Title
Comparative Structural Studies of Human Herpesvirus Capsids and Capsid-Associated Tegument Proteins

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Comparative Structural Studies of Human Herpesvirus Capsids
and Capsid-Associated Tegument Proteins

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Biomedical Engineering

by

Xinghong Dai

2015
Herpesviruses are a large group of double-stranded DNA viruses of animals. Nine species of herpesvirus take human as their primary host and cause diseases ranging from mild lesions to serious malignancies. Herpesviruses are grouped into three subfamilies, namely alpha-, beta-, and gammaherpesviruses. The herpesvirus smallest capsid protein (SCP) and capsid-associated tegument proteins are structurally and/or functionally divergent across the three subfamilies, but the mechanisms are not well understood due to the lack of detailed structures. Combining high-resolution cryo electron microscopy (cryoEM) with structure-guided mutagenesis, we compared virion structures of representative members of the three herpesvirus subfamilies, with a focus on the structure and function of the SCP and tegument proteins. Our main findings are summarized as following: 1. The SCP of betaherpesvirus human cytomegalovirus (HCMV) mediates the capsid-association of tegument protein pp150; 2. The SCP of gammaherpesvirus Kaposi’s sarcoma-associated herpesvirus (KSHV) bridges neighboring MCP molecules in the capsid.
hexons; 3. All herpesvirus SCPs are acting, alone or in coalition with tegument proteins, as cementing proteins like those in dsDNA bacteriophages to stabilize the capsid for genome packaging; 4. Gammaherpesvirus KSHV has capsid-associated tegument densities that are similar to those in alphaherpesviruses but different from those in betaherpesviruses, and the organization of the two components of these alpha- and gammaherpesvirus tegument is re-established. Our results provide new insights into the understanding of herpesvirus capsid and virion assembly, and are informative to searching for new antiviral targets.
The dissertation of Xinghong Dai is approved.

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Lily Wu

Leonard H. Rome

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University of California, Los Angeles

2015
This dissertation is dedicated to all my family members, especially my parents, Enjie Dai and Guilian Liu, and my wife, Chanjuan Shi, for their love, support and encouragement.
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PUBLICATIONS AND PRESENTATIONS


Chapter 1 INTRODUCTION
1.1 Herpesvirus taxonomy

Herpesviruses are a large group of double-stranded DNA viruses that are ancient and disseminated widely in nature. Their natural hosts include molluscs, fishes, amphibians, reptiles, birds, and mammals, including human beings (1). Originally grouped in one single family, the herpesviruses of reptiles, birds, and mammals were later found by sequence analysis to be only remotely related to those of molluscs and those of fishes and amphibians (2). Now, the *Herpesviridae* family only includes herpesviruses of reptiles, birds and mammals (1, 2).

The *Herpesviridae* family is further classified into three subfamilies, namely alpha-, beta- and gamma-herpesviruses. The classification was established originally on the basis of biological properties of the viruses and later was found to be remarkably consistent with information extracted from viral nucleotide sequences (1). The alphaherpesviruses have a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infection primarily – but not exclusively – in sensory ganglia (1). The betaherpesviruses have a restricted host range, relatively long reproductive cycle, and can establish latency in secretory glands, lymphoreticular cells, kidneys and other tissues (1). The gammaherpesviruses have the narrowest host range, and are usually specific for either T or B lymphocytes. They ordinarily establish latency in lymphoid tissue, and are uniquely associated with lymphoproliferative diseases and lymphomas (1, 3).
1.2 Human herpesviruses and diseases

Nine herpesviruses have been identified to have humans as their primary hosts. Table 1-1 lists the nomenclatures of all human herpesviruses, and summarizes the diseases etiologically associated with these human herpesviruses.

Human herpesviruses are ubiquitous and widespread in all parts of the world. For example, almost 90% of the US population is infected with the varicella-zoster virus (VZV) by the age of 10 years, and almost all of the population has antibody to the human herpesvirus 6 (HHV-6) by the age of 5 years (4). For many human herpesviruses, the serologic prevalence percentage is even higher in populations of developing countries with low socioeconomic status than in those of developed countries (4). The widespread of human herpesviruses is partly due to the fact that most human herpesvirus infections are asymptomatic or only have mild, unnoticed symptoms, but the patients with no symptoms shed infectious viruses as much as those patients do develop herpes symptoms (4, 5). Another reason is that all herpesviruses are capable of establishing latent, recurrent infections in their hosts (1, 6-8).

Human herpesvirus infection is a serious medical concern. They are especially dangerous for immunocompromised persons, including patients with acquired immune deficiency syndrome (AIDS) and organ-transplant recipients (4, 6). Transplacental transmission of herpesvirus from the pregnant woman to the fetus is a leading cause of birth defects in the newborns (8).

Despite currently available anti-herpesvirus therapies aimed at reducing the overall disease burden, more effective and safe, orally bioavailable antiviral drugs are needed (4, 8). Effective and safe herpesvirus vaccines are also much needed for universal vaccination. The development of new drugs and vaccines depends on deeper understanding of herpesvirus virology.
Table 1-1 Human Herpesviruses and diseases*

<table>
<thead>
<tr>
<th>Formal name</th>
<th>Abbreviation</th>
<th>Vernacular names and synonyms</th>
<th>Subfamily and genus</th>
<th>Genome size (Kbp)*</th>
<th>Pathophysiology</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Human herpesvirus 1</em></td>
<td>HHV-1</td>
<td>Herpes simplex virus [type] 1 (HSV-1)</td>
<td>α, Simplexvirus</td>
<td>152</td>
<td>Oral and/or genital herpes (predominantly orofacial)</td>
</tr>
<tr>
<td><em>Human herpesvirus 2</em></td>
<td>HHV-2</td>
<td>Herpes simplex virus [type] 2 (HSV-2)</td>
<td>α, Simplexvirus</td>
<td>155</td>
<td>Oral and/or genital herpes (predominantly genital)</td>
</tr>
<tr>
<td><em>Human herpesvirus 3</em></td>
<td>HHV-3</td>
<td>Varicella-zoster virus (VZV)</td>
<td>α, Varicellovirus</td>
<td>125</td>
<td>Chickenpox; Shingles</td>
</tr>
<tr>
<td><em>Human herpesvirus 4</em></td>
<td>HHV-4</td>
<td>Epstein-Barr virus (EBV)</td>
<td>γ, Lymphocryptovirus</td>
<td>172</td>
<td>Infectious mononucleosis; Burkitt's lymphoma; Post-transplant lymphoproliferative syndrome; Nasopharyngeal carcinoma</td>
</tr>
<tr>
<td><em>Human herpesvirus 5</em></td>
<td>HHV-5</td>
<td>[Human] Cytomegalovirus (HCMV, or CMV)</td>
<td>β, Cytomegalovirus</td>
<td>236/230</td>
<td>Infectious mononucleosis-like syndrome; Retinitis, Birth defects (congenital infection)</td>
</tr>
<tr>
<td><em>Human herpesvirus 6A</em></td>
<td>HHV-6A</td>
<td>HHV-6 variant A</td>
<td>β, Roseolovirus</td>
<td>159/170</td>
<td>Roseola infantum</td>
</tr>
<tr>
<td><em>Human herpesvirus 6B</em></td>
<td>HHV-6B</td>
<td>HHV-6 variant B</td>
<td>β, Roseolovirus</td>
<td>162/168</td>
<td>Roseola infantum</td>
</tr>
<tr>
<td><em>Human herpesvirus 7</em></td>
<td>HHV-7</td>
<td></td>
<td>β, Roseolovirus</td>
<td>145</td>
<td>Roseola infantum</td>
</tr>
<tr>
<td><em>Human herpesvirus 8</em></td>
<td>HHV-8</td>
<td>Kaposi’s sarcoma-associated herpesvirus (KSHV)</td>
<td>γ, Rhadinovirus</td>
<td>170/210</td>
<td>Kaposi’s sarcoma; Primary effusion lymphoma; Multicentric Castleman’s disease</td>
</tr>
</tbody>
</table>

* This table is adapted from Table 59.1 of Ref. (1) and information compiled in Ref. (4) and the Wikipedia webpage http://en.wikipedia.org/wiki/Herpesviridae.

# Different numbers may reflect differences in strains.
1.3 Herpesvirus virion and capsid structures

All herpesviruses share the same architecture for the assembly of their virions. A herpesvirus virion consists of four elements: a linear dsDNA genome, an icosahedral capsid containing the genome, a proteinaceous layer surrounding that capsid that is called the tegument, and a lipid bilayer envelope enclosing the capsid and the tegument layer (1, 6). More recent visualization of herpesvirus virion in three dimensions with cryo electron tomography has further revealed that the tegument layer is mostly unstructured and asymmetric in distribution, and the envelope is decorated with many envelope protein spikes (9, 10).

The herpesvirus capsid is a T=16 icosahedron consists of 150 hexons, 11 pentons, 1 portal complex and 320 triplexes. The portal occupies one of the twelve icosahedron vertexes, and was proposed to be a 12-mer of the portal protein. It is structurally and functionally similar to the portal complex of dsDNA bacteriophages (11-14). The pentons and hexons are each composed of five and six copies of the major capsid protein (MCP), respectively. They are connected by the triplexes. Each triplex is a hetero-trimer of two proteins - one Tri1 protein and two Tri2 proteins (6). The smallest capsid protein (SCP) is decorating the tip of MCPs with a 1:1 stoichiometry, and several evidences have suggested that it might only bind MCP in the hexons but not in the pentons (15-17).

1.4 Diversity of herpesvirus SCP

Among the several capsid proteins, SCP is the most divergent one across the three subfamilies of the Herpesviridae, in terms of molecular size, sequence similarity (as listed in Table 1-2), and function. In herpes simplex virus type 1 (HSV-1), an alphaherpesvirus, the SCP was found to be
Table 1-2 Comparison of capsid proteins across representative members of the three herpesvirus subfamilies.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Size (kDa)</th>
<th>Average % of identical amino acids (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HUD-1</td>
</tr>
<tr>
<td>MCP</td>
<td></td>
<td>HSV-1</td>
</tr>
<tr>
<td>VP5</td>
<td>149</td>
<td>UL86</td>
</tr>
<tr>
<td>VP19c</td>
<td>50</td>
<td>UL46</td>
</tr>
<tr>
<td>VP23</td>
<td>34</td>
<td>UL85</td>
</tr>
<tr>
<td>VP26</td>
<td>12</td>
<td>UL48.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

dispensable for replication of the virus in cell culture, though production of infectious virus in the nervous system of infected mice was affected (18). However, in beta- and gammaherpesviruses, the SCP was found to be essential for viral growth in cell culture: human cytomegalovirus (HCMV) bacterial artificial chromosome (BAC) construction with deletion of the SCP open-reading frame (ORF) did not give rise to infectious virus (19); SCP-deletion mutant of Kaposi’s sarcoma-associated herpesvirus (KSHV) also had a dramatically decreased viral titer in cell culture compared to that of the WT virus (20). Furthermore, the SCP in gammaherpesviruses was proposed to be essential for the viral capsid assembly: for KSHV and Epstein-Barr virus (EBV), capsid-like particles were successful assembled in insect cells infected with baculoviruses expressing the four capsid proteins (MCP, Tri1, Tri2 and SCP) and two extra proteins required for capsid assembly of all herpesviruses -- the scaffolding protein and the capsid maturation protease; however, when baculovirus expressing the SCP was omitted in the system, the capsid assembly was abortive (21, 22). In contrast, HSV-1 capsid-like particles were
readily assembled in a cell-free system with a mixture of only five proteins – MCP, Tri1, Tri2, the scaffolding protein, and the maturation protease, i.e., the SCP was not required for the assembly of HSV-1 capsid (23). The functional discrepancy of SCPs across the three subfamilies, and the exact role of SCP in the context of herpesvirus capsid assembly or viral growth are not fully understood.

1.5 Diversity of herpesvirus capsid-associated tegument proteins

Though the tegument layer of herpesvirus is mostly unstructured and nonsymmetrical (9, 10), cryo electron microscopy (cryoEM) three-dimensional (3D) reconstructions of some herpesvirus capsids and/or virions have revealed extra densities on the surface of the capsid that are attributable to some orderly-binding tegument proteins (24-29). The emergence of these capsid-associated tegument densities in the reconstruction suggests that these tegument proteins have very specific binding sites on the capsid surface and thus conform to the same icosahedral symmetry as the capsid.

Completely different patterns of capsid-associated tegument densities have been identified in alpha- and betaherpesviruses, but none has been observed yet in any gammaherpesvirus capsid or virion reconstructions (30-32). In alphaherpesviruses, exemplified by HSV-1 and Pseudorabies virus (PRV, a porcine herpesvirus), the capsid-associated tegument densities are restricted in the peripheries of capsid pentons; each of the five copies of filamentous tegument density surrounding a penton bridges on top of the two triplexes closest to the penton, and extends to the tip of the penton MCP (28, 29, 26, 27, 33). In contrast, the capsid-associated tegument densities are ubiquitous all over the capsid surface in betaherpesviruses, as exemplified by HCMV virion
reconstruction. In HCMV, three copies of filamentous tegument density sit on top of each triplex, and each extends to the tip of a neighboring hexon or penton MCP. The entire tegument densities form a cage-like structure enclosing the capsid (24, 25, 34).

Though still in controversial, the identities of capsid-associated tegument proteins in alpha- and gammaherpesviruses have been restricted to a few candidates. In HCMV, the tegument density was proposed to be composed of pp150, a betaherpesvirus-specific tegument protein (34). In HSV-1 and PRV, the tegument density was proposed to consist of at least two tegument proteins – UL17 and UL25 (26, 27, 33), which are actually conserved across all three herpesvirus subfamilies. Each of these candidates was demonstrated to be essential for viral growth in cell culture (35-37, 28, 38, 39). However, the detailed structures of these tegument proteins, and how they interact with the capsid and/or other viral proteins to affect the viral growth are not clear.

