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Incorporation of the Kaposi's sarcoma-associated herpesvirus capsid vertex-specific component (CVSC) into self-assembled capsids

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Abstract

Self-assembly of herpesvirus capsids can be accomplished in heterologous expression systems provided all six capsid proteins are present. We have demonstrated the assembly of icosahedral Kaposi's sarcoma-associated herpesvirus (KSHV) capsids in insect cells using the baculovirus expression system. Using this self-assembly system we investigated whether we could add additional capsid associated proteins and determine their incorporation into the assembled capsid. We chose the capsid vertex-specific component (CVSC) proteins encoded by open reading frames (ORFs) 19 and 32 to test this. This complex sits on the capsid vertex and is important for capsid maturation in herpesvirus-infected cells. Co-immunoprecipitation assays were used to initially confirm a bi-molecular interaction between ORF19 and ORF32. Both proteins also precipitated the triplex proteins of the capsid shell (ORF26 and ORF62) as well as the major capsid protein (ORF25). Capsid immunoprecipitation assays revealed the incorporation of ORF19 as well as ORF32 into assembled capsids. Similar experiments also showed that the incorporation of each protein occurred independent of the other. These studies reveal biochemically how the KSHV CVSC interacts with the capsid shell.

All herpesviruses assemble icosahedral capsid shell structures that serve as the protective coat for the virus genome. These multi-protein complexes are assembled in the nucleus of infected cells and are the substrates for the DNA packaging complex of proteins that insert a genome length of virus DNA into the interior of the shell (Baines, 2011; Brown and Newcomb, 2011; Cardone et al., 2012; Rixon and Schmid, 2014; Tandon et al., 2015). Kaposi's sarcoma-associated herpesvirus (KSHV) capsids consist of six proteins encoded by open reading frames (ORF) 25, 62, 26, 17, 17.5 and 65 (Deng et al., 2008; Nealon et al., 2001; Trus et al., 2001). The outer shell proteins are the major capsid protein (MCP–ORF25), the triplex proteins (ORF62/ORF26) and the small capsid protein (SCP–ORF65). The capsid subunits (hexons and pentons) are composed of the MCP and these are

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interconnected to each other by the triplex complex. The SCP decorates the capsid shell by virtue of its interaction with the MCP and is important for capsid assembly for KSHV (Dai et al., 2015; Lo et al., 2003; Perkins et al., 2008; Sathish and Yuan, 2010; Trus et al., 2001). Internal scaffold proteins (ORF17/17.5) are required for icosahedral capsid shell synthesis. Herpesvirus capsids become “decorated” with additional proteins and complexes involved in DNA and capsid stabilization as well as tegumentation. The capsid vertex in the icosahedral structure has become a focus of protein-protein interactions that are important for DNA packaging, maturation of the capsid and egress of the nucleocapsid from the infected cell. All herpesviruses encode a complex of two proteins that bind to the capsid vertex and are important for stabilization of the capsid, packaging and stabilization of the viral genome within the capsid interior, and potentially the interaction with the nuclear egress complex (NEC) (Cardone et al., 2012; Conway et al., 2010; Dai et al., 2014; Homa et al., 2013; Huet et al., 2016; Leelawong et al., 2011; McNab et al., 1998; Sae-Ueng et al., 2014; Thurlow et al., 2006; Thurlow et al., 2005; Toropova et al., 2011; Trus et al., 2007; Yang et al., 2014). The herpes simplex virus type 1 (HSV-1) complex is encoded by genes UL17 and UL25 and because it is found on all intra-nuclear capsids has been named capsid vertex-specific component (CVSC) (Conway et al., 2010; Toropova et al., 2011; Trus et al., 2007). ORFs 32 and 19 encode the KSHV orthologs of UL17 and UL25, respectively. These proteins may serve independent functions during capsid maturation but the capsid association of HSV-1 UL25 is dependent on its interaction with UL17 (Thurlow et al., 2006). Cryo-EM studies have mapped this capsid association at high resolution (Conway et al., 2010; Dai et al., 2014; Huet et al., 2016; Toropova et al., 2011; Trus et al., 2007). Although phenotypes for mutants in these genes in alpha- and beta-herpesviruses differ (Borst et al., 2016; Klupp et al., 2006; Kuhn et al., 2010; McNab et al., 1998; O’Hara et al., 2010; Preston et al., 2008; Stow, 2001), recent studies have begun to congregate to a role for this complex acting as a signal that is recognized by the nuclear egress complex and thus promotes capsid envelopment and nuclear exit (Leelawong et al., 2011; Yang and Baines, 2011).

