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Expression, purification, and characterization of full-length bovine leukemia virus Gag protein from bacterial culture

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ABSTRACT

In retroviruses, the Gag protein is a precursor from which the mature proteins matrix, capsid, and nucleocapsid are derived. Gag plays an important structural role in the assembly of virions at the plasma membrane. While Gag proteins from several different retroviruses have been purified for study *in vitro*, there has yet to be a report of successful purification of deltaretroviral Gag. In this paper, we report the cloning, expression and purification of full-length bovine leukemia virus (BLV) Gag from *Escherichia coli* using a combination of polyethyleneimine precipitation, ammonium sulfate precipitation, and affinity chromatography. Experiments using size-exclusion chromatography were also performed to analyze the oligomeric state of the Gag protein in solution, and results suggest that it exists primarily as a monomer but may oligomerize into higher-order complexes to a small extent. Molecular weight estimation by comparison of elution volume to a set of protein standards supports the hypothesis that BLV Gag adopts a slightly extended conformation in solution. The results are discussed in comparison to the solution structure and assembly pathways of other retrovirus genera.

Introduction

Retroviruses are a family of viruses that use a virally-encoded reverse transcriptase to convert their (+)-single stranded RNA genomes into double-stranded DNA prior to nuclear import and integration into the host cell's genome [1]. There are three genes common to all retroviruses: *gag*, *pol*, and *env*. The *gag* gene encodes the structural proteins of the virus; these include but are not limited to matrix (MA),[1] capsid (CA), and nucleocapsid (NC). During replication, these proteins are expressed as a single polypeptide, simply referred to as Gag. After assembly and release from the cell, Gag is proteolyzed into its constituent proteins in a process known as maturation. Prior to release of the immature virion from the cell, the Gag protein plays an important role in viral assembly and genome packaging [2–7]. In human immunodeficiency virus type 1 (HIV-1), a well-characterized retrovirus, the NC domain of Gag strongly binds to the genomic RNA while the MA domain binds to the plasma membrane of the cell through both electrostatic interactions and insertion of a covalently-attached myristoyl group into the lipid bilayer. The CA domain facilitates Gag–Gag interactions, promoting multimerization at the inner leaflet of the membrane [8–10].

While MA, CA, and NC have been successfully expressed and purified as individual proteins from a variety of different retroviruses, expression and purification of full-length Gag has proven to be much more challenging. This presents a significant roadblock for progress in understanding how retroviral assembly works, since pure protein is essential for biophysical studies that examine structure, binding, and oligomerization. HIV-1 Gag used for *in vitro* experiments typically contains several key mutations to preserve solubility and stability. First, the C-terminal peptide (p6) that exists immediately following the NC domain is deleted (commonly referred to as a Δp6 mutant). Compared to wild-type HIV-1 Gag, GagΔp6 is degraded to a much lesser extent when expressed in bacterial culture [11]. GagΔp6 can be purified to 85–90% homogeneity and with much better yields than the wild-type protein. Second, two mutations (W316A and M317A) are made to adjacent residues in the CA domain. This mutant, termed WM-Gag, has been shown to be predominantly monomeric in solution whereas the wild-type protein is primarily dimeric [12]. A third variant contains the Δp6 mutation as well as another deletion, Δ16–99, which has been shown to enhance *in vitro* assembly of Gag; the purification of this construct has been reported in great detail [13]. Interestingly, full-length Gag from feline immunodeficiency virus (FIV) has been successfully purified using affinity chromatography without any modifications, save the 6× His tag [14]. This result is surprising, since HIV-1 and FIV are both lentiviruses and their Gag proteins share sequence homology.

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[1] *Abbreviations used:* MA, matrix; CA, capsid; NC, nucleocapsid; FIV, feline immunodeficiency virus; HTLV-1, human T-cell leukemia virus type one; VLPs, virus-like particles; IPTG, isopropyl beta-D-1-thiogalactopyranoside.

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Several Gag proteins from other retrovirus genera have been purified from bacterial culture and used for *in vitro* experiments. Murine leukemia virus (MLV, a gammaretrovirus) Gag was isolated and its properties in solution were shown to differ significantly from what has been observed for HIV-1 Gag [15]. While evidence exists that HIV-1 Gag adopts a compact conformation [12,16,17], small-angle X-ray scattering experiments show that MLV Gag has a rigid, extended structure in solution. Wild-type MLV Gag is also predominately monomeric, while HIV-1 Gag exists in a monomer–dimer equilibrium. Gag protein from an alpharetrovirus, *Rous sarcoma* virus (RSV) has been purified in its native form with the exception of the C-terminal protease domain, which is normally translated as part of the Gag polyprotein in RSV [18]. This led to work showing that RSV Gag required nucleic acid to promote dimer formation as an intermediate step in assembly [19,20].

To date, successful purification of deltaretroviral Gag has not been reported. The most notable human deltaretrovirus is human T-cell leukemia virus type one (HTLV-1). HTLV-1 is endemic in many areas of the world, and is the causative agent of adult T-cell leukemia and HTLV-associated myelopathy/tropical spastic paraparesis [21]. Cell culture experiments have shown that, as for other retroviruses, elements of HTLV-1 Gag are critical for membrane binding and genome packaging [22,23]. Interestingly, there are some key differences between HIV-1 and HTLV-1 in terms of the behavior of Gag in solution; HTLV-1 Gag is primarily monomeric, and forms virus-like particles (VLPs) with a poorly organized Gag lattice compared to HIV-1 [24–26].