1.6 High-resolution structural studies of herpesvirus capsids

To understand the above mentioned mysteries regarding structural and functional diversities of herpesvirus capsid proteins and tegument proteins, high-resolution structures of herpesvirus capsids or virions are much needed. However, the structural study of herpesvirus capsid by cryoEM is stagnant in recent years despite of the rapid development of the cryoEM field reaching atomic resolution for reconstructions of other smaller viruses (40-44). The main obstacle for high resolution structure determination of herpesvirus capsid is its large size. To reconstruct the 3D structure of a large particle to a specific resolution, we need more sampling data points than reconstructing a smaller particle to the same resolution, which means a larger dataset is needed. It increases the burden not only experimentally (to collect more data), but also
computationally. Another important limiting factor caused by the large size of herpesvirus capsid is the so-called “Ewald sphere problem”, which dictates that for a particle as large as the herpesvirus capsid (>135nm) the Fourier transforms of 2D projections represent curved planes in 3D Fourier space due to the curvature of the Ewald sphere, especially at high resolution range (45, 46).

Despite of the difficulties, there are also opportunities brought by new technical developments in the cryoEM field that may help to push the envelope of herpesvirus capsid structure determination. The emergence of a new cryoEM image recording technique named direct electron detection has been revolutionizing the field. In the conventional way of recording cryoEM images with film or CCD (charge-coupled device) camera, due to the multiple-stage conversion of electron signals into light and then digital signals, a lot of noise is added to the cryoEM image which in itself is very noisy (47, 48). The noisy nature of the images limits the accuracy of center and orientation determinations of the images, damping the high resolution signals in the averages and 3D reconstructions. In contrast, with the new direct electron detection camera, the electrons are captured and converted to digital signals directly, with a much higher signal to noise ratio (SNR) than that in film or CCD cameras (49-52). Moreover, the electron-counting mode implemented in some of the direct electron detection cameras can help to further reduce the noise (52). Another benefit is brought about by the fast readout speed of the direct electron detection cameras, which has enabled fractionating one single exposure of a few seconds into tens of consecutive short exposures in the scale of milliseconds (48). Each of the short exposures produces an image, which is called a frame. Later, the whole stack of the frames can be aligned, and all or some of the aligned frames can be averaged to produce one single image for data processing. Aligning and averaging the frames corrects the small drifts of
consecutive frames which are resulted from beam-induced sample movement during the course of exposure (48). This drift-correction restores the high resolution information in the cryoEM images that is otherwise blurred by the beam-induced sample movement. The images recorded by a direct electron detection camera not only contains more high-resolution signals, but their high SNR also improves the accuracy of orientation determination of each image, leading to higher resolution in the final reconstructions. Since its launch in 2013, the direct electron detection technology has enabled atomic structure determination of low-symmetry (compared to icosahedral viruses) or even none-symmetric protein complexes (48, 53-56). To the date this dissertation is written, the record has been set to an astonishing 2.2Å resolution (57).

Taking advantage of the new direct electron detection technology, we have determined atomic-resolution structures and are building atomic models for three herpesvirus virions: HSV-1 of the alphaherpesviruses, HCMV of the betaherpesviruses, and KSHV of the gammaherpesviruses. However, this dissertation summarizes only some of the intermediate results we obtained on our itinerary to this final destination. In my opinion, these results well demonstrate how structures, even though at limited resolutions, can be combined with other molecular biology tools to guide our search efforts for answers to biological questions of herpesviruses.
Chapter 2 HCMV SCP MEDIATES PP150 BINDING
2.1 Abstract

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that causes birth defects in newborns and life-threatening complications in immunocompromised individuals. Among all human herpesviruses, HCMV contains a much larger dsDNA genome within a similarly-sized capsid compared to the others, and it was proposed to require pp150, a tegument protein only found in cytomegaloviruses, to stabilize its genome-containing capsid. However, little is known about how pp150 interacts with the underlying capsid. Moreover, the smallest capsid protein (SCP), while dispensable in herpes simplex virus type 1, was shown to play essential, yet undefined, role in HCMV infection. Here, by cryo electron microscopy (cryoEM), we determine three-dimensional structures of HCMV capsid (no pp150) and virion (with pp150) at sub-nanometer resolution. Comparison of these two structures reveals that each pp150 tegument density is composed of two helix bundles connected by a long central helix. Correlation between the resolved helices and sequence-based secondary structure prediction maps the tegument density to the N-terminal half of pp150. The structures also show that SCP mediates interactions between the capsid and pp150 at the upper helix bundle of pp150. Consistent with this structural observation, ribozyme inhibition of SCP expression in HCMV-infected cells impairs the formation of DNA-containing viral particles and reduces viral yield by 10,000 fold. By cryoEM reconstruction of the resulting “SCP-deficient” viral particles, we further demonstrate that SCP is required for pp150 functionally binding to the capsid. Together, our structural and biochemical results point to a mechanism whereby SCP recruits pp150 to stabilize genome-containing capsid for the production of infectious HCMV virion.
2.2 Introduction

Human cytomegalovirus (HCMV), the prototype of betaherpesvirus subfamily of the \textit{Herpesviridae}, is a leading viral cause of birth abnormalities and a major life-threatening pathogen in AIDS and organ transplant patients (58). HCMV virion shares a common architecture with other herpesviruses and consists of a polymorphic envelope, a tegument compartment and an icosahedral nucleocapsid enclosing a linear dsDNA genome. The HCMV genome is the largest amongst that of all human herpesviruses, and encodes a remarkable number of conserved proteins, as well as unique envelope and tegument proteins that lack homologs in alpha- or gammaherpesviruses (59). The HCMV capsid shell, similar to those of herpes simplex virus type 1 (HSV-1) and Kaposi’s sarcoma-associated herpesvirus (KSHV), is composed of four major proteins: the major capsid protein (MCP; encoded by UL86) (60), the minor capsid protein (mCP; encoded by UL85), the mCP binding protein (mC-BP; encoded by UL46) (61), and the smallest capsid protein (SCP; encoded by UL48.5) (61, 62). All herpesvirus capsids studied to date share a T=16 icosahedral assembly with pentons (MCP pentomers), hexons (hexamers of MCP), connecting triplexes (heterotrimers of two mCP and one mC-BP), and SCP attached to the tip of each MCP (63, 24, 64, 65, 30). While the other three capsid structural proteins are conserved, SCP is very divergent in size, amino acid sequence and function among different herpesviruses. In HCMV, SCP was shown to be essential for virus growth (19), but its function is still unknown.

CryoEM reconstruction also revealed different patterns of association between capsid and overlying tegument proteins in CMV and HSV. In HCMV, a layer of highly organized filamentous density of tegument proteins is attached to the pentons, hexons and triplexes of the underlying nucleocapsid (25). The three-dimensional (3D) reconstruction of the simian
cytomegalovirus (SCMV) capsid isolated from the cytoplasm of infected cells also revealed tegument proteins attached to the capsid (24), similar to HCMV. In contrast, HSV-1 ordered tegument proteins only bind pentons and those triplexes surrounding pentons (25, 19, 66). These observations indicate that viral proteins overlying the conserved capsid, such as tegument and envelope proteins, have evolved to have virus-specific structural and functional roles. Recently, biochemical and structural studies have assigned pp150 to the ordered filamentous tegument densities of CMV virion and suggested its function in stabilizing the dsDNA-filled C capsid (67, 3), but more structural details are needed to fully understand the molecular interactions between pp150 and capsid proteins.

Here, we report the 3D structures of HCMV capsid and intact HCMV virion at 6Å and 9Å resolution, respectively. Comparison of the capsid and virion structures reveals, at a secondary-structure level, that SCP mediates interactions between the capsid and tegument protein pp150. By constructing a ribozyme that inhibits SCP expression in HCMV-infected cells, and cryoEM reconstruction of the resulting “SCP-deficient” viral particles, we further demonstrate that SCP is required for pp150 binding to capsid and its absence results in only viral particles devoid of the DNA genome, thus revealing why SCP is essential for HCMV infection.

2.3 Materials and Methods

2.3.1 HCMV virion preparation

Human fibroblast MRC-5 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal bovine serum (FBS). 20 flasks (175cm²) of cells were grown to 90% confluence and infected with HCMV strain AD169 at a multiplicity of infection (MOI) of 0.1 - 1. At 6 days
post infection, when half of the cells were lysed, the media were collected and centrifuged at 10,000g for 15min to remove cell debris. The clarified supernatant was collected and centrifuged at 80,000g for 1hr to pellet HCMV virions. Pellets were resuspended in a total volume of 2ml phosphate buffered saline (PBS, pH7.4) and loaded on a 15%-50% (w/v) sucrose density gradient and sedimented at 100,000g for 1hr. Usually we observe three light-scattering bands – top, middle and bottom – containing mainly NIEPs, virions and dense bodies, respectively. The middle band was collected and diluted in PBS to a total volume of 13ml. Virion particles were pelleted again at 80,000g for 1hr and resuspended in 30μl PBS for cryoEM sample preparation.

### 2.3.2 HCMV capsid preparation

To obtain HCMV capsids, we infected 90% confluent MRC-5 cells at MOI=5. At 3 days post infection, when cytopathic effect reached 100%, cells were collected, pelleted by low-speed centrifuge at 1000g for 10min, and washed with PBS. The pellet was then resuspended in PBS containing 0.5% NP-40 (w/v) and incubated on ice for 5min. The mixture was centrifuged at 1000g for 10min to pellet cell nuclei. To break nuclear membrane, the pellet was then resuspended in PBS, subjected to three cycles of freezing (-80°C, 10 min), thawing (37°C, 3min) and vortexing, passed through a 23 gauge hypodermic needle for 20 times, and incubated in PBS with 2% NP-40 overnight at 4°C. The lysate was centrifuged at 1500g for 5min to remove large debris and then sedimented through a 30% sucrose cushion at 100,000g for 1hr. The pellet was resuspended in PBS containing 2% NP-40, diluted to a final volume of 13ml, and centrifuged again at 70,000g for 1hr. The pelleted capsids were resuspended in 30μl PBS and used for cryoEM sample preparation.
2.3.3 Construction and *in vitro* characterization of SCP-targeting and control ribozymes

Plasmids V482, pFL117 and pC102 contain the DNA sequences coding for variant V482 RNA, M1 RNA and mutant C102, respectively, driven by the T7 RNA polymerase promoter (68, 65). Mutant ribozyme C102 contains several point mutations at the catalytic domain (P4 helix). The DNA sequence coding for ribozyme TK1, which targets the mRNA of thymidine kinase of HSV-1, has been described (68). The DNA sequence encoding ribozyme SCP1 was constructed by PCR with V482 as the template. The 5’ and 3’ PCR primers were AF25 (5’-GGAAATTCTAATACGACTCACTATAG-3’) and M1SCP1 (5’-CCCCGCTCGAGAAAAATGGTGCTGAGCAAGTATACGCGTGTGGGAATTGTG-3’), respectively. The DNA sequence coding for ribozyme SCP2 was constructed by introducing into the DNA sequence coding ribozyme SCP1 with the point mutations (A<sub>347</sub>C<sub>348</sub>→C<sub>347</sub>U<sub>348</sub> and C<sub>353</sub>C<sub>354</sub>C<sub>355</sub>G<sub>356</sub>→G<sub>353</sub>G<sub>354</sub>A<sub>355</sub>U<sub>356</sub>) that were found in C102 and were shown to abolish the ribozyme activity (68, 65). The procedures for *in vitro* cleavage and binding analyses were carried out as described previously (68).

2.3.4 Construction of ribozyme-expressing cells

The DNA sequences encoding the ribozymes were subcloned into retroviral vector LXSN and placed under the control of the U6 RNA promoter. The retroviral DNA containing the ribozyme sequence was transfected into human U373MG cells, using protocols modified from Miller and Rosman (69). After 48–72 h of transfection, cells were incubated in culture medium containing 600 μg/ml neomycin. Cells were subsequently selected in the presence of neomycin for 2 weeks, and neomycin-resistant cells were cloned (68).
2.3.5 Northern and Western analyses of viral gene expression

Cells \((n = 1 \times 10^6)\) were either mock-infected or infected with HCMV at an MOI of 0.05–5 in 1.5 ml DMEM supplemented with 1% FBS. After 2h incubation, the inoculum was replaced with DMEM supplemented with 10% (v/v) FBS. The infected cells were incubated for 4–72 h and total cellular RNA or protein was isolated from the cells as described previously (68). Protein samples were also prepared from HCMV particles purified from the infected cells. The RNA fractions were separated in formaldehyde-containing 1% agarose gels, transferred to a nitrocellulose membrane, hybridized with \(^{32}\)P-radiolabeled RNA probes containing the HCMV sequences, and analyzed with a STORM840 PhosphorImager. The RNA probes used to detect M1GS RNA, HCMV IE 5-kb RNA transcript, and SCP mRNA were synthesized from plasmids pFL117, Cig27, and pSCP, respectively (68, 3). RNA probes were in vitro synthesized and radiolabeled using an in vitro RNA synthesis kit (Promega, Inc, Madison, IN).

In Western analysis experiments, protein samples were separated on SDS/7.5% polyacrylamide gels cross-linked with N,N’-methylenebisacrylamide, and then transferred electrically to nitrocellulose membranes. We stained the membranes using the antibodies against HCMV proteins in the presence of a chemiluminescent substrate (GE Healthcare), and analyzed the stained membranes with a STORM840 phosphorimager (68). Quantitation was performed in the linear range of RNA and protein detection.

2.3.6 Assays for inhibition of viral replication

Cells \((n = 1 \times 10^5)\) were infected with HCMV at MOI values specified in the Results section. The cells and medium were harvested at 1-day intervals throughout the 7 days after infection. Viral stocks were prepared by adding an equal volume of 10% (v/v) skim milk, followed by
sonication. The titers of the viral stocks were determined by infecting $1 \times 10^5$ human foreskin fibroblasts and counting the number of plaques 10–14 days after infection (68). The values obtained were averages from triplicate experiments.

2.3.7 Purification of SCP-deficient viral particles

Ribozyme SCP1-expressing U373MG cells were infected with wild type HCMV at MOI=1-5. After 2hr incubation, the medium was replaced with fresh DMEM plus 10% FBS to remove any free, extracellular viral particles. At 4 days post infection, viral particles were purified using the same procedure as described above for the wild type HCMV virion. Due to the significantly lower viral yield in SCP1-expressing cells, no clear light-scattering bands were visible in the density gradient. We therefore collected the fraction of the gradient corresponding to the range encompassing the three bands visible in the wild type virion purification. This fraction was diluted in PBS and centrifuged at 80,000g for 1hr to pellet SCP-deficient viral particles. The pellet was then resuspended in 10μl PBS, verified by negative staining electron microscopy to contain viral particles, and used for cryoEM sample preparation. The remainder gradient was also collected in fractions of 3ml each. Each fraction was diluted with PBS, pelleted, resuspended in 10μl PBS, and checked individually with negative staining electron microscopy to confirm the absence of viral particles.