Our simple goal in this study was to use a capsid self-assembly system to investigate how the proteins of the KSHV CVSC associate with proteins of the capsid shell and how they become incorporated into this higher order multi-protein assembly. We demonstrated the ability to assemble KSHV capsids in insect cells using baculoviruses expressing the six capsid shell proteins. These capsids could be easily purified using sedimentation methods and we were able to show the essential role of the small capsid protein, ORF65, in capsid assembly (Perkins et al., 2008), something that could not be done in KSHV infected cells until recently (Dai et al., 2015; Sathish and Yuan, 2010). Thus using this ex-vivo method to study essential functions of the structural proteins is a valid approach and has proven useful in the elucidation of these key functions. Our goal was to use KSHV capsids as a scaffold on which to add the proteins of the CVSC and determine how these proteins interact and bind with the shell.

We first cloned the genes encoding ORF19 and ORF32 into baculovirus transfer vectors. All ORFs were amplified using the KSHV BAC36 BAC genome (Gao et al., 2003) as a template. The ORF19 and ORF32 genes were cloned as BglII-SpeI (ORF19) or EcoRI-SpeI (ORF32) fragments into pFastBac1 (pFB1) baculovirus transfer vector (Invitrogen). We used a modified pFB1 that contained either a C-terminal V5 (pFB1CV5), hemagglutinin (HA)

(pFB1CHA) or green fluorescent protein (GFP) (pFB1CGFP) tag (Desai et al., 2012). We did this, as there are no monospecific antibodies to the KSHV ORF19 and ORF32 proteins. All PCR assays used either *Pfu* Ultra (Stratagene) or *Phusion* polymerase (Finnzyme-NEB) and the cloned genes were sequenced to check for authentic amplification.

We used the Bac-to-Bac system from Invitrogen and the *Escherichia coli* strain DH10BAC using both the manufacturer's protocol (Invitrogen) and modifications described by Okoye et al. (Okoye et al., 2006) to generate recombinant baculoviruses. *Spodoptera frugiperda* (Sf9 and Sf21) cells were grown in Grace's insect cell medium, supplemented with 10% fetal calf serum (Gibco-Invitrogen) and passaged as previously described (Okoye et al., 2006). The Bacmid DNA was transfected into Sf9 cells and viruses were amplified in the same cell type (Okoye et al., 2006; Perkins et al., 2008). Purified viruses were used to confirm the expression of each protein, following infection of Sf21 cells and analysis of protein accumulation after 48h of infection. Protein lysates were prepared in RIPA buffer (Desai et al., 2012) for this purpose. The western blots showed the accumulation of the polypeptide with the correct molecular weight using either V5, HA or GFP antibodies (Fig. 1). ORF19 encodes a 61.2 kD polypeptide whereas ORF32 encodes a 49.5 kD protein and GFP is a 27 kD polypeptide.

To examine whether the ORF19 and ORF32 proteins interact with each other as predicted from the studies on the HSV-1 CVSC, co-immunoprecipitation experiments were performed using the same methods described in Capuano et al. (Capuano et al., 2014) and Luitweiler et al. (Luitweiler et al., 2013). In these experiments we used a rabbit antibody to the GFP tagged protein to co-precipitate the V5 tagged polypeptide. The resulting precipitate was examined for the presence of the V5 tagged polypeptide using immunoblots. For both precipitations, the reciprocal V5-tagged ORF19 or ORF32 was detected in the precipitate, indicating a physical association between these two polypeptides in cells (Fig. 2A). In either case very little or none of the SCP control protein was precipitated under these conditions. We also tested for the ability of the CVSC proteins to bind the nuclear egress complex protein encoded by ORF69 under the same conditions. The virus expressing ORF69CV5 was made previously (Desai et al., 2012). As seen in the immunoblot, both ORF32 and ORF19 can associate with ORF69 indicating potential interactions (Fig. 2A).

