Assembly of monomeric human cytomegalovirus pUL104 into portal structures

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In order for human cytomegalovirus (HCMV) to replicate, concatemeric DNA has to be cleaved into unit-length genomes and packaged into preformed capsids. For packaging to take place and DNA to be translocated, a channel is required in the capsid. Viral capsid channels are generally formed by portal proteins. Here, we show by cross-linking, native gel electrophoresis of infected cells and gel permeation chromatography that the HCMV portal candidate protein pUL104 can form dimers and higher order multimers. Electron microscopy of purified monomeric pUL104 after 5 min incubation revealed that the protein had assembled into a multimeric form and that this form closely resembles complete portal assembly. This is the first study to show that pUL104 monomers have the ability to form portal complexes without additional viral proteins.

The maturation of herpesviruses, as well as double-stranded DNA (dsDNA) bacteriophages, is the first step in the viral life cycle and harbours a number of common features. Starting with the assembly of the viral procapsid, subsequent DNA replication leads to concatenated DNA that is cleaved into unit-length genomes and packaged into the preformed capsids (Adelman et al., 2001; Black, 1989; Bogner, 2002; Catalano, 2000). Packaging is an energy-conservative mechanism and with bacteriophages it was demonstrated that one molecule of ATP is required to package 2 bp of DNA (Guo et al., 1987; Morita et al., 1993). The viral nanomotor, which is involved in this process, is composed of the terminase and the portal. The human cytomegalovirus (HCMV) terminase consists of a large and a small subunit, called viral protein pUL56 and pUL89, respectively. The large subunit endows the terminase with ATPase activity and the small subunit is required for cleavage of concatemeric DNA (Bogner, 2002; Hwang & Bogner, 2002; Scheffczik et al., 2002; Scholz et al., 2003). In addition to terminate, the other critical packaging component is the portal protein. With bacteriophages and herpes simplex virus (HSV) type-1, the portal protein forms a homo-multimeric complex that serves as a channel for translocation of the DNA into the capsid (Valpuesta & Carrascosa, 1994; Newcomb et al., 2001, 2005). In addition to this, the portal plays a fundamental role in the assembly of capsids. Even though the portal proteins known so far share no sequence homology, the architecture of the portals is strikingly similar. All portal proteins occupy one vertex location in the capsid and are thought to be dodecamers upon integration (Guasch et al., 2002; Lurz et al., 2001; Trus et al., 2004; Valpuesta & Carrascosa, 1994); they are typically composed of a crown, a stalk and a wing domain. The crown and the stalk delineate a narrow central channel between which the wider wing domain is located (Agirrezabala et al., 2005; Poliakov et al., 2007).

With HCMV, previous studies have suggested that the open reading frame UL104 encodes the portal protein (Dittmer & Bogner, 2005). In vivo, pUL104 directly interacts with the large terminase subunit pUL56, which was shown to be essential for DNA insertion into the capsid (Dittmer et al., 2005). Using the recently developed compound tetrahalogena- nated benzimidale D-ribonucleoside (Cl₄RB), it was possible to entirely inhibit the interaction between pUL104 and pUL56 coinciding with a complete block of packaging (Dittmer et al., 2005), thus proving that pUL104 is critically required for DNA translocation into the procapsid and the subsequent formation of an infectious particle. To date, it is not known how pUL104 assumes this critical role. Moreover, direct evidence for the ability of pUL104 to self-assemble into a portal structure has not been reported.