2.3.8 CryoEM imaging and data processing

An aliquot of 2.5μl purified sample was applied to a 300 mesh Quantifoil R1.2/1.3 grid, blotted with filter paper, and plunge-frozen in liquid ethane. CryoEM images were collected at liquid nitrogen temperature in an FEI Titan Krios cryo electron microscope operated at 300kV with parallel illumination. The wild type HCMV virion and SCP-deficient HCMV particle images
were recorded on a Gatan 4k × 4k charge-coupled device (CCD) camera at an effective magnification of 97, 498× (nominal magnification 59, 000× on film plane), corresponding to an effective pixel size of 1.538Å/pixel at the specimen level. The HCMV capsid images were recorded on Kodak SO-163 films at a magnification of 59,000× and micrographs were digitized with Nikon Coolscan 9000ED scanner at a step size of 6.35μm/pixel, giving a pixel size of 1.076Å/pixel on specimen. In all cases, the electron dosage used in cryoEM imaging was ~25e−/Å². The defocus values were determined with CTFFIND (70) and are in the range of 0.5μm to 3μm underfocus.

Data processing and 3D reconstructions were accomplished with IMIRS (71, 10). Orientation and center parameters of each particle were refined against projections computed from 3D reconstructions in an iterative procedure until no further improvement in the reconstruction was obtained. Particles were selected based on the phase residues between the images and the projections. 3D reconstruction was obtained using the symmetry-adapted spherical harmonics method (10). The final capsid and virion reconstructions were obtained by averaging 20,502 particles (selected from 37,460 capsid images) and 11,863 particles (selected from 56,297 virion images), respectively.

Visualization and averaging of density maps were carried out with UCSF Chimera (72). Density regions to be averaged were segmented out as density cubes of similar size. These density cubes were then first manually aligned and subsequently computationally aligned by the “fit in map” function of Chimera. Averaged density was produced by executing the “vop add” command on the above aligned density cubes.

Secondary structure prediction of pp150 was performed with PSIPRED using the Protein Structure Prediction Server (73).
2.3.9 Data deposition

The cryoEM density maps of the capsid, the virion, and the SCP-deficient particles have been deposited in the Electron Microscopy Data Bank (EMDB) (accession code 5695, 5696 and 5697, respectively).

2.4 Results

2.4.1 CryoEM structural determination

Due to the large size of CMV particles and the difficulties to purify them, resolutions of previous cryoEM reconstructions were limited (25, 24, 65). In this study, highly purified HCMV capsid (no tegument, Figure 2-1A) and the intact virion (containing tegument proteins, Figure 2-1B) were obtained and imaged in a 300 kV Titan Krios high-resolution electron microscope. 3D reconstructions of HCMV capsid and virion were obtained at 6Å and 9Å resolution, respectively. The improved structures are a result of an exhaustive effort of processing more than 37,000 capsid images and 56,000 virion images. At these sub-nanometer resolutions, secondary structural elements, particularly α-helices, can be identified, as exemplified by the close-up views of a hexon in the capsid reconstruction (Figure 2-1C-E). Molecular boundaries can be established, allowing us to describe the interactions between the ordered tegument proteins and capsid proteins at secondary-structure level for the first time.
Figure 2-1 Comparison of 3D reconstructions of the HCMV capsid and virion.

(A, B) CryoEM images of HCMV capsid (A) and virion (B). (C) Radially colored surface representation of the 3D reconstruction of the capsid at 6Å resolution viewed along a 3-fold axis. Capsomers in an asymmetric unit, including a penton and three hexons, are labeled as “5”, “C”, “P” and “E”, respectively, as in the nomenclature of (74). (D, E) Close-up views of the C hexon demarked in the capsid reconstruction, viewed from outside (D) or inside (E) of the capsid. An α-helix with typical sausage shape is denoted in (E). (F, G) MCPud. The density map of the MCPud denoted by the square in (D) was extracted and radially colored (F). In (G), the same MCPud is shown in semi-transparent yellow and superimposed with the HSV-1 MCPud atomic model (magenta ribbon). Note all helices match in the two structures but the loops at the tip (arrow) and at the outer surface (arrowhead) of HSV-1 MCPud do not fit the cryoEM density map of HCMV MCPud, suggesting possible structural differences. (H) Radially colored surface representation of the 3D reconstruction of the HCMV virion viewed along a 3-fold axis. (I) Zoom-in view of the area denoted in (H). Structural components in an asymmetric unit are labeled, including a penton (“5”), three hexons (“C”, “P” and “E”), and six triplexes (“Ta”, “Tb”, “Tc”, “Td”, “Te” and “Tf”). Dashed square demarcates a region encompassing Te that is segmented out for averaging with similar regions around Tb and Td (see text and Figure 2.2).

2.4.2 Comparisons of 3D reconstructions of the capsid and the virion

The 6Å reconstruction of the HCMV capsid reveals the molecular boundaries among the 150 hexons, 12 pentons and 320 triplexes in the T=16 icosahedral particle (Figure 2-1C), allowing identification of individual molecules. In particular, the upper domain of an MCP monomer was extracted from a central hexon (Figure 2-1F) and superimposed with the crystal structure of the HSV-1 MCP upper domain (MCPud) (PDB accession code 1N07) (Figure 2-1G) (75). Except for very minor differences at the tip and the outer surface of the subunits (arrow and arrowhead in Figure 2-1G), excellent match of all the α-helices between HCMV and HSV-1 MCPuds is observed, indicating that the bulk of MCPud structure is conserved between the two viruses. This match also demonstrates the high quality of the map.

In addition, the fitting reveals that SCP molecules were lost in this highly purified capsid sample, probably due to the use of detergent during all purification steps (see Experimental
Procedures). In gently prepared capsid preparations, SCP binds MCP at the upper domain as shown in previous capsid reconstructions we obtained (65). The absence of SCP in the capsid reconstruction provides the advantage to identify the molecular boundary between SCP and MCP in the virion reconstruction (see below).

The 9Å reconstruction of HCMV virion shows a layer of filamentous tegument proteins bound to the capsid in an icosahedrally ordered fashion, like a net enclosing the entire capsid (Figure 2-1H). Three of these tegument densities sit on top of each triplex, forming a ‘group-of-three’, and extend to the top of the nearest subunits in the three surrounding capsomers. The location and appearance of these tegument densities are similar to those decorated by anti-pp150-antibodies (67, 3). In each asymmetric unit of the herpesvirus capsid, there are six quasi-equivalent triplexes, Ta, Tb, Tc, Td, Te and Tf (Figure 2-1I), following the nomenclature of (76). The group-of-three tegument densities on triplexes Tb, Td and Te are the most similar in structure. We averaged the densities within three cubes, each of which contains one of these group-of-three tegument densities (e.g., the region encompassing Te is outlined by the dashed square in Figure 2-1I) to improve the signal/noise ratio (Figure 2-2A). Helices in the tegument proteins can be resolved in the averaged density (as illustrated in Figure 2-2E). The same cubic regions from the capsid reconstruction were averaged for comparison (Figure 2-2B). This comparison allowed us to differentiate densities of MCP and triplexes from densities attributable to SCP and tegument proteins, and subsequently to segment out SCP and the tegument densities. The boundary between SCP and pp150 was established by referring to our pervious SCP-containing capsid reconstruction (65).
Figure 2.2 Structure of the capsid-interacting pp150 tegument protein.

(A, B) Averaged density of the regions surrounding triplexes Tb, Td and Te (as marked by the dashed square in Figure 2-1I) from the virion (A) or capsid (B) reconstructions. The two density maps are colored the same as in Figure 2-1H or 2.1C. (C) SCP (light blue) and tegument (green, magenta and cyan) densities segmented from the...
virion densities of (A) superimposed on the capsid densities (yellow) of (B). (D) Views of the three tegument densities of (C) after alignment to each other. The most prominent feature is the sausage-shaped densities due to helices. The dashed line denotes the boundary of the upper helix bundle (UHB) and the lower helix bundle (LHB).

(E) The cyan tegument density in (D) is shown semi-transparently and superimposed with cylinders representing helices. Helices in the upper helix bundle (magenta) and lower helix bundle (cyan) are connected by a 67Å-long central helix (CH, red). (F) Secondary structure prediction of pp150 based on its amino acid sequence. The putative location for the long central helix identified in (E) is indicated by the red line.

2.4.3 Structure of the capsid-interacting tegument protein, pp150

The three tegument densities in the averaged group-of-three exhibit a high level of similarity and appear nearly identical when displayed side by side (Figure 2-2D). This structural similarity among the three tegument densities suggests that three copies of the same tegument protein or protein complex associate with each triplex, which differs from the situation in SCMV, where only two copies of tegument densities were interpreted to bind to each triplex (24). In each tegument density of HCMV, we resolved two helix bundles, an upper one and a lower one, joined by a long central helix (~67 Å in length). The upper helix bundle (UHB) is composed of the central helix and five shorter surrounding helices. The lower helix bundle (LHB) only has three short helices surrounding the central helix (Figure 2-2E).

Previous studies of HCMV and SCMV particles have suggested pp150 as one of the candidates for the capsid-interacting tegument densities (25, 24, 3). Secondary structure prediction indicates that the C terminal half of pp150 is almost entirely coils, in contrast to the N terminal half, which contains many helices (Figure 2-2F). Among these predicted helices, the longest one has 47 residues from a.a.195 to a.a.241 (Figure 2-2F), which would span ~70Å as each amino acid in an α-helix gives an axial distance of 1.5Å. This ~70Å length of the longest
predicted helix and the measured ~67Å length of the central helix resolved in the cryoEM density correlate well with each other, and both are more than twice the length of any other predicted or resolved helices. Moreover, there are eight other predicted major helices with more than 3 helical turns (each turn = 3.6 a.a.) and their lengths (12-22 a.a.) also correlate with those of the eight shorter helices resolved in the tegument density. Therefore, we conclude that the resolved tegument density is contributed only by the N-terminal half of pp150 molecule, while the C-terminal half of pp150 may be disordered or flexible. This conclusion is consistent with previous biochemical data showing that N-terminal segment of SCMV pp150 was both necessary and sufficient to bind either SCMV or HCMV capsid in vitro (77).

2.4.4 SCP mediates interactions between pp150 and the capsid

We further identified the interface between the tegument density and the capsid. At one end of pp150, its LHB has direct contacts with the triplex (Figure 2-3A-C). At the other end, pp150 UHB interacts with the capsomer through one of its five short helices (arrowhead in Figure 2-3D and 3E). This interaction appears to be mediated by the 8kDa SCP molecule, which is situated in the cleft formed by the pp150 UHB and the upper domain of MCP (Figure 2-3D-E). The direct contact between the densities assigned to pp150 and SCP suggests direct binding of the two molecules, although at this resolution, one can’t rule out the unlikely possibility that binding of SCP to MCPud can in theory change conformation of MCPud, causing it to bind to pp150 directly.

2.4.5 Confirmation of the role of SCP by ribozyme inhibition

To assess the functional significance of SCP in mediating pp150 binding to the capsid, we constructed a cell line expressing a ribozyme that inhibits the expression of SCP when the cell
line is infected by HCMV. Then, we determined the consequence of this inhibition on pp150-binding to the capsid by cryoEM analyses of viral particles harvested from this cell line.

![Figure 2-3 SCP mediates pp150 binding to the capsid.](image)

**Figure 2-3 SCP mediates pp150 binding to the capsid.**

(A-C) Density slices showing that pp150 tegument protein binds to the capsid triplex with its LHB (lower helix bundle). The binding sites on the triplex are labeled with “*”. The LHB of one molecule in the group-of-three tegument densities also has contact with MCP. It is labeled with “#”. (D) A close-up, top view of the region demarcated by the dashed square in the inset, revealing the interactions between pp150 (cyan), SCP (light blue) and MCPud (yellow). (E) Same as in (D) but viewed from the direction indicated by the eye symbol in (D). Arrow in both (D) and (E) points to the α-helix in pp150 UHB (upper helix bundle) that interacts with SCP.
We constructed a ribozyme, called SCP1, by covalently linking the 3’ terminus of a previously established M1GS ribozyme variant (V482) (68) to an 18-nt guide sequence complementary to the targeted HCMV SCP mRNA sequence. Two other ribozymes, SCP2 and TK1, were also designed and used as controls. SCP2 contains the same guide sequence as SCP1 but has multiple point mutations at the catalytic P4 domain that abolish its catalytic activity (68), thus serving as a control for the antisense effect in our experiments. TK1 targets the mRNA of thymidine kinase (TK) of HSV-1 and serves as a control to determine whether M1GS RNA with an incorrect guide sequence could target SCP mRNA in tissue culture. We subsequently constructed cell lines expressing each of these three M1GS ribozymes and carried out the following three experiments.

First, we analyzed SCP mRNA expression in HCMV-infected cells by Northern blotting, using the level of viral immediate-early (IE) 5-kb mRNA as an internal control (Figure 2-4A). Based on radioactivity of 32P-labeled probes, we estimated that target mRNA expression level was reduced by 98 ± 8%, 7 ± 4%, and 3 ± 3% (average of three experiments) in cells expressing SCP1, SCP2 and TK1, respectively. Furthermore, the protein level of SCP, as determined by Western analyses with the MCP protein level as the internal and loading control, was reduced by 97 ± 9%, 8 ± 5%, and 2 ± 1% in cells expressing SCP1, SCP2, and TK1, respectively (Figure 2-4C, lanes 9-12). Thus, targeted cleavage of SCP mRNA by ribozyme SCP1 significantly reduced SCP expression in cells expressing SCP1, but not in cell lines expressing both control ribozymes. The low level of inhibition observed in SCP2-expressing cells was probably due to an antisense effect, as SCP2 has a target-binding affinity similar to that of SCP1 but is catalytically inactive.
Figure 2-4 Ribozyme-mediated inhibition of HCMV SCP expression and viral growth.