The proteins of CVSC were also examined for their ability to bind to members of the capsid shell, specifically the triplex proteins, ORF62 and ORF26 and the MCP encoded by ORF25. In each case the ORFs for the capsid proteins were fused to an N-terminal V5 tag. The baculovirus expressing ORF25 NV5 was described before (Capuano et al., 2014). For ORF26 and ORF62, the genes were derived as EcoRI-HindIII fragments from pFastBac1 cloned plasmids (Perkins et al., 2008) and moved into a modified pFastBac1 expressing an N-terminal V5 sequence (Capuano et al., 2014). These fusion proteins had been tested previously in a self-assembly assay and shown to support capsid shell assembly (data not shown). In co-immunoprecipitation assays, this time using a mouse monoclonal antibody to HA, both ORF32 and ORF19 could bind and precipitate the triplex proteins but not the SCP of KSHV (Fig. 2B). The affinity for ORF62 was lower than for ORF26 for both CVSC proteins as judged by the amounts of protein detected in the immunoblot. This observation was more pronounced in ORF32 immunoprecipitates. Similar experiments revealed both

ORF32 and ORF19 could bind to the MCP (ORF25) under these conditions (Fig. 2C). Again ORF32, as judged by the immunoblot, precipitates relatively less ORF25 than ORF19 even though there is more ORF32 protein present in the lysate. These data were consistent in several independent co-immunoprecipitations. Some of these interactions were confirmed using reciprocal immunoprecipitations and similar immunoblots (Fig. S1). This experiment showed ORF69 co-precipitated ORF19 and ORF32 but not ORF67A, a control protein that is part of the DNA packaging complex. ORF65 as expected did not bind any of the V5 tagged polypeptides. However, because of the close migration of ORF32 and especially ORF19 to the IgG heavy chain we cannot present clearly all the reciprocal co-immunoprecipitation data.

Our next experiments were aimed at discovering if the CVSC proteins expressed by recombinant baculoviruses in insect cells could interact with the assembled capsid shell in the same infected cell. To do this we made use of two proteins of the capsid shell. ORF65 tagged at the C-terminus with HA and ORF62 tagged at the amino terminus with a V5 tag. These tagged polypeptides when co-expressed in Sf21 cells with the other capsid proteins self-assemble to give icosahedral capsids in this system (Capuano et al., 2014 and data not shown). Our goal was to use ORF65 tagged with HA to immunopurify assembled capsids from fractions following sucrose gradient sedimentation of capsids from infected cell lysates. We have shown this before using 6XHIS tagged ORF65 (Capuano et al., 2014). The inclusion of ORF62 which has a fixed copy number in the capsid (320 copies) tagged with the same immunodetection epitope would allow us to determine relative incorporation of V5 tagged ORF19 and ORF32 as well as detect capsids using immunoblot methods. The aim was to co-infect insect cells with the viruses expressing all the capsid proteins and the proteins of the CVSC and then determine using immunoblots whether the ORF19 and ORF32 proteins specifically associate with the assembled capsids in the infected cells. By using an assembly minus control we could determine if capsid association was specific since the proteins could co-sediment regardless of capsid association or not.