Bovine leukemia virus (BLV), another deltaretrovirus, infects domestic cattle and is prevalent in dairy herds in the United States [27]. Although humans cannot be infected with BLV, infected cattle have shown reduced milk production compared to healthy animals [28], making study of BLV important from an agricultural standpoint. Additionally, antibodies reactive to BLV capsid have been discovered in human blood sera, presumably due to consumption of products from infected animals [29]. Finally, BLV may also be useful as an animal model for development of a vaccine for HTLV-1 due to the similarity of BLV and HTLV-1 [30,31]. Previous work has shown that BLV Gag relies on both its MA and NC domains for effective genome packaging; zinc-binding residues in the NC domain and basic residues in the MA domain appear to be especially important [32]. Furthermore, myristylation of the N-terminal glycine residue of Gag and the presence of a PPPY motif were also shown to be crucial for the production of VLPs [33]. While the role of the myristate moiety in this context is clear, less is known about the interaction of BLV Gag with the plasma membrane of the host cell. Both MA [34] and NC [35] of BLV have been purified and used in biophysical experiments that characterize nucleic acid binding, but the use of authentic Gag would provide a clearer and more accurate representation of the events that occur during replication.

In this paper, we report the first successful purification of a full-length deltaretroviral Gag protein. Cloning, expression, and purification procedures are described in detail, along with preliminary experiments designed to characterize the properties of BLV Gag in solution. Our results show that Gag has been purified to near homogeneity, and that it exists in solution as a partially-extended monomer.

Materials and methods

Cloning

The DNA sequence corresponding to full-length Gag was amplified by PCR using a plasmid template (pKB426, a kind gift from Dr. Kathleen Boris-Lawrie) which is a derivative of pBLV-SVNEO, a previously described construct [36]. The forward primer contained an XhoI restriction site at the 5′-end of the sequence coding for Gag, while the reverse primer contained an AvrII site positioned after the stop codon at the 3′-end. The sequences of the primers were as follows: 5′-GTGACACTCGAGATGGGAAATTCCCCC-3′ (forward) and 5′-GTGACACCTAGGTTAGTTTTTTGATTTGAGGG-3′ (reverse). Both primers were purchased from Integrated DNA Technologies (Coralville, IA) and used without further purification.

Following PCR amplification, the DNA product and the expression vector pET45b (EMD Millipore, Darmstadt, Germany) were each digested with XhoI and AvrII (New England Biolabs, Ipswich, MA) using standard molecular biology protocols. The digestion reactions were cleaned up using a PureLink PCR Purification kit (Life Technologies, Grand Island, NY); the High-Cutoff binding buffer provided was used to remove DNA less than 300 bp in length. The Gag DNA insert (25 ng) and linearized pET45b (20 ng) were incubated at 16 °C for 4 h in the presence of 2 Weiss units of T4 DNA ligase (EMD Millipore) in a total volume of 10 μL according to the manufacturer's instructions.

The entire ligation reaction was transformed into chemically competent NovaBlue *Escherichia coli* cells (EMD Millipore). Several colonies were screened for the presence of BLV Gag DNA by colony PCR; colonies that were positive were grown in liquid culture and plasmids were purified using a commercially available miniprep kit (Omega Bio-Tek, Norcross, GA). The presence of BLV Gag DNA was confirmed by restriction digests of the purified plasmids, and plasmids were sequenced to confirm that the correct sequence had been inserted. The expression plasmid was then transformed into chemically competent Rosetta-2 (DE3) pLysS cells (EMD Millipore), a strain that contains a plasmid encoding a number of tRNA genes corresponding to rare codons.

Protein expression and purification

A small culture (10 mL) of LB media containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol was inoculated with a single colony of Rosetta-2 (DE3) pLysS cells containing the BLV Gag expression plasmid. After shaking overnight at 37 °C, the small culture was added to 500 mL of LB media (with antibiotics at the concentrations used for the small culture) and incubated at 37 °C with agitation until the optical density of the culture at 600 nm reached 0.5. At this point, the culture was allowed to cool to room temperature before addition of isopropyl beta-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Expression was allowed to continue overnight at room temperature with shaking, and cultures were harvested by centrifugation for 20 min at 8000 rpm. Cell pellets were stored at −80 °C until purification took place.

Cell pellets (about 2.4 g wet weight, representing 500 mL of liquid culture) were thawed on ice and resuspended completely in 20 mL of lysis buffer (50 mM HEPES pH 7.0, 0.3 M NaCl, 1 mM tris (2-carboxyethyl) phosphine–HCl, 10% glycerol, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride). The cells were sonicated on ice using 10 cycles of the following sequence: 1 s on, 1 s off for 20 s; rest on ice for 40 s. Polyethyleneimine was then added to a final concentration of 0.15%, and the lysate was allowed to incubate on ice for 15 min to precipitate nucleic acids. After incubation, a thick white precipitate was observed. The lysate was centrifuged at 11,000 rpm for 20 min, and the pellet discarded. To the supernatant was added 1/2 volume of ammonium sulfate solution (saturated at room temperature), dropwise, on ice, with stirring. Following addition, the solution was allowed to incubate on ice for 30 min without stirring. The solution was then centrifuged at 11,000 rpm for 20 min. The supernatant from this step was discarded, and the solid white pellet was gently and completely resuspended on ice with 1 mL of column buffer (50 mM HEPES pH 7.0,