In this study, starting from purified monomers, the self-assembly of pUL104 was examined by chemical cross-linking, gel permeation chromatography and electron microscopy. In addition, oligomeric structures were probed for by Western blots in infected cell extracts. For cross-linking, recombinant pUL104 (170 ng) was incubated for 5 min at room temperature in PBS (pH 7.4) in a final volume of 20 µl. The cross-linking reaction was started by addition of glutaraldehyde to a final concentra-
tion of 0.01 % (w/v) in PBS. The reaction was stopped by addition of SDS loading buffer and incubation of the samples for 0, 2, 5 or 10 min at 95 °C. The proteins were separated by SDS-PAGE and analysed by Western blotting with the specific antibody pAbUL104 (Dittmer & Bogner, 2005). Cross-linking of pUL104 (Fig. 1) resulted in electrophoretic mobility shifts, indicating the formation of dimers after approximately 2 min (Fig. 1, lane 2) and multimers after 5 min (Fig. 1, lane 3) of incubation, i.e. pUL104 monomers are able to assemble into high molecular mass oligomers without the aid of additional viral proteins. In comparison, using the same approach with purified terminase subunit pUL56, only dimers could be detected (Savva et al., 2004). In order to establish whether the in vitro results would also be echoed by the in vivo situation, HCMV-infected (AD169 cells, m.o.i. of 1) human foreskin fibroblasts were harvested 72 h post-infection (p.i.) and the proteins were separated under non-reducing conditions. Western blot analysis with pAbUL104 only revealed a multimeric complex (Fig. 1, lane 6) of a mass comparable to the upper multimeric form observed by cross-linking (Fig. 1, lane 4). Taking these two results together suggests that pUL104 has the ability to autonomously form specific assemblies.

To investigate this possibility further, recombinant pUL104 obtained from insect cells (High five) infected with recombinant baculovirus was subjected to gel permeation chromatography. Briefly, High five cells (4 × 10⁸) expressing the recombinant pUL104 were harvested 48 h p.i. Sedimented cells were lysed in 50 ml cation-exchange buffer (20 mM MES, pH 6.5, 150 mM NaCl and protease inhibitors) and sonicated on ice. Cell lysates were sedimented and passed through a 0.2 μm filter prior to loading onto an equilibrated cation exchange column (HiTrap SP HP, 1 ml bed volume; GE Healthcare). The purification was performed at 4 °C by using AKTA FPLC (GE Healthcare), as described previously (Dittmer & Bogner, 2005). Fractions containing the protein were separated on a gel permeation chromatography column (HiPrep 16/60 Sephacryl S-200 HR; GE Healthcare). A total of 120 fractions were collected, analysed by gel electrophoresis, and fractions containing the protein were stored at −80 °C. The molecular mass markers thyroglobulin (Stokes radius, rₛ=8.5 nm), ferritin (rₛ=6.1 nm), catalase (rₛ=5.2 nm) and aldolase (rₛ=4.8 nm) were carried for calibration purposes. Two peaks (Fig. 2a) in the elution profile, labelled ‘a’ and ‘b’, were found to contain pUL104 with peak ‘a’ being composed of the high molecular mass form. The low molecular mass fractions (peak ‘b’ in Fig. 2a), were separated on 10 % polyacrylamide gels and stained with Coomassie brilliant blue. The gel in Fig. 2(b) shows that fractions 70–75 clearly contain a band at approximately 73 kDa, which equates to monomeric pUL104. Fraction 72 was used for a final assessment using electron microscopy. Prior to this, it was incubated for 5 min at room temperature to allow for assembly into higher order structures. Negatively stained specimens were prepared essentially as described by Valentine et al. (1968) but omitting any fixation steps and using an aqueous solution of 4 % (w/v) uranyl acetate (pH 4.3); the adsorption time was 20 s. Specimens were mounted onto 400 mesh copper grids and observed in a Tecnai F12 (FEI Company) transmission electron microscope operated at 120 kV. Micrographs were recorded with a 2K SIS digital camera at calibrated magnifications.

Fig. 3 summarizes the electron microscopic observations. First of all, it was noted that the high molecular mass forms of pUL104 form structures that bear striking similarity to portal assemblies described previously for bacteriophages and HSV (Agirrezabala et al., 2005; Trus et al., 2004). Viewed side-on (Fig. 3a, b, c), three distinct regions can be readily discerned: (i) the crown region with a diameter of 11 nm, (ii) the wing region with a substantially larger

![pUL104](image_url)