Sf21 cells (1.2×10^7 cells) in a 100-mm Petri dish were infected with 300 μ L of baculoviruses expressing ORF25/ORF17.5 (Capuano et al., 2014), ORF26/ORF62NV5 (This virus was made as described in Capuano et al. 2014 except that the pFastBac Dual vector contained a N-terminal V5 sequence cloned at the polyhedron multiple cloning site) and ORF17/ORF65CHA (Capuano et al., 2014) (BAC-ALL) as well as the viruses expressing ORF19 and ORF32 (tagged at the C-terminus with a V5 sequence). Similar cell cultures were also infected with the same viruses except the virus expressing ORF25/17.5 (-MCP/SCA) was not added. This was an assembly negative control as both the MCP and internal scaffold proteins are required for assembly. Sixty-eight to seventy two hours after infection the cells were harvested, and processed for sedimentation as described by Perkins et al. (Perkins et al., 2008). Capsids were harvested by side-puncture aspiration after visualizing the light scattering band using incident light. For the capsid-minus (-MCP/SCA) control gradients, material from the gradient at the same position where capsids sediment, was harvested by side-puncture.

The capsids were then reacted with anti-HA antibody coupled to agarose to further purify capsids and the associated proteins and the resulting immunocomplexes could then be

detected by immunoblots with anti-V5 antibody. To do this capsids were diluted 1:1 in RIPA buffer and pre-cleared with 50 μ l protein A/G agarose beads (Santa Cruz) for 1 hr with rotation at room-temperature. Cleared supernatants were then mixed with 50 μ l anti-HA agarose conjugate (Sigma) for 1 hr with rotation at room-temperature. Agarose beads were then washed 5-times with 0.5X RIPA, resuspended in 2X Laemmli buffer, and analyzed by immunoblot methods.

In the first experiments, an example of which is shown in Fig. 3A, we detected specific capsid association of ORF19/ORF32 with immunoprecipitated assembled capsids as judged by the detection of these two proteins in the assembly positive IPs (IP α HA lane BA +32/19^{V5}) but not in the assembly minus IPs (IP α HA lane -MCP+32/19^{V5}). Both proteins as well as ORF62 were expressed and detected in the co-infected cell lysates (lysate lanes) and even in the capsid fraction (capsid lanes) whether assembled capsids were present or not. ORF62 was not detected in the -MCP+32/19^{V5} lane of the IP α HA as expected. To test whether these proteins bind as a complex or individually similar infections were performed, some contained both ORF19/ORF32 and others contained only ORF19 or ORF32. Following IP of the capsid fraction with anti-HA agarose and resolution of the precipitated proteins, the V5 tagged proteins were detected again by immunoblots. The results of the blot shown in Fig. 3B demonstrates that either protein can associate with assembled capsids in this system, compare lane 1 to lanes 2 and 3 of IP α HA. Hence, for the KSHV CVSC proteins in this system, bi-molecular complex formation is not required for capsid association.

In summary, we have for the first time documented the biochemical properties and interactions of the KSHV CVSC proteins. Both ORF19 and ORF32 interact with each other as demonstrated for the alphaherpesviruses (Thurlow et al., 2006; Toropova et al., 2011; Trus et al., 2007) and also specifically with proteins of the capsid shell, which include the triplex and major capsid proteins but not the small capsid protein. Cryoelectron microscopy data have revealed at high resolution the localization of this complex at the each of five points surrounding the pentonal vertex (Dai et al., 2014; Homa et al., 2013; Huet et al., 2016). Both data for HSV and KSHV show connections between the two proteins and the triplex complex as well as adjacent hexons and tegument constituents. Biochemical data similarly show protein contacts between the CVSC and the capsid/tegument proteins (Szczepaniak et al., 2011). Thus this complex is likely to participate in many interactions as the capsid is assembled, packaged with a genome and matures into an infectious particle. Specific attachment of the CVSC at the pentonal site may occur because of the unique conformations of the capsid shell proteins at this site or the presence of interacting tegument proteins in virus infected cells. Our data showed the CVSC proteins ORF19 and ORF32 associate with capsids independently and are not dependent on each other for this function in an ex vivo model. This is different from what is reported in the alphaherpesviruses because UL17 is required for efficient UL25 incorporation (Thurlow et al., 2006). Similarly high-resolution cryo-EM data suggest a heterodimer complex of 1:1 for UL17 and UL25 (Conway et al., 2010; Homa et al., 2013; Huet et al., 2016; Trus et al., 2007) and the stable UL25nt whose crystal structure has been determined is a monomer (Bowman et al., 2006). Our data although subject to the limitations of our detection method indicates more of ORF32 than ORF19 in the capsids. These data may reflect real differences between the