(A) Northern analysis of HCMV mRNAs in infected cells. RNA samples were isolated from parental U373MG cells (lanes 1, 2, 5 and 6) or M1GS-expressing cells (lanes 3, 4, 7 and 8) that were either mock-infected (lanes 1 and 5) or infected with HCMV (MOI = 0.5–1; all other lanes) for 48 h, separated by denaturing gels, and transferred to membranes. Membranes were hybridized with radiolabeled probes containing the sequence of HCMV SCP mRNA (lanes 1-4) or IE 5 kb RNA (lanes 5-8). SCP1, ribozyme targeting HCMV SCP mRNA for degradation; SCP2,
control ribozyme that binds but cannot degrade HCMV SCP mRNA. (B) Growth of HCMV in U373MG cells and cell lines expressing M1GS RNA. Cells (5 \times 10^5) were infected with HCMV at MOI = 3. Values are means derived from triplicate experiments. Standard deviation is indicated by error bars. TK1, control ribozyme targeting HSV-1 thymidine kinase mRNA. (C) Western analysis of HCMV SCP and MCP proteins. Protein samples were either isolated from the parental U373MG cells or ribozyme-expressing cells (Infected cells, lanes 9-12) or from viral particle preparations purified from these cells (HCMV particles, lanes 13-15), separated in SDS-polyacrylamide gels, transferred to membranes, and reacted with antibodies against HCMV SCP (anti-SCP) and MCP (anti-MCP) (68).

Second, we assessed the effect of SCP-inhibition in viral yield by measuring viral titers of stocks from HCMV-infected cells that express the ribozymes. At 5 days post-infection, viral yields were reduced by at least 10,000-fold in cells expressing SCP1, whereas no significant reduction was observed in cells expressing SCP2 or TK1 (Figure 2-4B).

Third, to uncover the structural basis of the reduction of viral yield due to SCP inhibition, we imaged viral particles isolated from SCP1-expressing cells by cryoEM and compared its 3D structure with that of the wild-type HCMV virion. Using MCP as the internal and loading control, Western analyses showed that SCP was hardly detected in HCMV particles isolated from SCP1-expression cells but was readily found in viral particles isolated from cells that did not express any ribozymes or expressed control ribozymes SCP2 or TK1 (Figure 2-4C, lanes 13-15). CryoEM images of wild-type HCMV virion have the characteristic “fingerprint” appearance (Figure 2-1B), which is a hallmark of encapsidated genomic dsDNA (78, 25). In contrast, none of the cryoEM images of particles harvested from the SCP1-expressing cell culture media shows a fingerprint pattern (Figure 2-5A), indicating that they do not contain viral DNA genome and are thus non-infectious. The existence of non-infectious enveloped particles
(NIEPs) in this preparation indicates that the inhibition of SCP expression does not prevent capsid assembly and envelopment. Furthermore, 3D reconstruction at 20Å resolution of these SCP-deficient particles shows a structure with no visible tegument densities bound to the capsid (Figure 2-5C). In contrast, reconstruction of wild-type virion at the same resolution clearly shows tegument densities interacting with the underlying capsid (Figure 2-5B). This result clearly demonstrates that SCP is required for the functional binding of pp150 to capsid. Considering that pp150 may function in stabilizing the dsDNA-filled C capsid (67, 3), we reason that, in the absence of SCP, pp150 can no longer form the stabilizing network of density surrounding the capsid, thus preventing the formation of DNA-containing virion (Figure 2.5A). However, we cannot rule out the possibility that the lack of DNA in the SCP-deficient particle and failure to bind pp150 are two unrelated, downstream consequences of lacking SCP. It is also noteworthy that, although the absence of SCP prevents pp150 from binding to the capsid with icosahedral symmetry, it does not necessarily eliminate binding of pp150 to the capsid triplex in a non-icosahedrally ordered fashion, which could have also produced a cryoEM map without visible pp150 densities.
(A) Representative cryoEM images of SCP-deficient viral particles showing enveloped particles without the dsDNA genome. (B, C) Radially colored surface representations of 3D reconstructions of wild type (B) and SCP-deficient (C) viral particles. Figure 2-5 Confirmation of the role of SCP by structural comparison of SCP-deficient and wild-type viral particles.
(C) HCMV viral particles at 20Å resolution. Lower panels are zoom-in views of the region containing a triplex, revealing that pp150 is present in the wild-type structure but absent in the SCP-deficient viral particles. (D, E) 15Å-thick central slices extracted from reconstructions of wild type (D) and SCP-deficient (E) particles respectively. Concentric shells of density inside the capsid in (D) are attributable to the viral dsDNA genome, and they are uniformly spaced (23Å). A ring of scaffold densities are identified in (E), but there is no DNA density. Small bulge on tip of MCP in (D) corresponds to the density of SCP. There is no such bulge at the corresponding position in the SCP-deficient reconstruction.

2.5 Discussion

As mentioned above, among all human herpesviruses, HCMV has the largest dsDNA genome contained within a capsid of similar size. As a result, the distance between adjacent dsDNA duplex in HCMV capsid is 23Å (79), as compared to 26Å and 25Å for those in alphaherpesvirus (25) and gammaherpesvirus (16), respectively. It is conceivable that the electrostatic repulsion of the more densely packed genome in HCMV would exert higher pressure to the capsid shell, possibly rendering the DNA-containing capsid (i.e., “C capsid”) unstable. Indeed, throughout our cryoEM imaging of the HCMV capsid preparation, not a single intact DNA-containing capsid was observed among the over 30,000 particle images we examined (Figure 2-1A), in stark contrast to the situations of alphaherpesvirus (78) and gammaherpesvirus (16) where C capsids can be readily purified from the nuclei of infected cells. Upon tegumentation, including the addition of pp150, DNA-containing nucleocapsids are stabilized and thus are routinely found in HCMV virion preparations (Figure 2-1B).

Of the capsid structural proteins, SCP is the least conserved across different herpesviruses in size, amino acid sequence, and function. For example, the HSV-1 SCP has a
molecular weight of 12kDa and is dispensable for virus growth in cell culture (18). The 16kDa KSHV SCP is the largest and is essential for capsid assembly (21, 20). The 8kDa HCMV SCP is the smallest and is essential for virus growth (19), but its functional role is a long-standing mystery. Here, by using SCP-targeting ribozyme and cryoEM reconstruction, we show for the first time that the HCMV SCP is required for the functional binding of pp150 to the capsid, and thus indirectly stabilizes the capsid for the formation of infectious HCMV virion. To our best knowledge, this role is the only function of HCMV SCP identified to date. This result, when considered together with the absence of a pp150 homolog in both alpha- and gammaherpesviruses, indicates that SCP of herpesviruses has diverged in function though its location in different herpesviruses is conserved. Perhaps, SCP has a yet unknown, non-essential function conserved across all herpesviruses, but in HCMV, it is re-utilized by pp150 as a partner to stabilize DNA-containing capsid, an essential process for HCMV infection.

Taken into consideration the relatively small size and essential function, HCMV SCP clearly provides a potential target for intervention against HCMV infection. One possible way is to design SCP-mimicking peptides that act as competitive inhibitors of pp150 binding and functioning, thus preventing infectious viral particle formation.

2.6 Acknowledgments

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cytomegalovirus. PLoS pathogens 9(8):e1003525. Author contributions were as following: ZHZ and XY designed experiments. XD, XY and SS cultured and purified the wild type virus, XD, XY, HL acquired the cryoEM data, XD and XY processed the data. HG, XJ and GA performed the ribozyme inhibition and Western blot experiments. XD purified the SCP-deficient virus, imaged the virus and processed the data. WJB provided the anti-SCP and anti-MCP antibodies. XD, ZHZ, XY, FL and HZ wrote the paper. The authors thank Xiaorui Zhang for initial efforts in cell culture and HCMV sample preparation, Xing Zhang, Ivo Atanasov, and Jiansen Jiang for assistance in cryoEM, Paul Rider and Rachael Burchfield for ribozyme experiments, and Xiaokang Zhang for assistance in data-processing. This project was supported in part by grants from the National Institutes of Health (AI069015 to ZHZ). The authors also acknowledge the use of cryoEM facilities at the Electron Imaging Center for NanoMachines supported in part by NIH (1S10RR23057 to ZHZ).
Chapter 3 KSHV SCP CEMENTS CAPSID HEXONS
3.1 Abstract

With just one-eighth the size of the major capsid protein (MCP), the smallest capsid protein (SCP) of human tumor herpesviruses – Kaposi’s sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) – is vital to capsid assembly, yet its mechanism of action is unknown. Here, by cryo electron microscopy (cryoEM) of KSHV at 6Å resolution, we show that SCP forms a crown on each hexon and uses a kinked helix to crosslink neighboring MCP subunits. SCP-null mutation decreased viral titer by 1000 times, impaired but did not fully abolish capsid assembly, indicating an important but non-essential role of SCP. By truncating the C-terminal half of SCP and performing cryoEM reconstruction, we demonstrate that SCP’s N-terminal half is responsible for the observed structure and function while the C-terminal half is flexible and dispensable. Serial truncations further highlight the critical importance of the N-terminal 10 amino acids, and cryoEM reconstruction of the one with 6 residues truncated localizes the N-terminus of SCP in the cryoEM density map and enables us to construct a pseudo-atomic model of SCP. Fitting of this SCP model and a homology model for the MCP upper domain into the cryoEM map reveals that SCP binds MCP largely via hydrophobic interactions and the kinked helix of SCP bridges over neighboring MCPs to form non-covalent crosslinks. These data support a mechanistic model that tumor herpesvirus SCP reinforces the capsid for genome packaging, thus acting as a cementing protein similar to those found in many bacteriophages.
3.2 Introduction

The *herpesviridae* is a large family of dsDNA viruses containing several widespread human pathogens. It is classified into three subfamilies, namely alpha-, beta-, and gammaherpesviruses (80). Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is a member of the oncogenic gammaherpesvirus subfamily (81). All herpesviruses share the same architecture with a genome-containing capsid surrounded by a poorly defined tegument layer, which in turn is enclosed in a lipid envelope. The capsids of different herpesviruses are similar in composition and structure (64, 82, 31), each composed of at least five capsid proteins (encoding genes of KSHV given in parentheses): the major capsid protein (MCP, ORF25) in both hexameric and pentameric capsomers, triplex proteins 1 and 2 (ORF62 and ORF26, respectively) forming hetero-trimers in a 1:2 ratio to connect the capsomers, the smallest capsid protein (SCP, ORF65) previously shown to decorate tips of hexons (16, 65, 15, 83), the portal protein (ORF43) forming a dodecameric structure for DNA genome packaging at one of the twelve icosahedral vertices (12).

Assembly pathway of herpesvirus capsid resembles what has been established for dsDNA bacteriophages such as T4, λ, and P22 (84, 85): a spherical, porous intermediate named “procapsid” is firstly assembled around a core of scaffold proteins; the scaffold proteins are then cut by a maturational protease and extruded through the large holes of the procapsid shell; concurrently, DNA genome is packaged through the portal; the procapsid undergoes dramatic conformational changes and matures into a more stable, angularized icosahedral capsid (85).

The assembly of herpesvirus capsid was proposed to be a spontaneous process as the capsid of herpes simplex virus type 1 (HSV-1, an alphaherpesvirus) could be assembled in a cell-free system (23, 86). The minimum set of capsid proteins required for HSV-1 capsid assembly was
determined to include the MCP, the two triplex proteins, the scaffold protein and the 
maturational protease. The SCP of HSV-1 was dispensable for capsid assembly (23) and virus 
propagation (18). However, SCPs of KSHV and Epstein-Barr virus (EBV), the two known 
human pathogens of the gammaherpesvirus subfamily, have been demonstrated to be vital for 
capsid assembly in vitro (21, 22) and important for virus propagation (20). How SCP of tumor 
herpesviruses affects capsid assembly is poorly understood due to the lack of high-resolution 
capsid structures of these viruses.

Here we use cryo electron microscopy (cryoEM) combined with bacterial artificial 
chromosome (BAC) mutagenesis to determine structures of KSHV virion capsids bearing full-
length or truncated SCP. The results provide molecular and mechanistic insights into the role of 
this 170-residue small protein in KSHV capsid assembly and viral propagation.

3.3 Materials and Methods

3.3.1 Construction of KSHV mutant BACs

KSHV BAC16 genome was modified according to a previously described method (87). Briefly, 
for KSHV SCP-null mutant (KSHV-SCPnull), wild type sequence (ATC CCG CCT TTG AAT 
TCC ACC CAT CCT CCT CAG, nt. 112,532—112,564, GQ994935.1) in the ORF65 coding 
region was replaced by a mutant sequence (ATC CCG CCT Tag atc tca ACC CAT CCT CCT 
CAG), to introduce a stop codon which can abolish ORF65 protein translation and a Bgl II 
restriction site which will facilitate the screening of desired mutation. For KSHV SCP truncation 
mutants, shortened sequence with the corresponding amino acids removed was used to replace 
the wild type sequence by homologous recombination. The restriction patterns of BAC plasmids
were verified by comparing them with BAC16 to ensure their overall integrity without gross changes other than the expected ones. Fragments with the mutations in the middle were PCR amplified from the BAC plasmids, and sequenced to confirm that all mutations were correct. All mutant BAC plasmids were further introduced into iSLK-puro cells, followed by selection with 1,200 μg/ml hygromycin B, 1 μg/ml puromycin and 250 μg/ml G418 for one month to generate cell lines latently infected by the KSHV mutants.

3.3.2 Titration of infectious KSHV virus

To determine the concentration of infectious KSHV virions released from iSLK cells harboring KSHV WT or ORF65 mutant genome, the supernatants were collected from iSLK cell cultures three days after induction with 1 mM sodium butyrate plus 1 μg/ml doxycycline, centrifuged at 10,000 g for 15 min to remove cellular debris, serially diluted in DMEM with 10% FBS, and then used to infect 293T cells by spinoculation (3,000 g for 1 h at 30 °C). Because the KSHV BAC16 virus harbors a green fluorescent protein (GFP) expression cassette driven by the cellular EF1_promoter, 293T cells will express GFP once the viral genome is delivered into the cells, providing a means to measure the amount of infectious viruses present in the original supernatant solution. Three days post-infection, GFP positive cell clusters containing two or more cells were counted under fluorescence microscope to determine the titers of KSHV viruses. Infectious units (IU) are expressed as the number of GFP-positive cell clusters in each well at the time of analysis.
3.3.3 Western blotting and antibodies

Cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membrane, and further probed with rabbit polyclonal antibody against SCP (88) or mouse monoclonal antibody to actin (Abcam).