**Fig. 1.** Oligomerization of pUL104 by Western blotting. Lanes 1–4, purified protein was reacted with glutaraldehyde for the indicated times. Lanes 5 and 6, cell extracts of mock and infected human foreskin fibroblast cells were analysed under non-reducing conditions. Blots were incubated with monospecific pAbUL104. Molecular mass markers (M) are indicated.
projections have been observed with HSV-1 pUL6 portal assemblies (Nellissery et al., 2007). In Fig. 3(l), a schematic drawing is presented which highlights the basic features of the HCMV portal, i.e. the crown, wing and stalk domains related directly to the side-on projection in Fig. 3(a). The portal is approximately as tall as it is wide and displays distinct crown, wing and stalk domains, with the latter two being substantially smaller in diameter than the wing; all of these data are in excellent agreement with other reported portal structures, such as the one of bacteriophage φ for which a surface-rendered model is shown in Fig. 3(m) (Trus et al., 2004). In order to find out whether the dimensions of the structures observed concur with a dodecameric portal assembly, the mass of the observed particles was estimated using the volume–mass relationship of Zipper et al. (1971) relating 1.37 nm$^3$ to 1 kDa. Starting from the top, the crown measures 11 nm in diameter and 2.5 nm in height. Assuming its overall shape is a cylinder, its molecular mass can be estimated to 170 kDa. In the wing domain, densities for single putative subunits measured 4 × 6 nm; assuming an ellipsoidal shape, this equates to 37 kDa × 12 ≈ 440 kDa for a dodecameric assembly. The stalk domain measured, on average, 9 nm in diameter and 10 nm in length, resulting in a mass of 465 kDa. The oblique face-on projections in Fig. 3(g–k) are easily described as portals, as they exhibit prominent wing and stalk regions. More interestingly, all of the projections show a pronounced protein deficit at the centre of the stalk, which indicates that the previously reported channel runs along the central axis, from the top to the bottom of the portal (Orlova et al., 2003). This central protein deficit is also found in the axial projections Fig. 3(d–f), corroborating the notion that the channel runs from the crown to the tip of the stalk. For the mass estimate, it is important to deduct the volume (and mass) occupied by the central channel. The channel measures approximately 3.6 nm across for the entire length of the portal (19.5 nm) corresponding to a mass of ~145 kDa. With this, the total mass is 170 + 440 + 465−145 = 930 kDa, which agrees well with a dodecameric (73 kDa × 12 = 876 kDa) pUL104 oligomeric structure. For the bacteriophage P22, Lorenzen et al. (2008) demonstrated for the first time that a dodecameric quaternary structure exclusively constitutes a portal, thus closing a long-lasting debate on the oligomeric state of in vitro-assembled portals.

In conclusion, this study has demonstrated that monomeric pUL104 has the ability to assemble into portal structures. Portal protein structures were first characterized in detail for bacteriophages and HSV-1 (Agirrezabala et al., 2005; Guasch et al., 2002; Trus et al., 2004). Higher order assemblies of recombinant expressed portal protein were also found in bacteriophage P22 (Poliakov et al., 2007). While this bacteriophage may be more closely related to herpesviruses than the other phages, the data presented herein constitute the first structurally motivated study on HCMV pUL104 portal complexes.
Acknowledgements

This work was supported by the German Research Foundation (Bo 1214/15-1 to E.B.), the Alexander von Humboldt-Foundation (A.H.) and the Texas A & M University OVPR (A.H.). Fig. 3(a) from Trus et al. (2004) (Fig. 3m in this paper) is reproduced with permission from the American Society for Microbiology (license no: 2218300192373). We thank N. Bannert (Robert Koch-Institute, Berlin) for access to electron microscopy facilities and D. Krüger for continued support and interest in this work.

References


Fig. 3. Electron microscopy of negatively stained pUL104. (a–c) Complete portal assembly viewed side-on and highlighting the crown, wing and stalk regions of the complex. (d–f) Crown-biased (d) and stalk-biased axial (face-on) (e, f) projections of the assembly. In (g–k), oblique face-on (near axial) projections emphasize the stalk protruding from the wing. For each projection, a drawing is provided in which the arrow points in the direction of the mouth of the stalk, aiding in the identification of orientation. (l, m) A diagram of the HCMV portal (l) and a model of bacteriophage φ portal (m) showing the crown, wing and stalk regions. Bars, 20 nm. Fig. 3m is reproduced from the paper by Trus et al. (2004) with permission from the American Society for Microbiology (license no: 2218300192373).