different herpesvirus capsids or the different methods to analyze this. It is also possible that in the absence of other capsid-associated proteins in our assembly method it allows additional binding sites for these proteins to be exposed for capsid association, which may account for the differences. Nevertheless, we have shown again that an ex-vivo method (Perkins et al., 2008), which first established the essential role of the small capsid protein for assembly can also be used to investigate high-order assemblies of these complex particles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- First biochemical studies that examine and discover interactions of the Kaposi's sarcoma-associated herpesvirus (KSHV) capsid vertex complex proteins with themselves and with the triplex complex and capsid shell
- Use of an ex-vivo self assembly system to investigate interactions of the KSHV capsid and vertex complex
- New insight into the mode of incorporation of the capsid vertex complex specified by ORF19 and ORF32 into the assembled structure

Fig. 1.

Protein expression of tagged ORF19 and ORF32 polypeptides. Sf21 insect cells (1×10^6) were infected with baculoviruses encoding ORF19 and ORF32 tagged at the C-terminus with V5, HA and GFP sequences. Protein lysates were prepared following 48 h of infection, the proteins separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblot methods as previously described (Luitweiler et al., 2013). Different blots were probed with mouse monoclonal antibody to V5 (Invitrogen R960), rat monoclonal antibody to Flu HA (Roche clone 3F10) and mouse monoclonal antibody to GFP (Molecular Probes). Protein standards (kD) are shown in the first lane.

Fig. 2.

Interactions of the KSHV CVSC proteins with each other and with proteins of the capsid shell. Infected Sf21 protein lysates were prepared as described by (Desai et al., 2012) and used in co-immunoprecipitation assays to detect protein binding partners of ORF19 and ORF32. (A) Protein lysates containing ORF32CGFP or ORF19CGFP were mixed with extracts containing ORF32CV5, ORF19CV5, ORF69CV5 or ORF65CV5 and immunoprecipitated (IP) using anti-rabbit GFP antibody (Abcam). The precipitated complexes were resolved by 4–12% NuPage gels (Invitrogen) and the proteins following transfer to nitrocellulose were incubated with anti-V5 mouse antibody (Blot). The input lysates for the V5 and GFP tagged proteins were also examined by western blots. (B)

Similar immunoprecipitations were performed with lysates containing ORF32CHA or ORF19CHA and lysates containing ORF62NV5 or ORF26NV5 as well as ORF25NV5 (panel C) using mouse anti-HA antibody (12CA5 Babco). ORF65CV5 containing lysates were used as control lysates. Again the precipitated proteins and the input lysate proteins on membranes were probed with anti-V5 or anti-HA. Protein standards (kD) are shown in the leftmost lane. IgG heavy chain (hc) and light chain (lc) are labeled where evident.

Fig. 3.

Capsid incorporation of the KSHV CVSC. (A). Sf21 infected cell lysates that contain all six KSHV capsid proteins (BA) and the ORF19 and ORF32 proteins were sedimented through sucrose (20–50%) gradients and the light scattering capsid band visualized was harvested. Control lysates, which did not contain the major capsid/scaffold proteins (-MCP) were similarly sedimented and material from the same position where the capsids band was harvested. These fractions were precipitated with anti-HA agarose (Sigma-Aldrich Clone HA-7) and the precipitated proteins were resolved by 4–12% NuPage gels and transferred to membranes prior to probing with anti-V5 antibodies. Proteins detected with the V5 antibody in the starting lysate, the capsid fraction and the immunoprecipitated material (IP) are labeled. (B) Similar capsid purifications and immunoprecipitations were carried out as described above except that this time lysates contained either both ORF32/ORF19 or just

ORF19 or ORF32. Immunoblots carried out using anti-V5 antibody was used to detect the capsid associated proteins. For both panels protein standards are shown in the left lane and the IgG heavy (h) and light (l) chains are evident in all samples.

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