3.3.4 Transmission electron microscopy

The iSLK-WT, iSLK-SCPnull, or iSLK-SCPN86 cells were treated with 1 µg/ml doxycycline plus 1 mM sodium butyrate for three days to induce viral lytic replication. Then cells were collected and subjected to plastic embedding and transmission electron microscopy with the method previously described (89). Briefly, cells were washed with PBS, fixed with 2% glutaraldehyde in PBS for 1 h, post-fixed in 1% OsO4 for 1 h, en bloc stained in 2% uranyl acetate for 1 h, dehydrated in an ascending ethanol series, and embedded in Spurr’s resin. Approximately 75 nm sections were stained with saturated aqueous uranyl acetate and lead citrate, and examined with an FEI Tecnai F20 electron microscope operated at 200 kV.

3.3.5 Virion purification

The iSLK cell lines harboring WT or mutant KSHV BAC16 were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 1µg/ml Puromycin, 250µg/ml G418 and 1200µg/ml Hygromycin to 80% confluency, and then treated with 1mM Sodium Butyrate and 1µg/ml doxycycline in fresh DMEM plus 10% FBS for 3-5 days to induce viral lytic replication. WT and mutant KSHV virions were purified from supernatants of the culture, following the procedure developed previously for purification of HCMV virions (82, 90).
3.3.6 Cryo electron microscopy and 3D reconstruction

Viral sample was applied to holey carbon coated grids, plunge-frozen in liquid ethane, and imaged at liquid nitrogen temperature in an FEI Titan Krios electron microscope. We first imaged the KSHV-WT sample on Kodak SO-163 films at a magnification of 59,000, which were digitalized with Nikon Coolscan 9000ED scanner at 1.1 Å/pixel and used to obtain an 8Å resolution map. To improve the image quality, we subsequently imaged the KSHV-WT sample on a Gatan K2 direct electron detector at super resolution mode with a nominal magnification of 14,000, giving a pixel size of 1.03 Å/pixel. The KSHV-SCPΔN86 and KSHV-SCPΔN6 mutants were imaged on the Gatan K2 detector with the same condition. For the KSHV-SCPnull mutant, images were recorded on a Gatan UltraScan 4k x 4k CCD camera at a nominal magnification of 47,000, giving a pixel size of 1.89 Å/pixel. An accumulated electron dose of 25 e⁻/Å² was used in all cases. For all the K2 datasets, each image was fractioned into 26 frames, and all the frames were aligned and averaged for drift-correction with the method described elsewhere (48).

Defocus value of each image was determined with CTFFIND3 (70) to be ranging from 0.5 to 2.5µm underfocus. Particles were picked with Ethan (91) and preprocessed with EMAN (92). Center and orientation parameters of each particle were determined and refined iteratively with the common-line-based method using the IMIRS software package (71, 93). 3D reconstructions were carried out with the GPU program eLite3D (94). Resolution was determined based on the 0.143 Fourier shell correlation criterion (95). Visualization of the density maps was carried out with UCSF Chimera (72).
3.3.7 Pseudo-atomic model building

The pseudo-atomic model of KSHV SCP was built *ab initio* with Coot (96). The pseudo-atomic, homology model of KSHV MCP upper domain (MCPud) was firstly built with MODELLER (97) using the atomic model of HSV-1 MCPud (PDB ID: 1NO7) (75) as template, and then manually adjusted in Coot by matching the secondary structures resolved in the cryoEM map as described previously (98).

3.3.8 Data deposition

The cryoEM density maps have been deposited to EMDB under the following accession numbers: KSHV-WT, EMD-6038; KSHV-SCPnull, EMD-6212; KSHV-SCPN86, EMD-6213; KSHV-SCPΔN6, EMD-6214. The backbone model of KSHV SCP has been deposited to PDB under the accession code 3J9A.

3.4 Results

3.4.1 KSHV SCP crosslinks neighboring MCPs in the hexon

Sample availability has been a major hurdle for structural studies of human tumor herpesviruses. We purified infectious KSHV virion to high concentration with the newly developed BAC16 plasmid and the iSLK-puro cell line (99, 100). From ~5,000 particle images recorded on a direct electron detector with super-resolution mode, we reconstructed the structure of KSHV virion capsid to 6Å resolution (Figure 3-1A). Taking advantage of the known crystallographic structure of HSV-1 MCP upper domain (MCPud) (PDB ID: 1NO7) (75) and the simple fold of KSHV SCP (it is merely 170-residue long), we were able to identify the SCP-MCP boundary, segment
SCP monomers (Figure 3-1B-E) and trace the SCP backbone (Figure 3-1F), though identification of the N and C termini was problematic.

Figure 3-1 The cryoEM structure of KSHV virion capsid reveals that SCP crosslinks hexon MCPs. (A) The cryoEM reconstruction of KSHV virion capsid at 6Å resolution. The density map is radially colored. Note that the tegument proteins surrounding the pentons identified previously (101) are at low density level and thus not visible at the contour level optimal for displaying the capsid proteins. (B, C) Zoom-in views of a hexon in the KSHV virion capsid structure. For clarity, the six MCP subunits are all colored in yellow, the six SCP molecules are colored differently, while all other densities of the capsid are grey (C) or hidden. The dashed lines in (B) roughly mark boundaries of neighboring MCP subunits. Note that each hexon SCP binds two neighboring MCPs to crosslink them. (D, E) Zoom-in views of a penton in the KSHV virion capsid structure. The coloring scheme is similar as that in (B) and (C). (F) SCP monomers segmented out from the penton (left) or from the hexon (right). Secondary structural elements identified are labeled in the hexon SCP structure.
SCP densities were identified in both hexons and pentons (Figure 3-1BD), in contrast to previous notion that KSHV SCP only decorates hexons but not pentons (16). In the hexon, six copies of SCP form a crown-like rim of the hexon (Figure 3-1BC). Each hexon SCP density consists of a “stem helix” binding in a surface groove of the underlying MCP, flanked by a “bridging helix” extended over the neighboring MCP, and a “fold-back helix” and “pigtail coil” on the other side (Figure 3-1F). With their bridging helices, the six SCP molecules in a hexon crosslink and fasten the six MCP subunits (Figure 3-1BC). In the penton, five copies of SCP bind the MCP with their stem helices in a way similar to that in the hexon, but no crosslinking is observed for neighboring MCP/SCP subunits (Figure 3-1DE). Actually, no bridging helix and pigtail coil densities are visualized for penton SCP, suggesting structural flexibility of these segments. It is likely that due to an enlarged distance and/or relative rotation (75) between neighboring MCP subunits in the penton compared to that in the hexon (compare Figure 3-1D with 1B), the bridging helix of penton SCP can no longer reach to and interact with the neighboring SCP and MCP, rendering the bridging helices and pigtail coils of all penton SCPs become flexible.

3.4.2 Structure-guided mutagenesis establish SCP’s auxiliary role in capsid assembly

The above observed crosslinking of hexon MCPs by SCPs suggests that SCP may function by strengthening the integrity of hexons in the capsid. In this regard, previous studies with recombinant capsid proteins in a baculovirus expression system have suggested an essential role of SCP in KSHV and EBV in vitro capsid assembly (21, 22) and the “essential” region of SCP was further mapped to the N-terminal half by mutagenesis scan with the in vitro capsid assembly system (22, 102). To assess whether SCP is also essential for in vivo viral assembly and to better
Figure 3-2 Construction of KSHV SCP-null and SCP C-terminal truncation mutants.

(A) Schematic illustration of KSHV BAC16 plasmids at the region encoding SCP (ORF65). Single stop codon was introduced into 5’ end of ORF65 to generate the KSHV-SCPnull mutant; the coding sequence for the SCP C-terminal 84 a.a. was removed to generate the truncated mutant KSHV-SCPN86. (B) Expression of SCP proteins during KSHV reactivation. iSLK cells harboring KSHV-WT or mutants (KSHV-SCPnull or KSHV-SCPN86) were induced with sodium butyrate plus doxycycline for three days, and expression of SCP proteins were analyzed by Western blotting with anti-SCP antibodies. (C) Virus production from KSHV-WT or mutants. Supernatants from the cells were also collected, and titers of infectious virus were determined on 293T cells.
understand its mechanism of action, we performed both conventional transmission electron microscopy (TEM) of mutant virus-infected cells and cryoEM of purified viruses bearing SCP-null mutation or SCP C-terminal truncation.

We constructed an SCP-null mutant by introducing a stop codon at the 5’ end of ORF65 in the KSHV-BAC16 plasmid (KSHV-SCPnull). The SCP C-terminal truncation mutant was generated by truncating SCP at residue number 86 (KSHV-SCPN86) to delete C-terminal 84 a.a. (Figure 3-2A). Absence of SCP expression in iSLK cells harboring KSHV-SCPnull genome (iSLK-SCPnull) and expression of truncated SCP in cells harboring KSHV-SCPN86 genome (iSLK-SCPN86) were confirmed by Western blotting with anti-SCP antibodies (Figure 3-2B). We then tested the role of SCP in KSHV lytic replication by measuring progeny virus production of the two mutants. As shown in Figure 3-2C, compared to that of the wild type (KSHV-WT), viral titer of KSHV-SCPnull mutant decreased about 1,000 fold, much more significant than the previously reported 30-fold decrease of total viral particles released, which was estimated by real-time PCR of extra-cellular viral genome (20). By contrast, the KSHV-SCPN86 mutant only had a moderate decrease in viral titer (about 3-fold), suggesting that the C-terminal half of KSHV SCP plays only a minor role in viral propagation.

To verify whether the observed decrease of viral titer in absence of SCP expression is indeed due to deficiency of capsid assembly, we examined thin sections of iSLK-WT, iSLK-SCPnull, or iSLK-SCPN86 cells at three days after induction of KSHV lytic replication (Figure 3-3A). As expected, although capsid-like particles were occasionally observed in nuclei of some iSLK-SCPnull cells, the number of cells that contained assembled capsids, and the number of capsids per cell if any, were both much smaller than that of iSLK-WT cells (Table 3-1). Moreover, a higher percentage of empty capsids and a lower percentage of DNA-filled capsids were observed
in iSLK-SCPnull cells compared to iSLK-WT cells. Consistently, we also observed by cryoEM that the percentage of DNA-filled C-capsids in purified virion samples was much lower for KSHV-SCPnull mutant (~15%) compared to that for the KSHV-WT (over 90%). We also compared the number of assembled capsids in iSLK-SCPN86 cell nuclei with that in iSLK-WT cells. Indeed, these numbers were comparable, consistent with viral titer measurements (Figure 3-3A, Table 3-1). These ultra-structural observations confirm that the effects of SCP mutations on viral growth can be correlated to capsid assembly.

Table 3-1 Statistic of number of capsids observed in thin sections of cells infected with WT or mutant KSHV.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Number of representative sections counted</th>
<th>Average number of capsids in cell nucleus (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iSLK-WT</td>
<td>6</td>
<td>76±34</td>
</tr>
<tr>
<td>iSLK-SCPnull</td>
<td>9</td>
<td>16±9</td>
</tr>
<tr>
<td>iSLK-SCPN86</td>
<td>7</td>
<td>98±42</td>
</tr>
</tbody>
</table>

We then compared cryoEM reconstructions of purified KSHV-SCPnull particles and KSHV-SCPN86 particles with that of the WT. A structure of KSHV-SCPnull capsid at ~30Å resolution was obtained from 137 particle images. This structure resembles that of the WT filtered to the same resolution (Figure 3-3B). Together with the fact that DNA-packaged virions were observed in cryoEM images of KSHV-SCPnull, this result confirms that KSHV capsid is correctly assembled and capable of encapsidating viral genome in the absence of SCP. Therefore, in contrast to the suggested essential role of SCP in the in vitro assembly studies, KSHV SCP is not essential for capsid assembly in vivo, but rather dramatically enhances the efficiency of capsid assembly. It is also possible that capsids were also assembled in absence of SCP in previous in vitro studies but might have eluded detection due to their scarcity (21, 102). Our structure and in vivo mutagenesis studies support the notion that SCP is non-essential and plays a secondary or auxiliary role. We also obtained a 7Å resolution structure of KSHV-SCPN86 capsid from ~1,500 particle images (Figure 3-3C). Other than revealing less density, the structure of this mutant virus
(A) Capsid assembly in the nuclei of KSHV-WT or mutant infected cells during viral lytic replication. The iSLK cells harboring KSHV-WT or mutants (KSHV-SCPnull or KSHV-SCPN86) were induced with sodium butyrate plus doxycycline for three days, and viral capsid assembly in the nuclei was examined with plastic embedding, ultra-

Figure 3-3 Comparative structural studies of KSHV SCP-null and SCP C-terminal truncation mutants.
thin sectioning, and transmission electron microscopy. Red arrow, DNA-filled C-capsid; yellow arrow, empty A- or B-capsid. Scale bar, 500nm. (B) Comparing the cryoEM reconstruction of KSHV-SCPnull mutant with that of the KSHV-WT at 30Å resolution. The WT map was reconstructed from randomly selected, same number of particles as that of the mutant. The two structures are very similar except that a little bit more densities are present on tip of capsomers in the WT which are attributable to SCP (inset), suggesting correct assembly of the KSHV-SCPnull capsids. (C) Comparing the cryoEM reconstruction of KSHV-SCPN86 mutant with that of the KSHV-WT at 7Å resolution. Zoom-in view of one hexon instead of the entire capsid structure is shown to emphasize that the C-terminal half truncated SCP has the same density as the full-length SCP, suggesting the flexible nature of KSHV SCP C-terminal half in the WT virus.

containing the C-terminal-half truncated SCP is nearly identical to that of the WT at secondary structure level; particularly, the truncated form of SCP retains the same density as the full-length SCP (Figure 3-3C). It indicates that the SCP density visualized in KSHV-WT is only contributed by residues within the N-terminal half and the C-terminal half is flexible.

