The purification procedure is robust and reproducible, and may find general use for glycoprotein purifications from sources where the presence of serum is desirable.

Keywords

Empigen BB; gp120; HIV envelope protein; Surface plasmon resonance; Vaccinia virus expression

Introduction

Vaccinia virus (VV), a member of the orthopoxvirus family, is a powerful tool for heterologous gene expression in mammalian cells [1–3]. Advantages of the VV expression system include cytoplasmic site of expression, a broad host range, and a large cloning

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capacity [4]. Among the many proteins expressed using VV vectors are those derived from the HIV envelope gene [5–7]. The envelope protein (Env) is produced as a single chain polypeptide, gp160, and is intracellularly processed by host cell furin or furin-like proteases into the surface antigen gp120 and the membrane bound gp41 [8, 9]. The Env proteins present on the surface of HIV virions and HIV infected cells are non-covalently associated trimers of gp120/gp41 heterodimers [10, 11]. As the target of broadly neutralizing antibodies as well as non-neutralizing antibodies that may contribute to protection against infection, Env is the focus of all antibody based HIV-1 vaccine design. The RV144 trial in Thailand employing a poxvirus priming and a gp120 boosting strategy showed a 31% efficacy against HIV infection, and demonstrated for the first time that vaccine prevention against HIV infection is possible [12].

In part because of the results from the RV144 trial, interest in examining gp120 as a potential vaccine immunogen has increased. Since differences in cell lines, growth conditions, and the expression vectors may contribute to the properties of the gp120 produced [13, 14], it is important to develop methodologies to allow the preparation of highly purified gp120 from different expression systems so that the factors that may affect its immunogenic properties can be better defined. However, very little information is available in the literature describing the purification and characterization of HIV-1 gp120 from a mammalian source other than stably transfected CHO cells or transiently transfected human embryonic kidney cells. Our group has used different forms of Env proteins, including gp120, gp160, and gp140 (a truncated form of gp160 lacking the transmembrane sequence and the cytoplasmic tail [15]) from VV-infected African green monkey kidney cells, as the boost immunogen to demonstrate the advantages of the “prime-boost” immunization approach in preclinical models as well as early clinical trials [16–19]. How VV-produced Env compares with native material produced in HIV infections, or those generated from other recombinant sources, is not defined. It is likely that different posttranslational modifications (e.g. glycans) may play a significant role in the structural and biological properties of these proteins. Therefore, it is desirable to produce HIV gp120 from VV-infected cells in high purity to enable these comparative studies and in-depth analyses.

Here we report a new procedure to isolate gp120 monomer glycoprotein from VV-infected African green monkey kidney cells. The method is efficient, reproducible, and applicable to the purification of gp120 from diverse isolates of HIV-1. It is especially useful to prepare gp120 proteins from starting material where the presence of serum is desirable or unavoidable.

Materials and Methods

Construction of recombinant VV expressing HIV-1 gp120

The *env* genes of a clade C primary HIV isolate 1084i [20] and a monkey passaged primary isolate 1157ip [21] were kindly provided by Dr. Ruth Ruprecht (Harvard Medical School). The *env* gene of ADA was kindly provided by Dr. Joseph Sodroski (Harvard Medical School). The *env* genes of SF162 and HXBc2 were kindly provided by Dr. Leonidas Stamatatos (Seattle BioMed). The 1157ip K195N mutation was generated by site-directed mutagenesis. One or two stop codons were introduced at the primary gp120-gp41 cleavage site to generate the corresponding gp120 sequence. The transgenes were inserted into the thymidine kinase gene locus of the v-NY strain of VV under the control of a synthetic early-late promoter [4]. Construction and propagation of the recombinant VV were as previously described [22].

Culture condition and expression of gp120

Monolayer cultures of African green monkey kidney cells (BSC40) were propagated at 37 °C and 5% CO₂ in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% Penicillin/Streptomycin (Pen/Strep). After infection with VV at a multiplicity of infection (MOI) of 3, the cells were cultured in DMEM supplemented with 1% Pen/Strep and either 5% FBS or 10% FBS for an additional 48 h. The culture medium was then collected and the cells were removed by centrifugation at $2,645 \times g$ for 20 min at 4 °C. After addition of Empigen BB (Sigma) to a final concentration of 0.25%, the sample was used directly for purification without any further treatment.