Taken together with our structural data, these observations suggest that hexon crosslinking by SCP plays a role in stabilizing assembled capsids, and consequently, also enhancing viral genome containment, as discussed later.

3.4.3 Structural and functional mapping of the SCP N-terminus

To better understand how the SCP interacts with the MCP and with each other to function, it is necessary to map the SCP density to its sequence and build model for the SCP. However, the 6Å resolution of the density map does not provide enough structural details such as sidechain
densities to allow us to register the protein sequence directly. Particularly, we could not
determine unambiguously which end of the SCP density corresponds to the N-terminus of SCP.

To localize SCP N-terminus and to test the function of SCP N-terminal residues in the context of
KSHV replication, we constructed a set of mutant viruses with their SCP truncated in the N-
terminus for 3, 4, 6, 7, and 10 residues respectively (KSHV-SCPΔN3, KSHV-SCPΔN4, and etc.)
(Figure 3-4A). Successful SCP expressions were confirmed by western blotting with anti-SCP
antibodies (Figure 3-4B). Measurement of progeny virus production indicated that removal of 3
or 4 a.a. from KSHV SCP N-terminus did not affect viral propagation; but removal of 6 or 7 a.a.
exerted significant impact with virion production decreased 81.6% or 95.7% respectively; and
removal of N-terminal 10 a.a. reduced viral production to the level same as SCP-null mutation
(Figure 3-4C). We then selected the KSHV-SCPΔN6 mutant for cryoEM study because it showed
attenuated viral titer but still yielded adequate amount of virions for cryoEM reconstruction. A
7Å-resolution virion capsid structure was obtained from ~1,200 particles. As expected, fitting the
structure with that of the WT clearly showed missing of a short segment of density in the KSHV-
SCPΔN6 mutant at end of the pigtail coil (Figure 3-4D). This result unambiguously mapped the
N-terminus of SCP to the pigtail coil.
Figure 3-4 Structural and functional mapping of KSHV SCP N-terminal residues.

(A) Partial protein sequences of WT SCP and the N-terminal truncated mutants. (B)Expression of SCP in iSLK cells harboring BAC plasmids of KSHV-WT or the SCP N-terminal truncated mutants during KSHV reactivation. (C) Virus production from KSHV-WT or the mutants during KSHV reactivation. (D) Comparison of cryoEM reconstructions of KSHV-SCPΔN6 mutant and the WT. It is obvious that a small trunk of density (red arrows) is missing in the cryoEM map of the mutant, which corresponds to the truncated N-terminal 6 residues of SCP.
3.4.4 Pseudo-atomic models of SCP and MCPud and SCP-MCP interactions

With the N-terminus established, we then correlated the SCP density with the secondary structure prediction (Figure 3-5A) and built a pseudo-atomic model for KSHV SCP (Figure 3-5B). Taking advantage of the known crystallographic structure of HSV-1 MCPud and the fact that it is highly structurally-conserved with KSHV MCPud, we also built a homology model for KSHV MCPud (Figure 3-6, 3-5C).

The model shows that association of SCP to the capsid is primarily via its stem helix (a.a.39-65) binding in a groove formed by three helices at top surface of MCP, namely helices 763-778, 826-840, and 871-883 (Figure 3-5D). In hexon, the bridging helix (a.a.70-82) of SCP is also interacting with loop 837-856 of the neighboring MCP and pigtail coil a.a.10-20 of another SCP, forming the crosslink (Figure 3-5CD). The stem helix and bridging helix of each SCP can also be regarded as one long helix kinked by a flexible Glycine pivot point (G66 and G67). Pigtail coil a.a.1-10 of hexon SCP also makes specific contacts with the surface of underlying MCP (Figure 3-5C). As we have demonstrated, very short truncations in this region affected viral replication considerably (Figure 3-4C). Therefore, these 10 amino acids may help to maintain the entire pigtail coil in the right position and right conformation to form the crosslink.

Six residues – R14, D18, V25, R46, G66, and R70 – of KSHV SCP were demonstrated previously to be vital for capsid assembly: when any of these residues was mutated to alanine, capsid assembly was “abolished” or reduced (102). Based on our model, R14, D18, and R70 are likely directly involved in forming the crosslink (Figure 3-5D). G66, together with G67, may serve as a pivot point for the bridging helix to have some flexibility relative to the stem helix, since glycine is the smallest and most flexible amino acid. Conceivably, having a somewhat flexible kinked helix, instead of a rigid straight long helix sticking out, might be a good strategy
Figure 3-5 Pseudo-atomic models and interactions of KSHV SCP and MCPud.
(A) Secondary structure prediction of KSHV SCP sequence (103). The predicted secondary structures correlate well with the observed structure of SCP in the density map, and their assignments to the real structure are marked. (B) Pseudo-atomic model of KSHV SCP. The model is rainbow-colored from N’ (blue) to C’ (red). Key structural
segments as named in Figure 3-1F are also marked here. (C) Fitting the pseudo-atomic model of SCP (rainbow-colored) and the homology model of MCPud (magenta) into the cryoEM density map of KSHV-WT hexon (translucent grey). (D) KSHV SCP-MCP and SCP-SCP interactions revealed by the pseudo-atomic models. The orientation and coloring are the same as in (B). For clarity, only part of the MCPud is shown, including the three helices forming the SCP-binding groove and a loop involved in SCP crosslinking. Positions are marked for SCP residues R14, D18, V25, R46, A54, G66, and R70, which were demonstrated previously to be vital for KSHV capsid assembly (102, 104). (E, F) Hydrophobicity surface presentations of KSHV and HSV-1 MCPud. In (E), the KSHV SCP model is also shown and colored according to its sidechain hydrophobicity to demonstrate the complementarity between the SCP and MCP interfaces. The position of SCP residue A54 is marked, which is buried in a hydrophobic packet of the MCP groove. In (F), the two dash lines mark the most-likely binding area on HSV-1 MCPud for the “stem helix” of HSV-1 SCP. Note that this “SCP-binding groove” in HSV-1 is not as prominent as that in KSHV.

Interestingly, G67 is conserved among SCPs of several gammaherpesviruses (Figure 3-7) as discussed later in more details.

Hydrophobicity analysis of the model suggests that hydrophobic interactions might be an important driving force for SCP binding MCP. Highly hydrophobic patches are identified in the MCP surface groove, which are complementary to several hydrophobic or neutral residues on the groove-binding-side of SCP stem helix, especially the segment LVFLIA (a.a.49-54) (Figure 3-5E, marked position of A54). In support of this, it had been demonstrated that substitution of A54 with polar residue lysine abolished SCP binding and capsid assembly, while substitutions with hydrophobic residues leucine, valine, or even proline did not (104). In addition, L49K and I53K mutations also resulted in partially defective assemblies (104).
Figure 3-6 Comparison of KSHV and HSV-1 MCP upper domains.
The pseudo-atomic model of KSHV MCPud was initially calculated by homology modelling with the crystal structure of HSV-1 MCPud (1NO7) as template, and then adjusted according to the 6Å resolution cryoEM structure of KSHV. The side-by-side presentations of KSHV and HSV-1 MCPud models demonstrate the similar overall fold of the two structures. The major differences between the two structures that might affect the SCP binding are marked with black arrows. A surface loop in HSV-1 has become a major helix in KSHV, creating a deep groove (dash line) with other two helices (red arrows) for the binding of SCP stem-helix.

Although identities among SCP sequences from alpha-, beta-, and gammaherpesviruses are relatively low, the presence of an MCP-binding “stem helix” is likely a conserved structural feature among all SCPs (64, 105, 82, 33). However, the MCPs from different herpesvirus
subfamilies may have evolved different surface properties for SCP binding. Comparing surface presentations of MCPud from KSHV and HSV-1, it is obvious that KSHV MCP has a more prominent groove than that in HSV-1 MCP at the SCP-binding area (Figure 3-5EF). As seen from the models, the helix 763-778 contributing one cliff of the groove in KSHV MCP is replaced by a loop 767-781 in HSV-1 (black arrows in Figure 3-6), resulted to a not-so-prominent groove. We propose that evolution may have taken place to strengthen the MCP-binding of KSHV SCP for its pivotal role in viral propagation; or conversely, weakened evolutionary pressure due to dispensability of HSV-1 SCP might have led to weakened binding of HSV-1 SCP.

3.5 Discussion

Our 6Å KSHV virion capsid structure shows that SCP forms a crown at the rim of each hexon to crosslink neighboring MCP subunits. SCP-null mutation confirmed an important but non-essential role of SCP. Mutagenesis studies with C- and N-terminal truncations established that the N-terminal half is responsible for SCP to crosslink hexon MCPs. Integration of structural and functional studies has enabled us to construct a pseudo atomic model for SCP and to define the largely hydrophobic interactions between SCP and MCP. Taken together, this first description of SCP-MCP chemical interactions for a herpesvirus helps understand the functional roles of SCP in different subfamilies of herpesviruses.

Within gammaherpesvirus subfamily, secondary structure predictions of SCPS from four gammaherpesviruses – KSHV, EBV, RRV (rhesus monkey rhadinovirus), and HVS (herpesvirus saimiri) – show similar pattern of three-helix composition in their N-terminal segments (Figure
3-7). Besides, multiple-sequence alignment shows that their N-terminal segments are more conserved compared to their C-terminal segments (Figure 3-7), consistent with what we have observed here for KSHV SCP that the N-terminal half is functionally important but the C-terminal half is dispensable. Indeed, the SCP of EBV was also demonstrated to be important for capsid assembly in vitro, and only its N-terminal half was required and sufficient (22), strikingly similar to that of KSHV. Therefore, it is reasonable to expect that the crosslinking of hexon MCP by a crown of SCP we observed in KSHV is a conserved feature among many, if not all, gammaherpesviruses.

Figure 3-7 SCPs of different gammaherpesviruses share conserved sequences and secondary structures in their N-terminal halves.

Secondary structures of KSHV, EBV, RRV and HVS SCP were predicted from I-TASSER server (103). The predicted helices are labeled in red, β-strands in blue, and coils in black. The protein sequences were also subjected to multiple sequence alignment with Clustal Omega (106), and level of conservation is indicated by symbols. An “*” (asterisk) indicates identical amino acid in all sequences; a “:” (colon) indicates different but highly conserved amino acids; a “.” (period) indicates different amino acids that are somewhat similar.

The auxiliary role of SCP in gammaherpesvirus capsid stabilization mirrors that of “cementing” proteins often found in dsDNA bacteriophages, such as the Soc protein in phage T4.
(107, 108) and the gpD protein in phage λ (109), as proposed previously for VP26 of HSV (110). In phage λ, the 11.4kDa gpD forms a trimer and binds to capsid surface at quasi and icosahedral 3-fold axes after capsid maturation; it stabilizes the capsid structure by fastening six gpE (the major capsid protein) subunits from three neighboring capsomers (109). These non-essential auxiliary proteins in phages “cement” adjacent major capsid proteins to stabilize the mature capsid against extremes in pH and temperature as well as other factors in their hostile environment. They also help the thin capsid shell to withstand the pressure exerted by the packaged DNA (107, 109, 78, 111). In gammaherpesvirus, it is conceivable that the crosslinking by hexon SCP would also fasten the MCP subunits in hexons and thus increase the overall stability of the capsid. Indeed, we observed lower percentage of genome-containing C-capsids in SCP-null mutant-infected cells and also in cryoEM images of purified virion when compared to that of WT. It is possible that, in the absence of SCP, some capsids may collapse during and/or after DNA packaging.

Our finding of gammaherpesvirus SCP acting as an auxiliary cementing protein agrees with previous notions of similarities between herpesviruses and dsDNA bacteriophages (110, 85, 112). It has been well documented that these two groups of viruses utilize strategies of capsid assembly that bear many similarities, including the use of scaffold proteins, the formation of procapsids, and the incorporation of a portal complex at one unique capsid vertex for translocation of genome (85). Moreover, herpesvirus MCP contains an HK97-like fold in the capsid floor domain (112). All these similarities support a common ancestry for herpesviruses and tailed dsDNA bacteriophages as proposed before (112).

Across the three subfamilies of Herpesviridae, SCP is the least conserved among all capsid proteins in terms of the identities and sizes of their sequences. Nonetheless, our notion of SCP
acting as a cementing protein can be extrapolated to all herpesviruses. For alphaherpesviruses whose SCP is dispensable, the effect of SCP on capsid stability may have been somehow weakened over the course of evolution, possibly due to strengthened interactions among other capsid components. For betaherpesviruses, other auxiliary proteins, such as the capsid-associated tegument protein pp150 in human cytomegalovirus (HCMV), may work together with SCP to “cement” capsid proteins. Indeed, HCMV SCP is required for the association of pp150 to the capsid (82). In this scenario, the extensive cementing network formed by SCP and pp150 in cytomegalovirus may provide additional strength that is needed for the encapsidation of its genome, which is the largest among all herpesviruses (34).

3.6 Acknowledgments

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Chapter 4 KSHV CAPSID-ASSOCIATED TEGUMENT COMPONENTS
4.1 Abstract

Capsid-associated tegument proteins have been identified in alpha- and beta-herpesviruses to play an essential role in viral DNA-packaging. Whether and how such tegument proteins exist in gamma-herpesviruses have been a mystery. Here we report a 6Å resolution cryoEM structure of Kaposi’s sarcoma-associated herpesvirus (KSHV) virion, a member of the oncogenic gamma-herpesvirus subfamily. The KSHV virion structure reveals for the first time how capsid-associated tegument proteins are organized in a gamma-herpesvirus -- with five tegument densities cap each penton vertex -- a pattern highly similar to that in alpha-herpesvirus but completely different from that in beta-herpesvirus. Each KSHV tegument density can be divided into three prominent regions: a penton-binding globular region, a helix-bundle stalk region and a β-sheet-rich triplex-binding region. Fitting of crystal structure of the truncated HSV-1 UL25 (the KSHV ORF19 homolog) and secondary structure analysis of the full-length ORF19 establish that ORF19 constitutes the globular region with an N-terminal, 60 amino acid long helix extending into the stalk region. Matching secondary structural features resolved in the cryoEM density with secondary structures predicted by sequence analysis identifies the triplex-binding region to be ORF32, a homolog of alpha-herpesvirus UL17. Despite high-level of tegument structural similarities between KSHV and alpha-herpesvirus, an ORF19 monomer in KSHV, in contrast to a UL25 dimer in alpha-herpesviruses, binds each penton subunit, an observation that correlates with conformational difference in their pentons. This newly discovered organization of triplex-ORF32-ORF19 also resolves a long-standing mystery surrounding the virion location and conformation of the alpha-herpesvirus UL25.
4.2 Introduction

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is the causative agent of Kaposi’s sarcoma (81, 113), a cancer commonly occurring in AIDS patients (114). It is also associated with certain B-cell lymphoproliferative disorders like primary effusion lymphoma and multicentric Castleman’s disease (115). KSHV is a member of the gamma-herpesvirus subfamily of the Herpesviridae. Like all herpesviruses, the KSHV virion is composed of a double-stranded, linear DNA genome encased within an icosahedral capsid which is itself wrapped in a proteinaceous layer called the tegument, and then a lipid bilayer membrane called the envelope. Although the tegument compartment of herpesvirus is pleomorphic and has no distinguishable substructures, it is roughly divided into “outer tegument” and “inner tegument” layers (10, 9). Upon cell entry, the outer tegument proteins are immediately released into the host cell cytoplasm where some of them perform functions needed early in the infection process, while the inner tegument proteins remain associated with the nucleocapsid and participate in its transport to the nucleus via the microtubule/dynein motor system (116-121).

Of particular interest, some inner tegument proteins associate with the nucleocapsid in an ordered manner that enables visualization of tegument densities in three-dimensional (3D) reconstructions of the virion by cryo electron microscopy (cryoEM). In herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV), two representative members of the alpha-herpesvirus subfamily, tegument densities are present at the icosahedral vertices of the capsid and thus are named capsid vertex-specific component (CVSC) (25, 66, 27, 122, 111). In human cytomegalovirus (HCMV) and simian cytomegalovirus (SCMV), members of the beta-herpesvirus subfamily, the capsid-associated tegument protein pp150 forms a net enclosing the entire capsid, and presumably stabilizes the capsid to contain the large viral genome (3, 82, 25,
All these capsid-associated tegument components identified in alpha- and beta-herpesviruses have been demonstrated to be essential for viral propagation (35, 36, 67), but the underlying mechanisms are not clear.

Structural studies of gamma-herpesviruses are lagging behind due to more difficulties of sample preparation. Particularly, whether and how capsid-associated tegument proteins exist in gamma-herpesviruses have not been addressed.

Recently, a new KSHV bacterial artificial chromosome (BAC) plasmid, BAC16, was generated to facilitate the robust production of KSHV infectious virions and efficient genetic modification of KSHV genome (99). A high titer of BAC16-derived virus stock can be obtained with the use of a cell line, iSLK-puro, engineered to express a doxycycline (DOX)-inducible immediate early viral protein, RTA, that drives the lytic replication of KSHV (99, 100). Here, by using this iSLK-KSHVBAC16 system for KSHV virion production, we obtained a 6Å resolution structure of the KSHV virion, which reveals, for the first time, that KSHV also has capsid-associated tegument densities around the capsid vertices. Two components in the KSHV tegument density were identified, ORF19 and ORF32, which are homologs of alpha-herpesvirus UL25 and UL17 respectively. ORF32 mediates assembly of tegument proteins by anchoring to the capsid on penton-proximal triplexes and connecting to ORF19 through its N-terminal helix and possibly to other tegument proteins too. This result also clarifies the previous mystery surrounding the locations and structures of alpha-herpesvirus UL25 and UL17.
4.3 Materials and Methods

4.3.1 Sample preparation of KSHV virion

The iSLK-KSHVBAC16 cells (kind gift from Dr. Jae U. Jung) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 1µg/ml Puromycin, 250µg/ml G418 and 1200µg/ml Hygromycin. When cells reached ~80% confluency, the media was replaced with DMEM plus 10% FBS, 1mM Sodium Butyrate and 1µg/ml doxycycline. After 3 to 5 days, when 80% of the cells rounded up, the supernatant was collected to purify KSHV virion following procedure described previously (82).

4.3.2 CryoEM imaging and data processing

An aliquot of 2.5 µl purified KSHV virion suspension in Phosphate Buffered Saline (pH7.4) was applied to a 300-mesh copper grid coated with holey carbon film, blotted with filter paper, and plunge-frozen in liquid ethane. The sample was imaged at liquid nitrogen temperature in an FEI Titan Krios cryo electron microscope operated at 300kV with the Leginon software (123). The images were recorded on a Gatan K2 Summit direct detection camera at super resolution mode with effective magnification of 24, 300x (nominal magnification 14, 000x), corresponding to a pixel size of 1.03Å/pixel at the specimen level. Each selected sample area was exposed at dose rate of ~8e−/pixel/s on camera for 13s, resulting to a total dose of ~25e−/Å² on specimen. Each image was fractioned into 26 frames, with 500ms accumulation time for each frame. All the frames were aligned and averaged with the drift-correction software described in (111). The averaged images were used for following data processing and 3D reconstruction.

Defocus values of the dataset were determined with CTFFIND3 (70) and were in the range of 1-2µm underfocus. A total of ~40,000 particles were picked manually using EMAN (92).
Orientation and center parameters of each particle were determined and refined iteratively with the common-line method implemented in the IMIRS software package (71, 10), and 3D reconstructions were carried out with the GPU program eLite3D (103). The final reconstruction was obtained by averaging 20, 395 best particles selected based on phase residues between raw images and model projections. Resolution of the density map was measured to be 6Å based on the FSC=0.143 criterion (95). Visualization of the density map was carried out with UCSF Chimera (72).

Fitting of crystal structures into the cryoEM density map was done with the “Fit in Map” tool of UCSF Chimera (72). During the fitting, a simulated map was generated from the crystal structure at the same resolution as that of the experimental map. A cross-correlation coefficient between the simulated map and the experimental map was calculated and reported as a quantitative estimate of the quality of fitting.

Secondary structure predictions were performed with PSIPRED using the Protein Structure Prediction Server (73).

4.3.3 Data deposition

The cryoEM density map has been deposited to EMDB under the accession number EMD- 6038.

4.4 Results

4.4.1 Structure of KSHV virion at 6Å resolution

Aiming for resolving the tegument, we chose to purify intact virion particles from viral culture media for this study. Protected by the viral envelope, the virion encounters less structural
Figure 4-1 CryoEM and 3D reconstruction of KSHV virions.

(A) CryoEM image of purified KSHV virion. (B) 3D reconstruction of the KSHV virion at 6Å resolution. The structure is rainbow colored radially. The left half of the capsid is rendered at contour level (CL) of 2δ (δ is the standard deviation), while the right half is rendered at 0.5δ to show the presence of tegument densities. (C) One quarter of a central slice of the KSHV virion reconstruction. The white arrow points to a capsid-associated tegument density surrounding the penton. Intensity of the tegument density is measured to be ~30% of the peak intensity of the capsid shell. 2, 3, 5: icosahedral symmetry axes. (D, E) Zoom-in views of capsid surface areas as denoted with dash squares in (B). Black arrow in (D) points to a tegument density around the capsid vertex. Black arrows in (E) point to tegument density close to the 2-fold axis. 5: 5-fold axis. P: periphery-hexon. Ta, Tc: Triplexes. 2: 2-fold axis. E: edge-hexon. C: central-hexon. Tb,Te: Triplexes. (F) KSHV MCP upper domain (MCPud) density map (transparent grey) fitted with HSV-1 MCPud atomic model (red ribbon, PDB ID: 1NO7) (75).

disturbance and should have better preservation of its tegument structure compared to nucleocapsid purified from infected cell nuclei used in previous structural studies. We imaged the purified KSHV virion embedded in vitreous ice (Figure 4-1A) with a 300kV Titan Krios electron microscope equipped with an electron-counting direct detection camera (Gatan K2
Summit) operated at super-resolution mode. By averaging over 20,000 images of virion particles, we reconstructed the KSHV virion to 6Å resolution (Figure 4-1B).

The capsid structure of KSHV is similar to other published herpesvirus capsid reconstructions. It is composed of major capsid protein (MCP, ORF25) pentamers and hexamers joined by triplexes (hetero-trimers of one ORF62 and two ORF26 proteins) and decorated by the smallest capsid proteins (SCP, ORF65). Density of the MCP upper domain (MCPud) can be fitted well with the atomic model of HSV-1 MCPud (PDB ID: 1NO7) (75) (cross-correlation coefficient 0.47, see Materials and Methods), demonstrating good quality of the reconstruction, and also indicating structural conservation between MCP molecules of the two viruses (Figure 4-1F).

We identified extra densities other than capsid proteins in the KSHV virion reconstruction that are attributable to capsid-associated tegument components (CATC). The tegument densities are mainly distributed around the capsid vertices, with five copies binding each penton and its surrounding triplexes Ta and Tc (as in the nomenclature of (76)) (Figure 4-1D). These densities are weaker than capsid proteins. They can only be visualized when the map is displayed at low contour level (~0.5δ above the mean, δ is the standard deviation) but are hardly discernible when other capsid components are displayed at contour level of 2δ (Figure 4-1B). A central slice of the 3D reconstruction shows that intensity of these tegument densities is only about 30% of the highest intensity of capsid proteins (Figure 4-1C). The weak intensity of these tegument densities explains why we failed to detect them previously in a low-resolution cryoEM reconstruction of murine herpesvirus 68 (MHV-68) virion with only a few hundreds of particles (10).

Other minor tegument densities are also observed around the two-fold symmetric axis, roughly at the midpoint between triplexes Tb and Te, E-hexon and C-hexon (Figure 4-1E). These
minor densities are very weak and broken, resulted from either very low occupancy or high flexibility. Given their similarity in bridging over two triplexes, we propose that these densities are the same kind of tegument proteins as those surrounding the capsid vertices but occupying a secondary, lower-affinity binding site.

4.4.2 Capsid-associated tegument components in KSHV are similar to those in alpha-herpesviruses but different from those in beta-herpesviruses

Comparison of KSHV CATC with tegument components in alpha- and beta-herpesvirus reconstructions revealed its similarity to those in alpha-herpesviruses and difference from those in beta-herpesviruses. Distribution of visible tegument densities in published HSV-1 and PRV reconstructions is limited to particle vertices, and thus these densities were named as capsid vertex-specific component or CVSC (122). The binding pattern and overall shape of KSHV CATC densities are similar to those of HSV-1 and PRV CVSC. To illustrate these similarities, we compare our KSHV structure side-by-side with a recently published 9Å resolution reconstruction of PRV C-capsid (EMDB-5650) (111) (Figure 4-2A, B, D, E, G and H). The tegument density in both KSHV and PRV can be divided into three regions in the same way: a globular region in the upper end binds the penton MCP; a bridge-shaped region in the lower end binds two triplexes closest to the penton, triplex Ta and Tc; a stalk region in the middle connects the penton-binding region to the triplex-binding region (Figure 4-2G, 2H and 3A).

By contrast, the KSHV and PRV tegument densities bear no similarity to the tegument densities of cytomegalovirus (beta-herpesvirus subfamily). In cytomegaloviruses, three filamentous, pp150-containing densities bind to each triplex and its surrounding capsomers (compare Figure 4-2AB with Figure 1H in our previously published paper (82)).
These observations suggest that KSHV inner tegument is very similar to that of alpha-herpesvirus but has nothing in common with that of beta-herpesvirus.

Figure 4-2 Comparison of capsid-associated tegument densities in KSHV and pseudorabies virus (PRV). (A, B) Densities around capsid-vertex in 6Å KSHV virion reconstruction (A) and 9Å PRV C-capsid reconstruction (B, rendered from EMDB-5650 published by Homa FL et al. 2013 (33)). In both structures, capsid proteins are
rainbow colored radially, tegument densities are grey and they are displayed at lower contour level than that of the capsid because they are weaker than capsid densities. (C) Schematic representation of penton and periphery-hexons in KSHV and PRV. The PRV penton has a 30° clockwise rotation when its periphery-hexons are aligned to those of KSHV. (D, E) Zoom-in views of a single capsid-associated tegument density in KSHV and PRV. Their positions are denoted with dash squares in (A) and (B). Note the slightly different orientations of the tegument density relative to the penton MCP. In KSHV (D), a single copy of HSV-1 UL25 atomic model (magenta ribbon, PDB ID: 2F5U) can be fitted into the tegument density, while in PRV (E) two copies (magenta and blue ribbons) can be accommodated. (F) Two copies of HSV-1 UL25 atomic model (magenta and blue ribbons) fitted in tegument density of PRV virion reconstruction (light blue) (EMDB-5655, Homa FL et al. 2013). Note the good match between overall shape of the density map and that of the model. (G, H) Side views of tegument density interacting with penton and triplexes Ta, Tc in KSHV (G) and PRV (H) respectively.

4.4.3 In-situ structure of full-length ORF19 revealed in the KSHV virion reconstruction

In alpha-herpesviruses, one of the capsid-associated tegument components was shown to be UL25, a homolog of KSHV ORF19 (27, 111). Structure of the N-terminal-truncated (missing 133 a.a.) UL25 of HSV-1 has been solved by crystallography (PDB ID: 2F5U) (29). We find that this HSV-1 UL25 atomic model fits well with the penton-binding globular region of the KSHV tegument density (cross-correlation coefficient 0.49, see Materials and Methods) (Figure 4-3C, supplement movie S1). This observation indicates that the penton-binding globular region is made up by a monomer of the ORF19 protein. The structural agreement between HSV-1 UL25 model and KSHV tegument density further demonstrates structural conservation between gamma- and alpha-herpesvirus tegument proteins up to the level of secondary structures.

The N-terminal segment of a.a. 1-133 is missing in the UL25 crystal structure. Secondary structure predictions of the full-length KSHV ORF19 and HSV-1 UL25 sequences reveal similar secondary structures for their N-terminal segments. Both of them have a short β strand connected to a long helix of about 60 residues (Figure 4-3E). We suggest that this predicted long helix corresponds to the magenta helix observed at the surface of the stalk region (Figure 4-3A). This
helix spans ~100Å from the penton-binding globular region to the right-bottom of the triplex-binding region, with a kink in the middle (Figure 4-3A, B and D). The length of this helix agrees with that expected for the predicted N-terminal helix containing 60 amino acids (~90Å in length, given an axial rise of 1.5Å/residue). At 6Å resolution, the β strand and loop predicted at the very end of the N-terminal segment are not resolved in our structure, but could be part of the large β-sheet in the triplex-binding region (the one in Figure 4-3H).