Purification of gp120

All purification steps were handled at 4 °C using an ÄKTA 10/100 purifier (GE Life Sciences). The sample was loaded at 1 ml/min onto a 10-ml GNA column (*Galanthus nivalis* lectin-coupled agarose, Vector laboratories) pre-equilibrated with binding buffer (150 mM NaCl, 20 mM Tris-HCl, 0.25% Empigen BB, pH 7.5). After washing with 10 column volumes (CVs) of high salt wash buffer (500 mM NaCl, 20 mM Tris-HCl, 0.25% Empigen BB, pH 7.5) followed with 10 CVs of binding buffer, the bound protein was eluted with GNA elution buffer containing methyl- α -D-mannopyranoside (MMP, Sigma) (150 mM NaCl, 20 mM Tris-HCl, 0.25% Empigen BB, 1 M MMP, pH 7.5). Peak fractions were pooled and dialyzed overnight against DEAE binding buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0) followed by one additional buffer exchange for another 3h. The dialyzed sample was loaded at 1 ml/min onto a pre-packed 5-ml DEAE Sepharose column (GE Healthcare) and the flow through was collected. After concentration with an Amicon centrifugal concentrator (Millipore), the sample was loaded onto a 320-ml HighLoad 26/600 Superdex 200 column (GE Life Sciences) for size exclusion chromatography (SEC) and run at 1.2 ml/min in PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). Peak fractions were pooled and concentrated. Protein concentration was determined by BCA assay (Pierce) using BSA as the standard.

Polyacrylamide gel electrophoresis (PAGE) analysis

To estimate protein purity, 1 μ g, 2.5 μ g, and 5 μ g of the final product were denatured and analyzed by SDS-PAGE along with the PrecisionPlus Dual Color Protein Standards (Bio-Rad). The SDS-PAGE gel was stained with SimplyBlue SafetyStain, a highly sensitive, colloidal Coomassie G-250-based protein staining solution which detects less than 7 ng of protein according to the product manual (Invitrogen). After destaining by H₂O, the gel was scanned with a Bio-Rad gel documentation system (Universal Hood II) and the image was analyzed with imaging software Quantity One (Bio-Rad, version 4.6.7). Any protein band on the gel that did not appear to be 120 kDa was regarded as an impurity. The calculated purities from the 3 samples were averaged to give the final purity value. The apparent molecular weight of the non-denatured gp120 was analyzed by blue native PAGE (Invitrogen) following the manufacturer's instruction using the NativeMark Protein Standards as the marker (Invitrogen). A pooled human serum from HIV-positive donors was used for Western blot to evaluate protein integrity.

Surface plasmon resonance (SPR) binding analysis

SPR analysis of gp120 interactions with CD4-binding site ligands was performed at 25 °C on a Biacore T100 instrument (GE Life Sciences). Two immobilization formats were used for the two separate ligands, IgG1 b12 (b12) [23] and soluble two-domain CD4 (sCD4) [24]. For b12, two flow cells of a CM5 chip were immobilized with 30 μ g/mL goat-anti human Fc-specific antibody (Jackson ImmunoResearch Laboratories Inc.) in 10 mM sodium acetate (pH 5.5) to a density of 15,000 RU by standard amine coupling. Flow cell 1 was used as the

reference surface, and flow cell 2 was used to capture b12 at a level of 50 RU by injecting 0.2 µg/mL b12 for 30 s at 10 µl/min. For sCD4, flow cell 1 of a separate CM5 chip was blank immobilized (activated with EDC/NHS and capped with ethanolamine) for the reference surface, and flow cell 2 was immobilized with sCD4 at 1 µg/mL in 10 mM sodium acetate (pH 5.5) to a target level of 200 RU by amine coupling. Duplicate samples of gp120 were prepared independently by diluting either SF162 or 1084i gp120 into HBS-EP+ running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% P-20, pH 7.4) using a two-fold concentration series. Buffer blanks and gp120 samples were injected using a contact time of 240 s and variable dissociation times of 720 s and 1200 s for b12 and a constant dissociation time of 900 s for sCD4, all at a flow rate of 40 µl/min. Surfaces were regenerated using a 30 s injection of 10 mM glycine (pH 1.5) at a flow rate of 10 µl/min. Double reference-subtracted binding curves were fit globally using a 1:1 Langmuir binding model in Biacore T100 Evaluation Software version 2.0.2 (GE Life Sciences).

Results

Purification of gp120 in the presence of Empigen BB

We used a 3-step purification procedure beginning with a GNA affinity column to capture gp120 from cell culture medium. We started with 1 liter VV culture medium expressing 1157ip K195N gp120. Empigen BB was added to a final concentration of 0.25% to the sample to reduce VV infectivity. The same concentration of detergent was also included in the GNA binding, washing, and elution buffers to reduce non-specific binding to the column. Empigen BB was removed from the eluted sample by dialysis and the following DEAE and SEC steps did not involve use of the detergent.

As shown in Fig. 1a, gp120 was enriched to 60% pure by GNA affinity chromatography. The DEAE anion exchange chromatography removed a few contaminating proteins, improving the purity further to 78%. The Superdex 200 column was critical for separating gp120 monomer from dimer and other higher order oligomers (Fig. 1b). The gp120 monomer preparation was apparently free of any dimer contamination, as evident on the native PAGE (Fig. 1c). As demonstrated in Fig. 1d, the monomer appeared to be homogeneous when evaluated on a SDS-PAGE gel. There was no contaminating protein band detected in up to 5µg sample used (~500 × the detection limit). In contrast, a few contaminating protein bands were present in the dimer population (Fig. 1d) and they were not immuno-reactive towards the anti-HIV serum (Fig. 1e). The nature of these contamination proteins is not known at this moment. The compositional purity of 1157ip K195N monomer and dimer is 100% and 83%, respectively as assessed by SDS-PAGE (Table 1).

Additional gp120s including 1084i (clade C), ADA (clade B), HXBc2 (clade B), and SF162 (clade B) were purified with similar results, suggesting purification is generalizable among HIV isolates. Their yields and purities are listed in Table 1.

Changes in serum concentration alter gp120 yield but do not impact purity

We routinely culture BSC40 cells in DMEM supplemented with 10% FBS and reduce the serum concentration to 5% during VV infection, in order to minimize serum protein contaminations of gp120 preparation. To evaluate the impact of serum concentration on gp120 purification, SF162 gp120s were prepared from VV culture containing either 5% or 10% FBS. As shown in Table 1 (SF162 2nd prep vs. 3rd prep), no difference in monomer purity was observed between the two preparations, although the monomer yield was slightly better from the sample containing 10% FBS. Interestingly, the dimer/monomer ratio calculated from integrated peak areas of the SEC chromatograms (data not shown) increased

from 0.31 to 0.47 after the serum concentration was switched from 5% to 10%. Whether this trend holds true remains to be seen when more gp120s are prepared. We conclude that the presence of 10% FBS commonly used in mammalian cell culture has no negative impact on gp120 purification using the procedure.

Purified gp120s are recognized by sCD4 and b12

To ensure that the purified gp120 proteins retained their structural integrity, we examined binding of gp120 1084i as well as gp120 SF162 to sCD4 and b12 by SPR. As shown in Fig. 2, the calculated K_D was 20 nM for SF162 and 66 nM for 1084i, in line with previously reported results [25, 26]. We also tested binding of the two gp120 proteins to b12, which is a neutralizing antibody against the CD4 binding site [27]. SF162 gp120 displayed a high affinity towards b12, with a calculated K_D of 2.8 nM. In contrast, 1084i appeared to interact weakly with b12, with fast association and dissociation rates, and a binding curve that fitted poorly to a 1:1 interaction model (Fig. 2). The weak interaction of gp120 1084i with b12 is consistent with its resistance to neutralization by b12 [28] and the low potency of this antibody against clade C isolates [29].

Discussion

We have developed a new procedure to isolate VV expressed HIV gp120 from serum-containing cell culture medium. The monomeric gp120s from 4 out of 6 constructs (6 out of 8 total preparations) were purified to apparent homogeneity. To the best of our knowledge, this study represents the first example of milligram scale of homogeneous HIV envelope (Env) protein preparation from any serum-containing sample. The gp120s reported here were mostly produced in BSC40 cells cultured in 5% FBS to allow efficient expression of the HIV-1 protein from the VV infected cells. The purification procedure appears to be robust, since no decrease in purity of the final product was observed when the method was used with culture medium containing 10% FBS.

Lectin affinity chromatography provides an efficient way to generate gp120 with sufficient purity. GNA has been used previously for the purification of gp120 and gp140 [30–32]. However, most soluble Env protein purifications described to date were performed with samples generated under serum-free conditions either by using cells adapted to serum-free growth or by replacing supplemented medium with serum-free medium at the stage of protein production [33–37]. This invariably increases the operation cost, as serum-free adaption is time consuming and serum-free medium costly. The presence of serum components not only compromises the performance of GNA, it also makes regeneration of the resin difficult. Detergent such as Triton X-100 has been used to reduce non-specific binding during the affinity step [38]. Empigen BB, a zwitterionic detergent, has a critical micelle concentration (CMC) value of 1.6–2.1 mM [39]. The relatively high CMC makes the detergent removal possible by dialysis. At 0.25% (9.2 mM), it is well-tolerated by the GNA resin [30]. The resin can be regenerated and used for identical protein purification at least 3 times without compromising its performance. Empigen BB is inexpensive, feasible to use for large-scale protein purification applications. More importantly, it has been shown safe to prepare protein antigens for vaccine application. For example, Jennings and Erturk demonstrated in mice studies that Empigen-solubilized HSV-1 antigen preparations elicited greater antibody responses and greater protection against HSV-1 challenge than the NP40-solubilized preparation [40]. Lowthert et al. found Empigen BB to be the most efficient detergent for the solubilization of keratins while preserving their antigenicity [41]. We and others have used this detergent to prepare Env proteins for *in vivo* immunization studies in mice and macaques [30, 42, 43].