4.4.4 Localization of ORF32 in KSHV virion

A second constituent of alpha-herpesvirus tegument density was determined to be UL17, a homolog of KSHV ORF32 (122). It was assigned to mainly occupy the stalk region and a small portion of the triplex-binding region closest to the penton (122).

Based on correlation of secondary structural feature of the remaining un-assigned density in the KSHV tegument with the secondary structure prediction of ORF32, we assign it to be the entire triplex-binding region (Figure 4-3A, B and F). Density of the triplex-binding region in KSHV tegument is well-separated into two domains, with each domain having a large β-sheet in the core (Figure 4-3F, G and H). This β-sheet-rich structural feature correlates well with secondary structure prediction of ORF32, which is also rich in β-strands (Figure 4-3I).

In previous GFP-labeling studies of HSV-1 UL17, the GFP tag was attached to the C-terminal and produced extra density at the penton-proximal end of triplex-binding region in the cryoEM reconstruction (green □ marks in Figure 4-3B and F) (122). Because the cryoEM densities for the triplex-binding regions of capsid-associated tegument are similar in KSHV and HSV-1, by analogy, we assign this penton-proximal domain in KSHV to be the C-terminal half of ORF32, and the penton-distal domain to be the N-terminal half (Figure 4-3F).
4.4.5 Other constituent in KSHV capsid-associated tegument structure

The remaining three helices in the stalk region have no obvious density connectivity to either the penton-binding region ORF19 or triplex-binding region ORF32 (Figure 4-3A, B and J). Given the still limited (6Å) resolution of the current structure, and the lack of biochemical data, we cannot make a definitive protein assignment to these three helices in the helix bundle.
Previously, it was proposed in alpha-herpesviruses that VP1/2 (encoded by UL36) is present in the capsid-associated tegument densities and constituting the penton-binding region (124, 111). Biochemical studies in HSV-1 and PRV also showed that VP1/2 was recruited into nuclear assemblons and specifically bound capsids via its C-terminus and this binding required UL25 (118, 125, 126). However, as shown above, the proposed location of VP1/2 - the penton-binding region - has been determined unambiguously to be occupied by UL25. Whether the three-helix-bundle in the stalk region could be part of ORF64, the KSHV homolog of VP1/2, awaits further investigations.

**4.4.6 Stoichiometric difference of capsid-associated tegument proteins in alpha- and gamma-herpesviruses**

When applying the above determined organization of tegument proteins in KSHV to PRV, we further identified a stoichiometric difference in the capsid-associated tegument proteins between the two viruses. The penton-binding globular region of PRV tegument is roughly twice the size of that in KSHV (Figure 4-2D and E). By fitting with HSV-1 UL25 atomic model, we confirmed that this region in PRV can indeed accommodate two UL25 molecules (Figure 4-2E). We double-checked the fitting in PRV virion reconstruction (EMDB-5655) (111), which has poorer resolution but better preservation of tegument densities than the capsid reconstruction. As expected, overall shape of the density in the penton-binding globular region matches that of the two UL25 models (Figure 4-2F). We further examined tegument densities in HSV-1 reconstructions published to date. Two bulbs of density similar to that in PRV virion reconstruction can be easily identified [compare Figure 4-2F with Figure 3 in (25) and Figure 4A in (124)]. We propose that it is a conserved feature among all alpha-herpesviruses that two UL25 molecules, in contrast to only one ORF19 in KSHV, are present in each capsid-associated
tegument component. In support of our proposal, Conway et al. had found in their HSV-1 UL25 GFP-labeling study that the GFP density could actually accommodate two GFP molecules (27). Moreover, approximate 2:1 ratios of UL25 to UL17 were reported for HSV-1 and PRV C-capsids respectively [see Table S2 of (66) and Figure 4b of (111)]. The UL25 homolog in HCMV, UL77, was also demonstrated to form a dimer (127).

Figure 4-4 Schematic presentation of capsid-associated tegument component organizations in gamma-herpesvirus (A) and alpha-herpesvirus (B).

The main differences are: 1. only one ORF19 molecule is present in each gamma-herpesvirus tegument density, compared to two copies of UL25 in each alpha-herpesvirus tegument density; 2. ORF19 binds only one penton MCP in gamma-herpesvirus, while each UL25 in alpha-herpesvirus binds two penton MCPs due to a 30° clockwise rotation of the penton as denoted in Figure 4-2C.

In contrast, the stoichiometry of alpha-herpesvirus UL17 and KSHV ORF32 appears to be the same. In KSHV, an ORF32 monomer occupies the triplex-binding region of the capsid associated tegument component. The similar size of this region in KSHV and PRV (Figure 4-2D, E, G and H) indicates that only one copy of UL17 exists in each alpha-herpesvirus CVSC too.
The stoichiometric difference of the UL25/ORF19 proteins in alpha- and gamma-herpesviruses may correlate with structural difference in their pentons. ORF19 in KSHV and UL25 in PRV bind pentons in different ways. More specifically, KSHV ORF19 binds only one penton MCP, while each of the two PRV UL25 molecules binds two MCPs at the same time, and the binding area on penton MCP is different from that in KSHV (Figure 4-2D, E, G and H). By superimposing the five-fold axis of the KSHV and PRV reconstructions, we find that the penton in the two structures does not fit with each other, although their periphery hexons and triplexes are aligned well. By fitting the HSV-1 MCPud atomic model (PDB ID: 1NO7) (75) into the penton MCPs and marking positions of the same structural element in KSHV and PRV, we measured that there is roughly a 30° clockwise rotation of the PRV penton compared to that in KSHV (Figure 4-2C). Conceivably, rotation of the PRV penton exposes alternative binding area on the MCP to its UL25 protein and also results in more space between the penton and its adjacent hexon to accommodate a UL25 dimer than that required for an ORF19 monomer in KSHV.

4.5 Discussion

Alpha-herpesvirus tegument has been studied extensively and cryoEM of HSV-1/PRV capsids with GFP-tagged UL25 confirmed its identity as one contributor to the CVSC. However, both the exact location and in-situ conformation of the UL25 protein inside alpha-herpesvirus virions remained hitherto puzzling. It was mysterious why the crystal structure of HSV-1 N-terminal-truncated UL25 did not fit the density designated as UL25 in a 9Å-resolution cryoEM reconstruction of PRV C-capsids (111). Two possibilities were offered to explain this
discrepancy: first, PRV and HSV-1 UL25 proteins have different structures; or second, UL25 has a conformation inside the virion that is different from that in the crystal (111). One can safely rule out the first possibility, given the rule-of-thumb of structural biology that sequence identity of over 30% usually leads to nearly identical folds and the high sequence identity between PRV and HSV-1 UL25 proteins (50% identity and 60% similarity). The structure presented here has allowed us to rule out the second possibility too, thus resolving the long-standing mystery surrounding the exact location and the in-situ structure of UL25 and its homolog in gamma-herpesvirus.

Specifically, the newly resolved structure of N-terminal segment not present in the crystal structure of HSV-1 UL25 explains why the previous labeling studies had led to the mystery and the eventual incorrect assignment of UL25 to the triplex-binding region of CVSC (27, 111, 122). The crystal structure of UL25 contains the globular domain encompassing a.a. 134-577 but not the extended N-terminal segment (Figure 4-3D and E). In the GFP-labeling studies of HSV-1 and PRV UL25, the GFP tag was inserted between residues 50 and 51, and produced extra density on top of the CVSC densities (27, 111), roughly at the position marked by a “*” in our KSHV structure (Figure 4-3A and D). Similarly, the position of N-terminus of HSV-1 UL25 was labeled by a tandem affinity purification tag and visualized by cryoEM (122). A small extra density was identified at one side of the triplex-binding region [see Figure 8 of (122)], which agrees with our assignment of the UL25 N-terminus extending to the triplex-binding region. In all these cases, the N-terminal of UL25 was correctly identified by labeling; however, the poor resolutions of the cryoEM structures have unfortunately led to the misplacements of the globular domain of UL25 to the density largely belonging to UL17.
The direct structural comparison presented here shows that a similar, but not identical, organization of capsid-associated tegument proteins exists in alpha- and gamma-herpesviruses, as depicted in Figure 4-4. This organization suggests an important structural role for ORF32/UL17. This tegument protein mediates assembly of tegument proteins by anchoring to the capsid on penton-proximal triplexes and connecting to ORF19/UL25 through its N-terminal helix and possibly to other tegument proteins too. This notion is supported by previous finding in HSV-1 that UL17 is required for efficient binding of UL25 to capsid (37).

UL17 and UL25 genes are conserved among all three herpesvirus subfamilies. We show here that the capsid-association pattern of UL17 and UL25 homologs is similar in gamma-herpesviruses and alpha-herpesviruses. In addition to binding to pentons as visualized here, HSV-1 UL25 has also been shown to bind the portal protein UL6 (128), a component of the DNA-packaging machinery. The portal only occupies one of the 12 capsid vertices and is not visible in the reconstruction shown here due to imposition of icosahedral symmetry. The capability of UL25 homologs to bind DNA-packaging machinery suggests the possibility that they are directly involved in the processes of genome packaging, retention, and/or releasing (36, 129, 28, 128, 39). The presence of UL25 improved the efficiency of packaging full-length genome (28, 129, 38). In beta-herpesvirus HCMV, the UL17 and UL25 homologs (UL93 and UL77, respectively) are also required for efficient virus growth in cultured fibroblasts (16, 130). We have shown previously that the most abundant capsid-associated tegument protein in HCMV is pp150 (3, 82), a beta-herpesvirus-specific tegument protein not found in alpha- and gamma-herpesviruses. The binding of pp150 might have prevented association of the UL17/UL25 homologs around the capsid locations as those in alpha- and gamma-herpesviruses. However, the UL25 homolog in HCMV was demonstrated to be a structural protein associated with the capsid
and interact with portal protein UL104 (the alpha-herpesvirus UL6 homolog) (127). It is conceivable that in HCMV, the HSV-1 UL17 and UL25 homologs could also bind to the portal-specific vertex, which, like in the case of alpha- and gamma-herpesvirus virions, were not visible in the icosahedral reconstructions due to the imposition of symmetry during cryoEM reconstruction.

4.6 Acknowledgments

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Chapter 5 SUMMARY AND PERSPECTIVE
Herpesviruses are grouped into three subfamilies based on their biological properties and also genome sequence similarities. Members of different subfamilies share a lot in common, but also have properties that seem to be specific for the subfamily. Structurally, all herpesviruses share the same virion architecture and capsid/virion assembly scheme, but their SCPs and capsid-associated tegument proteins differ across the three subfamilies. Using cryoEM single particle reconstruction, we compared virion structures of representative members of the three subfamilies, focusing on their SCPs and capsid-associated tegument proteins.

In Chapter 2, we compared betaherpesvirus HCMV capsid and virion reconstructions, and discovered that HCMV SCP mediates the association of its tegument protein, putatively pp150, to the capsid. We further verified this structural message by designing a ribozyme inhibiting SCP expression in HCMV-infected cells, reconstructing the resulted “SCP-deficient” viral particles, and confirming the absence of pp150 density in these SCP-deficient particles.

Chapter 3 reports the cryoEM reconstruction of KSHV virion, which is the first sub-nanometer resolution structure of any gammaherpesvirus. We found that KSHV SCP bridges neighboring MCP molecules in the hexon, a configuration that seems to cement and stabilize the capsid. We further mapped the functional domain of KSHV SCP by constructing C- or N-terminal truncations of the SCP molecule with the bacterial artificial chromosome (BAC) system. We measured viral titers of the mutants and examined capsid assembly in cells infected by these mutant viruses. We found that KSHV SCP is not essential for capsid assembly as previously proposed, instead it promotes capsid assembly possibly by stabilizing the capsid structure. Only the N-terminal half of SCP is important for its function, while the C-terminal half is flexible and dispensable. By comparing cryoEM reconstructions of the SCP-truncated mutants with those of the WT, we successfully traced the backbone of the SCP molecule and built
pseudo-atomic model of the SCP to show the details of its interaction with the underlying capsid. We discovered that KSHV SCP binds to an MCP surface groove with its “stem helix” via hydrophobic interactions, and bridges another neighboring MCP with its “bridging helix”.

Combining results of Chapter 1 and Chapter 2, we propose that herpesvirus SCP functions as a cementing protein to stabilize the capsid for genome packaging, as those cementing proteins do in dsDNA bacteriophages. Unlike gammaherpesvirus SCP that works alone to exercise this function, SCP in the betaherpesvirus seems to function together with the pp150 tegument protein to stabilize the capsid.

In Chapter 4, we report the first discovery of orderly binding tegument proteins on the KSHV capsid surface. Comparison of these tegument densities with those in HSV-1 and PRV reconstructions revealed the similarity between alpha- and gammaherpesvirus capsid-associated tegument proteins. Combining our new structure with previously published molecular labeling studies of HSV-1 and/or PRV tegument densities, we re-established the model of how the two components of these tegument densities are organized in alpha- and gammaherpesvirus capsids. This result is informative for future investigations of the mechanism how these tegument proteins essentially affect herpesvirus genome packaging and viral growth.

In summary, we have looked into the structural and functional aspects of herpesvirus smallest capsid proteins and capsid-associated tegument proteins with a combination of structural and molecular biology tools. The abundant structural information we have gathered is intriguing for further researches of herpes virology. Just to name one, we have designed and synthesized peptides mimicking the KSHV SCP stem helix, which are expected to be able to bind the MCP surface groove but unable to bridge/crosslink neighboring MCP molecules in the capsid hexons as the WT SCP does. We will test the idea of using these synthesized peptides to
compete with, and thus inhibit, WT SCP in binding and stabilizing the capsid. Our final goal is to search for potent and specific inhibitors of herpesvirus infection that would possibly lead to development of new anti-herpesvirus drugs.
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