The gp120 expressed by VV contains both monomer and dimer and the ratio between them varies among constructs. The presence of stable gp120 dimers is not unique to VV expression as it has been reported before in transiently-transfected HEK293T cells [44]. The dimers are formed intracellularly, mediated via V2 loop and N-termini interactions, and linked by disulfide bonding [38, 44]. Better expression construct designs are needed to minimize dimer formation as important epitopes on the gp120 inner domain and the chemokine receptor-binding surface are altered or occluded by gp120 dimerization [44]. This has been suggested to have implications for immunogenicity of V2-directed responses [45].

Although both gp120 monomer and dimer run as a 120 kDa protein on the SDS-PAGE, their apparent molecular weights estimated from the blue native PAGE based on the protein standards are about 240 kDa and 480 kDa, respectively, off by a factor of two from their anticipated molecular weights. This discrepancy could result from the charge on the gp120 protein due to the presence of glycan molecules, as surface charge on a protein plays a significant role in its electrophoretic mobility on native PAGE. However, the calculated molecular weights of gp120 monomer and dimer from SEC are also off by two folds (266 kDa and 495 kDa, respectively). These results are in agreement with the recently reported observations of Guttman et al. (2013) that the molecular weights estimated from SEC and native PAGE are often inaccurate for proteins with high glycan content (in some cases off by a factor of two) [46]. It is well documented that many classic techniques for molecular weight determination of proteins and complexes yield inaccurate estimates when applied to proteins with significant glycan content [47, 48]. We have confirmed the sizes of the purified monomeric gp120s by dynamic and static light scattering to be on the order of 100 kDa (Thaddeus Davenport and Kelly Lee, unpublished data). Static light scattering, in particular, has been shown to be a straightforward and reliable method of molecular weight determination for proteins and glycoproteins [46, 49].

In conclusion, we have developed a robust and reproducible procedure for the purification of HIV-1 Env gp120 from serum containing culture media to apparent homogeneity. The availability of highly purified gp120 from the VV expression system should allow in depth characterizations of this protein and comparative studies with material produced in other expression systems. The method described here should also find general application for the purifications of glycoproteins from sources where serum-free media is undesirable or unavailable.

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Highlights

- We developed a new method to purify vaccinia virus-expressed HIV gp120 from serum-containing culture.
- Six out of eight gp120 monomers were purified to apparent homogeneity.
- Structural integrities of purified gp120s were confirmed by surface plasmon resonance analysis.

Fig.1.

Purification of 1157ip K195N gp120. (a) SDS-PAGE analysis of the gp120 samples saved at each purification step (5 μ g protein per lane). GNA: *Galanthus nivalis* lectin affinity chromatography. (b) Size exclusion chromatogram. Monomer and dimer were pooled as indicated by the arrows. (c) Native PAGE analysis of the purified proteins (5 μ g protein per lane). (d) SDS-PAGE analysis of the purified proteins. (e) Western blot (0.5 μ g protein per lane).

Fig.2.

Kinetic analysis of binding of sCD4 and b12 to gp120s. The recorded sensorgrams are shown in blue and the fitted curves in red. k_a : on-rate constant; k_d : off-rate constant; K_D : dissociation constant.

Table 1

Purification of HIV gp120s from 1L VV-infected BSC40 cell culture medium

Construct	Monomer			Dimer		
	total protein (mg)	purity (%)	gp120 (mg)	total protein (mg)	purity (%)	gp120 (mg)
1157ip K195N	1.96	100	1.96	2.01	83	1.67
1157ip	2.02	100	2.02	n/a	n/a	n/a
1084i	1.99	98	1.96	0.62	88	0.55
HXBc2	3.73	93	3.47	1.19	83	0.98
ADA	2.59	100	2.59	0.56	83	0.47
SF162, 1 st prep 2 nd prep 3 rd prep ^a	1.49	100	1.49	0.63	96	0.60
	2.96	100	2.96	1.36	86	1.17
	3.32	100	3.32	1.70	96	1.63

^aThis sample was prepared from culture medium containing 10% FBS and the rest were prepared from culture medium containing 5% FBS.